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THE ROLE OF DIETARY FATS IN THE PATHOGENESIS OF GALLSTONES

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1. ABSTRACT

Gallstone disease is exceptionally common, occurring especially in Western populations, with cholesterol gallstones predominating. Currently, it is believed that one of the essential factors in the pathogenesis of cholesterol gallstones is a physical–chemical event that results primarily from alterations in the lipid composition of gallbladder bile. Cholesterol supersaturation is due principally to excessive secretion of cholesterol into the bile. Several biochemical defects, as well as diet, might cause hypersecretion of cholesterol. The precise effects of diet on cholesterol supersaturation of bile have not been clearly established, although epidemiological, clinical, and animal studies indicate that diet plays an important role in cholesterol gallstone formation. This review summarizes current information on the role of dietary fat in the modulation of cholesterol gallstone formation.

2. INTRODUCTION

Gallstone disease (GD) is exceptionally common in Western populations and is a major source of morbidity in the United States (1), other Western countries (2), and Latin American countries, such as Chile(3) and Mexico(4). In these countries, the economic impact of GD is high (1,2), and the effective prevention of gallstone formation is therefore an important objective. Epidemiological studies have identified risk factors for cholesterol gallstones(2,5-6), some of which are environmental, such as obesity and diet.

The most important factor involved in gallstone formation is increased biliary secretion of cholesterol from the liver, producing cholesterol-supersaturated bile. Subsequently, biliary cholesterol precipitates as cholesterol monohydrate microcrystals, which grow and agglomerate to form macroscopic stones in the gallbladder (7). Cholesterol is an essential constituent of living tissues and plays a critical role as a structural component of most biological membranes and as a precursor of vitamins, hormones, and bile acids (8). It is of critical importance, therefore, that the cells of the major tissues of the body are assured a continuous supply of this molecule. Metabolic alterations in hepatic cholesterol secretion combine with changes in gallbladder motility and intestinal bacterial degradation of bile salts to destabilize cholesterol carriers in the bile and produce cholesterol crystals (9). This review focuses on the importance of dietary fat in the development of GD, analyzing epidemiological, nutritional, metabolic and genetic studies that reveal the importance of dietary fat in GD. We believe that future investigations in this area will lead to an increased understanding of the pathogenesis of cholesterol gallstones, which may help provide interventions to interrupt the earliest stages of gallstone formation.

3. EFFECTS OF CALORIC INTAKE

There is evidence of an association between high energy intake and risk of GD. At the end of the 1960s, Sarles *et al*(10) demonstrated that an increased intake of total calories, irrespective of dietary composition, increases the risk of GD compared with age-matched controls. These results have been confirmed in other multicenter cohort studies (11,12), with Stampfer *et* al (12) finding a strong positive correlation between high energy intake and risk of clinical GD in a study carried out with more than 60,000 subjects.

The most critical variable in lithogenic bile formation is the relative mass of free cholesterol fluxing through the bile. In humans, this correlates with the absolute load of calories consumed (13-15) and the resulting lipoprotein cholesterol that must eventually return to the liver and pass into the bile as bile acids or, failing that, as free cholesterol(16). Not surprisingly, obesity represents the greatest risk factor for gallstones. Females whose body mass index (BMI) exceeds 32 have six times the risk of those with a BMI of less than 22 (13). Equally remarkable is the increased risk of gallstones associated with increased caloric consumption in non-obese women (13).

The association of gallstones with obesity (and caloric overconsumption) presumably reflects cholesterol production, or flux, which is substantially elevated both in obese people and in people with gallstones (17,18). This is further complicated by increased insulin levels in obesity

(16); hyperinsulinemia itself is a risk factor for gallstones (19). Several studies have shown that hyperinsulinemia is associated with high triglyceride and low high density lipoprotein (HDL) cholesterol levels, mainly the HDL2 subclass. In addition, there is evidence for an association between GD and higher serum insulin levels. High insulin levels are common in obese people: obesity and insulin resistance are major contributory factors to premature death worldwide, and these conditions are characterized by increased circulating levels of insulin (20-22). In recent decades, hyperinsulinemia has been proposed as a risk factor for GD and there are some studies that support this hypothesis. For example, Scragg *et al* (23), in a case–control study, observed the relation between plasma insulin and GD risk. They found mean fasting insulin levels to be higher in cases of GD in both sexes, independent of age and triglyceride levels. Laakso *et al* (24) also found that subjects with GD had significantly higher levels of insulin than controls. Furthermore, Haffner *et al* (25), in the San Antonio Heart Study, observed increasing hyperinsulinemia and high prevalence of GD in both Mexican Americans and non-Hispanic Whites. In Mexico, Gonzalez Villalpando *et a l* (25) found higher fasting insulin levels in women with GD than in controls, but no such relation was found in men. Finally, Ruhl and Everhart (27) in an elegant study have confirmed this association of GD with higher fasting serum insulin and C-peptide levels in women and showed that the association is independent of fasting glucose levels and other covariates related to GD.

Some possible mechanisms by which insulin may play a role in gallstone formation are through increasing cholesterol saturation of bile or decreasing gallbladder motility, two factors that are, at present, considered very important in the pathogenesis of gallstones. In addition, it has been suggested that high concentrations of insulin could increase the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) (28), the rate limiting enzyme in hepatic synthesis of new cholesterol, or by activating low density lipoprotein (LDL) receptors, resulting in greater hepatic uptake of low density lipoprotein cholesterol (29). By inhibiting basal and cholecystokinin-stimulated gallbladder motility, insulin might also increase the risk of developing gallstones through an effect on gallbladder motility (30).

Increased hepatic cholesterol synthesis and food intake have been linked to biliary cholesterol secretion in humans in certain circumstances (18). On the other hand, inhibition of cholesterol synthesis by lovastatin in humans depresses bile acid synthesis and secretion, and biliary cholesterol secretion (31,32). Moreover, studies in LPN hamsters, in which the incidence of gallstones is 65% when fed a lithogenic diet, have shown that the addition of β-cyclodextrin, a cyclic oligosaccharide (produced by the enzymatic cleavage of amylomaize starch) that contains seven glucopyranose units all linked in the β-position, to the lithogenic diet produces a fall in the molar proportion of cholesterol and phospholipids in gallbladder bile, which is reflected in a lower cholesterol saturation index (CSI) (33).

In essence, the liver of obese people must cope with an increased flux of cholesterol derived both from newly synthesized cholesterol, associated with elevated hepatic lipoprotein secretion, and from increased exogenous cholesterol entering the lipoprotein cholesterol pool from the diet. As a consequence, free biliary cholesterol rises to dangerous concentrations: the CSI of gallbladder bile becomes supersaturated (>1.0) and readily exceeds the solubilization threshold, setting the stage for cholesterol nucleation and stone formation (34).

4. DIETARY FAT

The average daily intake of cholesterol in the Western diet is about 300–500 mg (35). More than twice this amount of cholesterol may be secreted into the duodenum through the bile, and an estimated additional 300 mg is derived from sloughed intestinal cells (35). The role of dietary fat as an etiological factor for cholesterol GD has received considerable attention, but remains unresolved. We will discuss the role of fat in different animal models used to study the pathogenesis of cholesterol gallstone. In all animal models, fat is essential for the induction of cholesterol gallstones. Several models of cholesterol-based GD have been studied, for example, the squirrel monkey (36), prairie dog (37,38), hamster (39-41), and mouse (42). Hamsters are small, easy to handle and have a bile acid composition similar to that of humans,(1) which makes this model useful. Two hamster models of cholesterol-based GD have been described by Dam (43) and by Pearlman *et al* (40). The Syrian golden hamster (*Mesocricetus auratus*) is one of the most commonly used animal models for studying cholesterol gallstone development because it has important aspects of cholesterol and bile acid metabolism that are similar to that of humans. In addition, hamsters develop lithogenic bile and cholesterol gallstones in response to a variety of dietary manipulations. Generally, hamsters fed on purified diets with a modest level of fat (40–59 g/kg) and excessive amounts of cholesterol (3–4 g/kg) develop cholesterol gallstones, but the incidence is variable, probably due to genetic and dietary factors (42,44,45).

In hamsters, saturated dietary triglycerides augmented the suppressive effect of cholesterol on hepatic LDL receptor-dependent transport and lead to increased plasma LDL levels (46). In contrast, n-6 polyunsaturated triglycerides have the reverse effect, diminishing the suppressive effect of dietary cholesterol on hepatic LDL receptors (46).

Studies in prairie dogs (*Cynomus ludovicianus*) (47) fed with three different diets (a control diet with no added cholesterol and 5% of calories from corn oil; 1.2% cholesterol with 5% of calories from corn oil; or 1.2% cholesterol with 40% of calories from corn oil) showed that cholesterol feeding increases biliary cholesterol and phospholipid concentrations compared with controls, and those results were substantially higher in the group receiving 40% of calories from fat (47). Additionally, animals fed a 5% corn oil-cholesterol diet had a low incidence of cholesterol monohydrate crystals after one week, in contrast with the high prevalence of cholesterol monohydrate crystals in the animals fed with the

cholesterol-40% corn oil diet (47). In investigating the effects of a high cholesterol diet on biliary excretion of cholesterol and bile acids, Smit *et al* (48) found that, after feeding rats a cholesterol-free (control) or a high cholesterol diet (1% wt/wt) for two weeks, the cholesterol induced a 20% increase in plasma cholesterol concentration, a threefold increase in hepatic bile acid synthesis and a 27% increase in bile acid pool size, whereas biliary excretion of cholesterol was decreased by 50%. After cholesterol feeding, chylomicron remnant cholesteryl ester is more efficiently converted to bile acids, a mechanism that may contribute to the resistance of rats to diet-induced elevation of plasma cholesterol. In contrast, biliary excretion of free cholesterol, the second main excretory pathway, is significantly decreased by the high-cholesterol diet (48). It was concluded that chylomicron remnant uptake by the liver is efficiently coupled to bile acid synthesis and biliary excretion, thus providing an efficient pathway for removal of intestine derived cholesterol (48). The same groups of researchers (49) studied cholesterol metabolism in rats fed purified diets supplemented (9% wt/wt) with either fish oil (FO, n-3 fatty acids) or corn oil (CO, n-6 fatty acids) for four weeks. FO as compared with CO induced a lowering of plasma cholesterol levels by 38% and of triglyceride levels by 69%. This reduction in plasma lipids in FO rats was accompanied by increased biliary excretion of cholesterol (51%). The results show that FO induces changes in transport and metabolic pathways of cholesterol in the rat liver, which result in a more rapid disposition of plasma-derived cholesterol into the bile. Also, when these researchers investigated in more detail the relation between cholesterol and bile acid secretion in rats with chronic bile diversion fed purified diets supplemented (9% wt/wt) with either FO or CO for two weeks, the effects of FO on biliary cholesterol secretion (+400% as compared to CO after 14 days) were much more pronounced than previously observed in rats with intact enterohepatic circulation (+50%). Biliary bile acid (+30%) and phospholipid (+120%) secretion were increased to a much lesser extent than that of cholesterol, resulting in the formation of bile supersaturated with cholesterol. They concluded that, in rats, dietary fish oil increases the disposition of cholesterol into bile by potentiating bile acid-dependent cholesterol secretion, presumably by facilitating the recruitment of bile-destined cholesterol (50).

In humans, Méndez-Sánchez *et al*(1) demonstrated the importance of diet composition in obese women losing weight with the consumption of n-3 polyunsaturated fatty acid (PUFA) by these patients showing a beneficial effect on bile composition. Cholesterol nucleation time decreased significantly in the placebo group, but not in the n-3 PUFA group. None of the women in the n-3 PUFA group had developed gallstones at six weeks. These results suggest that n-3 PUFA maintains the CSI and nucleation time in obese women during rapid weight loss, which probably results in the prevention of cholesterol gallstone formation. The mechanisms by which n-3 PUFA improves the bile involve changes in fatty acid composition via enrichment with eicosapentaenoic acid and docosahexaenoic acid-containing species (1). Furthermore, in this study we observed a decrease in the serum cholesterol and LDL concentrations.

On the other hand, a very interesting study carried out in a two-strain (LPN and Janvier) hamster model was published by Férézou and colleagues (51). They investigated the cholesterol, bile acid, and lipoprotein metabolism in two strains of hamster that differed markedly in their response to a sucrose-rich/low fat diet. Under basal conditions, hamsters from the LPN strain differed from Janvier hamsters by showing lower cholesterolemia, higher post-prandial insulinemia, and more active cholesterogenesis in the liver. They found that the two-strain hamster model is analogous to the well-accepted notion of an inverse association between plasma cholesterol levels and cholesterol gallstones in humans that is often reported in epidemiological studies (52). In their experimental model, the etiology of cholesterol gallstone formation is basically related to a strain difference in both lipid and glucose metabolism. This contrasts with other studies using lithogenic diets containing significant quantities of added cholesterol and fat (53). Dietary fat alters the distribution of cholesterol between vesicles and micelles in hamster bile (54). In the LPN hamsters, which are genetically predisposed to cholesterol gallstones, induction is by a sucrose diet. However, in both experimental models the homeostasis of cholesterol is altered by different diets. Surprisingly, LPN hamsters have lower hepatic expression of LDL receptor, even when the sucrose-rich diet induces a significant fall in LDL cholesterol in this strain. Similarly, LPN animals also have reduced plasma HDL cholesterol levels and type I scavenger receptor (SR-BI) expression, especially under basal conditions, as compared with Janvier hamsters, which are resistant to cholesterol gallstone induction. This resistance is due to greater active synthesis of cholesterol associated with a limited capacity of the liver to store and transform cholesterol into bile acids.

Also, it is interesting to point out that, in inbred strains of mice in which the genes *Lith1* and *Lith2* have been identified, the induction of gallstones is through dietary cholesterol and fat (55). Gallstone-susceptible mice fed a lithogenic diet show a relative hypersecretion of cholesterol, as compared with phospholipids and bile acids. The lithogenic diet used contains large amounts of fat and cholesterol, which, after absorption, enter chylomicrons. The precise mechanism by which their remnants are cleared from the blood appears to involve a complex interplay of apolipoprotein E (ApoE), the LDL receptor, the LDL receptor related protein, hepatic lipase, and heparan sulfate proteoglycans (56,57). A recent preliminary study (58) showed that biliary secretion of cholesterol carried in chylomicrons is more rapid in gallstone-susceptible C57L mice. Together with decreased biliary cholesterol secretion rates and reduced gallstone prevalence in ApoE-deficient C57BL/6 mice fed a lithogenic diet,(59,69) these data indicate that the chylomicron pathway most likely participates in cholesterol transport to intracellular pools, providing cholesterol for biliary excretion.

SR-BI is the cell surface HDL receptor that recognizes ApoE. It appears that the SR-BI receptor also binds LDL, but its role in the metabolism of apoprotein B-

containing lipoproteins is not well understood. Selective uptake of HDL cholesterol esters is a process by which the core cholesterol ester is taken up without degradation of the HDL apoprotein. This type of selective transport may be the major source of cholesterol ester delivery into hepatocytes and steroidogenic tissues (61). Fuchs *et al*(62) have suggested that up-regulation of hepatic SR-BI and caveolin-1 expression is associated with biliary cholesterol hypersecretion and gallstone formation in gallstone-susceptible C57L mice, compared with resistant AKR mice. In addition, analysis of plasma lipoproteins showed striking differences between C57L and AKR mice, with substantially lower HDL cholesterol levels in the C57L mice, confirming an earlier report (55). This indicates a role for the HDL receptor in the hepatic uptake and biliary secretion of cholesterol.

Another component of lithogenic diets is the bile acids. In the mouse, bile acids, usually cholic acid, and cholesterol are required for dietary induction of cholesterol gallstones (42,63). Surprisingly, several animal models of pigment gallstones also require diets high in cholesterol (39,64-68). Interestingly, we have shown that orally ingested unconjugated bile acids, particularly UDCA and to a lesser extent nodeoxycholic acid (CDCA), and added dietary cholesterol resulted in increased secretion of bilirubin into bile attributable to enterohepatic cycling of bilirubin as well as elevating plasma unconjugated bilirubin levels in two rodent species (69).

In addition, the effects on the bile salts when a model animal is fed a cholesterol-diet are quantitative and qualitative. For example, in cholesterol-fed rats, the bile acid pool contained a larger proportion of chenodeoxycholic acid and of muricholic acids than of cholic acid, probably indicating that chenodeoxycholic acid, which is rapidly converted to muricholic acids in the rat liver, is absorbed more efficiently than cholic acid when the bile acid pool is enlarged (48). It has been suggested that bile acid pool size and its composition is regulated not only at the bile acid biosynthesis level in the liver, but also at the intestinal level with regulation of active ileal transport (70). In prairie dogs, the increase in the lithogenicity in the gallbladder induced by cholesterol feeding, as estimated from the cholesterol concentration and the CSI, were associated with shifts in the cholate/chenodeoxycholate ratio and with changes in the proportion of individual molecular species of biliary lecithin. These changes in molecular species of lecithins and bile salts are probably consequences of excess biliary cholesterol secretion.(47)

Recently, Wang and Tazuma (71) investigated the effects of beta-muricholic acid and ursodeoxycholic acid (UDCA) as biliary cholesterol-desaturating agents to prevent cholesterol gallstones and on the dissolution of gallstones in gallstone-susceptible male C57L mice fed for eight weeks with a lithogenic diet (2% cholesterol and 0.5% cholic acid), with or without 0.5% UDCA or beta-muricholic acid. All the mice fed the lithogenic diet formed cholesterol gallstones. Addition of beta-muricholic acid and UDCA decreased gallstone prevalence to 20% and 50%, respectively, through significantly reducing biliary secretion rate, saturation index, and intestinal absorption of cholesterol. In addition, they found that eight weeks of beta-muricholic acid and UDCA administration produced complete gallstone dissolution rates of 100% and 60% when compared with standard chow (10%).

On the other hand, as mentioned before, ApoE plays a key role in lipoprotein metabolism and may have other important biological functions (72). ApoE is a major apolipoprotein constituent of chylomicrons, and plays a critical role in the hepatic catabolism of lipoproteins, acting as a high-affinity ligand for the low-density lipoprotein receptor (LDLR)/LRP mediated pathway. Both human genetic studies and transgenic and gene knockout (KO) animal models have established the importance of ApoE in chylomicron remnant metabolism. Amigo *et al* (60) showed the importance of ApoE using an ApoE deficient mouse model. ApoE KO mice, after being fed a high-cholesterol diet for four weeks, exhibited a 40% increase in plasma cholesterol concentration, compared with no change in wild-type mice. When mice were fed with a lithogenic diet, plasma cholesterol increased in both the wild-type and ApoE KO mice compared with chow-fed animals. However, the plasma total cholesterol concentration was significantly higher in ApoE-deficient versus the wild-type mice. These data demonstrate that ApoE plays a critical role in controlling the response to dietary cholesterol of plasma lipoprotein cholesterol concentration and profile, as well as cholesterol storage in the liver. On the chow diet, biliary lipid secretion was not affected by ApoE deficiency. After four weeks on the high-cholesterol diet, cholesterol and bile salt output were significantly increased in wild-type mice, with the most dramatic change seen in biliary cholesterol secretion, and a lesser effect on bile acid output. In contrast, significantly lower increases in biliary cholesterol (2.2-fold increase) and bile acid (1.4-fold increase) outputs were observed in ApoE KO mice receiving the high-cholesterol diet. After two weeks of feeding the lithogenic diet, wild type mice had mucinous bile containing abundant isolated and aggregated liquid cholesterol crystals. Cholesterol monohydrate crystals were observed in all these mice. At four weeks, all the gallbladders of lithogenic diet-fed wild-type mice contained mucinous bile with abundant aggregated liquid crystals and multiple isolated and aggregated cholesterol monohydrate crystals and 88% of wild-type mice had formed many fragile sand-like and/or hard stones. Cholesterol crystallization and gallstone formation sequences were significantly different in the lithogenic diet-fed ApoE KO mice. ApoE expression appears to be critical for gallstone induction by a lithogenic diet. The central role of ApoE in the overall regulation of cholesterol metabolism and biliary cholesterol secretion in mice has raised the possibility that it may also be involved in the pathogenesis of cholesterol gallstones in humans (60).

In summary, we can conclude that, according to the results from those experimental studies carried out in gallstone-susceptible C57L mice, LPN hamsters, and wild-type C57BL/6J mice, dietary fat is a very important factor in inducing changes in biliary lipid secretion, gallbladder

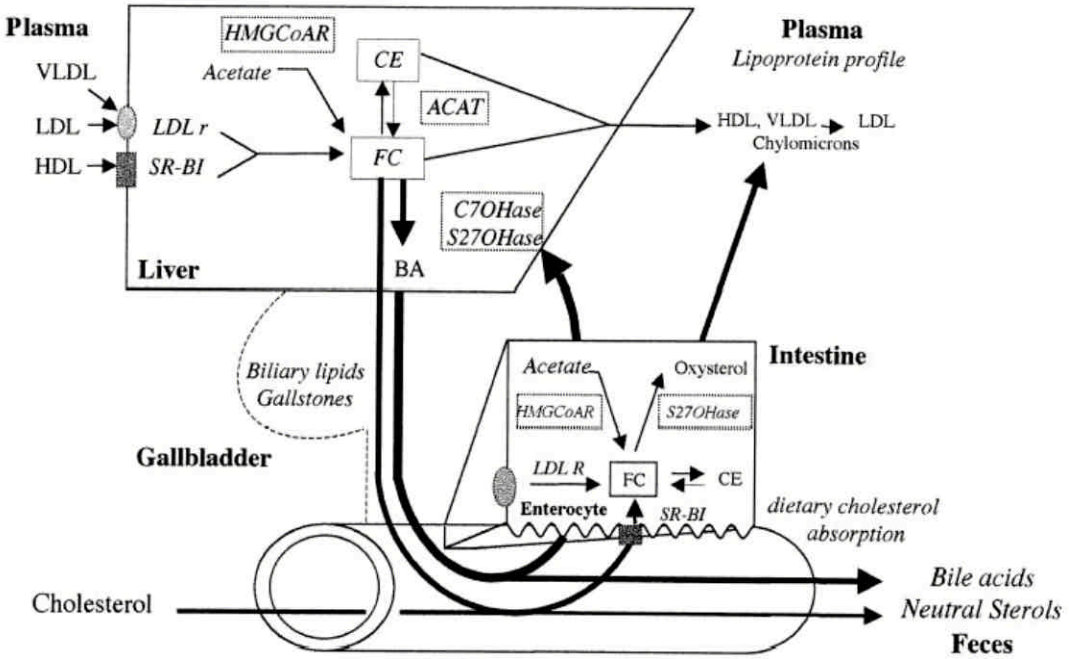

Figure 1. Scheme for hepatic, biliary, and intestinal cholesterol metabolism (51). (ACAT, acyl-coenzyme A:cholesterol acyltranferase; BA, bile acids; CE, cholesteryl esters; FC, free cholesterol; HMG-CoAR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDLr, LDL receptor; SR-BI, scavenger receptor class B type I; C70Hase, cholesterol 7α-hydroxylase; S27Ohase, sterol 27-hydroxylase). The biochemical and molecular changes induced by dietary fat appear to affect both the LDL and the SR-BI HDL receptors, and HMG-CoA reductase. These changes are important in the pathogenesis of cholesterol gallstones in gallstone-susceptible C57L mice, LPN hamsters, and wild-type C57BL/6J mice used to establish the role of ApoE (Reproduced with permission).

bile lipid composition, and serum lipid and lipoprotein metabolism (Figure 1). These changes appear to be a result of alterations in both LDL and SR-BI HDL receptor expression, and changes at the level of HMG-CoAR. Finally, these changes are important in the pathogenesis of cholesterol gallstones.

5. PERSPECTIVE

The studies in the animal models have not only confirmed the present advances of gallstone formation, but also have helped in the identification of novel genes in humans that might play an important in pathogenesis of the disease. A "gallstone map" for the mouse has been reported, showing genes that confer gallstone susceptibility, as well as candidate genes. In the future, it will be very important to examine if there is a relation between the gallstone genes in mice and genes that may be present in humans.

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