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Alcohol Protects Against Cholesterol Gallstone Formation

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Epidemiologic studies have suggested that alcohol intake may protect against cholelithiasis. Gallstone formation was studied in 20 prairie dogs fed a 0.4% cholesterol-supplemented liquid diet. In ten animals, ethanol provided 35% of total calories. In ten pair-fed controls, ethanol was replaced with isocaloric maltose. After 3 months the gallbladders were inspected for gallstones, and gallbladder bile was analyzed. Cholesterol macroaggregates were present in all controls and pigment concretions were noted in five. No stones were observed in ethanol-fed animals. Bile in the ethanol group contained less cholesterol than the controls (5.60 ± 0.71 *vs.* 9.16 ± 0.61 mmol/L, $p < 0.05$) while phospholipids, total bile acids, and bilirubin were unchanged. The resulting cholesterol saturation index was reduced in the ethanol group (0.81 *vs.* 1.22, $p < 0.05$). The ratios of trihydroxy to dihydroxy bile acids were also different (2.07 ± 0.25 in ETOH *vs.* 3.29 in controls, $p < 0.05$). The bile calcium concentration was higher in control animals presumably secondary to the use of complex sugars (5.36 ± 0.37 *vs.* 3.77 ± 0.32 mmol/L, $p < 0.05$). These results confirm that ethanol inhibits cholesterol gallstone formation. They further suggest that this effect is dependent on reductions of biliary cholesterol and selective changes in bile acid concentrations.

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THE INFLUENCE of ethanol on cholelithiasis is still incompletely understood. In autopsy studies the prevalence of gallstones appears to be increased when alcoholic cirrhosis is present.[1,2] This finding is explained largely by an increment in pigment stone formation.[3,4] Cholesterol stones, in contrast, are reported only infrequently in cirrhotic patients who undergo cholecystectomy.[5,6] Moreover, epidemiologic surveys have suggested the intriguing possibility that biliary disease may actually be decreased by alcohol ingestion. In the Framingham study, the amount of alcohol consumed appeared to have an inverse relationship to the incidence of definite gallbladder disease, although this effect did not reach statistical significance.[7] Similarly, data from the Kaiser-Permanente cohort indicated that heavy drinkers had a risk of hospitalization for cholelithiasis, which was only one-third that for nondrinkers.[8] More recently, Scraggs et al. have reported a case control study of 267 patients with gallstones carefully matched to both community and hospital controls. They also noted that the consumption of alcohol was associated

with a substantial decrease in the prevalence of gallstones.[9]

Because the prairie dog has been shown to be a useful model for the study of various aspects of lithogenesis, the effects of oral ethanol in this animal were investigated. Ethanol administered as 35% of total calories prevented cholesterol stone formation. This protective effect was accompanied by changes in both bile acid distribution and biliary calcium concentration as well as by a relative cholesterol desaturation.

Materials and Methods

Animals and Diet

Twenty adult male prairie dogs (cynomys ludovicianus) weighing 0.6–1.4 kg were obtained from Otto M. Locke (New Braunfels, TX) and individually housed in a thermoregulated room (23–25 C). During a 2-week adaptation period the animals were fed a standard laboratory chow containing negligible cholesterol (Teklad, Madison, WI). They were then divided into pairs with the members matched in weight as closely as possible. Animals were pair-fed a commercially prepared, nutritionally complete semisynthetic liquid diet as the sole source of oral intake (Bioserv, Inc., Frenchtown, NJ). Experimental animals received a diet containing 0.4% cholesterol by weight to which ethanol was added to provide 35% of total calories. Previous experiments have demonstrated that this diet results in peak serum ethanol levels of 240–320 mg/dL. Control animals received an identical cholesterol-supplemented diet except that ethanol was replaced by equicaloric amounts of maltose–dextrin. Both diets were kept refrigerated in darkness in their dry form to minimize cholesterol oxidation and were reconstituted on alternate days.

At the end of 90 days, the prairie dogs were anesthetized and a celiotomy was performed. The common bile duct was cannulated with polyethylene tubing, the cystic duct ligated, and the gallbladder aspirated. One drop of gallbladder bile was examined under light and polarizing microscopy for biliary crystals.[10] The remaining gallbladder bile was frozen and stored for subsequent analysis. Measurements of hepatic bile flow were conducted for 1 hour. Blood was collected by cardiac puncture, allowed to clot and then centrifuged, after which serum was carefully aspirated and frozen. The gallbladders were removed, opened longitudinally, and inspected for stones. The animals were then killed with an intracardiac injection of T-61 Euthanasia Solution (Hoechst, Somerville, NJ).

Biochemical Analyses

Prior to analysis, frozen bile was rewarmed in a 37 C water bath and centrifuged for 5 minutes at 2000 rpm. Cholesterol content was determined using a commercial colorimetric assay kit based on the enzymatic oxidation of cholesterol (Sigma Diagnostics, St. Louis, MO). Total phospholipids were also measured enzymatically using the color reagent described by Qureshi et al.[11] and the method of Takayama et al.[12] Bile acids were determined by high performance liquid chromatography (HPLC) as described previously.[13] The column was a Microsorb C18 column (0.5 × 25 cm, Rainin Instrument Co., Inc., Woburn, MA), and the mobile phase was acetonitrile–methanol – 0.03 mol/L phosphate buffer, pH 3.4 (10:60:30). The flow rate was 1 mL/min and detection was at 204 nm using a LDC Spectromonitor D variable wavelength absorbance detector. Conjugated and unconjugated bilirubin were also determined by the method described previously.[13] HPLC was performed on a 25 cm Microsorb C18 column using 0.1 mol/L dioctylamine acetate in methanol as the eluting solvent. The flow rate was 0.75 mL/min and detection was by absorbance at 435 nm. All bilirubin analyses were conducted in dim red light. Total calcium concentrations were determined by atomic absorption spectrophotometry (Varian Instrument Co., Palo Alto, CA).

Quantitative Treatment of Data

All data are reported as mean ± SEM. Statistical comparisons of mean values among groups were assessed by the unpaired Student's t-test. Proportions were evaluated by the chi square test. Cholesterol saturation indices (CSI) were calculated from bile lipid data using the computer program of Kuroki et al.[14] as derived from the critical tables of Carey.[15]

Results

All animals appeared to tolerate the liquid diet without difficulty. Changes in total body weight recorded at the end of the experiment were small, and there was no significant difference in the weight changes (−0.10 ± 0.04 kg for control animals *vs.* −0.13 ± 0.06 kg for ethanol-fed animals).

Gallstone Formation

Upon gross inspection, the gallbladders of all control animals were found to be packed with aggregated cholesterol crystals or cholesterol stones. In addition, several small black concretions were visible in five of the ten control animals. Infrared spectroscopic analysis of one of these black deposits showed that it was composed of approximately 40% calcium bilirubinate by weight. By contrast, no cholesterol macroaggregates or pigment deposits or crystals were found in any of the ethanol animals by either gross or microscopic observation ($p < 0.001$).

TABLE 1. *Gallbladder Bile Composition*

	Control	Ethanol
Cholesterol (mmol/L)	9.2 ± 0.7	5.6 ± 0.7*
Bile salts (mmol/L)	146.9 ± 10.0	152.2 ± 11.1
Phospholipids (mmol/L)	20.0 ± 1.5	16.7 ± 1.3
Total lipids (mmol/L)	176.1 ± 11.7	174.4 ± 12.5
CSI	1.16 ± 0.02	0.80 ± 0.06*
Cholesterol:phospholipid ratio	0.46 ± 0.01	0.34 ± 0.03*
Total calcium (mmol/L)	5.49 ± 0.38	3.77 ± 0.35*
Unconjugated bilirubin (μmol/L)	10.8 ± 1.4	11.2 ± 2.6
Conjugated bilirubin (μmol/L)	183.5 ± 27.3	162.2 ± 29.0

* $p < 0.05$.

Hepatic Bile

Bile flow rate was measured at the time of hepatic bile collection. The difference found between the flow rates of the two groups approached but did not reach significance (0.7 ± 0.2 mL/h for control animals *vs.* 1.2 ± 0.2 mL/h for alcohol animals).

Gallbladder Bile

Analytic data for the major components of gallbladder bile in both control and ethanol groups are presented in Table 1. The mean cholesterol level was lower in the ethanol-fed animals by a factor of almost 2. Total bile salt concentrations were the same in the two groups, while phospholipid values were slightly, but not significantly, lower in the alcohol group. Total lipid concentrations were equal. The difference in cholesterol levels resulted in a significant difference in the CSI for the biles of ethanol and control animals. In the case of controls, the CSI of all biles was greater than 1, while in the ethanol group only one bile had a CSI greater than 1 (Fig. 1). In addition, the cholesterol:phospholipid ratio was significantly lower in the alcohol group. Although no difference was found in the concentration of total bile salts in the two experimental groups, marked changes did occur in the levels of specific bile salts. The changes in the major bile salts are summarized in Table 2. A significant increase in the total amount of dihydroxy bile salts was found in ethanol-fed animals. This increase was due in large part to a doubling in the mean concentration of taurochenodeoxycholic acid. At the same time a small decrease in the total amount of trihydroxy bile salts was found in the alcohol group compared to controls. These changes resulted in a decrease of the trihydroxy:dihydroxy bile salt ratio from a value of greater than 3:1 in controls to approximately 2:1 in the ethanol group ($p < 0.005$).

Total calcium concentrations in gallbladder bile were significantly lower in ethanol-fed animals when compared to controls. Unconjugated bilirubin values were the same in both groups. The biles of ethanol animals were found to have a small, but not significant, decrease in the concentration of conjugated bilirubin. The monoconjugated form of the pigment accounted for more than 95% of total bile pigments.

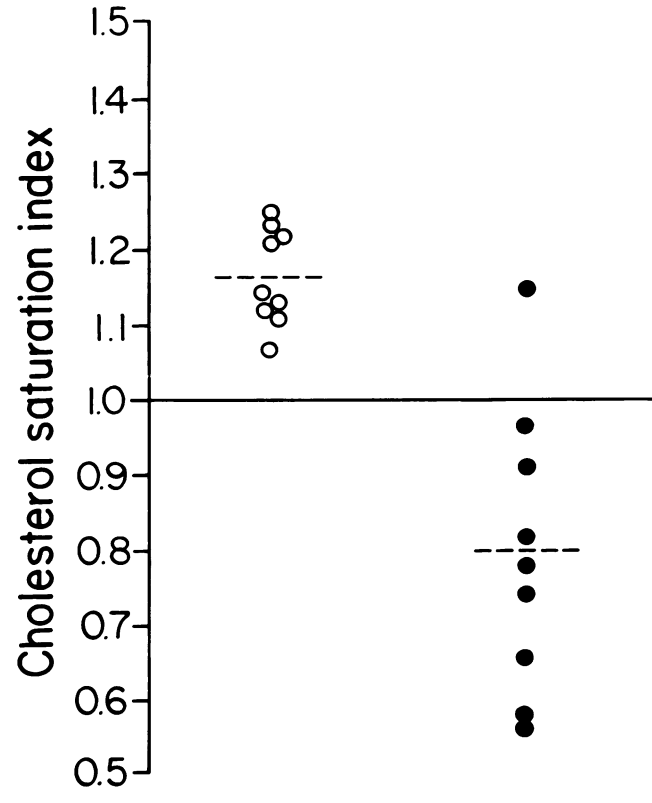

FIG. 1. CSIs are plotted for individual animals. Mean values (----) for control (O) and ethanol-fed (●) groups are significantly different ($p < 0.05$).

Discussion

Although epidemiologic studies have indicated that moderate alcohol intake reduces the risk of biliary disease, investigations of the mechanisms responsible for this effect have been hampered by serious methodologic problems. To overcome these difficulties, the prairie dog was chosen as the best model for study. In this animal the biliary lipid composition is similar to that of humans and becomes supersaturated with cholesterol when a lithogenic diet is provided.[16] As in humans, cholesterol

TABLE 2. *Bile Salts in Gallbladder Bile*

	Control	Ethanol
Taurocholic acid (mmol/L)	110.0 ± 7.5	97.5 ± 8.1
Taurochenodeoxycholic acid (mmol/L)	16.6 ± 1.4	35.6 ± 5.4*
Taurodeoxycholic acid (mmol/L)	18.8 ± 2.6	16.5 ± 3.3
Trihydroxy:dihydroxy ratio	3.19 ± 0.16	2.07 ± 0.3*

* $p < 0.005$.

lithogenesis then proceeds from the supersaturation phase through both crystal nucleation and stone growth phases. In these experiments, a liquid diet was used to overcome the natural aversion of prairie dogs to alcohol and to allow accurate pair feeding.[17] This approach ensured that each group received equal calories and nutrients; thus, the effect of nutritional intake alone should be minimized.

At the end of the 3-month study period, it was clear that ethanol administration had effectively prevented cholesterol precipitation and stone formation. In fact, bile samples from the gallbladders of ethanol-fed animals demonstrated no microscopic evidence of cholesterol crystals, indicating a high degree of solubilization. These results parallel those seen when other substances are used to arrest stone formation in the prairie dog such as hyodeoxycholic acid,[18] aspirin,[19] and the bile acid analog, ursodeoxyoxazaline.[20] The diversity of these litholytic substances and their putative mechanisms of action underscores the newly appreciated complexity of cholesterol gallstone formation.

In the current study, multiple factors appeared to be affected by alcohol administration. Notably, the CSI, a numerical approximation of the cholesterol-holding capacity of bile, was significantly lower in the ethanol group. All but one of the biles tested from this group were below unity, the defined point of cholesterol saturation. Conversely, all control biles were supersaturated with cholesterol. On this basis alone one would expect that no cholesterol monohydrate crystals would precipitate in ethanol-treated animals because cholesterol saturation is a necessary although not sufficient prerequisite for stone formation.[21] These results are similar to those of Thornton et al.[22] who found a decreased CSI in the bile of healthy human volunteers maintained for 6 weeks on moderate alcohol intake.

The reduction of the CSI in this model was largely accounted for by a significant decrease in the concentration of cholesterol in gallbladder bile. Decreases in biliary cholesterol output have previously been observed in association with ethanol administration in biliary fistula dogs[23] and in patients with cirrhosis.[24] Such a decrease in bile cholesterol promotes its complete solubilization by incorporation into either mixed micelles or phospholipid vesicles. This effect of long-term ethanol administration could be caused by a variety of mechanisms including a decrease in the intestinal uptake of cholesterol or modification of the hepatic regulatory pathways. Little information is available concerning cholesterol absorption during periods of alcohol ingestion. However, because it is predominantly a passive process, it is not likely to be greatly changed.[25] In one of the few reported studies, a slight enhancement of cholesterol uptake was noted to occur following the acute administration of ethanol to rabbits.[26]

Conflicting data have been published concerning the effect of ethanol on hepatic rate-limiting enzymes. The activity of HMG-CoA reductase, the enzyme responsible for hepatic cholesterogenesis, has been found to be both increased or decreased in rats fed ethanol for 3 weeks or more.[27,28] This study provides no specific information on this point. Interestingly, there is general agreement among these same authors that ethanol induces a reduction in the activity of the other major rate-limiting enzyme, cholesterol 7-alpha-hydroxylase, which is responsible for the degradation of cholesterol to bile acids. It is not known how this change affects the biliary dynamics of the prairie dog model, although it is cited as a contributory cause for cholesterol accumulation in the liver during ethanol feeding.[29]

Bile salts are essential to cholesterol solubilization because of their ability to form mixed micelles. In this study, the total bile salt concentrations in the gallbladder were not statistically different between groups. Other investigators have noted that chronic ethanol feeding results in increased bile acid output in animals.[30,31] However, Nestel et al.[32] found no change in normal humans who consumed ethanol as 37% of calories. Moreover, once cirrhosis develops, the size of the total bile acid pool is decreased mostly as a result of selective reductions in deoxycholic acid and cholic acid.[24]

Importantly, the size of the bile acid pool is also not the only critical factor in the solubilization of cholesterol, but the specific distribution of bile acids must also be considered. Thus, Carulli et al.[33] have observed that the hydrophilic–hydrophobic balance of bile salts helps to determine the capacity of bile to maintain cholesterol in solution. The trihydroxy bile acids, because they are more hydrophilic than the dihydroxy bile acids, are less effective solubilizers of cholesterol. In this regard, the ratio of trihydroxy to dihydroxy bile acids was significantly decreased by ethanol intake. The level of chenodeoxycholate, one of the major dihydroxy bile acids was significantly elevated in animals receiving ethanol. This selective increment is important because of the specific desaturating potential of this agent.[34] The reason for the enhancement of chenodeoxycholate in association with alcohol intake is not known. It is possible that either increased synthesis occurs through an alternate enzymatic pathway[35] or that bacterial dehydroxylation in the intestine is suppressed.[36] Thus, this accumulated data and the work of others suggest that qualitative changes in the bile acid composition of ethanol-treated animals may contribute substantially to the prevention of cholesterol gallstones.

Physicochemical factors other than supersaturation are also important in the modulation of cholesterol solubility, and these may be affected by ethanol. Recently, increasing attention has been focused on crystal nucleation and the role played by the bile salt-independent

vesicular mode of cholesterol solubilization. In model systems of supersaturated bile the key determinants of the nucleation time have been found to include the total lipid concentration, the cholesterol:phospholipid ratio, and the Ca^{+2} concentration.[37] In this study, the concentration of total lipids was unchanged, but the cholesterol:phospholipid ratio was significantly lower in the ethanol group, a finding consistent with a prolonged nucleation time. This decrease indicates that fewer cholesterol molecules are incorporated into each phospholipid vesicle resulting in a more stable carrier state.[38]

The finding of an elevated biliary calcium in the control animals and pigment deposits in 50% of the same group was not unexpected; others have noted similar findings in cholesterol-fed prairie dogs and guinea pigs.[16,39] Based on a recent study by Conter et al.[40] it seems likely that the calcium bilirubinate concentrations in the control animals are related to the use of a complex sugar as the equicaloric substitute for ethanol. Conter et al.[40] noted increased concentrations of phospholipid, calcium, and bilirubin in the gallbladder bile of animals on a high carbohydrate diet. In this study, the concentration of calcium was elevated in the control animals when compared to ethanol-fed animals. In addition, values from previous experiments using prairie dogs maintained on nonmodified diets were the same as in the alcohol groups (unpublished observations). The calcium elevation seen in the controls may have been sufficient, however, to result in a calcium–bilirubin solubility product that exceeded the point of saturation. It is also possible that the elevated bile calcium in control animals actually promoted the more rapid nucleation of cholesterol crystals. Calcium is reported to significantly increase the metastability of unilamellar phospholipid–cholesterol vesicles[37] and acts as a pronucleating agent by accelerating cholesterol aggregation and precipitation in model bile solutions.[41]

Overall, these studies document that a major mechanism by which alcohol can protect against cholesterol gallstone formation is to induce changes in the concentration and solubilization of cholesterol itself. Still unresolved is the issue of whether alcohol administration causes significant additional perturbations of other important factors such as nucleation time, gallbladder motility, mucin production, or bile protein secretion.

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Discussion

DR. HENRY PITT (Baltimore, Maryland): First, let me state that I concur with the author's conclusions and believe that this paper is an important contribution that adds to other data which Dr. Schwesinger has reported on the influence of alcohol on bile composition. I have one concern, however, about the control group that was used in this experiment. You compared a high cholesterol/high alcohol diet to a high cholesterol/high carbohydrate diet. This control group may have been appropriate, but were the groups comparable in terms of the relative proportions of carbohydrates, fats, and proteins?

To fully interpret your data, I would have liked to have seen other control groups. For example, a low cholesterol group with and without alcohol, a high cholesterol group without alcohol, and a low cholesterol/high carbohydrate group would have added considerably to your study. If data from these additional groups support your observations, the conclusions you have drawn would be even more secure.

In reviewing the manuscript, I was also looking for other data. For example, do you have data from hepatic bile on biliary lipids, calcium, or bilirubin? With those data you could speculate as to whether the changes that you have observed are occurring in the gallbladder or in the liver. Also, do you have data on serum bilirubin and calcium? With this information, you might be able to state whether there were differences in intestinal absorption. You did provide data on serum cholesterol, which was lower in the animals receiving ethanol. In the discussion of your paper, however, you suggested that ethanol was not affecting cholesterol absorption. How, then, do you explain the lower serum cholesterols in the ethanol-fed animals?

I was also looking for the gallbladder volumes of the two study groups. You reported hepatic bile volumes and stated that their differences were not statistically different. Frequently, when the gallbladders of these animals become filled with stone and crystals, the gallbladder volume may change. Were the gallbladder volumes different in your two study groups? Finally, your own very elegant work has demonstrated that ethanol can cause hemolysis. With hemolysis, biliary levels of bilirubin should be increased. How, therefore, do you reconcile your previous observations with the present study in which biliary bilirubin levels were similar in the two study groups?

DR. FRANK G. MOODY (Houston, Texas): Dr. Schwesinger and his colleagues are to be commended for looking at a very important issue: the prevention of gallstones. Very soon we are going to have a large population of patients in which, for surgeons, this is going to be an important issue.

There are many alternatives to cholecystectomy currently being touted, and soon you will read about the German trial where over 160 patients were treated by extracorporeal shock wave lithotripsy with bile salt adjuvant therapy. You will be surprised to learn that 100% of those treated in this way during the course of a year were rendered free of their presumed cholesterol gallstones.

In addition, percutaneous techniques are being developed which employ mechanical lithotripsy or dissolution by methyl-tert-butyl-ether. Furthermore, stones can be fragmented within the common duct by extracorporeal shock wave or direct mechanical lithotripsy. And the newest technique, tunable dye laser lithotripsy, is proving to be effective and safe. These advances will no doubt rapidly transform our approach to the treatment of gallstones.

DR. JAMES PATRICK O'LEARY (Dallas, Texas): I would like to compliment Dr. Schwesinger on his paper and to thank him for allowing me to read it ahead of time. I commend it to your perusal when it is in its final published form.

I would like to ask several questions. The serum cholesterol levels were low. Lower in the ethanol group than in the control group. Bile cholesterol was also low.

That raises the question: Are we dealing with a problem of absorption? Where is the cholesterol going? This gets back to the way the diet was constructed. If you were putting the alcohol with the cholesterol in the diet, then perhaps we have some local effect in the gut lumen that might be impacting on cholesterol absorption. Therefore, the animals may not be challenged with quite the same amount of cholesterol.

Do these animals conjugate their bile salt with taurine. In humans, about 80% of bile salts are with conjugated glycine. If the percentage of conjugation is different, would this make a difference in the study?

My third question has to do with the histology of both the gallbladder wall and the liver. I suspect that you looked at both of these and I was wondering if cholecystitis or changes in hepatic architecture were present.

DR. WILLIAM CHEADLE (Louisville, Kentucky): I, too, would like to compliment the authors on a very elegant and clinically relevant study in an appropriate model with proper analytic methods. It is certainly interesting that pigment stones account primarily for the probable increased incidence of gallstones in cirrhotic patients; however, ingestion of moderate amounts of alcohol seem to be protective against cholesterol stone formation.

Dihydroxy bile acids such as taurochenodeoxycholic acid have been shown to have a detergent effect on hepatocytes which can cause feathery hepatocyte degeneration. The increase in the dihydroxy to trihydroxy bile acid ratio seen in these animals, therefore, might eventually cause low-grade chronic hepatotoxicity. Have you examined liver tissue histologically in this model or others, and have you analyzed common duct bile acid composition which might be different from gallbladder bile and more indicative of that which is in contact with the hepatocytes?

DR. GORDON L. HYDE (Lexington, Kentucky): I, too, would like to welcome the authors to the Southern Surgical Association and commend their work.

I do question how this applies to human physiology and have some concerns that this paper will be misinterpreted by many to mean that drinking alcohol is good for you and will prevent gallstones.

You touched on the fact that the study was performed in dogs; are we certain that species differences might not account for the findings?

Secondly, according to my calculations, ethanol provided 35% of the