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ETHANOL AND BILE CALCIUM EXCRETION IN THE RAT

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Abstract

Acute ethanol (5 g/kg BW, ip.) induced hypocalcaemia and reduction in bile flow, bile calcium and bile salt secretion in the rat. Changes in bile calcium concentration paralleled changes in plasma calcium concentration. Plotting the relationship of bile salt secretion and bile calcium secretion showed that, the amount of calcium secreted per unit change of bile salt secretion (0.03 μmol Ca/ μmol bile salt) and the bile acid independent fraction of calcium (0.5 μmol/kg/min) remained unchanged after ethanol administration. Thus the reduction in bile calcium secretion was due to a reduction in bile acid dependent fraction of calcium. Concerning the components of bile flow, ethanol caused decreased bile flow by suppressing the bile acid independent bile flow possibly by decreasing liver plasma membrane Na-K-ATPase activity. The bile acid dependent bile flow was not reduced despite diminished bile salt secretion because of an increased choleretic property of bile salts.

Introduction

Ethanol has been shown to cause hypocalcaemia which could not be explained by increased loss of urinary calcium[1] or suppression of PTH secretion.[1-3] Because the investigators working on the effect of calcitonin on calcium accumulation in liver[4] and biliary calcium secretion[5-6] have suggested a possibility of biliary calcium secretion constituting a route for short term plasma calcium regulation, it was interesting to study how the ethanol-induced change in bile flow and bile calcium secretion may be related to the ethanol-induced hypocalcaemia. Available reports on ethanol effects on hepatic functions included the increase in bile acid secretion following chronic ethanol ingestion[7,8] whereas acute ethanol administration was shown *in vivo* in rat[8] and dog[9] to diminish bile acid secretion and bile flow. Neither the mechanism of suppression of bile flow

nor components of bile flow (bile acid dependent and independent bile flow) altered by acute ethanol treatment has been elucidated. At present, it is not known whether or not the biliary calcium secretion is altered by ethanol, although recent evidence[10] showed that at the usual rate of bile acid secretion, biliary calcium is bile acid dependent. Thus, the present study was designed to (i) find out if there was a change in bile calcium secretion after acute ethanol administration, (ii) evaluate the relationship between plasma calcium and bile calcium concentration for information concerning mechanism of bile calcium secretion and (iii) study how acute ethanol administration may affect the two components of bile calcium, namely, the bile acid dependent fraction and the bile acid independent fraction of biliary calcium secretion.

Materials and Methods

Animals:

Male Wistar rats weighing between 180-200 g, supplied by the Animal Centre of Salaya Campus, Mahidol University, were maintained on standard laboratory diet (Gold Coin Ltd., Singapore) and kept in cleaned hanging steel cage under 12 h dark-light cycle.

Animal Preparation :

Rats, fasted overnight with access to water, were anaesthetized with 40 mg/kg BW sodium pentobarbital (Vetanacol, Veterinaria, Zurich) intraperitoneally. Preparation included tracheotomy, cannulation of femoral vein and artery, midline lapalotomy and cannulation of common bile duct at the liver hillus with PE 10. Bile secretion was collected every 30 min period by allowing to flow freely into a preweighed cup. In experiments where infusion of calcium was performed, the other femoral vein was also cannulated with PE 50 connected to a Harvard infusion pump (Harvard Apparatus Co. Dover, Massachuset).

A constant body temperature (37℃) was monitored and maintained by a Tele-thermometer Controller (Yellow Spring Instrument Co., Ohio) connected to a rectal probe.

Experimental Protocols

The effect of ethanol on plasma calcium and bile calcium secretion : Animals were divided randomly into 4 groups which received an ip. injection of either normal saline or 1, 3 and 5 g/kg BW ethanol as 20% (V/V) solution immediately after collection of the control blood (0.5 ml) and bile samples.

The effect of 5 g/kg BW ethanol on bile flow and bile calcium secretion at varying levels of plasma calcium : In this series of experiments the effect of 5 g/kg BW ethanol on bile flow and bile calcium secretion was investigated when plasma calcium was kept at normal

or hypocalcaemic level. Normocalcaemia after ethanol injection was maintained by an intravenous priming dose of 1 mg Ca as calcium gluconate per 200 g rat with concurrent continuous infusion of 1.08 mg Ca as calcium gluconate in 0.54 ml per 30 min for the first 30 min, and 0.52 mg Ca in 0.26 ml per 30 min for the rest of the experiment. Hypocalcaemia without ethanol was produced by overnight thyroparathyroidectomy (TPTX) which was performed by blunt dissection under ether anaesthesia. Only those rats which exhibited plasma calcium concentration of less than 2 mM at the time of experiment were used. Blood and bile samples were then collected at 30 min intervals for 120 min.

The effect of 5 g/kg BW ethanol on Na-K-ATPase activity in crude liver plasma membrane : After an overnight fast, animals were divided randomly into 2 groups which received an ip. administration of normal saline or 5 g/kg BW ethanol. Two hours later, the animals were decapitated and the liver was removed. Liver plasma membrane was isolated as described by Song *et al.*[11] for determination of the enzymic activities.

Analytical Procedures

Bile volume was measured gravimetrically assuming a density of 1.0 g/ml. Bile salt concentration was determined enzymatically with 3α -hydroxysteroid dehydrogenase (Sigma Chem. Co., St. Louise) as previously described[12]. Plasma and bile samples were analysed for total calcium using atomic absorption spectrophotometry (Varian AA 575). ATPase activities were determined by inorganic phosphate release by the Fiske-SubbaRow Method[13]. Protein content of plasma membranes was determined by the method of Lowry *et al.*[14].

Statistical Analyses

Results were presented as mean ±SEM. Student's t-test was used to determine the significance of difference between the means with significance level set at 0.05. Correlation between plasma and bile calcium concentrations, bile flow versus bile acid secretion and bile calcium versus bile acid secretion were calculated using linear regression analysis by the method of least squares. The Y-intercept of the regression line calculated for the relationship of bile flow to bile acid secretion was used to represent the bile acid independent bile flow while the slope of the regression line represented the amount of bile flow per μmol bile salt (choleretic property of bile salt)[15,16]. Bile acid dependent bile flow (BADBF) at each time interval was calculated from the bile salt secretion (μmol/kg/min) times the choleretic property of bile salt (represented by slope of the regression line, μl/ μmol). Bile acid independent fraction of calcium output was obtained from the Y-intercept of the regression line for the relationship of bile calcium secretion and bile salt secretion.

Results

The acute effects of ethanol on plasma and bile calcium concentration, bile flow and bile calcium secretion.

The effects of 5 g/kg BW ethanol in normal rats are shown in Fig. 1. In control experiment, the plasma calcium concentration and bile flow remained constant throughout the 120 min experimental period. The total bile calcium was reduced at 120 min due to a gradual decline in bile calcium concentration at 90 and 120 min. On the other hand, administration of 5 g/kg BW ethanol always caused a significant reduction ($P < 0.01$) in the plasma and bile calcium concentrations when compared to the corresponding control. The additional suppressive action of ethanol on bile flow causing decrease in the total bile calcium secretion was seen at 90 and 120 min. The doses of 1 and 3 g/kg BW ethanol also exhibited similar effects on these parameters in a dose-dependent manner (results not shown).

Relationship between the plasma and bile calcium concentrations

As shown in the representative data (Fig 2), the bile calcium concentration seemed to change in parallel with plasma calcium concentration. When ethanol-induced hypocalcaemia was elevated to a slightly hypercalcaemic level by calcium infusion (ethanol +Ca), the bile calcium concentration at 30 min also increased to exceed normal values. When hypocalcaemia of similar degree to that observed in ethanol-treated group was induced by prior thyroparathyroidectomy, the bile calcium concentration at 30 min was the same as in ethanol-treated group. It was also noted that the bile calcium concentration in every group even in the control group, appeared to gradually decrease with time during the experimental period. However, reduction in bile flow was observed only in ethanol-treated group, resulting in a more rapid reduction of total bile calcium secretion.

The dependency of the bile calcium concentration on the plasma calcium was clearly demonstrated by a linear relationship in Fig. 3. Fig. 3A represents pooled data taken from groups without ethanol administration i.e. normal saline control, normal saline plus calcium infusion, and TPTX groups. Fig. 3B represents pooled data from various ethanol-treated groups. Administration of ethanol did not influence the relation between plasma and bile calcium concentration since both Y-intercepts and slopes were nearly identical (without ethanol $Y = -0.96 + 1.09\,x$, $r = 0.86$, $P < 0.01$; with ethanol, $Y = -0.95 + 1.11\,x$, $r = 0.87$, $P < 0.01$). For every 1 mM change in plasma calcium concentration, there was also a corresponding change of approximately 1 mM in bile calcium concentration.

Relationship between bile calcium secretion and bile salt secretion

In Fig. 4, it can be seen that the relationship between bile calcium secretion and

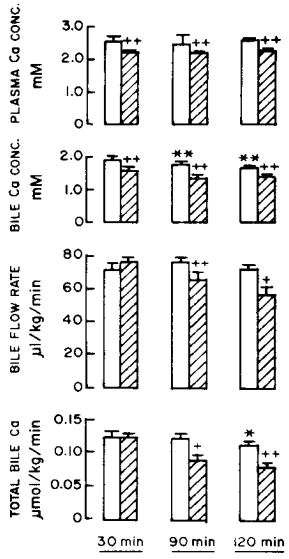

Figure 1. Changes in plasma and bile calcium concentrations, bile flow and total bile calcium secretion at 30, 90 and 120 min after an i.p. adminstration of normal saline control (□, n = 7) or 5 g/kg BW ethanol (▨, n = 11). *P < 0.05, **P<0.01, paired t-test the 30 min control value. $^{+}$P 0.05, $^{++}$P 0.01 unpaired t-test between the alcohol group with corresponding saline control value.

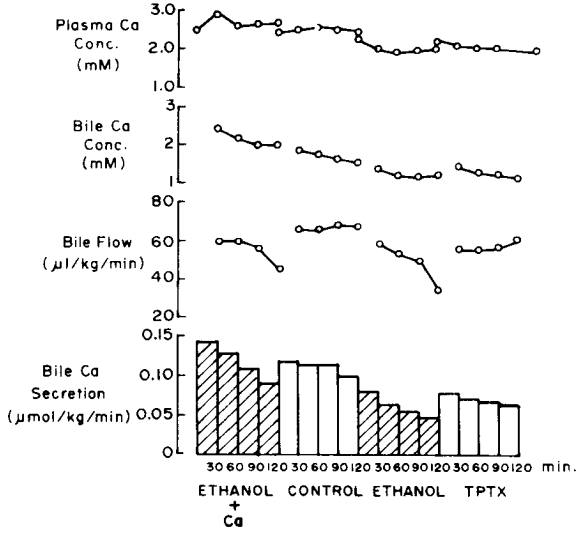

Figure 2. Representative data showing the changes in plasma and bile calcium concentration, bile flow and total bile calcium secretion at various time intervals in 5 g/kg BW ethanol-treated group infused with calcium gluconate, normal saline control, 5 g/kg BW ethanol-treated group and TPTX group.

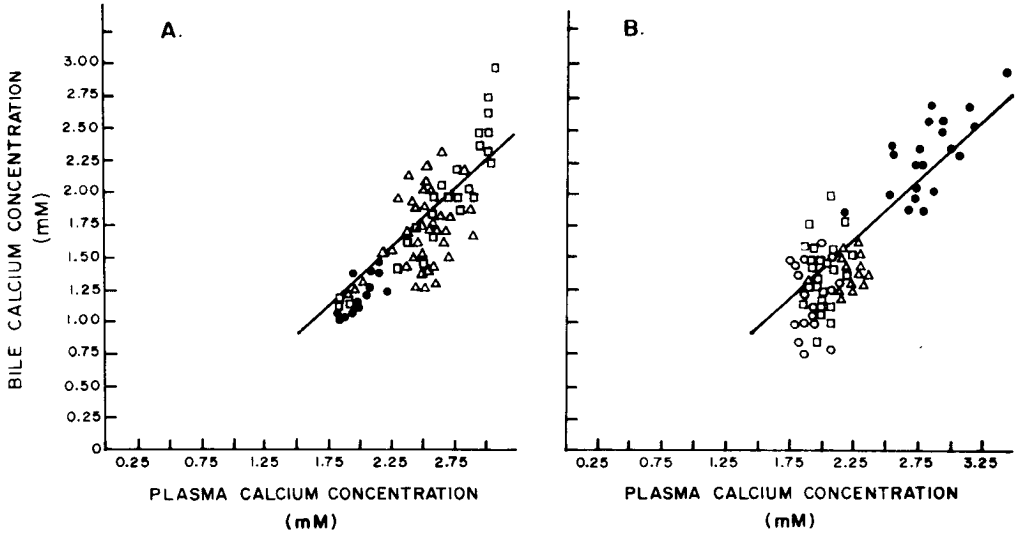

Figure 3. Relationship between plasma and bile calcium concentration. Data represent results obtained from 30 min collection period of individual rat at various time intervals. A = Data from saline control (Δ , n = 38), saline + Ca infusion (□, n = 25), and TPTX (●, n = 13). B = Data from 1 g/kg BW ethanol-treated group (Δ , n = 14), 3 g/kg BW ethanol-treated group (□, n = 24), 5 g/kg BW ethanol-treated group (o, n = 17), and 5 g/kg BW ethanol-treated + Ca infusion group (●, n = 21).

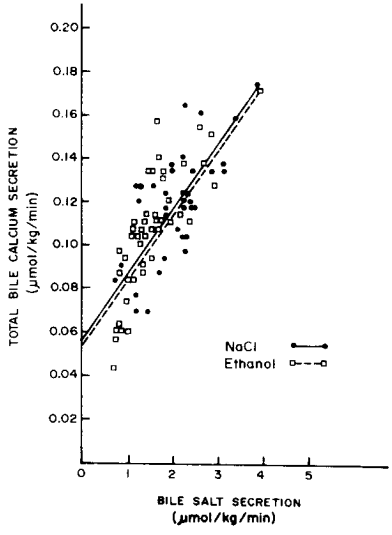

Figure 4. Relation between bile salt and bile calcium secretions from samples collected during each 30 min for 120 min in saline control and 5 g/kg BW ethanol-treated rats. The linear least square equation (Y = MX + C) was applied. The r values for both regression lines were 0.80 and 0.82 respectively. P-value < 0.0005 in both cases.

bile salt secretion was the same in saline control and ethanol-treated group. In both groups, the amount of bile calcium secreted at zero value of bile salt secretion (bile acid-independent fraction of bile calcium or Y-intercepts) were nearly identical being 0.057 and 0.053 μmol/kg/min in control and ethanol-treated group, respectively. The relationship between secretion of bile calcium and bile salt (slope), 0.03 μmol/ μmol bile salt, was unaffected by ethanol administration.

The effect of 5 g/kg BW ethanol on bile flow and bile composition

Table 1 shows the changes in total bile salt secretion (μmol/kg/min) at various times in control and 5 g/kg BW ethanol-treated group. Total bile salt secretion in control tended to decrease with time from 2.4 ± 0.1 μmol/kg/min to 1.8 ± 0.2 μmol/kg/min at 90 min and to 1.6 ± 0.2 μmol/kg/min at 120 min possibly due to interruption of the enterohepatic circulation of bile salt. Acute ethanol administration led to a more abrupt decrease in total bile salt secretion which was markedly decreased with the values of 1.9 ± 0.2 μmol/kg/min at 30 min to 1.2 ± 0.1 μmol/kg/min at 90 min and 1.0 ± 0.1 μmol/kg/min at 120 min, all of which were also significantly lower ($P < 0.05$) than the corresponding control values, respectively.

Since there has been no report on the effect of acute ethanol administration on bile acid dependent bile flow (BADBF) and bile acid independent bile flow (BAIBF), bile flow and bile salt secretion obtained at 30, 90 and 120 min collection periods from the same treatment group were plotted separately. The values of Y-intercept and slope at 30, 90 and 120 min are also presented in Table 1. When considering the saline control group alone, it could be seen that the values of Y-intercept, which were used to define the BAIBF, gradually increased from 41.50 μl/kg/min (58% of total bile flow) at the first 30 min to 45.57 μl/kg/min (60.8%) and 48.24 μl/kg/min (67.2%) at 90 and 120 min respectively while the slope which represents the amount of bile secreted with 1 μmol bile salt (choleretic property of bile salt) remained relatively constant i.e. being 11.0, 12.15 and 11.49 μl/ μmol at 30, 90 and 120 min respectively. On the other hand, 5 g/kg BW ethanol seemed to drastically change the relation between bile salt secretion. BAIBF dropped from 53.74 μl/kg/min during the first 30 min collection period to 42.95 μl/kg/min at 90 min and 29.82 μl/kg/min at 120 min. However, the slope or the choleretic property of bile salt increased from 12.85 μl/ μmol at 30 min to 20.11 μl/ μmol at 90 min and 28.85 μl/ μmol at 120 min.

Effect of ethanol on liver plasma Na-K-ATPase activity

Two hours after the administration of 5 g/kg BW ethanol, the activity of Na-K-ATPase in the crude preparation of liver plasma membrane was reduced from 5.25 ± 0.87 to 2.46 ± 0.39 μmol Pi/mg protein/hr ($P < 0.01$) while the activity of Mg-ATPase which is probably not involved in bile secretion and is regarded as a control remained unaffected at 47.77 ± 3.77 μmol/Pi/mg protein/hr.

Table 1. Changes in bile salt secretion (BS) and slope and y-intercept of the relationship between bile salt secretion and bile flow at 30, 90 and 120 min after an i.p. administration of normal saline or 5 g/kg BW ethanol in intact rats.

	30 min			90 min			120 min		
	Bite Salt Secretion (B.S.) μmol/kg/min	BS : Bile Flow		Bile Salt Secretion (B.S.) μmol/kg/min	BS : Bile Flow		Bile Salt Secretion (B.S.) μmol/kg/min	BS : Bile Flow	
		Slope μl/μmole	Y-intercept μl/kg/min		Slope μl/μmole	Y-intercept μl/kg/min		Slope μl/μmole	Y-intercept μl/kg/min
Control (n = 11)	2.4 ± 0.1	11.0 r=0.73,	41.50 P < 0.0005	1.8 ± 0.2*	12.15 r = 0.76,	45.57 P < 0.05	1.6 ± 0.2**	11.49 r = 0.73,	48.24 P < 0.01
Ethanol (n = 13)	1.9 ± 0.2$_{+}$	12.85 r=0.83,	53.74 P<0.0005	1.2 ± 0.1$^{*}_{+}$	20.11 r = 0.74,	42.95 P<0.005	1.0 ± 0.1$^{**}_{++}$	28.85 r = 0.70,	29.82 P<0.025

* P<0.05, ** P<0.01, paired t-test with the 30 min control value.

$^{+}$ P<0.05, $^{++}$ P< 0.01, unpaired t-test with control

Discussion

Biliary calcium secretion has been shown to constitute a mechanism by which calcitonin, a hypocalcaemic hormone, regulates the plasma calcium concentration.[5,6] Although the mechanism of ethanol-induced hypocalcaemia is still not completely understood, it has been shown that it was not due to urinary calcium loss[1], or suppression of PTH secretion[1-3] or reduction in bone calcium release[17] The present work has demonstrated that it was not caused by increase in bile calcium secretion. In fact, acute administration of ethanol resulted in a significant reduction in both plasma calcium concentration and biliary calcium output, of which the latter was a result of reduction in both bile calcium concentration and bile flow rate (Fig. 1,2). Moreover, the present data indicate that plasma calcium concentration has a direct influence over the bile calcium concentration. This positive correlation between plasma and bile calcium concentration was not altered by ethanol (Fig. 3). The data also provided information on how the two components of bile calcium, namely bile acid dependent fraction and bile acid independent fraction of calcium output may be affected by ethanol. In control group, the biliary calcium secretion correlates well with bile salt output ($r = 0.8$, Fig. 4) which is in agreement with a recent report[10]. By analysis of the relation between total bile calcium secretion and bile salt secretion, the amount of calcium secreted at zero bile salt output (the bile acid independent fraction of bile calcium) was 0.057 μmol/kg/min (Y-intercept) which accounted for approximately 47% of total bile calcium output. In this respect, our results are somewhat different from those of Cumming and Hofmann[10] who reported that at the usual rate of bile salt secretion, most biliary calcium is bile acid dependent. Perhaps the difference in animals used in our procedure with the interruption of enterohepatic circulation of bile salt might alter the ratio of these two fractions.

As noted in Fig. 3, bile calcium concentration and total bile calcium secretion decreased with time in all preparations. In the control and TPTX groups without ethanol, the interruption of enterohepatic circulation might lead to a lower bile salt secretion with time, and thus, the reduction of bile acid dependent fraction of calcium output as manifested in the reduction of bile calcium concentration with time. In the acute ethanol treated group, ethanol neither altered the bile acid independent calcium output, nor changed the relation between bile salts and bile calcium secretion (slope, Fig. 4) i.e. the bile calcium output still remained 0.03 μmol/μmol of bile salt after ethanol administration. Since bile salt reduction by ethanol treatment was more pronounced than the reduction by interruption of enterohepatic circulation alone (control), the bile acid dependent fraction of bile calcium was reduced. The latter is thus attributed to a faster decrease in total bile calcium excretion.

The mechanism by which ethanol affected bile flow seemed complicated. Previous reports demonstrated that acute ethanol significantly suppressed bile flow.[8,18,19]

However, there has been no information on how the two components of bile flow (bile acid dependent and independent bile flow) may be changed by acute ethanol administration. Maddrey and Boyer[8] showed in the isolated perfused rat liver that ehtanol caused a reduction in bile flow. Since in the latter preparation bile salt secretion was negligibly low, one might predict that the reduction in bile flow must have been due to a reduction in bile salt independent bile flow. However, Marin and coworkers[9] showed that in the dog, where enterohepatic circulation was interrupted, ethanol suppressed bile flow could be restored by bile salt administration indicating that bile salt could counteract the effect of ethanol on bile volume.

By using the analysis of relationship between bile flow and bile salt secretion that the slope of the regression line represents the change in bile flow induced by one micromole of bile salt or choleretic property of bile salt while the Y-intercept defines the bile acid independent component of bile flow (BAIBF), our present time-course study shows that the volume of bile secreted per micromole of bile acid in control group was constant and in the range of 11-12 µl (slope of control, Table 1). The BAIBF accounted for about 60 % of total bile flow which was in accordance with previous reports.[15,20-23] The total bile salt secretion gradually decreased with time due to the interruption of the enterohepatic circulation of bile salts. Such reduction in bile salt secretion with no change in its choleretic property indicated a reduction in bile acid dependent bile flow (BADBF or bile salt secretion X choleretic property of bile salt). Since the total bile flow remained unchanged throughout the experimental period (Fig. 1) despite a gradual reduction in BADBF (Table 1), bile flow must have been maintained by a proportional increase in BAIBF. In actual fact, the values of BAIBF as reflected by the Y-intercept of the relationship between bile flow and bile salt output were found to increase during the experimental period. However, the mechanism responsible for this compensatory increase in BAIBF is not known.

In contrast to the control group, acute ethanol administration resulted in a significant reduction in bile salt secretion when compared to control (Table 1). However, this reduction in bile salt secretion did not necessarily indicate a reduction in the BADBF since when the increased choleretic property of bile salt (slope) in ethanol-treated group was also taken into consideration, the BADBF was not decreased. The reduction in bile flow after acute ethanol administration was infact due to marked suppression of BAIBF (Y-intercepts, Table 1). Little is known about the direct acute effect of ethanol on bile acid synthesis and secretion but chronic ethanol administration was reported to cause increased bile acid secretion and enhanced BAIBF if the ethanol level was low at the time of the study.[7,8] Although there has been no direct evidence, it has been postulated that chronic ethanol administration may induce BAIBF by stimulating the Na-K-ATPase activity.[8] Correlations obtained between BAIBF and Na-K-ATPase activity under various experimental conditions[24] indicate that Na-K-ATPase which is found in the

basolateral membrane[25] may be involved in bile secretion by maintaining a sodium gradient for hepatic uptake of bile acid and other secretagogues[26] or by inducing water flow through intercellular space and tight junctions into the canaliculus.[27] As for the acute effect of ethanol on bile flow the present data demonstrate for the first time that the reduction in BAIBF by acute ethanol administration was partly due to suppression of the Na-K-ATPase activity in the liver plasma membrane. Since the mechanisms responsible for BAIBF are still subject to speculation, the mechanisms of ethanol-induced suppression of BAIBF may also involve other processes, for instance the paracellular transport of electrolytes and water which has been suggested as an important component of BAIBF.[26]

In conclusion, the present results demonstrate that acute ethanol administration caused the reduction of total bile calcium secretion which was a result of a more significant decrease in bile calcium concentration through hypocalcaemia and suppression of total bile flow by the reduction of BAIBF, the latter may be partly explained by the suppression of the liver plasma membrane Na-K-ATPase activity. Though the total bile salt secretion was decreased, the choleretic property of bile salt in ethanol-treated group was actually increased, so that the BADBF was not reduced by ethanol. Therefore, the diminished bile flow was mainly due to reduction in BAIBF. On the other hand, relationship between bile salt secretion and bile calcium secretion showed that both amount of calcium secreted per μmol change in bile salt secretion and the bile acid independent fraction of calcium were not altered by ethanol while total bile salt secretion was reduced. Therefore, the bile acid dependent fraction of calcium must have been reduced and this could also contribute to the reduction in total bile calcium secretion.

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บทคัดย่อ

จากการทดลองในหนูขาว พบว่าแอลกอฮอล์ (5 กรัม/กก. ฉีดเข้าช่องท้อง) มีผลลดระดับแคลเซี่ยมในพลาสมา ลดอัตราการไหลของน้ำดีและอัตราการหลั่งเกลือน้ำดีและแคลเซี่ยม ความเข้มข้นของแคลเซี่ยมในน้ำดีมีการเปลี่ยนแปลงขนานกับระดับแคลเซี่ยมในพลาสมา เมื่อดูความสัมพันธ์ระหว่างการหลั่งเกลือน้ำดีและแคลเซี่ยมพบว่าปริมาณแคลเซี่ยมที่หลั่งต่อ 1 ไมโครโมลเกลือน้ำดีมีค่าเท่ากับ 0.03 ไมโครโมล และแคลเซี่ยมส่วนที่เรียกว่า bile acid independent fraction มีค่าเท่ากับ 0.5 ไมโครโมล/กก./นาที ทั้งในกลุ่มควบคุมและกลุ่มที่ได้รับแอลกอฮอล์ แสดงว่าปริมาณแคลเซี่ยมในน้ำดีที่ลดลงนั้นเป็นผลจากการขับแคลเซี่ยมในส่วนที่เรียกว่า bile acid dependent fraction

อนึ่ง การที่แอลกอฮอล์มีผลลดอัตราการไหลของน้ำดีโดยยับยั้ง bile acid independent bile flow นั้นอาจมีสาเหตุเนื่องมาจากการลดการทำงานของเอ็นไซม์ Na-K-ATPase ส่วน bile acid dependent bile flow ไม่เปลี่ยนแปลงถึงแม้นปริมาณเกลือน้ำดีที่หลั่งจะลดลง ทั้งนี้เพราะเกลือน้ำดี 1 ยูนิตมีความสามารถกระตุ้นการหลั่งน้ำดี (choleretic property of bile salt) ได้ดีขึ้นในหนูที่ได้รับแอลกอฮอล์