# EFFECT OF TAURODEHYDROCHOLIC ACID ON THE HEPATIC EXPRESSION OF ABCG5 AND ABCG8 AND BILIARY PARAMETERS IN THE FISTULA RAT.

**Running head:** Taurodehydrocholic acid and hepatic Abcg5g/8 expression

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## ABSTRACT

**Introduction:** Biliary secretion of phospholipids and cholesterol is mainly dependent on bile-salt secretion although the exact mechanisms involved are not clearly defined.

**Aims:** This study was to investigate the effect of oral administration of turodehydrocholic acid (TDHC) on cholesterol metabolism.

**Materials and Methods**: TDHC was administered orally at a concentration of 10mM twice a day to rats for 7 days in order to mimic the in vivo situation in gallstone patients on bile salt therapy. Bile duct was cannulated and bile was collected for 30 mins on ice and subjected to analysis for total cholesterol, total bilirubin, alkaline phosphatase (ALP) and Gamma glutamyl transferase (GGT). Liver samples were subjected to real time PCR and the gene expression of the adenosing triphosphate-binding cassette transporter heterodimer Abcg5/g8 was determined.

**Results:** TDHC significantly increased the expression of Abcg5 whilst the level of Abcg8 was significantly decreased. No changes in biliary cholesterol, ALP and GGT were observed, however the total bilirubin was significantly increased in the bile of TDHC administered rats.

**Conclusions:** Increased expressıon of Abcg5 may be one mechanism by which TDHC stimulates the secretion of biliary cholesterol.



K**eywords:** Taurodehyroxycholic acid; Abcg5 transporter; Abcg8 transporter; bile; biliary cholesterol


## Introduction

Biliary lipids mainly consist of cholesterol and phospholipids that are synthesised in the hepatocytes and are thought to be transferred into bile by vesicular and non-vesicular mechanisms. Bile salts have a predominate effect on both biliary cholesterol and phospholipid secretion such that when bile salt secretion increases there is a parallel increase in secretion of biliary cholesterol and phospholipids [(1)]. By contrast, when the hepatic influx of bile salts is low, for example following interruption of their enterohepatic circulation, there is an associated decrease in the secretion of biliary lipids in man and in animals [(2)]. Hydrophobic bile salts such as chenodeoxycholate, deoxycholate and their conjugates lead to an increase in lipid secretion into the bile when compared to hydrophilic bile

salts such as cholate and ursodeoxycholate and their conjugates[3-6]. The more hydrophilic non-micelle-forming bile salts, such as taurodehyrocholate (TDHC) do not have the ability to provoke biliary lipid output [7,8].

Hepatocytes obtain biliary lipid by three sources namely (i) biosynthesis, (ii) lipoproteins and existing lipid molecules drawn from intracellular membranes and (iii) newly synthesised biliary lipids; these accounting for less than 20% of the total lipids [9]. Trans hepatic cholesterol trafficking into bile is still not fully elucidated. However, it has been reported that some intracellular transport proteins localized to the bile canaliculus, the apical plasma membrane of hepatocytes, have important functions in the biliary secretion process [10].

Adenosine triphosphate (ATP)-binding cassette transporter B4 (ABCB4) is the key protein for the biliary output of phospholipids and the formation of mixed micelles, which are two important events for the efficient biliary excretion of sterols (10). ABCG5 and ABCG8 are half-size ATP-binding cassette (ABC) transporter proteins that function together as a heterodimer (ABCG5/G8) and have an important physiological function in biliary cholesterol secretion and intestinal absorption of sterols [11-14]. There is evidence that biliary cholesterol secretion is mostly mediated by ABCG5/G8[15,16] after this cholesterol is transported to the canalicular membrane of the hepatocyte; however, contradictory results have also been reported. The other cholesterol which contributes between 10% to 30% of total biliary cholesterol secretion is thought to be transferred into bile by vesicular mechanism without these transporter proteins [17-20].

An increase in biliary lipit secretion can have implications for hepatic disorders such as cholesterol gallstone formation and atherosclerotic cardiovascular disease (CVD). Hence the mechanisms is involved in the biliary secretion of lipids and the role of ABCG5/G8 transporter may lead to a better understanding of such hepatic disorders[10]. Most of the studies investigating the role of bile acids in inducing biliary lipid output have utilised either isolated perfused rat livers or one pass infusion of bile acids in fistula rats. In the present study we report the effects of oral dosing of TDHC to rats for seven days, this was performed in order to mimic the situation in gallstone patients on bile

acid therapy. TDHC is a non-micelle-forming bile salt analogue which does not by itself produce appreciable lipid output [21] but may, however, be capable of causing lipid movements within the hepatocyte.

The aim of this study was thus to investigate the effect of oral administration of TDHC on cholesterol metabolism in the fistula rat by measuring the expression of hepatic Abcg5/g8 transporter by real time qualitative polymerase chain reaction and biliary parameters in order to understand better the mechanisms involved in the control of biliary lipid secretion.

## Materials and methods

Upon the approval of the experimental protocol by the Animal Ethics Committee of the Ankara University (Protocol #: 15.12.2004-2004/48), animals, obtained from Ministry of Health, Serum Production Farm, Ankara, Turkey, were cared in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twelve male Wistar-Albino rats age five months and weighing 250-300 g were housed in separate cages at 25°C and subjected to a 12:12-h light:dark cycle. The rats were randomly divided into two groups: Control group (Group 1), subjected to 1ml distilled water administration by gavage for 7 days; TDHC group (Group 2), subjected to 1 ml 10 mM TDHC administration by gavage twice a day for 7 days. All animals were allowed food ad libitum and consumption a standard laboratory diet and had free access to water throughout the experimental period. The rats in all groups were anesthetized on day 7 with sodium pentobarbitale (6mg/100 g of body weight, intraperitoneal) and bile collection made as stated below. Then animals were hepatectomized and the liver was separated for the determination of the Abcg5/g8 transporter proteins.

### Cannulating the bile duct

Following general anaesthesia, an incision was made at the upper abdomen and the bile duct was located. The bile duct was cannulated using pp 10 tubing and the bile was collected on ice for 30 min and stored at -80 $^{o}$C for analyses of biochemical analysis.

### RNA isolation and PCR detection

Total RNA was isolated from approximately 30 mg of frozen liver tissue by using RNeasy Mini Kit (Qiagen, Maryland, USA) following the homogenization of the tissue. The RNA precipitate was dissolved in 75 μL of RNAse free water.

**First-strand cDNA synthesis**

Total RNA was used for the synthesis of single-strand complementary DNA (cDNA) by using antisenses and 100 units avian myeloblastosis virus reverse transcriptase (Q-Biogene, Irvine, Canada) for 60 minutes at 42ºC in a final volume of 20 μL. This cDNA pool was used as control for the qualitative detection of Real-Time PCR.

**Real-Time PCR**

The expression of Abcg5/g8 was determined by Real-Time PCR, using Lightcycler Instrument (Roche Diagnostics, Mannheim, Germany). A previously described method using a SYBR green I dye was used after making some modification for Real-Time PCR [22]. Briefly, Fast Start DNA Master SYBR Green mix (Roch Diagnostics, Mannheim, Germany) containing hot start Taq DNA polymerase, 5 μL cDNA, 0.5 μM of each gene specific Abcg5: sense 5'-GAG GTT ACT TAA TAG CCT ACG-3', antisense: 5'-GAA CAC CAA CTC TCC GTA AG-3'; Abcg8: sense 5'-GCT CAG TTC AAG TTA CCG TG-3', antisense: 5'-GTC AAG TCC ACG TAG AAG TC-3' and 3 mM magnesium chloride in a final volume of 20 μL in glass capillaries were run in duplicate. The reaction mixture was preheated at 95 ºC for 10 mins, followed by 45 cycles at 95 ºC for 15 second and at 60 ºC for 1 minute. Glyceraldehyde-3-phophate dehydrogenase (Gapdh) a housekeeping gene was also amplified in each sample by using Gapdh: sense 5'- ACC ACA GTC CAT GCC ATC AC-3', antisense: 5'- TCC ACC ACC CTG TTG GTG TA-3' [23]. All cycle threshold (Ct) values of studied gene expressions were adjusted according to the Ct value of Gapdh expression profiles.

**Biochemical analyses of bile**

Bile was collected and stored at -80°C until analyses and was analysed for total cholesterol, total bilirubın, alkaline phosphatase (ALP, EC3.1.3.1) and gamma glutamyl transferase (GGT. EC

2.3.2.2) activities. These parameters were analysed by using commercially available kits in an auto-analyzer (Cobas Integra 800; Roche Diagnostics GmbH; Mannheim, Germany).

### Statistical analysis

Data was subjected to 2-tailed paired t-test and P values < 0.05 were considered as statistıcally significant.

## Results

### Expression of hepatic Abcg5 and Abcg8 mRNA

In TDHC administered rats the level of hepatic Abcg5 mRNA expression was significantly higher than control. In contrast, the level of Abcg8 mRNA was significantly decreased when compared with the control values (Table 1). Standardization of all data were made by Gapdh expression and values are expressed as means ±SEM of 6 animals, compared with the control. Abcg5/g8 and Gapdh were determined by Real-Time PCR as described in materials and methods section.

### Biliary parameters

The biliary parameters analysed are presented in Table 2. The biliary cholesterol in control bile was present at a concentration of 16.14 mg/dl and this increased fractionally to 18.40 mg/dl after oral dosing of TDHC. In contrast, the amount of total bilirubin present in bile was 5.52 mg/dl and this significantly increased to 6.89 mg/dl ($p< 0.05$) on TDHC dosing (Table 2). There was no significant difference observed in the activity of biliary ALP and GGT activity between the values observed in control bile and bile from TDHC administered rats (Table 2).

## Discussion

Bile salts play an important role in the regulation of biliary cholesterol and phospholipid secretion. The characteristics of the bile salt associated lipid secretion are dependent on the physical properties of the bile salts. The more hydrophobic bile salts, such as chenodeoxycholate,

deoxycholate and their conjugates, stimulate biliary lipid secretion at lower bile salt concentrations than do hydrophilic bile salts such as cholate and ursodeoxycholate and their conjugates [21]. The more hydrophilic bile salts such as TDHC, a non-micelle forming bile salt analogue does not appear to be able to provoke biliary lipid out. However, earlier experiments have shown that TDHC increases the speed of delivery of lipids probably by increasing the movement of vesicles to the canalicular membrane[21] where they can be removed by the action of the more hydrophobic bile salts. TDHC is also reported to selectively increase cholesterol output into bile[21]. The mechanisms involved in the secretion biliary lipids especially cholesterol is not clearly defined but is important due to its association wıth gallstone disease. The aim of the present study was to investigate whether oral dosing of TDHC to fistula rats affects the ATP-binding cassette subfamıly G member 5 and 8 (ABCG5/G8) as majority of cholesterol secretion depends on this protein[10]. This approach was adopted to mimic the in vivo situation in gallstone patients on bile salt therapy.

To stimulate biliary cholesterol secretion TDHC was administered to rats at a concentration of 10mM in one ml dose twice a day for 7 days. The expression of the Abcg5/g8 was determined and is presented in Table 1. TDHC administration led to a significant increase in the expression of Abcg5 (approx. 35%). In contrast an intriguing result was observed when the expression of Abcg8 was determined, there was a significant down expression of thıs gene by 30% (Table1). Abcg5/g8 are ABC half transporters that form an obligate heterodimer for full functionality [10]. Mutations in either ABCG5 or ABCG8 have been characterized as the genetic substrate for sitosterolaemia, a disease that is characterized by an accumulation of plant sterols within the body due to increased intestinal absorption and diminished biliary excretion in the absence of functional ABCG5/G8 expression[24]. In addition, patients with sitosterolaemia display accelerated atherosclerotic lesion formation[25]. Evidence also points to the fact that certain mutations in ABCG5/G8 have also reproducibly been associated wıth genetically conferred susceptibly to cholesterol gallstone formation, demonstrating that the functionality of this heterodimeric transporter pair is also of prime relevance for gallstone disease[26-27]. In the studies reported here when bile salt secretion was continouslly stimulated by oral dosing of rats with TDHC a selective expression of Abcg5 was observed. This is in agreement with

data reported by Dikkers et.al who observed that bile salt stimulated increase in biliary cholesterol secretion is fully dependent on Abcg5 expression [10]. TDHC did not increase the expression of Abcg8 but the opposite effect was observed and was unexpected (Table 1). It may well be that during TDHC administration the majority of cholesterol secreted into bile is driven by Abcg5 protein hence the liver switches to its expression whilst down regulating Abcg8. It has to be remembered that in rodents cholesterol secreted into bile has been shown to be derived from high-density lipoproteins where as the remaining part is supplied by de novo cholesterol synthesis[28]. Administration of TDHC may also be increasing alternative pathways of biliary cholesterol secretion such as via lysosomal output [21], also up to 30% biliary cholesterol secretion is independent of ABCG5 and ABCG8 activity [29] and the pathways contributing to this may also be increased at the expense of the down expression of Abcg8. The decrease in the expression of Abcg8 after TDHC administration needs to be investigated further.

Biliary parameters total cholesterol, total bilirubin, ALP and GGT were also measured and are presented in Table 2. The total biliary cholesterol in control rats was 16.14 mg/dl and this increased just under 10% to 18.40 mg/dl. It has been previously shown that TDHC increases the speed of delivery of lipids, probably by increasing the movement of vesicles to the canalicular membrane [1]. Although the output of cholesterol into bile was stimulated by TDHC however, these studies were performed in an isolated rat perfused liver system and the experiments reported here were conducted in a fistula rat to investigate the physiology of the whole body system. O'Maille and Hofmann have shown that in the fistula rat, infusion of TDHC did not increase biliary phospholipids and cholesterol levels above basal values [30]. Our results are in agreement with this observation and it is likely that no increase in biliary lipid output was observed in the experiments reported here as the background levels of biliary lipid concentration would be higher in the whole animal when compared to the isolated perfused rat livers.

One of the main components of bile is bilirubin and the concentration of total bilirubin in control bile was 5.52 mg/dl and this was significantly increased to 6.89 mg/dl. Bilirubin, the end product of heme catabolism, needs to be taken up into hepatocytes and is then glucuronidated within

the cells prior to its excretion via bile. Members of the SLC21A family in the sinusoidal membrane of hepatocytes selectively mediate the uptake of unconjugated bilirubin and of bilirubin conjugates. After conjugation within the hepatocyte by UGT1A1, yielding monoglucuronosyl and bisglucuronosyl bilirubin, the conjugates are transported across the canalicular membrane into bile by the apical conjugate export pump MRP2, a member of the ABCC subfamily of ATP-dependent transporters. MRP2 also mediates the export of a number of other amphiphilic anions and anionic substances including xenobiotics conjugated with glutathione, glucuronate, or sulfate. Mutations in the ABCC2 gene leading to the absence of a functional MRP2 protein from the canalicular membrane, are the molecular basis of Dubin-Johnson syndrome in humans. Its likely that TDHC administration to the rats increases the expression of ABCC subfamily of ATP-dependent transporters such as MRP2 thus resulting in an increases in total bilirubin in TDHC administered rats. Recent results show that the expression of MRP2 is regulated by several nuclear receptor-mediated pathways including the FXR, the PXR, and the CAR receptor and its probable that TDHC or its metabolites may be exerting their affect on one of these pathways.

There was no significant difference between biliary ALP and biliary GGT in control bile and the bile from TDHC administered rats. This indicates that TDHC administration did not alter the hepato-biliary physiology. The location of hepatic ALP on the sinusoid membrane suggests involvement in transport function. Increased ALP synthesis is observed during cholestasis with increased bile acid concentration caused by intrahepatic or extra hepatic obstruction of the biliary tree. Hepatobiliary disease is the predominant source of increased serum GGT activity. Increases are associated with all forms of primary and secondary hepatobiliary disorders. Elevations are moderate (2 to 5 times) with diffuse hepatic cell injury due to toxic or infectious hepatitis[30]. Cholestasis due to intrahepatic or extrahepatic biliary obstruction causes higher serum levels (5 to 30 times)[30]. Increases occur earlier and persist longer than ALP in cholestatic disorders. Since no differences were observed in these two enzymes this indicates that no hepato-biliary damage occurred on TDHC administration.

In conclusion, TDHC administration significantly increased the expression of Abcg5 whilst decreasing the expression of Abcg8. A significant increase in total bilirubin was also observed whilst no changes were seen in the levels of total cholesterol, ALP and GGT between the bile of control rats and TDHC administered rats. TDHC may increase biliary cholesterol output via an increase in the expression of Abcg5 and may also increase bilirubin output via an increase in the Mrp2 protein.

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**Legends**

**Table 1.** Expression of hepatic Abcg5 and Abcg8 mRNA in Control and TDHC administered rats

Analysis of hepatic Abcg5/g8 mRNAs in control and TDHC administered rats. Abcg5/g8 were determined by RT-PCR. All data were standardised for Gapdh and values are expressed as means ±SEM for 6 animals, *P< 0.05 signficantly from control.

**Table. 2** Effect of TDHC administration on biliary output in the fistula rat.

TDHC was administered orally at a concentration of 10mM twice a day for 7 days. Rats were anasthetised and bile duct was cannulated and bile collected for 30 mins on ice. Values are ± SEM for 6 animals, *P < 0.05 significantly from control.

**Table 1.** Expression of hepatic Abcg5 and Abcg8 mRNA in Control and TDHC administered rats

| | Control | TDHC Adminstered |
|---|---|---|
| | Control Cycle Threshold (Ct) | TDHC Cycle Threshold (Ct) |
| Abcg5/Gapdh | 1.29±0.08 | 0.84±0.03* |
| Abcg8/Gapdh | 0.72±0.05 | 0.94±0.07 |

**Table 2.** Effect of TDHC administration on biliary output in the fistula rat.

| | Control Bile | TDHC Administered Bile |
|---|---|---|
| Total Cholesterol (mg/dl) | 16.14±0.59 | 18.40±2.20 |
| Total Bilirubin (mg/dl) | 5.52±0.29 | 6.89±0.40* |
| Alkaline Phosphatase (U/L) | 13.83±2.12 | 17.4±0.81 |
| Gamma Glutamyl Transferase (U/L) | 125.03±4.37 | 114.12±9.28 |