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Bile Obstruction is associated with increased CYP7A1 and MIR33a expression and activation of alternative bile acid synthesis pathway: A research analysis

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Abstract

Introduction: MicroRNAs are small molecules of RNA which play a predominant role in controlling the expression of genes, including the genes involved in synthesis of bile acids from cholesterol. **Materials and Methods:** In this study we sought to explore the effect of bile duct ligation on the expression of 7α-hydroxylase and MIR33a. We also explored whether increased accumulation of bile acid inside liver owing to blockade of bile duct, activates the secondary pathway. A group of 16 male Sprague-Dawley rats was divided into two groups. One group underwent bile duct ligated while the other was left un-ligated as control. The two groups were further divided into a group of 4 rats each based on the content of fat in their diet. Expression level of MIR33a and 7α-hydroxylase in liver were assessed by real time quantitative PCR. Serum concentrations of 7α-hydroxycholesterol and 27-hydroxycholesterol were determined by High Performance Liquid Chromatography (HPLC) and Gas Chromatography – Mass Spectrometry (GC-MS) respectively). **Results:** Our results indicate that intra-hepatic expression of CYP7A1 and MIR33a are up-regulated in bile ligated rats. The expression level is also directly correlated with the fat content in feed. Furthermore, bile acid accumulation inside liver activates the secondary 27-hydroxylase pathway as indicated by increased serum concentrations of 27-hydroxycholesterol and decreased 7α-hydroxycholesterol. **Conclusion:** Bile duct obstruction up-regulates CYP7A1 and MIR33a expression as well as activating secondary bile acid synthesis pathway.

Keywords: MicroRNAs, bile acids, PCR, CYP7A1 and MIR33a.

Introduction

Conversion of dietary cholesterol intake as lipoprotein particles into bile acids is the main metabolic pathway for clearance of these lipoproteins from a person's blood (Davis, et al., 1988; Schwartz, et al., 1978). This process is carried out in liver and is the major pathway for eradication of cholesterol from the body. Rate of synthesis of bile acids is directly related with many factors like increased synthesis of cholesterol in liver (Mackinnon, et al., 1987), increased uptake of lipoproteins mediated by cell surface receptors (Junker & Davis, 1989; Mitropoulos, et al., 1973) and cholesterol rich diet (Straka, et al., 1990). These data suggest that changes in availability of hepatic cholesterol result in compensatory alterations in bile acid synthesis pathways and hence controlling the cholesterol homeostasis (Greim, et al., 1972; Siperstein, et al., 1952).

Cholic acid and chenodeoxycholic acid are the main products of bile salt synthetic pathway in Humans and many other species and are referred to as primary bile salts (Lefebvre, et al., 2009). Once inside small intestinal compartment, these primary bile acids are converted to secondary bile acids by the action of intestinal microbial flora, e.g cholic acid is converted to deoxycholic acid and chenodeoxycholic is converted to lithochlic acid (Lefebvre, et al., 2009). The most important and rate limiting step in conversion of cholesterol to bile acids is the hydroxylation step catalyzed by 7α-hydroxylase (CYP7A1). CYP7A1 belongs to the cytochrome P450 family of enzymes and its structure and function have been well characterized both in humans and other experimental animals.

Almost 50% of the daily turnover of cholesterol is eliminated by conversion into bile acids in a chain of enzymatic reactions. This series of reactions converts the cholesterol into amphipatic compounds (Insull, 2006). Being more water soluble as compared to cholesterol, these amphipatic compounds are easy to be removed from the body. This enzymatic conversion of cholesterol to bile acids is restricted to hepatocytes primarily the perivenous hepatocytes (Twisk, et al., 1995). Perivenous hepatocytes are the cells which surround central hepatic vein. The CYP7A1 reaction is highly regulated and a feedback mechanism is responsible to make sure that this biosynthetic pathway functions properly. If the turnover of bile acids in the intestine exceeds its elimination capacity, bile acids are re-circulated back to liver via hepatic portal system. It has been estimated that approximately 95% of bile acids reaching the small intestinal compartment are re-circulated back to liver. It has been shown that bile acids suppress the expression of 7α-hydroxylase. Hence the higher concentrations of bile acids in the liver would result in reduced expression of 7α-hydroxylase while in times of depletion of bile acids, CYP7A1 expression might be increased to speed up the cholesterol conversion reaction (Insull, 2006). This way, the cholesterol and bile acid homeostasis is regulated.

The regulation of cholesterol by this feedback loop is believed to act at the level of gene transcription. Research has shown the presence of nuclear receptors specific for bile acids (Wang, et al., 1999) and it is believed that bile acids mediate their transcription control through these receptors (Russell, 1999).

Research has shown that CYP7A1 deficiency in humans results in increased incidence of hypercholesterolemia and atherosclerosis (Pullinger, et al., 2002). However, scientists have also reported an alternative pathway for the biosynthesis of bile acids from cholesterol. Instead of 7 hydroxylation, 27 hydroxylation of side chain is carried out by this pathway. This 27 hydroxylation reaction is mediated by mitochondrial enzyme sterol 27-hydroxylase (Bjorkhem, 1992). It is also important to note that this reaction is carried out in extra-hepatic tissue instead of hepatocytes. This pathway is used for conversion of cholesterol to bile acids when the main pathway is not yet mature in infant life (Javitt, 1994; Setchell, et al., 1998). It has also been reported that this pathway replaces the main pathway in case of some impairment like congenital mutation in the 7α-hydroxylase gene (Setchell, et al., 1998).

In this study we studied the effects of bile ligation on the expression of CYP7A1 and 27-hydroxylase in rat livers. We hypothesized that in the event of impairment of main pathway, secondary pathway is activated. To check the activation of secondary pathway, we monitored the serum levels of 7α-hydroxycholesterol and 27-hydroxycholesterol.

Materials and Methods

Animals and diet

Sprague-Dawley rats weighing 300-350 g were kept on a 12 hour light / 12 hour dark cycle. Rats were divided into two groups based on the diet they

received. One group was fed with routine laboratory chow diet containing 5% fiber, 20% protein and 5-10% fats. The second group was fed on adjusted calories diet, Western Diet (0.2% cholesterol and 42% calories from fats, 88137, Harlan Teklad, Madison, WI).

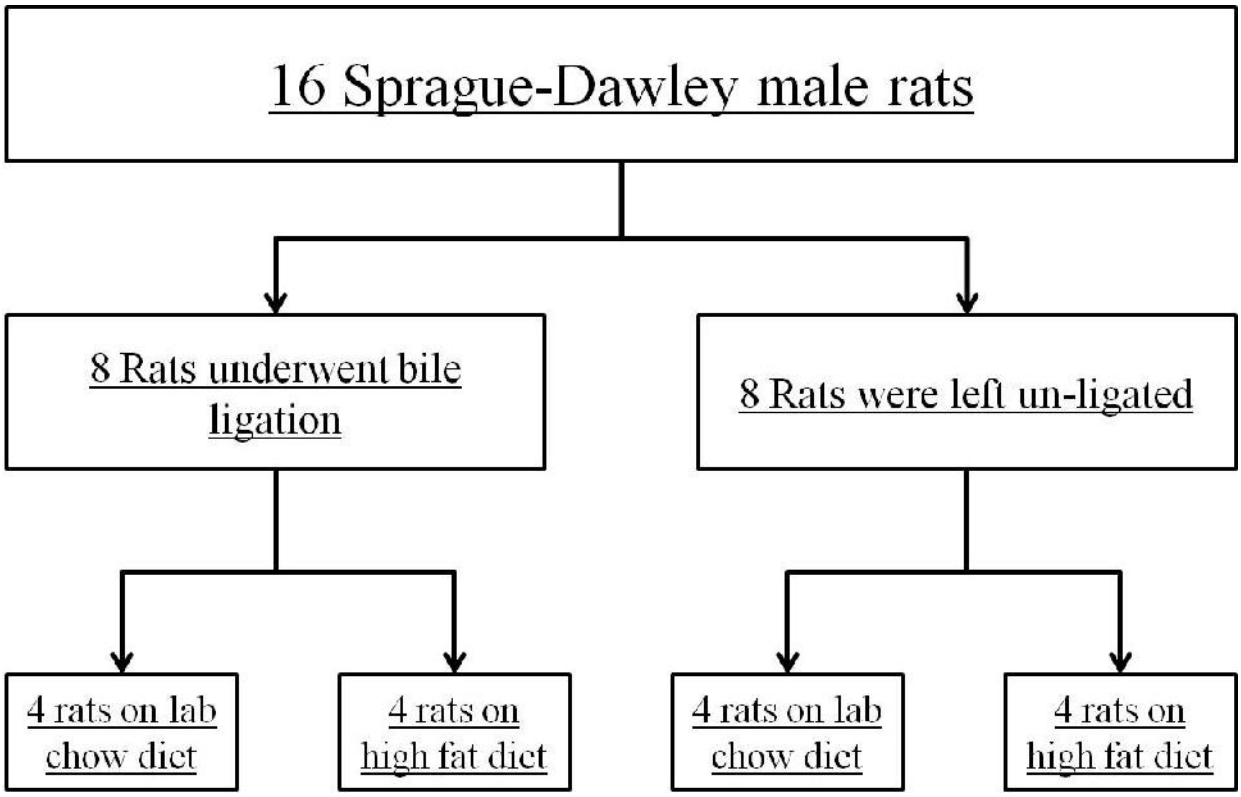

Figure 1: Animal Groupings

Ligation of bile duct in rats

Bile duct ligation of rats was carried out as previously described (Lauterburg & Bircher, 1976). In short, after the rats had gained required weight, they were anesthetized with 50mg/kg BW dose of pentobarbitone. A double ligature was used to block the bile duct. After incising 1cm section of bile duct, the abdominal incision was closed and the rats were reared for another 4 weeks of 12 hour light / 12 hour dark cycle before being killed. Control group of rats underwent similar procedure. The control rats underwent laprotomy similar to the other group but their bile ducts were not ligated. After closing incision, the control group rats were also kept in similar conditions for another four weeks before being killed.

Isolation of RNA and real time PCR

Expression levels of CYP7A1 and three bile acid hepatic transporters (Abcg5, Abcg8 and Ldlr) was carried out by quantitative real time PCR using RNA extracted from the rat livers. Using Trizol reagent (Invitrogen, Carlsbad, CA) total RNA was extracted from the livers of killed rats, according to manufacturer's protocol with slight modifications. Briefly, 50 mg tissue samples were homogenized in 1 ml Trizol reagent and left on room temperature for 5 hours. RNA was extracted using 0.2 ml of chloroform and precipitated using 0.5 ml 100% isopropanol. The pellets were washed with 75% ethanol and after air drying samples were suspended in 30ul of water (RNAse free). M-MuLV reverse transcriptase (Thermo Scientific, Waltham, MA) was used to synthesize first strand cDNA, as per manufacturer's protocol. 2ul of the cDNA was used as a template in real time PCR using SYBR Green dye (Life Technologies, Carlsbad, MA). The primers for all the amplified regions are shown in Table 1.

Table 1: Primer Sequences for Real Time Quantitative PCR

Gene	Forward Primer	Reverse Primer	Ref
Abcg5	5'-TTGCGATACACAGCGATGCT-3'	5-TGACTGCCTCTACCTTCTTGTTGT–3'	(Song, et al., 2010)
Abcg8	5'-CCGTCGTCAGATTTCCAATGA-3'	5'-GGCTTCCGACCCATGAATG-3'	-do-
Ldlr	5'-GCTCCATAGGCTATCTGCTCTTCA-3'	5'-CTGCGGTCCAGGGTCATC-3'	-do-
CYP7A1	5'-CCATGATGCAAAACCTCCAAT-3'	5'-ACCCAGACAGCGCTCTTTGA-3'	-do-
MIR33a (Probe sequence)		5′- GUGCAUUGUAGUUGCAUUG-3′	(Li, et al., 2013)

For amplification and quantification of Mir33a, separate RNA extraction protocol was used. We used miRNA kit, E.Z.N.A. (Omega Bio-tek, Norcros, U.S.A.), according to manufacturers' protocol. Similarly for cDNA synthesis for Mir33a we used TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) using Mir33a specific primer. The protocol for reverse transcription was as reported by (Baselga-Escudero, et al., 2013). Briefly, 3ng/ul RNA was used a template for reverse transcription and reaction conditions were as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. 2ul of obtained cDNA was used in qRT-PCR. The quantitative real time PCR was performed using TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain). The sequence of probe used for Mir33a is given in Table 1. U6 snRNA was used as an internal control and the mRNA level was calculated by 2-ΔΔCt (ΔCt = Ct miRNA – Ct U6, ΔΔCt = ligates rats' samples - unligated rats' samples) (Livak & Schmittgen, 2001).

Measurement of 27-hydroxycholesterol and 7α-hydroxycholesterol in serum

Before killing animals, venous blood samples were collected and stored at 4 °C till further use. Serum 7α-hydroxycholesterol levels were measured HPLC as described by (Maeda, et al., 1997). Briefly, 200ul serum was incubated 37 °C for 10 minutes, in 0.1M Potassium phosphate buffer (pH 7.4) in the presence of 1µU cholesterol oxidase. After vigorous shaking for 2-3 minutes, the sample was centrifuged at 3000 rpm for 5 minutes. This reaction produces 7a-hydroxy-4-cholesten-3-one. Extraction was carried out by 5 ml n-Hexane and the samples were evaporated to dryness. Dried samples were dissolved in 100ul isopropanol an aliquot of which was analyzed on HPLC with Fine Pack Sil columns (4.6x250mm; Crawford Scientific, Lanarkshire, Scotland, UK). n-Hexane:isopropanol (80:20, v/v) solvent system was used and the samples were run at a flow rate of 1 ml per minute. The concentration of 7a-hydroxy-4-cholesten-3-one in the effluents was measured by absorption at 240 nm.

27-hydroxycholesterol was measured by isotope dilution gas chromatography – mass spectrometry by the method described by (Bertolotti, et al., 2001). The rats were 19-hydroxycholesterol was used as an internal standard.

Statistical Analysis

All the statistical analysis was carried out using SPSS v16 (IBM Armonk, NY). Linear regression analysis of expression levels was carried out plotting CYP7A1 on X-axis and comparing the expression with individual hepatic bile acid transporters plotted on Y-axis. Average relative expression levels of each gene (Abcg5, Abcg8, Ldlr and CYP7A1) in each group of rats were compared with each other.

Results

7α-hydroxylase and Mir33a expression increases in bile ligated rats

In total there were 16 rats, out of which eight rats underwent the bile ligation. Similarly, four rats from each group (ligated and non-ligated) were fed on regular laboratory chow with low fat content while the other four were fed on high fat western diet (see Figure 1) An analysis of 7α-hydroxylase mRNA levels in the rat livers revealed marked increase in the

expression of CYP7A1 in bile ligated rats while the non ligated rats showed a lower expression level. It was also observed that the rats on high fat diet had (WD) had higher levels of CYP7A1 mRNA in livers than the ones on regular laboratory chow, both in ligated and non-ligated groups. The messenger RNA levels for 7α-hydroxylase are shown in Figure 2.

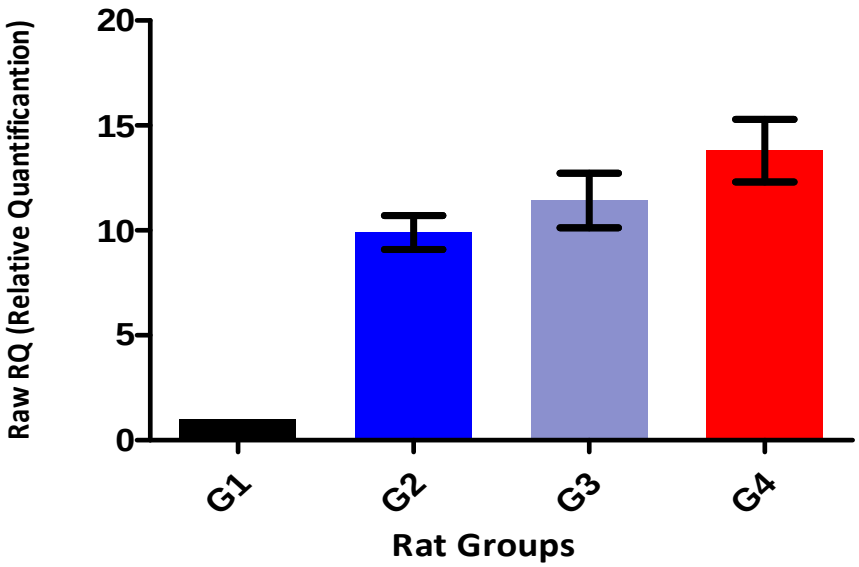

Figure 2: The figure represents the relative expression level on CYP7A1 in rat liver normalized against the control group 1 (un-ligated rats on regular laboratory chow). See material and methods for calculations and further details.

Similar patterns were observed for Mir33a expression in rat livers i.e the ligated ones showing high expression as compared to non-ligated ones and rats on high fat diet in each group (ligated or un-ligated) showing lower Mir33a expression levels (Figure 3)

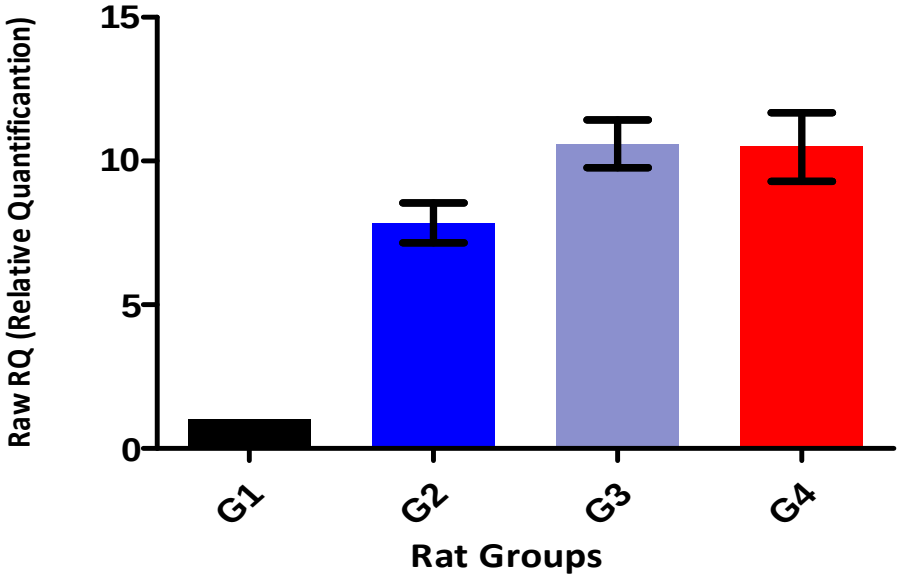

Figure 3: Individual bars represent expression of intrahepatic Mir33a in each rat. The relative quantification of Mir33a was carried out by Real Time PCR and the calculations were made using the 2-ΔΔCt formula. See materials and methods for more details

Expression of bile acid transporters in rats

Three of the bile acid transporters, namely; Abcg5, Abcg8 & Ldlr, were selected for this study and their expression levels were checked in rat livers through quantitative real time PCR using SYBR green chemistry. Our analysis revealed that there were no marked differences in expression levels of any of three transporters between ligated and un-ligated groups.

However, when a similar comparison was made between two groups of rats on different diets (high fat vs low fat), the group being fed on high fat WD showed significantly higher levels of all the three messenger RNAs. The results of real time PCR for this analysis are presented as ΔΔCt (where Ct refers to the threshold cycle at which amplification is observed). Results of this analysis are shown in Figure 4.

Figure 4: Expression of Hepatic bile acid transporters

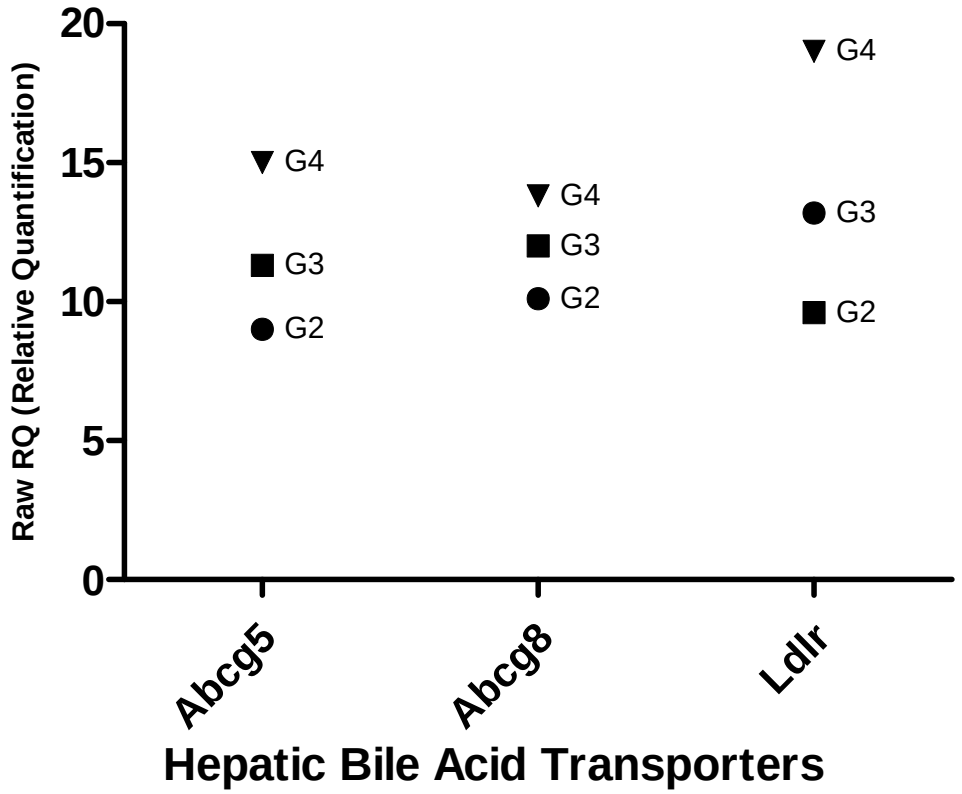

Figure 4: Intra-hepatic expression of three bile acid transporters, Abcg5, Abcg8 and Ldlr were analyzed by real time PCR. The figure shows a scatter plot of averaged group values. The expression levels were normalized against the control group (G1, un-ligated and on low fat lab cow diet). Results indicate that expression of bile acid transporters is directly correlated with intrahpetic bile accumulation as well as fat content in the diet.

Serum 7α-hydroxycholesterol and 27-hydroxycholesterol levels.

Serum analysis of 7α-hydroxycholesterol levels reveals that the rats which underwent bile ligation had significantly decreased levels of 7α-hydroxycholesterol as compared to non-ligated group. However the reverse was observed for 27-hydroxycholesterol serum concentrations. A comparison between rats on low and high fat diets revealed that serum levels of either of the two cholesterol derivatives correlated directly with the fat content in the diet (rats on high fat diet showed higher levels of 27-hydroxycholesterol in both ligated and non-ligated groups when compared with the rats on low fat regular laboratory chow in the same group). See Table 2 for more details.

Table 2: Serum concentration of cholesterol derivatives

Diet	Serum 7α-hydroxycholesterol levels (nmol/ml)	
	Ligated	**Un-Ligated**
Regular Chow	0.8 ± 0.1	1.4 ± 0.3
Western Diet	1.2 ± 0.5	2.4 ± 0.7

Diet	Serum 27-hydroxycholesterol levels (ug/dl)	
	Ligated	**Un-Ligated**
Regular Chow	22.2 ± 1.6	5.6 ± 0.5
Western Diet	28.4 ± 2.5	16 ± 0.8

Correlation of CYP7A1 and Hepatic Bile Transporters' Expression

Figure 5 (a, b and c) show the linear regression analysis of CYP7A1 expression and the expression Abcg, Abcg5 and Ldlr. Our results indicate that CYP7A1 expression is directly correlated with mRNA levels of all the three hepatic bile transporters. Best fit slope values for individual correlations were calculated as 1.540 ± 0.01345 (Abcg5) 0.9321 ± 0.1321 (Abcg8) 2.413 ± 0.02205 (Ldlr).

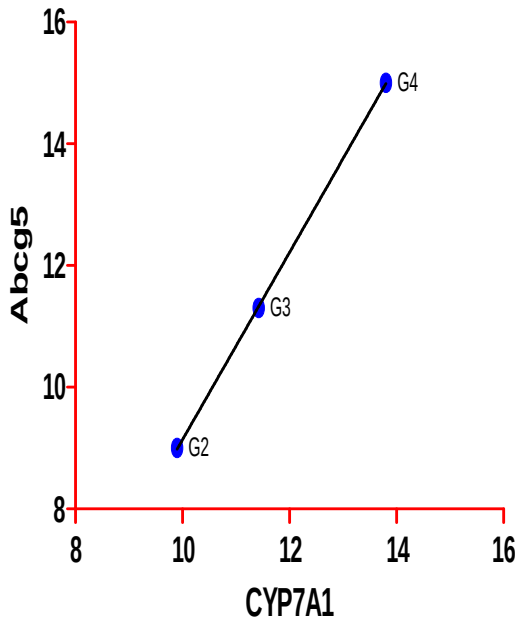

Figure 5a

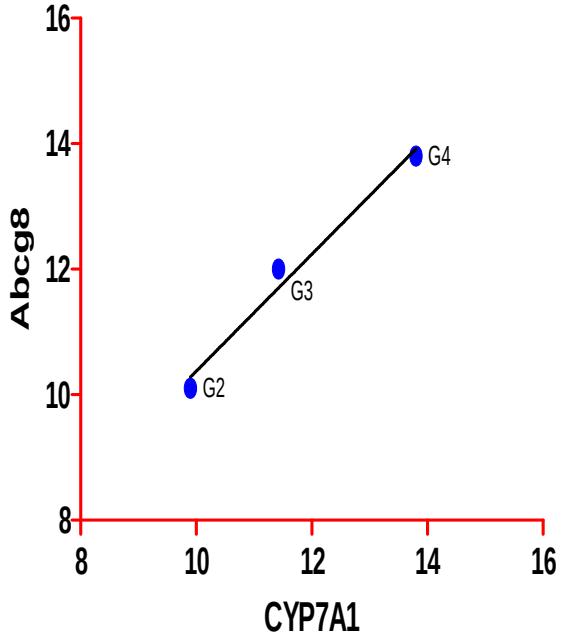

Figure 5b

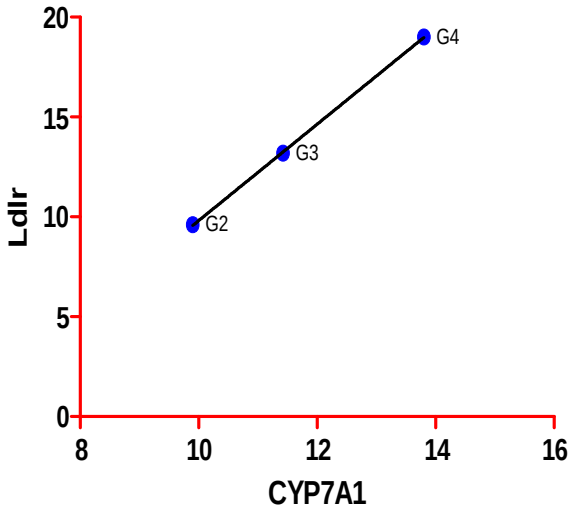

Figure 5c

Figure 5: Data shows that expression of CYP7A1 was directly correlated with all the three hepatic bile transporters; Abcg5 **(Figure 5a)**, Abcg8 **(Figure 5b)**, Ldlr **(Figure 5c)**

Discussion

Homeostasis of dietary cholesterol in mammalian body is maintained by converting it into bile acids in liver (Bertolotti, et al., 2001). These bile acids are transported to intestine via hepatic portal system and perform key functions in the digestion of lipids and fats. It has long been known that the bile acids are involved in the formation of micelles within the intestine where they perform digestion of dietary fatty acids (Hofmann & Borgstroem, 1964). The first step in conversion of dietary cholesterol to bile acids is the addition of a hydroxyl group at the 7th position. This 7-hydroxylation is mediated by cytochrome p-450 class enzyme 7α-hydroxylase (CYP7A1) and represents the major pathway for bile acid synthesis (Galeazzi & Javitt, 1977). However, in case of any impairment in this pathway, a secondary pathway can also be activated which employs 27-hydroxylase and the hydroxyl group is added to the position 27 of side chain (Galeazzi & Javitt, 1977). The CYP7A1 mediated bile synthesis pathway is under tight negative and positive feedback control. It has been observed that increased accumulation of bile acids inside liver as a consequence of any kind of bile duct obstruction, result in suppression in the synthesis of bile acids. Bertolotti, et al reported that in cholestasis patients, bile acid synthesis is markedly suppressed (Bertolotti, et al., 2001).

Our results indicate that in spite of the expected decreased production as well as increased accumulation of acids in the liver as a result of bile duct ligation expression of CYP7A1 increased significantly. Our results are consistent with the observation made by Bertolotti, et al in human cholestasis patients (Bertolotti, et al., 2001) in which it was observed that CYP7A1 expression increased in cholestatic patients in comparison with normal controls.

Role of Mir33a in the cholesterol homeostasis has recently been highlighted (Najafi-Shoushtari, et al., 2010). In order to check that whether increased CYP7A1 levels corresponded to an increased expression of its other co-mediators, we checked the mRNA level of Mir33a. Previous studies have found the link between over expression of CYP7A1 and steroid response element binding protein 2 (SREBP2). Li, et al. reported that in transgenic mice expressing increased amounts of CYP7A1, SREBP expression is markedly increased as revealed by microarray data (Li, et al., 2013). Intron 16 of SREBP gene encodes Mir33a so in case of increased CYP7A1 levels and subsequent increase in SREBP expression, Mir33a levels should also increase. Our findings proved our hypothesis and confirmed the results of previous studies (Li, et al., 2013).

High fat diet is correlated with increased cholesterol production in the blood. Therefore, it can be hypothesized that animals on high fat diet should express increased levels of CYP7A1 for increased demand of cholesterol conversion. Consistent to the hypothesis, CYP7A1 levels showed a marked increase in rats on high fat Western Diet. This correlation with the fat content of diet was observed in both ligated and non-ligated groups. Similarly, Mir33a levels also increased markedly in rats on high fat which is consistent with the expected results.

Adenosine triphosphate-binding cassette G5/G8 (Abcg5 & Abcg8) are heterdimeric cholesterol efflux transporters which help to transport hepatic cholesterol to bile. The two genes lie very close to each other with only a 180 nucleotide long intergenic promoter between them (Berge, et al., 2000). In spite of increased expression of CYP7A1 and Mir33a, no marked increase in the expression levels of these two efflux transporters was observed. The results can be interpreted in the light of the fact that there is no increased bile production and hence the demand for increased expression of transporters, therefore these two transporter proteins did not show increased expression levels. However similar to other observations, rats receiving high fat diet showed significantly higher expressions of both the genes as depicted by hepatic mRNA levels.

We also analyzed whether there is any correlation between CYP7A1 expression and the expression levels of hepatic bile transporters. Our analysis indicates that CYP7A1 expression is directly correlated with the mRNA levels of all the three hepatic bile transporters we analyzed. This interesting finding might provide the basis for further research as it is evident from data presented in this research that increased expression of CYP7A1 might send some kind of signals indicating the imminent increase in bile acid production and hence increased demand for bile acid transporters. This signal then causes the hyper-expression of bile acid transporters. However the signaling molecules and pathways involved in this seemingly positive feedback mechanism are not known. Further research might be needed to reveal the exact mechanism and the signaling pathways involved in this complex network of genes.

As has been discussed above, in addition to CYP7A1 pathway, alternate pathway for the conversion of cholesterol to bile acids is activated in times of impairment to main pathway. As the main CYP7A1 pathway is under the control the feedback from the intestine, in case of bile duct blockade, such feedback signals are disrupted. Our results indicate that in such times of impaired release of bile acids to intestine, secondary pathway may be activated. Ligated rats showed increased CYP7A1 levels while 27-hydroxylase in the serum of such rats was high as compared to non-ligated group.

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