약학석사학위논문

비알콜성 지방간 랫트 모델에서 Cyp2b1, Cyp3a, Ugt1a6, Mdr1 의 간 발현양의 변화에 관한 연구

Altered Expressions of Hepatic Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 in Non-Alcoholic Fatty Liver Disease in Rats

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서울대학교 대학원

약학과 약제과학 전공

조 성 준

Abstract

Non-alcoholic fatty liver diseases include simple fatty liver (SFL) and non-alcoholic steatohepatitis (NASH). These liver disease states may affect the expression and function of various hepatic drug metabolizing enzymes and transporters (DMETs), potentially leading to altered pharmacokinetics, therapeutic efficacy and toxicity. This study focused on the investigation of changes in the expression of hepatic CYP2B1, CYP3A, UGT1A6, and MDR1 which have not yet been clarified sufficiently in rat models of SFL and/or NASH. Male Sprague-Dawley rats were randomly placed into one of three diet treatment groups: (1) normal diet (control) group for healthy liver, (2) high fat diet (HFD) group to induce SFL and (3) methionine-choline deficient diet (MCD) group to induce NASH. To confirm the induction of SFL and NASH, liver histology in these groups was evaluated by haematoxylin and eosion (H&E) staining. The mRNA and protein expression levels of DMETs studied were determined by real-time PCR and Western Blot analysis. Compared with control rats,

significant increases in GOT, GPT level were observed in MCD groups. Several discernible histological differences in H&E staining were observed in HFD and MCD rats. The mRNA and protein expression levels of hepatic CYP3A and MDR1 in MCD rats were significantly higher than those in the other two groups. For CYP2B1, its mRNA expression levels in both HFD and MCD rats were significantly higher than those in control rats, while its protein expression levels were significantly higher only in MCD rats than those in the other two groups. For UGT1A6, its mRNA expression levels in both HFD and MCD rats were significantly higher than those in control rats, while its protein expression levels were significantly higher only in MCD rats than those in the other two groups. In conclusion, animal models of NAFLD including SFL and NASH were established. The NAFLD animal model showed altered expressions of Cyp2b1, Cyp3a, Ugt1a6 and Mdr1, which may indicate the possibility of altered expressions of enzymes in human NAFLD and of changing the pharmacokinetics of their substrate drugs.

key words : non-alcoholic fatty liver disease, high fat diet (HFD), methionine-choline deficient diet (MCD), simple fatty liver (SFL), non-alcoholic steatohepatitis (NASH), rats, drug metabolizing enzymes, drug transporters.

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국문초록

비알콜성 지방간에는 단순지방간과 비알콜성 지방간염을 포함한다. 이 질병은 간에 존재하는 여러 대사효소나 수송체들의 발현양을 변화시킬 수 있고, 이는 특정 약물의 약동학적 특성을 바꾸게 되어 효과나 독성까지도 바꿀 수 있는 원인이 될 수 있다. 본 연구의 주제는 간에 존재하는 대사효소와 수송체중 하나인 Cyp2b1, Cyp3a, Ugt1a6, Mdr1의 발현 양이 아직 연구가 명확히 이루어 지지 않은 랫트의 지방간염 모델에서 얼마나 변하는지에 대해 살펴보는 것이다. 수컷 Sprague-Dawley 랫트를 임의로 세 그룹으로 나눈 뒤, 일반 사료와, 고지방사료(HFD) 그리고 메치오닌-콜린이 결핍된 사료(MCD)를 주어서 단순지방간과 지방간염을 유도하였다. 제대로 질병상태가 유도되었는지를 확인하기 위해, H&E 염색과 혈액검사를 진행하였다. 혈액검사결과를 보면, GOT와 GPT 수치가 MCD 그룹에서 유의성 있게 증가한 것을 확인 할 수 있었고, H&E 염색에서도 각 그룹간에 병리학적인 차이를 확인할 수 있었다. mRNA와 단백질의 발현 정도를 확인하기 위하여 real-time PCR과 Western Blot을 진행하였다. CYP3A와 MDR1의 경우, MCD 그룹이 다른 두 그룹에 비해 mRNA와 protein이

증가한 것을 확인하였다. CYP2B1는 mRNA의 발현정도가 HFD, MCD 두 그룹에서 증가한 것을 확인했고 단백질의 발현양은 MCD 그룹에서만 유의하게 증가하였다. UGT1A6 역시 mRNA의 발현정도는 HFD, MCD 두 그룹에서 증가하였고 단백질의 발현양은 MCD 그룹에서만 유의하게 증가하였다. 결론적으로, 이번 연구를 통해 랫트에서 지방간과 지방간염 모델을 확립 할 수 있었다. 그리고 이 모델에서 Cyp2b1, Cyp3a, Ugt1a6 그리고 Mdr1의 발현 양이 변화한다는 것을 확인하였다. 본 연구의 결과는 인간의 비알콜성 지방간에서도 해당 단백질의 발현양이 변화되어 기질약물의 약동학적 특성이 바뀔 수 있다는 가능성을 암시하고 있다.

주요어

비알콜성 지방간, 고지방 사료, 메치오닌-콜린이 결핍된 사료, 단순 지방간, 비알코올성 지방간염, 랫트, 약물대사효소, 약물 수송체

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has been increasingly severe throughout the world as people get more fatty diet. As a result, the number of reported cases of Non Alcoholic Fatty Liver Disease (NAFLD) has been increased tremendously over the last decades, and that the understanding of the NAFLD also has been getting important.

There are spectrums of symptoms in NAFLD ranging from simple fatty liver (SFL) to steatohepatitis (a fatty liver with inflammation and progressive fibrosis) [1]. Cirrhosis and liver cancer can occur when steatohepatitis gets severe. The prevalence of NAFLD is estimated to be between 14% and 24% in the world population, and on contrary to the thought that NAFLD is an adult disease, children are also known to show NAFLD as well [2]. Simple fatty liver is characterized by micro and macrovesicular steatosis and makes liver more susceptible to more severe non-alcoholic steatohepatitis (NASH) [3]. NASH is histologically characterized by micro and macrovesicular steatosis, lobular inflammation, fibrosis, and hepatocellular damage, including accumulation of Mallory's hyaline, ballooning degeneration, necrosis, and fibrosis (zone 1 and zone 3)

[4].

Liver plays a major role in eliminating drugs from body. About 75% of top 200 selling drugs are eliminated mainly by metabolism and drug-metabolizing enzymes (DMEs), and drug-transporters (DTs) are critical in this metabolism and hepatic clearance. It is shown that NAFLD can change the expression and function of various DMEs and DTs which may alter the metabolism of their substrate drugs. Altered expression of cytochrome P450 enzymes and hepatic transporters were observed in viral and alcoholic hepatitis patients, and these changes have been associated with variability in drug response, following altered drug efficacy and toxicity [5, 6].

In this study, NAFLD was recapitulated in two of its prominent symptoms, SFL and NASH. A high-fat diet (HFD) model was used for the SFL model, while a methionine and choline deficient diet (MCD) was used to induce NASH model in rat. MCD diet could induce NASH in rats histologically similar to NASH in human. This model of "fibrosing steatohepatitis" was characterized by the development of fibrotic strands that surround hepatocytes in a way identical to those in human disorders of lipid-assoicated hepatic fibrosis. Additionally, a sequence of events including steatosis, chronic hepatocyte injury, heptatic inflammation, and fibrosis by

several weeks was analogous to that which occurs in human NASH [7]

It was known that CYP2B6 was mainly expressed in the liver, accounting for 6% of total microsomal CYPs. Interest in this enzyme has been stimulated by the discovery of polymorphic and ethnic differences in CYP2B6 expression [8]. CYP3A4 was known to be the most abundant CYP enzyme in the liver and accounts for the metabolism of over 50% of clinically used drugs. UGT1A6 was also reported to be one of the major UGT isoforms in human liver that catalyzes glucuronidation of a diverse range of drugs, carcinogens and endogenous substrates. MDR1 (also known as P-glycoprotein) was well characterized efflux transporter and largely determine drug absorption, distribution and excretion. Thus, this study focused on changes in the expression of hepatic CYP2B1, CYP3A, UGT1A6, and MDR1 because these proteins have not yet been clarified sufficiently in rat models of SFL and/or NASH.

2. Materials and Methods

2.1 Materials

Bupropioin and hydroxylbupropion were purchased from Sigma-Aldrich (St-Louis, USA). Normal rodent diets were purchased from Agribrands Purina Korea (Pyeongtaek, South Korea) and high-fat diet (HFD) and methionine-choline deficient diet (MCD) were purchased from Central Lab. Animal Inc (Seoul, Korea). Zoletil and Rumpun were purchased from Virbac Inc (TX, USA) and Bayer Inc (Leverkusen, Germany).

2.2 Animal treatment

Male Sprague-Dawley rats of 6 weeks age were purchased from Orient Bio, Inc (Seong-Nam, Korea) and housed in a temperature-, light- and humidity controlled environment. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Seoul National

University, Seoul, Korea. After a week for acclimation, rats (n=3) were randomly placed into one of three diet groups; control groups were fed normal diet to simulate healthy liver. HFD groups were fed HFD diet to simulate simple fatty liver and MCD groups were fed MCD diet to simulate NASH. Each group was fed their own diet *ad libitum* for 8 weeks.

2.3 Blood test and H&E staining.

Blood was collected through abdominal artery after anesthetization with Zoletil (Tiletamine and Zolazepam) and Rumpun (Zylazine). Plasma was collected by centrifugation at 3000 rpm for 15 min and stored in −70℃ until analysis. Blood tests were conducted by Laboratory Animal Center, College of Pharmacy, Seoul National University (Seoul, Korea). Liver slices were frozen rapidly with liquid nitrogen and stored at −70℃ until analysis. Liver slices were fixed in formalin solution for more than 12 hour and staining was performed by Maxdiagnostics Inc (Seoul, Korea).

2.4 Total RNA isolation and cDNA synthesis.

Total RNA was isolated from the liver tissues using TRIzol reagent (Invitrogen, CA) according to manufacturer's instructions. The concentration and purity were measured spectrophotometrically at 260/280 nm. To synthesize cDNA, approximately 1 ug of total RNA was used using 1st strand cDNA synthesis kit (Takara Shuzo Co., Ltd, Shiga Japan) according to manufacturer's instructions. The reactions were conducted by incubating mixture at 42℃ for 1 hour, heating to 95℃ for 5 min, and subsequent cooling to 4℃.

2.5 Real-time PCR analysis.

To determine the relative expression of each mRNA gene, real-time PCR was performed using Light Cycler 1.5 system (Roche, IN). Sequences of each primer were listed in Table 1. Gapdh gene was used to normalize gene expression in all samples. The Light cycler FastStart DNA Master SYBR Green1 kit (Roche, IN) was used for quantitative PCR analysis. The cycling conditions were performed as follows. Initial pre-

incubation at 95℃ for 10 min, followed by 45 cycles of denaturation at 95℃ for 10 s, annealing at 60℃ for 10 s, and elongation at 72℃ for 10 s. The temperature transition rate was set at 20℃ s^{-1}. To determine whether the non-specific amplification was carried out, a melting curve was constructed from the amplification reaction obtained by maintaining the temperature at 65℃ for 15 s, followed with a gradual temperature increase rate of 0.1℃/s to 95℃. For this study, the signal acquisition mode was set at "continuous". The relative quantification of the amount of the target mRNA in the liver tissues was obtained by measuring C_t and calculated using the $\Delta\Delta$ method [9]

2.6 Protein extraction and Western blot assay

To prepare protein samples for Western Blot, liver tissues with RIPA buffer (Biosesang Inc, SeongNam, Korea) containing protease inhibitor cocktail tablet (Roche, Manhein Germany) were homogenized using glass homogenizer. After centrifugation at 12000 rpm for 10 min, supernatants were

collected and stored at −70℃ until analysis. Protein concentrations were measured using BCA study kit (Thermo Inc, IL, USA). Aliquot (50 ug) of protein was mixed with loading buffer composed of 0.5 M Tris−HCl (pH 6.8), glycerol, 10% SDS, 2−b−mercaptoethanol, and bromophenol blue. Then, 50ug of proteins were loaded on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and electrophoretic separation was carried out. The gel was then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk (BD Inc, MD, USA) following primary antibody incutation for 12 hours ; Antibodies of beta−acitn (sc−8313,), Cyp2b1 (sc−53244) , Cyp3a (sc−258425) and Mdr1 (sc−8313) were purchased from Santacruz Inc (Santacruz, CA). An antibody of Ugt1a6 (ab157476) was purchased from abcam (Cambridge, UK). After washing 4 times with PBS−Tween buffer, either peroxidase−conjugated anti−rabbit or anti−mouse immunoglobulin G (Santa Cruz Biotechnology, CA) was used as a secondary antibody. The bound antibodies were detected using chemiluminescence detection system (GE

Health care, Sweden)

2.7 Statistical Analysis

Statistical significance between normal, HFD, MCD diet rats was determined using one-way ANOVA (Graphpad Prism 5) followed by tukey's post-hoc test. A p-value of less than 0.05was considered to be statistically significant.

3. Results

3.1 Blood test and H&E staining

Figure 1 showed the H&E stained liver sections of each group. It demonstrated the effects of the respective high-fat and MCD diets on liver histology after 8 weeks. Notably, the hepatic steatosis was apparent in both HFD and MCD diet fed rats while MCD fed rat showed more severe hepatic steatosis. Arrow in MCD diet designated inflammation related cells whose nuclear size was smaller than that of hepatocytes. Table 2 showed the blood test results of each group. GPT and GOT values of MCD diet group were much higher than that of other groups while TG and T-CHO values of MCD diet group were lower than other groups.

3.2 mRNA levels in liver

Figure 2 shows the effect of the respective high-fat diet and MCD diets on Cyp2b1, Cyp3a, Ugt1a6 and Mdr1. For Cyp2b1, mRNA level in both HFD and MCD diet fed groups increased

compared to control group while HFD fed rat showed more increased mRNA level than that of MCD group. When it comes to Cyp3a, Ugt1a and Mdr1, mRNA levels of all three proteins were increased in both HFD and MCD diet groups. However, there was no statistical significance between control and HFD group in Mdr1 mRNA level.

3.3 Protein levels in liver

After 8 week of feeding on the control, HFD and MCD diets, there were discernible difference in protein levels of Cyp2b1, Cyp3a, Ugt1a6 and Mdr1. In MCD rats, Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 protein expression levels were increased while HFD showed no statistically significant changes.

4. Discussion

The current study showed the altered expressions of Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 in experimental NAFLD models in rats. The clinical relevance of these topics was underlined by the fact that the NAFLD patient population who uses a great quantity and variety of prescription medications, was expected to continue growing at an alarming rate [10, 11]. To expand this comprehension and potentially develop better pharmacologic treatment for this disorder, it would be necessary to establish accurate animal models. It was thus pertinent to note that the current models of NAFLD (in this study, high-fat and MCD diets) provided accurate histological representations of SFL and NASH, respectively.

HFDs and MCD diets were chosen to induce simple fatty liver(SFL) and NASH in rats individually. HFDs contains higher fat contents(60%) than normal diets does (20%). This diet could induce SFL by increasing the amount of fat input to liver. As a result. HFD fed rats were reported to become heavier and had microvescular steatosis and larger size of

adipose tissues compared to normal rats [3]. MCD diets were known to develop chronic steatohepatitis that resulted in hepatic fibrosis in a pattern resembling that found in human NASH [7]. The MCD diets could make NASH in rats because they impaired Very Low-Density Lipoprotein (VLDL) synthesis, which later increased fatty acid uptake to liver. Although MCD fed rats showed reduced body weight and hypoglycemic conditions, they replicated the histological features of human steatohepatitis, and the sequence of steatosis, inflammatory cell injury and fibrogenesis [7].

To elucidate whether HFD and MCD diets induced SFL and NASH properly, H&E staining and blood biochemistry test were conducted because checking elevated liver enzymes and liver biopsy would be the most widely used diagnostic method. Especially, liver biopsy (tissue examination) is the only test widely accepted as a way to definitively distinguish NASH from other forms of liver disease and can be used to assess the severity of the inflammation and resultant fibrosis [12].

In blood biochemistry test, GPT and GOT values were

elevated in MCD groups while no significant changes were observed in HFD groups. The results of blood biochemistry showed that the liver of MCD fed rats were severely impaired. This tendency was consistent with that of human SFL (No significant change) and NASH (Elevated liver enzyme level).

In H&E staining, compared to the livers of control rats, HFD and MCD diet groups showed microvesicular steatosis while only MCD diet groups showed inflammation related cells. The results represent that HFD and MCD diets made SFL and NASH disease state, respectively, considering that inflammation is the physiological property of NASH.

Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 were selected as target enzymes in the current study. The previous studies showed that there was no clear interpretation of the effect of NAFLD on Cyp2b family. Few studies in human patients have reported on the effect of NAFLD on CYP2B6 expression. The effects of NAFLD on UGT1A6 were also not fully elucidated especially in NASH animal models. Although CYP3A and MDR1 were relatively more studied proteins, there has also been no

determined interpretation of the effect of NASH in rat models.

The mRNA and protein expressions were examined through real-time PCR and western blot analysis. As shown in Figure 2, the mRNA expressions of all four proteins were increased in both HFD and MCD group while Mdr1 in HFD showed no statistically significant changes compared to control groups. The altered protein expressions of four enzymes were similar to the mRNA results (Figure 3). In MCD diets, Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 were all increased compared to control and HFD groups. This increasing tendency was similar to that of mRNA.

For Cyp2b1, increased expressional tendency was similar to the previous studies. Published studies in mouse models reported increased Cyp2b10 expression and activity [13, 14]. Fisher et al [15] found NAFLD progression increased CYP2B6 mRNA in the absence of any effect on protein or activity. However, there are studies which reported decreased expressions of CYP2B6 [16, 17]. Thus, further studies are necessary to elucidate the tendency of altered Cyp2b

expression in NAFLD definitely.

Increased mRNA and protein expression in Ugt1A6 was observed in this study which was partially consistent to the previous studies. UGT expression in mouse liver was reported to be increased in obesity-and fasting- induced steatosis [18]. Rat obese models showed increased expression of Ugt1A6 [19]. However it was reported that expression of Ugt1A6 was decreased [20].

Cyp3A and Mdr1 also showed increased expressions in this study. The result of Mdr1 was consistent with the human data [21]. Thus, MCD diet-fed rats could be appropriate animal model to study the altered expression of Mdr1 and their contribution to drug distributions in NAFLD patients. For Cyp3A, rat models, to date, have predominantly showed decreased expression or activity while increased expression of several CYP3A isoforms have been observed in rat NASH models, using forced intragastric feeding [22] whose results are similar to the current study.

5. Conclusion

The results of this study indicated that animal models of NAFLD showed altered expressions of Cyp2b1, Cyp3a, Ugt1a6 and Mdr1 in liver. Blood biochemistry and H&E staining results represented that high fat diets and methionie-choline deficient diets could induce simple fatty liver and non-alcoholic steatohepatitis, respectively. Both mRNA and protein expressions of these four proteins were elevated in the SFL and NASH models. The results may indicate the possibility of altered expressions of these four enzymes and changed pharmacokinetics of their substrate drugs in human NAFLD.

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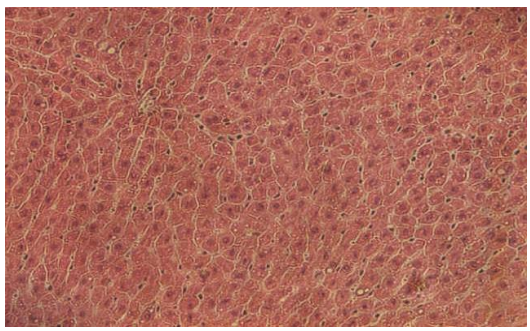
Table1. Sequences of primer pairs, annealing temperature, Tm, size of PCR products, and cycle numbers used for RT-PCR analysis of UGT1A6, CYP2B1, CYP3A, Abcb1a

Gene	Primer seqhences(5'→3')	Annealing temperature(℃)	Product length (bp)	Cycle number (number)
Cyp2b1,2	CTCCAAAAACCTCCAGGAAATCCTC	60	118	45
	GTGGATAACTGCATCAGTGTATGGC	60		45
Ugt1a6	CTTCATTGCTAACATCTTGGA	60	85	45
	TCTCTTGAGGAGGTCTGA	60		45
Cyp3a	CACACAGCTAAGAATAAAGG	60	170	45
	TTTAGGTCCAGAAATCCC	60		45
Mdr1a	TTGGCTGGACAGATTTTAC	60	148	45
	AGCATTGTAGAAACTCATACT	60		45
Gapdh	CAGTCAAGGCTGAGAATG	60	190	45
	GAGATGATGACCCTTTTGG	60		45

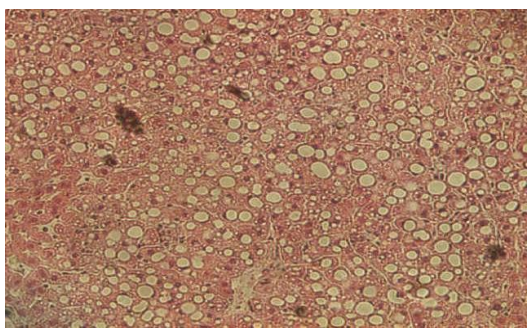
Table2. The results of blood test.

Group	GGT(U/L)	GPT(U/L)	GOT(U/L)	Glucose(mg/dl)	TG(mg/dl)	Total-CHO(mg/dl)	H-DLC(mg/dl)	Total-BIL(mg/dl)
control	10±1.5	19±1.5	66±26.5	96±11	48±7.8	36±11	27±6.8	0.4±0.1
HFD	7±1	17±0.5	54±6.65	95±4	37±17.15	43±3	33±0.5	0.3±0.1
MCD	13±2.5	101±7	209±63	51±11	22±6.24	30±3	18±2.64	0.6±0.1

(A)



(B)



(C)

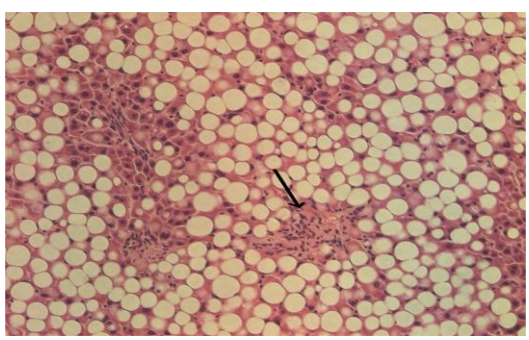


Figure 1. H&E stained liver histopathology in SD rat fed Standard diet (SD) (A), or High –fat diet (HFD) (B) or Methionine-Choline Deficient diet (MCD) (C) for 8weeks. An Arrow indicates gathered inflammation cells.

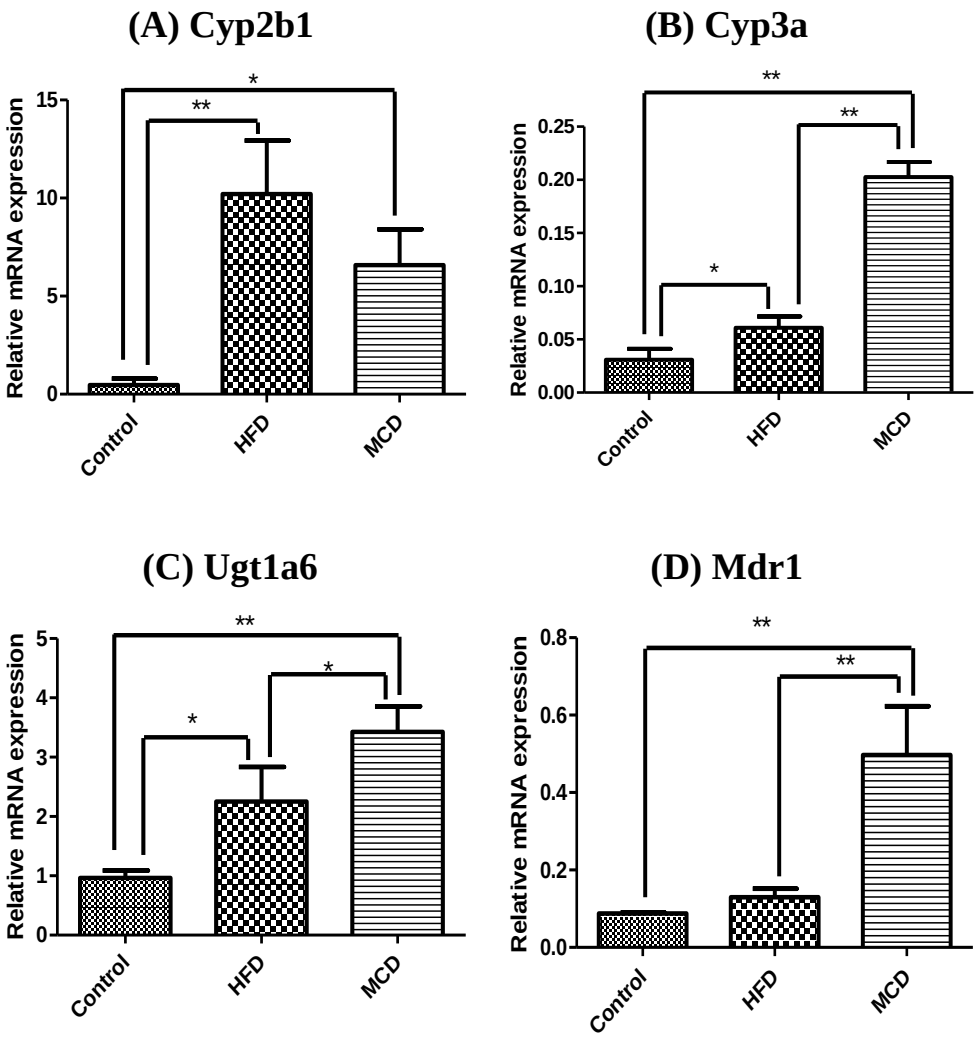


Figure 2. mRNA expression levels of hepatic Cyp2b1 (A), Cyp3a (B), Ugt1a6 (C) and Mdr1 (D) in control, HFD and MCD rats (*n* = 3). * and ** significantly different with $p < 0.05$ and $p<0.01$ respectively

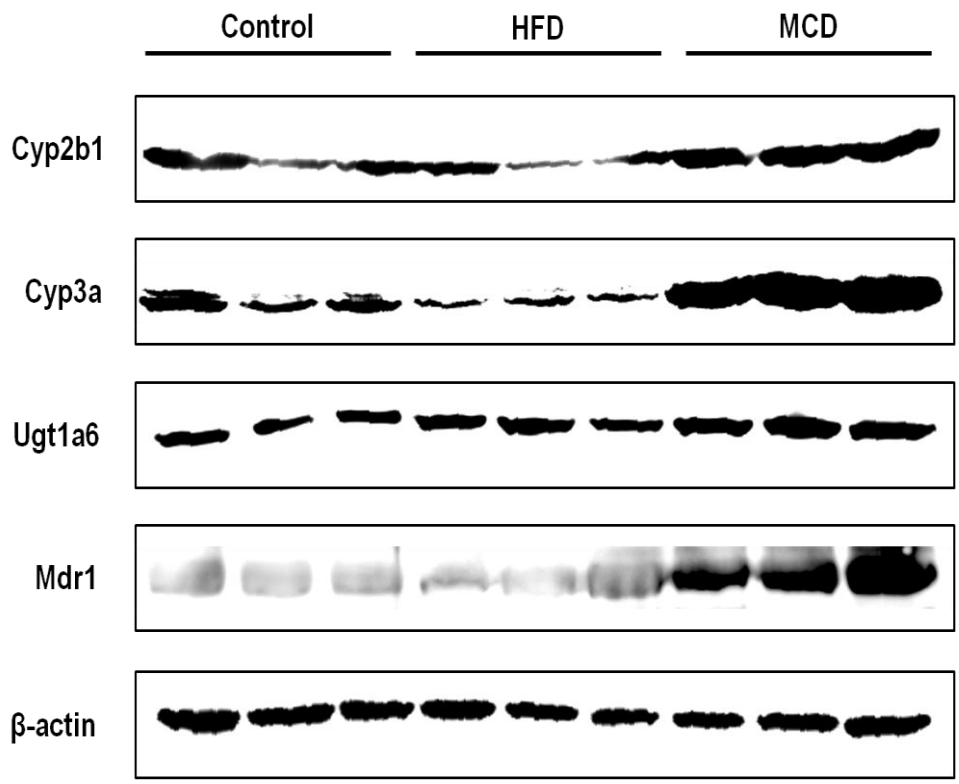


Figure 3. Protein band expression levels of hepatic Cyp2b1, Cyp3a, Ugt1a6, and Mdr1 in control, HFD and MCD rats (*n* = 3).

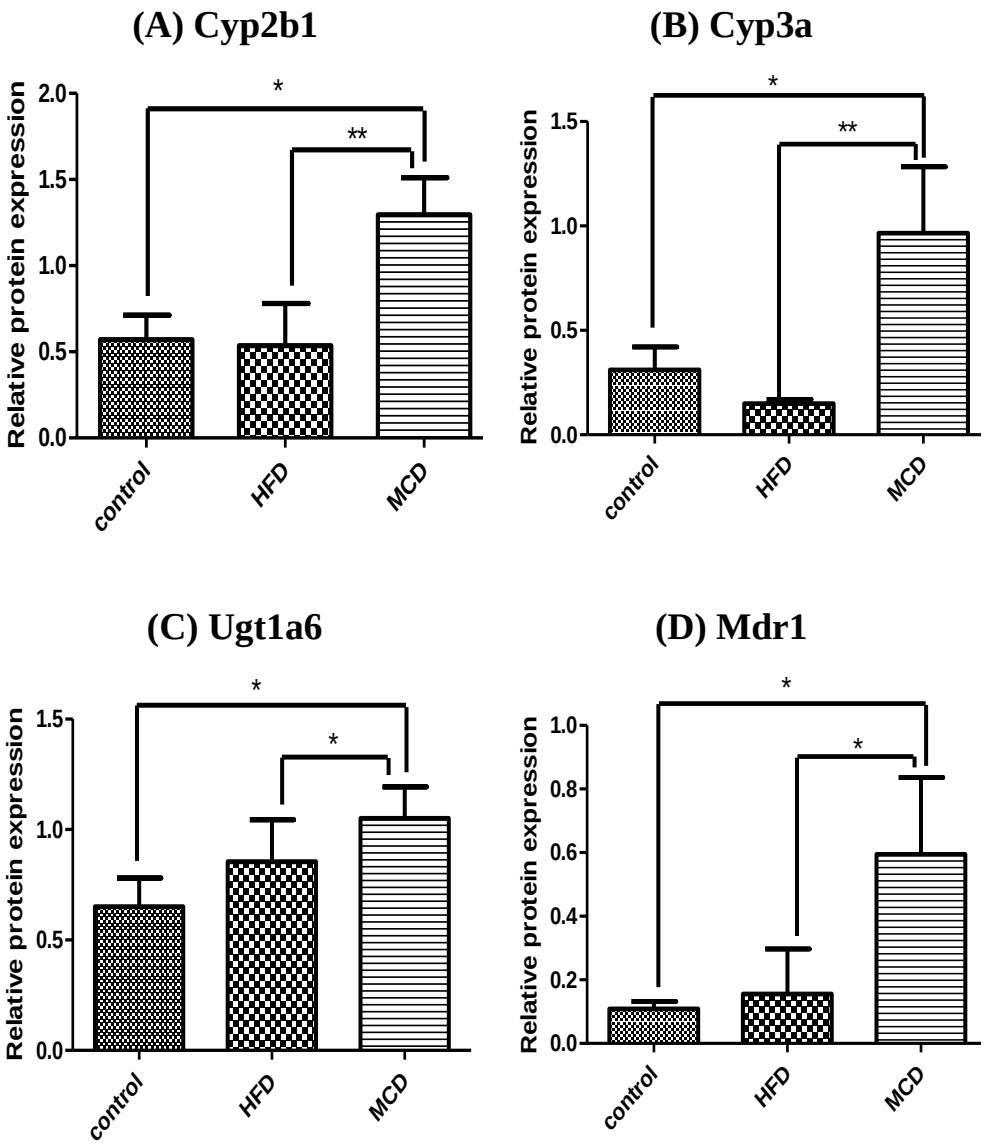


Figure 4. Protein expression levels of hepatic Cyp2b1 (A), Cyp3a (B), Ugt1a6 (C) and Mdr1 (D) in control, HFD and MCD rats (*n* = 3). * and ** significantly different with p < 0.05 and<0.01 respectively.