Original Article
Early changes in blood biochemistry and CYP450 metabolism in a rat model of alcoholic fatty liver disease

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Abstract: Background: Alcoholic fatty liver disease (AFLD) is an early sequel of heavy alcohol consumption. It is the most common alcoholic diseases seens in clinical practice. This study aims to investigate early biochemical changes in blood and changes in metabolic activity of cytochrome P450 (CYP450) in AFLD rats. Methods: Thirty-two Sprague-Dawley Rats were randomly divided into two groups: Control (n=13) and AFLD (n=19). The AFLD model rats were generated by intragastric alcohol administration. Blood biochemical indices of liver, kidney and lipid metabolism were analyzed. The metabolic activity of six CYP450 isoforms were investigated by a Cocktail method. Pathologic changes in liver and mRNA expression of CYP450 were studied to verify the development of the AFLD model. Result: An AFLD model was developed successfully according to morphologic observation. The albumin-globulin ratio, bile acid levels, total cholesterol and high density lipoprotein cholesterol levels were significantly increased. Alkaline phosphatase and apolipoprotein b levels were significantly decreased in the AFLD-group. The $AUC_{(0-t)}$, MRT, $t_{1/2}$ and C_{max} of tolbutamide (one of probe drugs in Cocktail method) were increased in the AFLD-group (p<0.05). RT-PCR showed significant inhibition of mRNA expression level of CYP3A2, CYP3A1, CYP2B1/2, CYP2D1, CYP2A1 and CYP2C11 in the AFLD rats. Conclusion: Lipid metabolism disorders were the first metabolic changes seen in early stages of AFLD in a rat model. Six isoforms of CYP450, particularly CYP2C11, were inhibited in the AFLD rat.

Keywords: Alcohol, liver, rat, cytochrome P450, blood biochemistry

Introduction

Alcoholic liver disease (ALD) is a major cause of morbidity and mortality world-wide, particularly in industrialized and developing countries [1] [2]. In 2012, the World Health Organization estimated that, approximately 6% of all global deaths were attributable to alcohol consumption [3]. Heavy alcohol consumption is positively associated with upper aerodigestive tract cancer [4], hemorrhagic stroke [5] and primary liver [4, 6].

ALD is characterized by degrees of hepatic injury, ranging from alcoholic fatty liver disease (AFLD) to more advanced damage, including hepatitis, fibrosis, cirrhosis, and hepatic carcinoma [7]. AFLD is therefore considered an early response to heavy alcohol consumption. There is extensive published literature on ALD, most

of which concerns severe alcoholic hepatitis, fibrosis, alcoholic cirrhosis and hepatic carcinoma [4-6]. However, in clinical practice, the morbidity associated with AFLD is much more common than the later, more sever stages. Therefore, we believe study the early changes in AFLD would make a useful contribution to the human being.

To date, there are few studies of AFLD, particularly concerning early changesin blood chemistry and metabolic ability of cytochrome P450 (CYP450) enzymes. The blood chemistry of interest includs lipid levels, liver functions and renal functions, all which are important in clinical practice. CYP450 enzymes are a super family that are critical for metabolism of a variety of chemicals, drugs, and endogenous factors [8]. The enzyme is expressed throughout the liver. The most important CYP enzymes, those that

play important roles in drug metabolism in humans, are CYP2A1, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 [9, 10]. The aim of this study is to investigate early blood biochemical change and metabolic ability of CYP450 enzyme in AFLD rats.

Materials and methods

Animals and chemicals

A total of 32 Male Sprague-Dawley (SD) rats, aged 8 weeks (200-250 g) were purchased from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China). All experimental procedures were conducted according to the Animal Care and Use Committee of Wenzhou Medical College, and were in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed individually in standard plastic cages and maintained on normal rat Purina chow and fresh water in a room controlled for temperature (23-25°C, RH 55 ± 10%) with a natural light-dark cycle. After the 1-wk acclimatization period, the rats were used for experiments, Every effort was made to minimize any animal suffering.

Edible 95% alcohol was purchased from Lizhi chemical co., LTD (Production license number QS320015060009, Shanghai, China) and diluted to 40% edible alcohol with ultrapure water. Six probe drugs, including omeprazole, tolbutamide, buproprion, phenacetin, metoprolol and testosterone (all > 98 %) and the internal standard diazepam (IS) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). A Millipore Milli-Q purification system (Bedford, USA) was used to prepare Ultrapure water (resistance > 18 mΩ). HPLC grade methanol and acetonitrile were from Merck Company (Darmstadt, Germany). Trizol was purchased from Invitrogen. Superscript First-Strand Synthesis Kit was from Promega, Madison,WI, USA, and SYBR® Green PCR Master Mix from Applied Biosystems, Warrington, UK. All other chemicals were of analytical grade.

Experimental design

The rats were randomly divided into 2 groups: Alcoholic Fatty Liver Disease (AFLD) group (n=19), and control group (n=13). Rats in the AFLD group received intragastric administration of 40% edible alcohol 0.5 ml/(100 g·d)

twice every day in the first week, 0.8 ml/(100 g·d) twice every day in the second week, 1 ml/(100 g·d) twice every day in the 3-4 week, 1.2 ml/(100 g·d) twice every day in the fifth week. From sixth to fourteenth weeks, the rats were free to drink 40% edible alcohol. Rats in the control group were given normal saline with the same administration scheme.

Early blood biochemistry changes and metabolic activity of CYP450 enzymes were measured in the 4 th week. In the fourteenth week, the development of the AFLD model was validated by pathological examination. The mRNA of CYP450 enzymes was extracted and analyzed.

Blood biochemistry test

In the fourth week, 0.5 mL blood samples were drawn from the caudal vein of each rat, then placed in a tube containing 80 IU heparin. The plasma samples were separated immediately and centrifuged at 8000 rpm for 10 min. Liver function tests, kidney function tests, and lipid levels were analyzed in a fully automated biochemistry analyzer (Hitachi 705/717). The indices of blood chemistries in both groups were analyzed with independent-samples T test by a statistics package for social science (SPSS) 16.0.

Metabolic change of CYP450

Administration of probe drugs: Six probe drugs; omeprazole, tolbutamide, bupropion, phenacetin, metoprolol and testosterone (10, 1, 10, 10, 10, and 10 mg/kg, respectively) were simultaneously administered to rats by gavage. Blood samples (0.3 mL) were then collected from the caudal vein in heparinized 1.5 mL polythene tubes at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h. The samples were immediately centrifuged at 8000 rpm for 5 min. The plasma was separated and stored in -80°C until LC-MS analysis.

Pharmacokinetic analysis: Based on the development of the LC-MS method, the plasma concentrations of six probe drugs were determined and analyzed. The pharmacokinetic parameters of the probe drugs were calculated using a two-compartment model and 1/cc weight coefficients by Drug and Statistics 2.0 software (DAS 2.0). The statistical differences of pharmacokinetic parameters between two groups were analyzed by SPSS 16.0 software.

Table 1. Sequences of primers used for the RT-PCR analysis

Isoforms	Primer sequence (50-30)	
	Forward	Reverse
CYP3A2	TAAAGCCCTGTCTGATGTTG	TAGGTAGGAGGTGCCTTACTCG
CYP3A1	ATGGAGATCACAGCCCAGTC	TAGGTGGGAGGTGCCTTATT
CYP2B1/2	GGCCTCCTCAATTCCTTC	TGTCTGTCCCACATAGCAT
CYP2D1	TGAATTTGCCACGCATCAC	ATCCTTCAGCACGGACGAC
CYP2A1	CACCTCACTGAATGGCTTCC	TCTCACTCAGGGTCTTGTCG
CYP2C11	TGATAGTATCGCTGTCATCC	CAAATCCACTGATAGCTGGT
β-actin	AGAGGGAAATCGTGCGTGAC	TTCTCCAGGGAGGAAGAGG

Pathological observation

In order to validate generation of the AFLD model, all rats were examined by pathological observation. Rats were anesthetized with 10% chloral hydrate (20 mg/kg, i.p.). Liver tissues were then rapidly isolated and fixed in 4% w/v formaldehyde. After fixation, liver tissues were embedded in paraffin to prepare histologic sections which were stained with hematoxylin and eosin according to routine HE methods.

mRNA expression of CYP450

The level of gene expression in liver tissues was analyzed by real-time quantitative PCR. Total RNA was extracted from liver by using Trizol (Invitrogen, CA), and used as the substrate for the reverse-transcription reaction to produce cDNA using a Superscript First-Strand Synthesis Kit (Promega, Madison, WI, USA). All PCR reactions were performed using SYBR® Green PCR Master Mix kit and an ABI 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK). We selected an endogenous housekeeping gene (β-actin) as an internal loading control and normalized the relative expression of all genes to the β-actin mRNA level. The primers of CYP3A2, CYP3A1, CYP2B1/2, CYP2D1, CYP2A1, CYP2C11 and β-actin are displayed in **Table 1**. The differences of mRNAexpression between these six CYP450 isozymes were analyzed by one sample *t* test.

Results

Early blood biochemistry change

A total of 26 indices of blood biochemistry were analyzed in the third week (**Table 2**). Of these, six indices showed statistically significant dif-ferences (P<0.05). These included albumin-globulin ratio, bile acid, total cholesterol, high density lipoprotein cholesterol, alkaline phosphatase and apolipoprotein-b. Levels of bile acid, total cholesterol and high density lipoprotein cholesterol were significantly increased. The level of apolipoprotein-b was decreased, suggesting that lipid metabolism maybe an early blood biochemistry change associated with AFLD.

Pharmacokinetics of probe drugs

The mass spectra and typical chromatograms results suggest no presence of interfering endogenous substances at the retention times of the probe drugs. The UPLC-MS/MS method gave good linearity, accuracy and precision. This method is discussed in detail elsewhere [11, 12].

The concentrations of omeprazole, bupropion, phenacetin, metoprolol and testosterone were all below the lowest detectable limit of the UPLC-MS/MS method at 8 hours. Therefore, the concentration-time profile of these compounds is displayed from 0 to 8 hours (**Figure 1**). According to DAS analysis, the pharmacokinetic parameters of the six probe drugs were calculated and displayed in **Tables 3** and **4**.

There was no obvious statistical difference between groups for omeprazole, bupropion, phenacetin, metoprolol and testosterone with respect to the pharmacokinetic parameters AUC, MRT, $t_{1/2}$, CL, T_{max} and C_{max}. However, there were statistical differences (P<0.05) for tolbutamide; the $AUC_{(0-\infty)}$ and $t_{1/2}$ was increased and CL was shortened in the AFLD group, suggesting that metabolism of tolbutamide was inhibited.

Morphological changes of liver

According to pathological examination *in vivo,* there were significant morphologic differences between the control group and the ALFD group. Under both low and high magnification, there was no morphologic damage observed in the control group. The structure of hepatic lobules, portal area and central vein were normal. Liver

Table 2. Statistical analysis of serum biochemical indexes in rats of Control-group (n=13) and ALFD-group (n=19)

Parameters Mean		Control-group		ALFD-group		P-value
		Mean	SD	Mean	SD	
Total bilrubin	TBIL	2.43	2.36	2.26	2.79	0.86
Indirect bilirubin	IBIL	1.08	2.31	0.43	0.39	0.23
Direct bilirubin	DBIL	1.35	0.53	1.84	2.48	0.49
Alanine aminotransferase	ALT	57.31	17.11	64.21	27.89	0.43
Total protein	TP	71.55	5.42	70.79	6.52	0.73
Albumin	ALB	29.15	3.73	30.13	2.62	0.39
Globulin	GLO	42.41	2.80	40.66	5.10	0.27
Albumin-globulin ratio	A/G	0.67	0.09	0.74	0.08	0.03
Aspartate aminotransferase	AST	244.00	34.80	253.53	91.94	0.73
R-glutamyl transpeptidase	GGT	0.00	0.00	0.05	0.23	0.42
Alkaline phosphatase	AKP	268.77	82.84	206.68	41.18	0.01
Bile acid	BA	11.45	5.72	15.89	5.64	0.04
Creatine kinase	CK	732.62	201.89	654.58	173.53	0.25
Lactic dehydrogenase	LDH	1960.70	261.25	1778.70	406.39	0.17
Hydroxybutyrate Dehydrogenase	HBDH	328.92	87.32	331.32	128.21	0.95
Creatine kinase isoenzyme MB	CK-MB	1403.40	399.51	1210.10	308.64	0.16
Urea nitrogen	BUN	8.35	3.38	7.49	0.79	0.29
Creatinine	CR	20.38	2.06	20.16	6.03	0.90
Uric acid	UA	99.15	18.80	88.63	18.69	0.13
Total cholesterol	TCHO	1.56	0.22	1.78	0.34	0.03
Triglyceride	TG	1.39	0.24	1.40	0.20	0.91
High density lipoprotein cholesterol	Hdl-c	0.93	0.15	1.09	0.20	0.02
Apolipoprotein A1	Apo-A1	0.0015	0.0038	0.0005	0.0023	0.3510
Apolipoprotein b	Apo-b	0.0231	0.0086	0.0153	0.0117	0.0490
Low density lipoprotein cholesterin	LDL-C	0.38	0.04	0.36	0.07	0.33
The ratio of aspartate aminotransferase to alanine aminotransferase	ALT/AST	0.71	0.23	0.79	0.14	0.22

cells were tightly connected with one other and arranged radially along central veins.

Deapite the absence of obvious structural disturbances such as regenerative nodules and fibrosis, local lesions were observed in the AFLD group. Fat vacuoles were clearly seen in some of liver cells However, there was no ballooning degeneration or alcoholic hyaline of Mallory observed. The structures of hepatic lobules were intact and could be recognized easily. No infiltrating inflammatory cells were found in the portal area and hepatic sinusoid. The nuclei of liver cells were round, clear and displayed fine luster. In short, fatty degeneration developed in the AFLD group (**Figure 2**).

Expression of CYP450 mRNA

The effects of alcohol consumption on mRNA expression of CYP450 isoforms is shown in **Figure 3**. After pretreatment with long-term alcohol consumption, mRNA expression of CYP3A2, CYP3A1, CYP2B1/2, CYP2D1, CYP2A1 and CYP2C11 was significantly inhibited (P< 0.05). These data suggest that long-term alcohol consumption can induce the inhibition of several CYP enzymes at the mRNA level, which may result in a decrease in enzymatic activity.

Discussion

According to the analysis of indices of blood biochemistry, the albumin-globulin ratio, bile acid, total cholesterol and high density lipoprotein cholesterol levels were significantly increased, but the level of alkaline phosphatase and apolipoprotein-b were significantly decreased. This suggests that lipid metabolism may be an early change in AFLD. There was a significant morphologic difference between the control group and the ALFD group. Most heavy drinkers do not progress beyond steatosis of the liver. A few studies indicated that approxi-
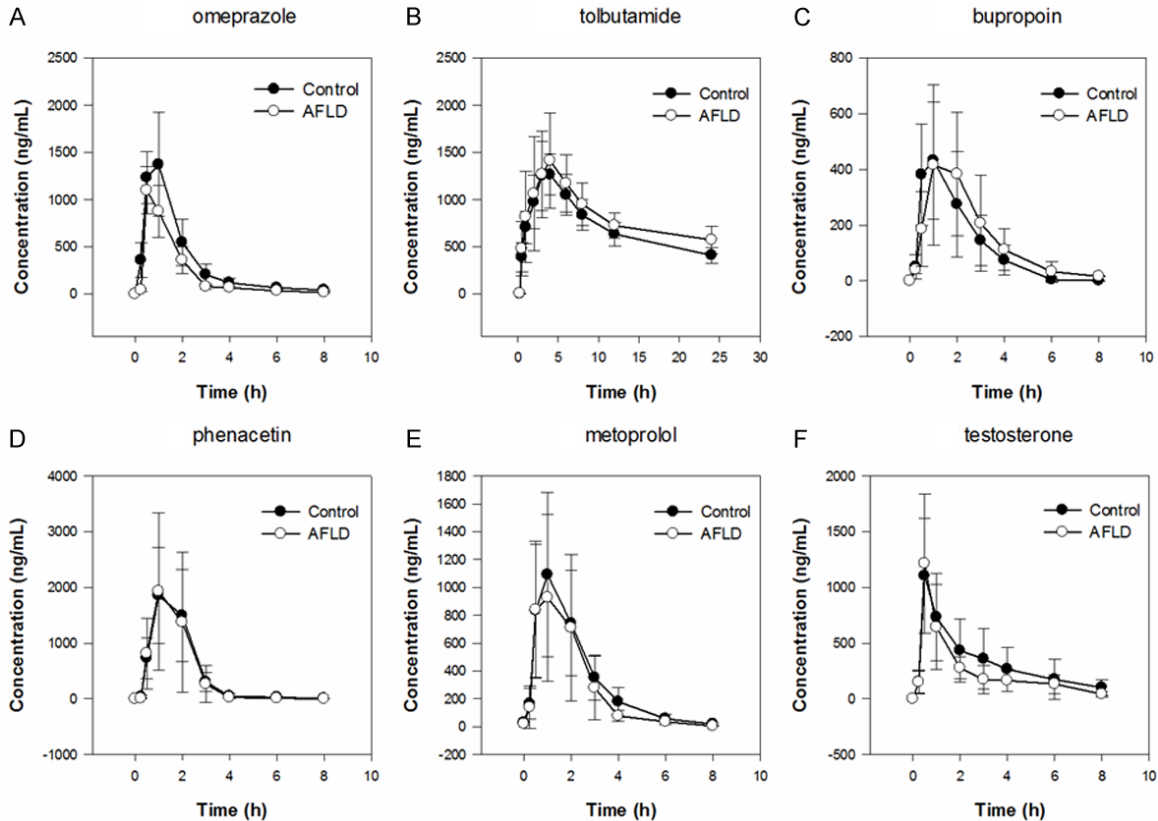
Figure 1. The pharmacokinetic parameters of six probe drugs in rats omeprazole (A), tolbutamide (B), bupropion (C), phenacetin (D), metoprolol (E), testosterone (F), Data were presented as mean ± SD in each group.

mately 8%-20% of heavy alcohol consumers with steatosis progress to steatohepatitis, fibrosis and cirrhosis, and some (3%-10%) ultimately develop hepatocellular carcinoma (HCC) [2, 13]. Thus early diagnosis of AFLD is important for the prevention of alcoholic liver disease. However, numerous studies suggest that other contributing factors, such as hepatitis virus infection [14], smoking [15], diabetes [16] and genetics, influence development and progression of AFLD [17]. Despite important advances having been made over the last several years, modifying factors and the mechanisms in the pathogenesis of alcoholic liver injury remain largely unknown. Because of its complex pathogenic mechanisms, there are still no effective treatments. Many patients are diagnosed at advanced stages of ALD, but few effective programs for early diagnosis exist.

CP450 is widely present in many tissues, such as liver, lung, kidney, brain, heart, and intestine [9, 18]. A study by Gramenzi A, et al. has shown that alterations in expression of ethanol metabolizing enzymes gave a predisposition to AFLD [7]. Several studies suggest that CYP2E1,

which catalyzes the oxidation of alcohol to acetaldehyde [19], plays an important role in the metabolism of alcohol [13, 20]. CYP2E1 catalyzes the oxidation of alcohol to acetaldehyde and acetaldehyde to acetate, which generates several reactive oxygen species such as superoxide, hydroxyl radicals, hydroxyethyl radicals and hydrogen peroxide [19]. Tsedensodnom O, et al. found homologous ADH and CYP2E1 expression in zebrafish liver and their metabolism of alcohol, which results in hepatic damage [21]. However, few studies have focused on the relationship between AFLD and other CYP enzymes, such as CYP3A2, CYP3A1, CYP2B1/2, CYP2D1, CYP2A1 and CYP2C11.

To our knowledge, our study is the first to report toxicological effects of heavy alcohol consumption on CYP450 enzymes in rats *in vivo*. According to the pharmacokinetics of probe drugs, the metabolism of tolbutamide was decreased in the ALFD group, because the $AUC_{(0-t)}$ and C_{max} were decreased, but $MRT_{(0-\infty)}$, $t_{1/2}$ and Vz/F were all increased. Furthermore, CLz/Fof omeprazole and Vz/F of testosterone were increased and $MRT_{(0-t)}$ of bupropion was

Table 3. Pharmacokinetic parameter of Six probe drug in Control-group rat

Parameters		Bupropion	Phenacetin	Metoprolol	Tolbutamide	Omeprazole	Testosterone
$AUC_{(0-t)}$	ug/L*h	1002.60 ± 506.42	3618.58 ± 1473.15	3305.24 ± 1318.44	17003.399 ± 2241.73	2665.08 ± 311.98	2594.82 ± 792.15
$AUC_{(0-\infty)}$	ug/L*h	1077.03 ± 537.84	3630.51 ± 1475.84	3762.37 ± 1248.14	24426.587 ± 8796.03	2737.04 ± 2313.56	3453.83 ± 1548.38
$MRT_{(0-t)}$	h	1.78 ± 0.32	1.69 ± 0.14	2.61 ± 0.38	9.782 ± 0.67	1.68 ± 0.41	2.47 ± 0.64
$MRT_{(0-\infty)}$	h	2.10 ± 0.45	1.72 ± 0.15	3.34 ± 0.87	22.455 ± 13.62	2.06 ± 0.73	3.32 ± 1.76
$t_{1/2}$	h	1.17 ± 0.49	0.88 ± 0.40	1.80 ± 0.59	13.556 ± 10.51	1.33 ± 0.81	2.09 ± 1.48
T_{max}	h	0.89 ± 0.42	1.15 ± 0.38	1.85 ± 0.80	3.462 ± 1.05	0.73 ± 0.26	1.04 ± 0.99
CLz/F	L/h/kg	11.70 ± 5.62	3.16 ± 1.17	2.94 ± 0.99	0.044 ± 0.01	4.81 ± 1.85	3.46 ± 1.49
Vz/F	L/kg	19.48 ± 10.84	3.95 ± 2.48	7.78 ± 3.40	0.743 ± 0.27	8.86 ± 5.47	10.73 ± 9.85
C_{max}	ug/L	483.85 ± 186.46	1903.71 ± 802.52	1149.23 ± 564.02	1396.875 ± 255.80	1562.88 ± 1242.35	1185.10 ± 495.43

Table 4. Pharmacokinetic parameter of Six probe drug in AFLD-group rat

| Parameters		Bupropion	Phenacetin	Metoprolol	Tolbutamide	Omeprazole	Testosterone
$AUC_{(0-t)}$	ug/L*h	1196.41 ± 658.21	3504.98 ± 2848.73	2851.76 ± 1581.42	19634.62 ± 3666.75	1732.29 ± 1187.35	2029.15 ± 796.28
$AUC_{(0-\infty)}$	ug/L*h	1219.20 ± 662.42	3506.62 ± 2848.24	2981.05 ± 1583.32	52956.99 ± 39411.13[*]	1757.86 ± 1194.60	2651.83 ± 1355.53
$MRT_{(0-t)}$	h	2.33 ± 0.37	1.56 ± 0.17	2.60 ± 0.54	10.49 ± 1.43	1.58 ± 0.40	2.19 ± 0.38
$MRT_{(0-\infty)}$	h	2.50 ± 0.51	1.58 ± 0.18	2.92 ± 0.81	52.14 ± 43.74	1.84 ± 0.55	3.58 ± 1.59
$t_{1/2}$	h	1.14 ± 0.50	0.59 ± 0.30	1.29 ± 0.66	34.78 ± 30.46[*]	1.29 ± 0.65	3.97 ± 3.43
T_{max}	h	1.11 ± 0.33	1.00 ± 0.00	2.11 ± 0.60	4.56 ± 2.01	0.61 ± 0.22	0.56 ± 0.17
CLz/F	L/h/kg	10.23 ± 4.88	6.11 ± 5.98	4.41 ± 2.55	0.03 ± 0.01[*]	8.90 ± 6.09	4.59 ± 2.05
Vz/F	L/kg	17.03 ± 12.03	7.26 ± 11.08	10.01 ± 10.21	0.84 ± 0.33	14.88 ± 10.59	21.51 ± 11.13
C_{max}	ug/L	422.49 ± 246.94	1930.40 ± 1409.21	980.26 ± 564.97	1467.69 ± 448.44	1114.91 ± 648.99	1282.64 ± 557.93

[*]Compared with Control group P<0.05
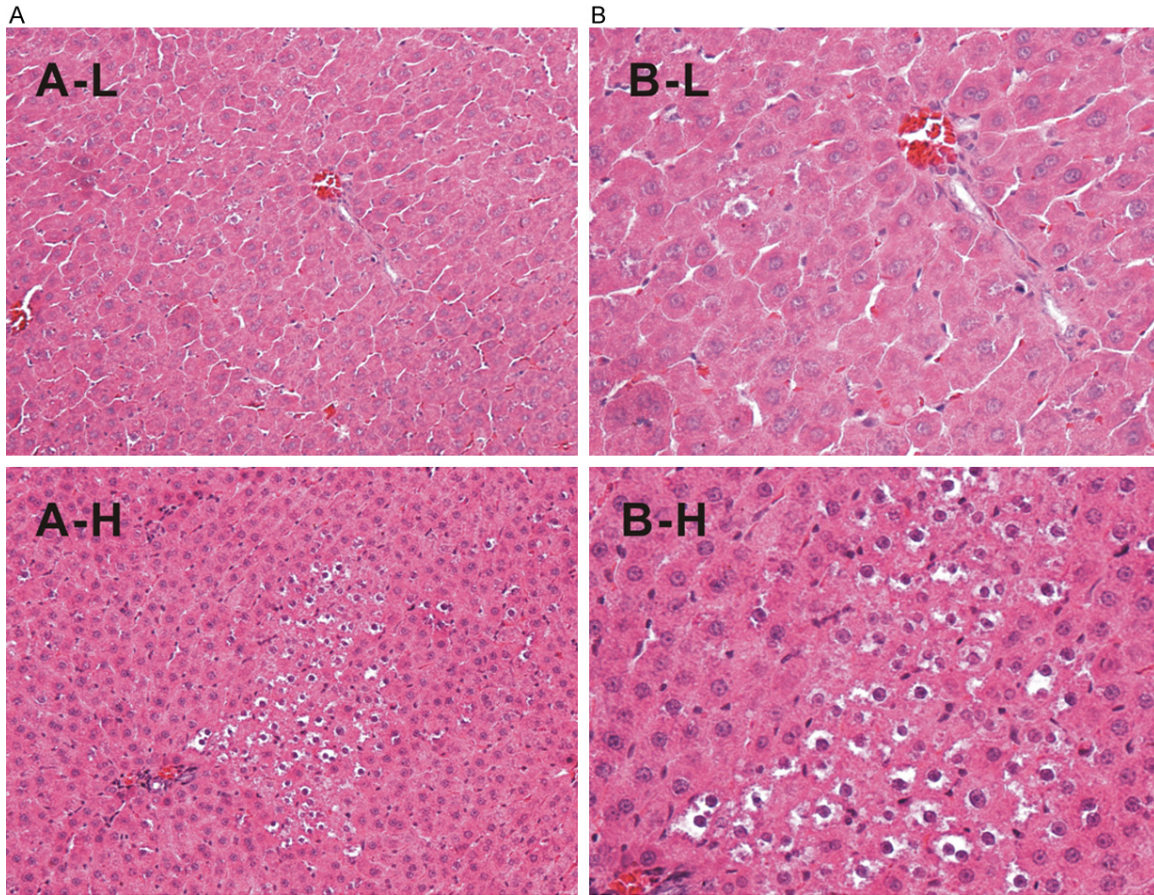
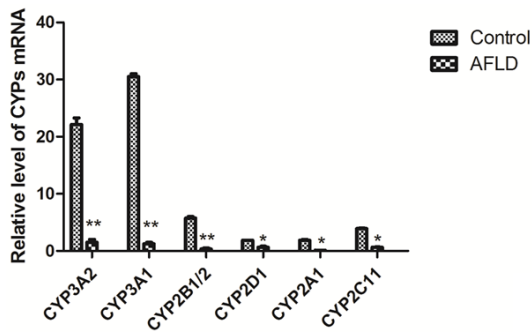Figure 2. Morphological changes of liver in Control-group (A) and AFLD-group (B) (hematoxylin-eosin, L×100, H×400).

Figure 3. Expression of CYP450 mRNA (* Compared with Control group P<0.05, ** Compared with Control group P<0.01).

increased. The effects of heavy drinking on probe drugs of CYP2C11 metabolism suggest that heavy drinking had significant inhibition effects on CYP2C11 while CYP3A2, CYP3A1, CYP2B1/2, CYP2D1 and CYP2A1 were not significantly influenced. However, in the analysis of expression of CYP450 mRNA, as a result of heavy alcohol consumption CYP3A2, CYP3A1, CYP2B1/2, CYP2D1, CYP2A1 and CYP2C11

were all inhibited. The mRNA expression levels of CYP isoforms were not in accordance with pharmacokinetics, with the exception of CYP 2C11. This suggests that, in the early stage of ALD, toxicological effects of heavy alcohol consumption is largely reflected by mRNA levels of CYP isoforms but not in protein levels. Further studies are needed to investigate the underlying mechanism of the toxicological effects of heavy alcohol consumption on protein levels of CYP isoforms.

Conclusion

A rat model of AFLD was generated by intragastric alcohol administration. The lipid metabolism disorders were the first metabolic change in early stage of AFLD rat. Moreover, six isoforms of CYP450, particularly CYP2C11, were inhibited in the AFLD rat.

Acknowledgements

Disclosure of conflict of interest

None.

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