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The Study of Alcoholic Liver Diseases

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The Study of Alcoholic Liver Diseases

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by

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ABSTRACT

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Excessive alcohol consumption is the primary contributing factor in the development of alcoholic liver diseases (ALD). Nicotine contained in tobacco is a major addictive alkaloid, which enhances the effects of ALDs. The major enzyme involved in nicotine metabolism is cytochrome P450 2A5 (CYP2A5) which is produced in the liver. Alcohol can stimulate the CYP2A5 enzyme. We utilized *cyp2a5*$^{-/-}$ knockout mice in this research to examine the effects of CYP2A5.

The *cyp2a5*$^{-/-}$ mice and wild-type (WT) mice were fed liquid ethanol diet with or without nicotine to induce ALD. Nicotine enhancing effects on ALD were observed in WT mice but not in *cyp2a5*$^{-/-}$ mice. Oxidative stress was stimulated by alcohol and further increased by nicotine in WT mice but not in *cyp2a5*$^{-/-}$ mice. Microsomal ROS production during microsomal metabolism of nicotine was increased in WT mice but not in *cyp2a5*$^{-/-}$ mice. These results suggest that nicotine enhances ALD is CYP2A5 dependent.

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CHAPTER 1

INTRODUCTION

The effects of excessive consumption of alcohol in the United States cost an estimated $249 billion in 2010, which is equivalent to $807 per person. The costs of excessive drinking extend beyond health implications. Alcohol abuse results in workplace losses and decreased output and productivity. Approximately 72% of total loss is related to criminal activities and motor vehicle crashes. Healthcare constitutes 11% of cost, while the remaining costs are varied across many widespread categories. (CDC 2015). According to a report from National Survey on Drug Use and Health (NSDUH) published in 2014, 139.7 million current alcohol users are 12 years of age or older, with 23% being binge drinkers and 6.2% being heavy daily drinkers. Excessive use of alcohol increases a risk of developing serious health problems, including brain and liver damage, heart disease, hypertension, and fetal damage in pregnant women (NSDUH 2014). Alcohol abuse is the third largest factor resulting in disability, diseases, and death. An estimated I 2.5 million deaths can be directly attributed to alcohol consumption each year, which is the equivalent of 4% of the total deaths worldwide (CDC 2015).

Alcoholic Liver Disease (ALD) is one of the major problems affecting the world and is the primary cause of at least 60 major types of systemic diseases, according to the World Health Organization on alcohol in the year 2011 (WHO 2011). Daily consumption of 60–80 g of alcohol in men and 20 g in women for a period of 10 years or longer will cause an advanced form of liver disease in about 40% of those contracting liver diseases (Alba et al. 2014). ALD is a major cause of severe liver injuries all over the world. In the United States, 48% of cases of cirrhosis associated liver diseases result in mortality.

Alcohol Dehydrogenase (ADH) is a major alcohol metabolism enzyme. This enzyme breaks down alcohol in the liver to form acetaldehyde and free radicals, which cause ALD. Aldehyde dehydrogenase (ALDH) converts acetaldehyde to acetate. The acetate will be further metabolized into carbon dioxide and water which releases energy (Lu and Cederbaum 2009).

The cytochrome P450 enzymes (CYP) functions as a metabolic synthesizers and degrader of endogenous molecules in cells. Examples of endogenous molecules include cholesterol, bile acids, steroid hormones, and fatty acids. The enzymes can also degrade or metabolize xenobiotic substances. Furthermore, Cytochrome P450 2E1, also known as CYP2E1, is also a part of the mixed–function oxidase system of cytochrome P450/CYP2E1 involved in the metabolic processes of xenobiotic substances in the body.

CYP2E1 can be induced by alcohol consumption and has been known to play the leading role in the development of ALD (Lu et al. 2011). Cytochrome P450 2A6 (CYP2A6) is also a part of the mixed–function oxidase system of cytochrome P450. CYP2A6 is the main enzyme responsible for the oxidation of xenobiotic substances including cotinine and nicotine and other forms of coumarin-type alkaloids in the body. CYP2A6 enzymes are primarily found and stimulated in humans. CYP2A6 is elevated in alcoholic patients and effects their hepatic system. CYP2A5, a mouse orthologue of human CYP2A6, is also alcohol inducible, and alcohol induction of CYP2A5 is CYP2E1 dependent (Lu et al. 2008).

There are many crucial factors that contribute to ALD susceptibility including diet, heredity, gender, environment and other comorbidities. Tobacco smoking can enhance ALD, and the nicotine present in tobacco causes it to be very addictive (NIDA 2018). The average cigarette produces 2mg of absorbed nicotine, a major component of tobacco. The concentration of 50-100mg of nicotine can be very harmful to human health (Mayer 2014). Nicotine ingestion can affect both e-cigarette and typical cigarette smokers. The ingestion of nicotine can increase pro-inflammatory cytokine production, which can lead to liver injury. CYP2A5 is a major nicotine metabolic enzyme (Raunio et al. 2008).

In this study, we investigated the effects of CYP2A5 on nicotine, ethanol, and ethanol-nicotine combined that may lead to oxidative stress, which can cause nicotine-enhanced ALD.

Hypothesis and Aims

Aims:

- To determine the induction of CYP2A5 in hepatic microsomes.
- To determine ROS generation in hepatic microsomes.
- To examine whether CYP2A5 affects nicotine-enhanced ALD.

Hypothesis:

- The nicotine-induced ROS generation will be lower in knock-out (*cyp2a5*$^{-/-}$) mice than in wild type (WT) mice
  Nicotine-enhanced alcoholic fatty liver is CYP2A5-dependent.

CHAPTER 2
MATERIAL AND METHODS

Animals

C57BL/6 mice were used for this study. The female mice were selected because they are more susceptible to liver damage than male mice. All the mice were supplied by Dr. Xinxin Ding, SUNY College of Nanoscale Science and Engineering, Albany, NY, United State of America.

Liquid Lieber-DeCarli Diet Preparation and Feeding

The Lieber-Decarli dextrose diets were made by mixing with appropriate volume of water alone and Lieber-Decarli ethanol liquid diets were mixed with water and ethanol based on the manufacturer's prescription. The dextrose liquid diet and the ethanol liquid diet contain equal number of calories. The control Lieber-Decarli liquid diet was initially fed to all the mice for a period of 3 days allowing the mice to adapt to the liquid diet. The control groups continued with the dextrose diet and the ethanol groups were switched to ethanol diets. The calories from ethanol were gradually increased from 10% to 15%, 20%, 25%, 30%, and finally 35%. All the mice in the ethanol groups were fed each concentration for 3 days for a total 18–day period. The nicotine groups were fed Nicotine hydrogen tartrate salt mixed with the appropriate liquid diets at 30 mg/L. All the mice have free access to food sources for each group. All the mice were sacrificed after 6 h of fasting. The livers were collected and aliquoted for pathological evaluation and biochemical assays.

Grouping and Treatments

Table 1. Grouping and Treatments

Group	**Treatment**	**Number**
WT mice	Dextrose diet	4

WT mice	Dextrose-nicotine diet	4
WT mice	Ethanol diet	4
WT mice	Ethanol-nicotine diet	4
cyp2a5$^{-/-}$ mice	Dextrose diet	4
cyp2a5$^{-/-}$ mice	Dextrose-nicotine diet	4
cyp2a5$^{-/-}$ mice	Ethanol diet	4
cyp2a5$^{-/-}$ mice	Ethanol-nicotine diet	4

Homogenization of Liver

The liver samples (about 200 mg) were placed in 1.5 ml of KCL (0.15 M) in test-tubes and were homogenized by gentle up and down motions for 45 seconds by using Servodyne Mechanical Mixer Controller.

Isolation and Preparation of Hepatic Microsomes

The homogenates were centrifuged at 9,000 × g at 4°C for 20 minutes to exclude nuclei and mitochondria. The supernatants were further centrifuged at 105,000 × g at 4°C for 60 minutes, and the pellets containing microsomes were re-suspended in a 50 mM sodium phosphate buffer (pH7.4) on ice.

Protein Assay

Bovine serum albumin (BSA) stock solution was prepared at 2 mg/ml, which was then serially diluted to concentrations of 1.0, 0.5, 0.25 mg/ml. The BSA and samples were added into each test tube, while Reagent A and reagent B (Bio-Rad) were added to each test tube, respectively. Each test tube was incubated for 10 minutes for each step and the absorbance was measured using a spectrophotometer at a wavelength of 750 nm. A standard curve is shown in Fig. 1, in which case protein concentration in samples (mg/ml) = 6.7491 × OD -0.6795.

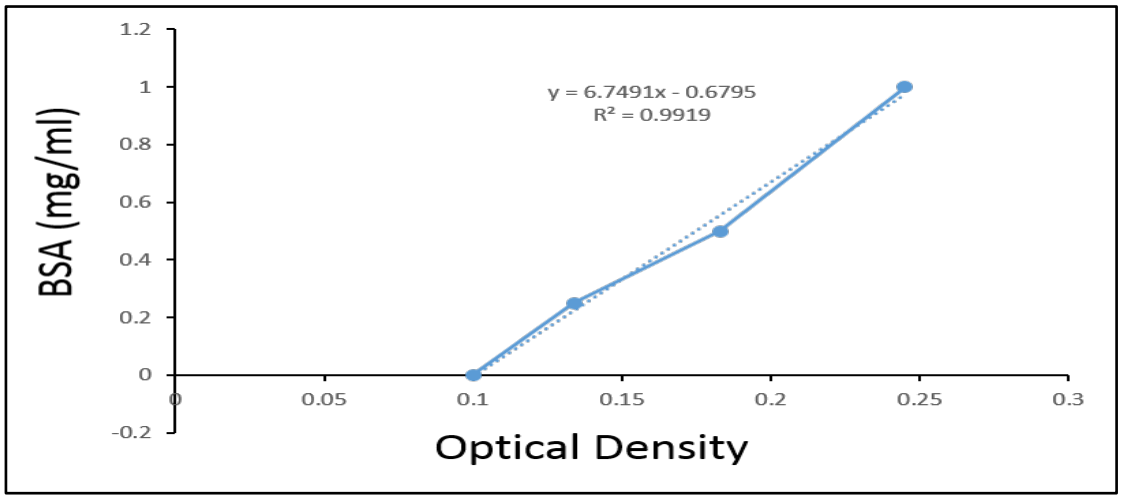

Fig 1. BSA standard curve

Microsomal CYP2A5 Activity Assay

Assay systems containing 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 µM coumarin (substrate of CYP2A5) and 200 µg of microsome (CYP2A5 enzyme) in 100 µl 0.1 M Kpi were incubated at 37°C in a water bath for 10 minutes, and then 30 µl 20% (w/v) trichloroacetic acid (TCA) was added to stop the reaction and to precipitate the protein. After a 3000 × g centrifugation at 4°C for 10 minutes, the resulting supernatants (100 µl) were mixed with 2 ml of 1.6 M glycine-NaOH, pH 10.3, and the fluorescence at 390/440 (emission/absorption) was read in a fluorescence spectrophotometer, which is described in the following section 2.7.

Fluorescence Spectrometer Machine

A fluorescence spectrometer machine measures the emitted fluorescent light produced from a sample at different wavelengths (Fig. 2). The fluorescence spectrum of the sample is recorded, which involved the scanning of the emission monochromator after excitation light with a constant wavelength. Fluorescence emission can be measured at right angles by the incident beam, short-lived emission that occur are known as fluorescence. (Kommu and Naresh 2014). It measures the change based on the range of the wavelength. It can be used to determine specifically the presence of molecules of

interest in a medium. In this research, it was used to measure CYP2A5 and ROS activities in the microsomes.

ROS Production by Nicotine in Microsomes

ROS assay systems containing 0.5 mM NADPH, 10 μM nicotine, 50 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA), and 200 μg of microsomes in 100 μl 0.1 M Kpi were incubated at 37°C in a water bath for 10 minutes, and then 30 μl 20% TCA were added to stop the reaction and to precipitate the protein. After a 3000 × g centrifugation at 4°C for 10 minutes, the resulting supernatants (100 μl) were mixed with 2 ml of 1.6 M glycine-NaOH, pH 10.3, and the fluorescence at 503/529nm (emission/absorption) was read in the fluorescence spectrophotometer described in the section 2.7.

Liver Histology and Immuno-histochemical Staining (IHC)

Unstained blank paraffin liver sections (5 μm) and hematoxylin & Eosin staining were performed by the Department of Pathology, Quillen College of Medicine at ETSU. The liver sections were subjected to de-paraffin with xylene followed by hydration. For IHC staining, the liver sections were autoclaved at 121°C for 20 minutes to retrieve antigen. The endogenous peroxidase was treated with 3% hydrogen peroxide solution for 20 minutes followed by being blocked with 10% normal goat serum-PBS for 30 minutes at room temperature. The anti-3-NT and anti-MDA antibodies (dilution of 1: 250 in PBS) were incubated on the liver sections in a wet chamber at 4℃ for overnight. Biotinylated secondary antibody and streptavidin were incubated on the samples at room temperature for 30 minutes, respectively. The liver sections were washed 3 times with PBS after each incubation. The DAB staining reaction was performed followed by counter staining with hematoxylin.

Serum ALT Assay

The whole blood of each mouse was centrifuged at 6600 × g for 10 minutes for serum separation. 10 μl of each sample were mixed with 50 μl of ALT reagent and incubated at 37°C for 30 minutes following incubating with DNPH color solutions at 37°C for 10 minutes.

Furthermore, 200 µl of NaOH (0.5M) were added to each well and incubated at 37°C for 5 minutes and absorbance for each sample was read at wavelength of 510 nm.

Liver Triglyceride Protocol

Standard preparation table 96-well procedure

Table 2. Liver Triglyceride standard preparation

No	1mM STD + H_2O	Vol (µl)	Glycerol (mM)
1	50 µl + 950 µl	1000	0.050
2	30 µl + 970 µl	1000	0.030
3	15 µl + 985 µl	1000	0.015
4	0 µl + 1000 µl	1000	0

The 10 µl of liver homogenates and 10 µl of the standard were pipetted into separate plates. The addition of 100 µl of working reagent was pipetted to each of the plates and mixed. The plates were incubated for 20 minutes at room temperature and read at 530 nm in a spectrophotometer.

Statistics

Results for these experiments were expressed as mean ± SEMs. The statistical evaluation was done by using one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test to show differences between the groups using IBM SPSS Statistics Data Editor. $P<0.05$ was considered as statistical significance.

CHAPTER 3
RESULTS

CYP2A5 Induction by Ethanol

After ethanol feeding, activities of CYP2A5 were increased by 3 times in microsomes from WT mice, but the addition of nicotine did not further increase CYP2A5 activities. Nicotine alone did not induce CYP2A5 in WT mice (Fig. 3). CYP2A5 were nearly undetectable in microsome from *cyp2a5*$^{-/-}$ mice, either fed with dextrose control diets or with ethanol diets (Fig. 3). These results suggest that ethanol induces CYP2A5 in WT mice, and nicotine has no effects on the ethanol induction of CYP2A5.

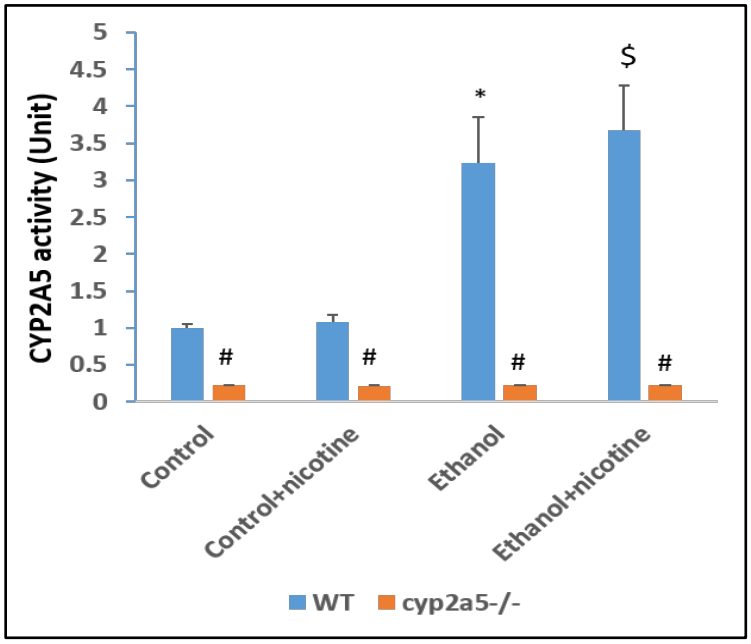

Fig 3. CYP2A5 activity in WT and *cyp2a5*$^{-/-}$ mice. * P<0.05, compared with Control group; # P<0.05, compared with corresponding WT group; $ P<0.05, compared with Control +nicotine group.

Microsomal ROS Production by Nicotine

In the presence of ROS, DCF-DA converts to fluorescent DCF, which is detectable at 503/529nm (Bai et al., 1999). The conversion of DCF–DA to DCF during nicotine metabolism in microsomal systems was measured to evaluate ROS production. Nicotine incubation of microsomes from WT mice resulted in a 4-time elevation of DCF fluorescence, but microsomes from c*yp2a5*$^{-/-}$ mice did not produce DCF (Fig. 4). These results suggest that microsomal CYP2A5 metabolizes nicotine and produces ROS.

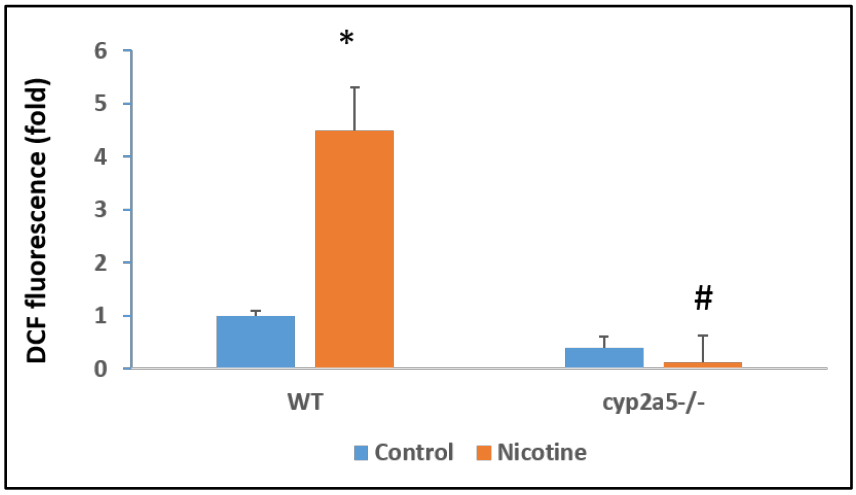

Fig4.ROS Production in Microsomes by Nicotine. * P<0.05, compared with WT Control group; # P<0.05, compared with WT Nicotine group.

Fatty Liver is Enhanced by Nicotine

All four diet groups were compared between the WT and *cyp2a5*$^{-/-}$ mice respectively. Liver sections with H&E staining were used for pathological evaluation. After ethanol feeding, lipid droplets on the liver sections from the WT mice were comparable with those from *cyp2a5*$^{-/-}$ mice (Fig. 5). Consistently, TG accumulations in liver were increased equally in both WT mice and *cyp2a5*$^{-/-}$ mice (Fig. 6). However, after feeding with nicotine plus ethanol, lipid droplets on the liver sections from the WT mice

were larger than those from *cyp2a5*$^{-/-}$ mice (Fig. 5). Likewise, TG accumulations in liver were increased in WT mice but not in *cyp2a5*$^{-/-}$ mice (Fig. 6). These results suggest that nicotine enhanced alcoholic fatty liver in WT mice but not in *cyp2a5*$^{-/-}$ mice.

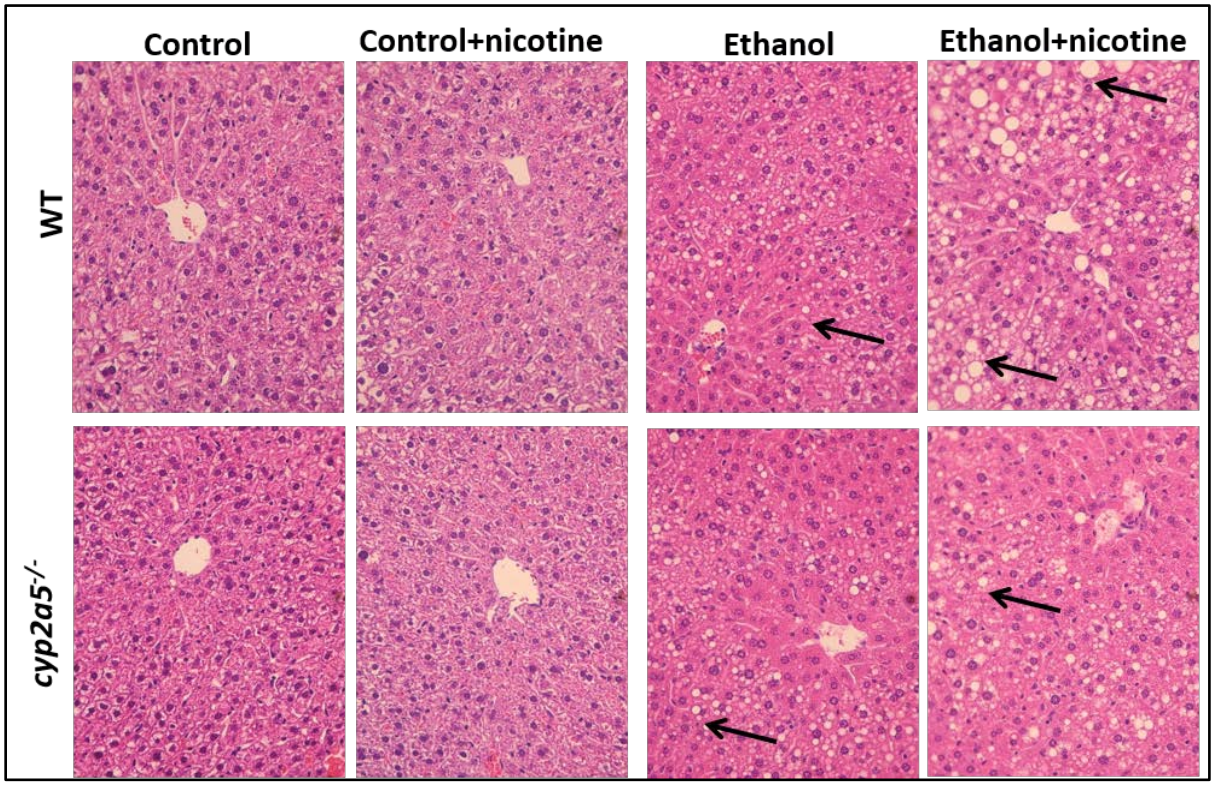

Fig 5. Nicotine enhanced ethanol-induced fatty liver in WT mice but not in *cyp2a5*$^{-/-}$ mice. H&E staining in liver sections. The black arrows show macro-vesicular lipid droplets.

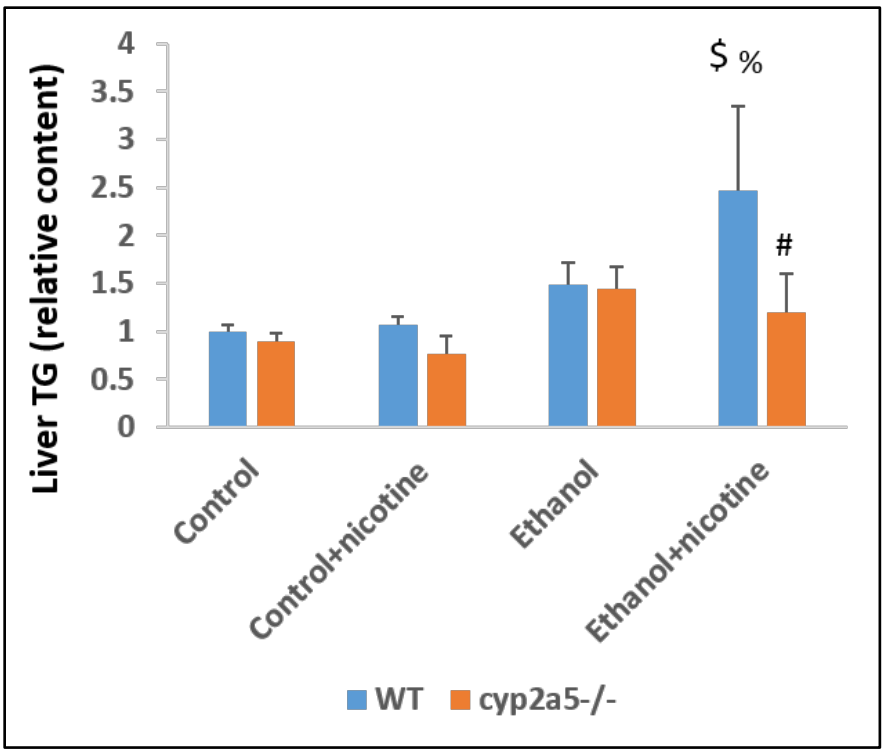

Fig 6. Nicotine plus ethanol increased hepatic TG contents in WT mice but not in *cyp2a5*$^{-/-}$ mice. $ P<0.05, compared with Ethanol group; % P<0.05, compared with Control+ Nicotine group; # P<0.05, compared with WT group.

Ethanol feeding induced serum ALT in WT mice to a similar extent as in *cyp2a5*$^{-/-}$ mice (Fig. 7). However, nicotine did not further increase serum ALT in both WT and *cyp2a5*$^{-/-}$ mice (Fig. 7), suggesting that nicotine did not enhance ethanol-induced liver injury.

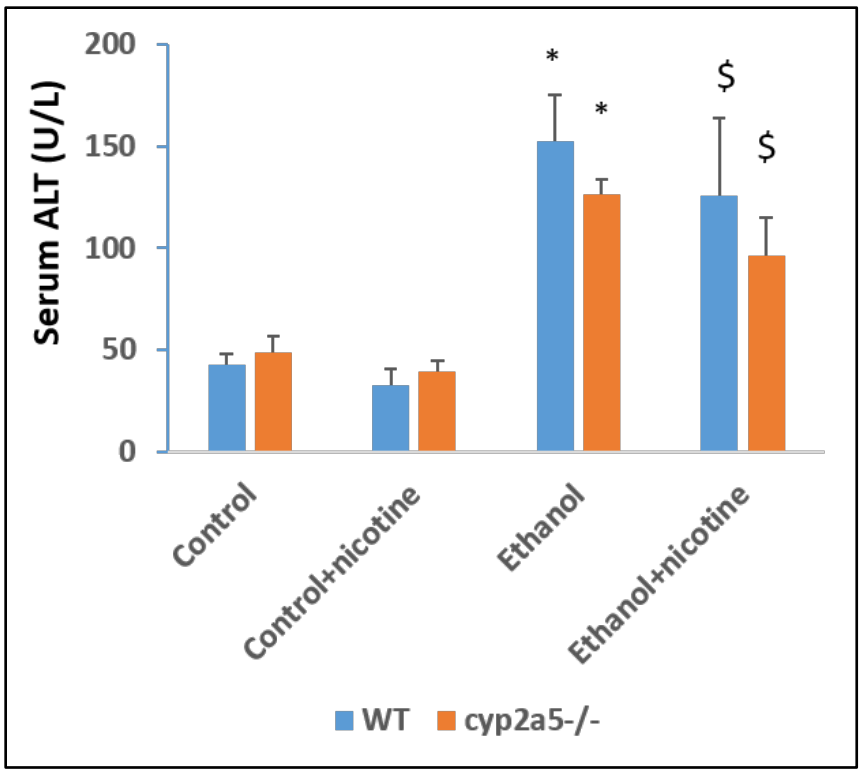

Fig 7. Nicotine did not further increase serum ALT in both WT and *cyp2a5*$^{-/-}$ mice. $ P<0.05, compared with control +nicotine group; * P<0.05, compared with control group.

Alcohol-induced Oxidative Stress is Enhanced by Nicotine

Alcohol-induced oxidative stress plays an important role in the development of ALD (Cederbaum et al. 2009). The 3-nitrotyrosine (3-NT) and malondialdehyde (MDA), markers of oxidative stress, were evaluated by IHC. As shown in Fig 8, after ethanol feeding, 3-NT was stained mainly surrounding central veins in liver sections in both WT mice and *cyp2a5*$^{-/-}$ mice. Staining of 3-NT was enhanced by nicotine in WT mice but not in *cyp2a5*$^{-/-}$ mice (Fig 8). Similarly, comparable staining of MDA was observed in liver sections in the WT and *cyp2a5*$^{-/-}$ mice, but nicotine enhanced MDA staining in WT mice but not in *cyp2a5*$^{-/-}$ mice (Fig 9).

These results suggest that nicotine enhances alcohol-induced oxidative stress in WT mice but not in *cyp2a5*$^{-/-}$ mice.

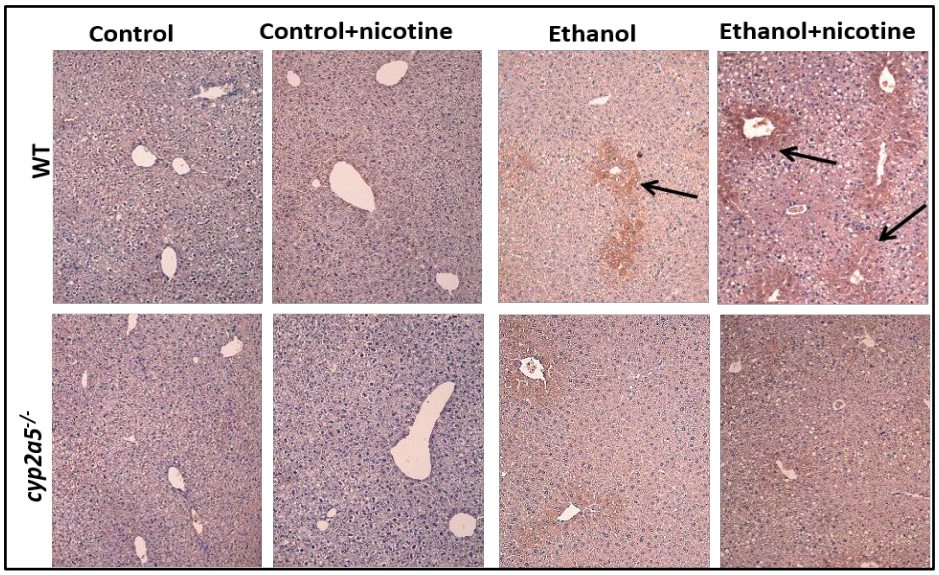

Fig. 8. IHC staining for 3-NT in liver sections. Black arrows show positive staining**.**

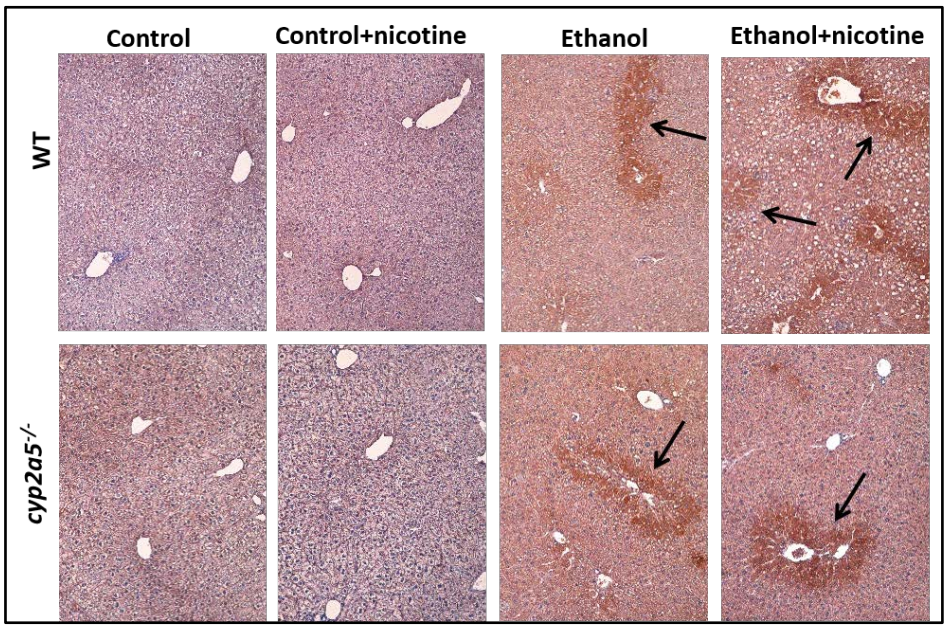

Fig 9. IHC staining for MDA in liver sections. Black arrows show positive staining.

CHAPTER 4
DISCUSSION/CONCLUSION

One of the primary purposes of this research was to determine if the loss of CYP2A5 would reduce nicotine-induced ROS generation. Our results showed that ROS was produced in the microsomes isolated from WT mice but not in the microsomes isolated from *cyp2a5*$^{-/-}$ mice, suggesting that nicotine-induced ROS generation is CYP2A5-dependent, which agrees with our hypothesis that the nicotine-induced ROS generation will be lower in *cyp2a5*$^{-/-}$ mice than in WT mice. Another hypothesis is that nicotine-enhanced alcoholic fatty liver is CYP2A5-dependent, which is supported by our observation that nicotine enhanced alcoholic fatty liver and oxidative stress in WT mice

In this research study, we observed the effects of specific diets in groups of WT mice and *cyp2a5*$^{-/-}$ mice. The diets utilized were: ethanol-nicotine diet, nicotine-control diet, ethanol diet and a control Dextrose diet. There was a clear CYP2A5 induction by ethanol in WT mice although nicotine has no effect on the ethanol induction of CYP2A5. CYP2A5 is a major nicotine metabolic enzyme (Raunio et al. 2008). Nicotine metabolism by CYP2A6 in cell culture can produce ROS and induce oxidative stress (Ande et al. 2012). Here, we show that nicotine metabolism by CYP2A5 can also produce ROS. Nicotine might also be metabolized by other cytochrome P450s (Yue et al. 2009), but microsomal metabolism of nicotine produces ROS in a CYP2A5-dependent manner, suggesting that only being metabolized by CYP2A5, nicotine may produce significant amount of ROS.

Interestingly, nicotine enhanced oxidative stress in WT mice but not in cyp2a5−/− mice, which agrees with CYP2A5-mediated ROS generation during nicotine metabolism. In bodies, the half-life for plasma nicotine is only 6–7 min in mice, and cotinine is a major nicotine metabolite (Matta et al. 2007). Compared with nicotine, cotinine has a long half-life in plasma (6–7 min vs 15–40 h) (Davis et al. 2009). Cotinine also is metabolized by CYP2A5 (Matta et al. 2007). It is possible that cotinine promotes alcohol-induced oxidative stress rather than nicotine.

Nicotine plus ethanol feeding increased liver TG accumulation and lipid droplets, which was observed in WT mice but not in cyp2a5−/− mice. These results suggest that nicotine-mediated oxidative stress contributes to nicotine enhanced alcoholic fatty liver. CYP2A5 is mainly located in endoplasmic reticulum (ER), so ROS produced in ER may attack protein and cause accumulation of unfolded and misfolded protein in the ER i.e. ER stress. In the ER of the liver, synthesized TG bind to apolipoprotein B (apoB) and then form matured very low density lipoprotein (VLDL) for secretion into blood (Fisher and Ginsberg 2002). Under oxidative stress and ER stress, misfolded apoB may retain in the ER and cause fat accumulation in liver (Pan et al. 2004). Thus, nicotine-induced oxidative stress and alcohol-induced oxidative stress may combine and make the alcoholic fatty liver exacerbated.

Alcohol and tobacco are frequently co-abused and tobacco smoke can enhance alcoholic fatty liver and oxidative stress (Bailey et al. 2009). Nicotine is a major addiction forming ingredient in tobacco smoke. Moreover, nicotine is being used as a surrogate of tobacco cigarettes, such as e-cigar, nicotine gum, nicotine patch, etc. This finding suggests that alcohol and nicotine co-abuse is a risk factor for alcoholic fatty liver, regardless of the source of nicotine.

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	The Study of Alcoholic Liver Diseases: Activities of CYP2A5 in Alcohol-induced Liver Damage (Thesis 2018).
	Production of the Extracellular Protease Enzyme Using *Zymomonas mobilis* Microorganism.