International Immunopharmacology 56 (2018) 98–104

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The therapeutic effect of fraxetin on ethanol-induced hepatic fibrosis by enhancing ethanol metabolism, inhibiting oxidative stress and modulating inflammatory mediators in rats

Xiaowei Chen[a], Xiaozhou Ying[b], Weiming Sun[a], Huijia Zhu[a], Xin Jiang[a], Bin Chen[a,*]

[a] Department of ultrasound imaging, the First Affiliated Hospital of Wenzhou Medical University, China
[b] Department of Orthopaedic Surgery, the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325000, China

ARTICLE INFO

Keywords:
Fraxetin
Antioxidant
Anti-inflammation
Hemeoxygenase-1 (HO-1)
Alcoholic liver fibrosis

ABSTRACT

The present study was designed to investigate the possible protective effects of fraxetin against ethanol induced liver fibrosis in rats. Rats were underwent intragastric administration of ethanol (5.0–9.5 g/kg) once a day for 24 weeks. Effect of fraxetin against ethanol induced liver fibrosis was investigated by giving 20 or 50 mg/kg fraxetin. At the end of experiment, the livers were collected for histopathological analyses, protein extraction, and enzymatic activities. Our results indicated that fraxetin significantly corrected ethanol-induced hepatic fibrosis, as evidenced by the decrease in serum ALT and AST, the attenuation of histopathological changes. Fraxetin also expedited ethanol metabolism by enhancing the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities. Besides, fraxetin alleviated lipid peroxidation, enhanced hepatic antioxidant capabilities, inhibited CYP2E1 activity, and reduced the inflammatory mediators, including TNF-α and IL-1β via up-regulation of hemeoxygenase-1 (HO-1) protein. In summary, the hepatoprotection of fraxetin is mostly attributed to its antioxidant capability, alcohol metabolism, and anti-inflammation effect.

1. Introduction

Alcohol abuse and chronic alcohol consumption remain as a global public health problem. Excessive alcohol consumption is one of the critical causative factors leading to alcoholic liver disease (ALD) [1]. Alcoholic liver fibrosis (ALF) is regarded as a turning point in ALD because it can lead to cirrhosis [2]. Oxidative stress, an imbalance of oxidation and antioxidation, has gained increasing attention in the study of ALD [3]. Oxidation of ethanol by microsomal oxidizing enzyme cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase results in the formation and accumulation of reactive oxygen species (ROS) in the liver [4]. Alcohol obviously increases free radicals and reactive oxygen species, oxidizing biological membrane to promote lipid peroxidation and triggering oxidative stress [5,6]. It has been demonstrated that profibrogenic cytokines, such as TNF-α and IL-1β, play a critical role in the progression of liver fibrosis [7]. Hemeoxygenase-1 (HO-1) is an inducible form of the rate-limiting enzyme involved in heme catabolism. Other studies have indicated that HO-1 was conferred cellular protection through inhibition of apoptosis, inflammation and cell proliferation in preclinical models of tissue injury [8,9]. Therefore, it is of interest to screen pharmacological agents with the potential to inhibit cytokine secretion and oxidative stress so as to identify effective treatments for liver fibrosis.

Recently, some natural products have been reported as having inhibitory effect on ethanol absorption, thus being an alternative to synthetic medicines in the prevention of alcohol provoked disorders [10]. Coumarins comprised a group of phenolic compounds widely distributed in natural plants, such as citrus fruits, tomatoes, vegetables, and green tea. Their popularity has increased because of the range of pharmacological properties demonstrated, such as antithrombotic [11], anti-inflammatory [12], antiviral [13], and antitumor properties [14]. Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a coumarin derivative, has been reported to possess antioxidative, anti-inflammatory and neuroprotective effects [15–18]. Fraxetin exhibits its antioxidant effect through increasing the level of GSH and reducing oxidative damage in a *Drosophila melanogaster* experimental model [15]. Previous we reported that fraxetin could alleviate carbon tetrachloride induced hepatic fibrosis via inhibition of oxidative stress and inflammation [19].

However, to date, few studies have investigated the effects of fraxetin directly on the ethanol induced ALF. Thus, the aim of this study is to explore the effects of fraxetin on ethanol induced liver fibrosis in rats toward the following topics: 1) antioxidant capacities; 2) alcohol

* Corresponding author at: Department of Ultrasound Imaging, the First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang streets, Ouhai, Wenzhou 325000, China.
E-mail addresses: wzchenbin@yeah.net, chenbin@wmu.edu.cn (B. Chen).

https://doi.org/10.1016/j.intimp.2018.01.027
Received 22 September 2017; Received in revised form 6 January 2018; Accepted 17 January 2018

metabolism; 3) inflammatory response.

2. Materials and methods

2.1. Chemicals

Fraxetin (7,8-dihydroxy-6-methoxy coumarin) was obtained from Sigma Chemicals Company. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GSH-Px) kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Other required materials are outlined in the following sections.

2.2. Animals and treatments

Male SPF-Wistar rats (200 ± 20 g) were purchased from the Experimental Animal Center of Wenzhou Medical University (Wenzhou, China). The research was conducted according to protocols approved by the institutional ethical committee of Wenzhou Medical University. After a period of 1 week, the rats were divided into five groups with 10 rats per group as follows: one untreated group (GI) and four groups (GII–GV) treated with ethanol gavage once a day. The doses of ethanol were increased gradually according to the method of Zhang et al. [20]: 5.0 g/kg/day from 1 to 4 weeks, 7.0 g/kg/day from 5 to 8 weeks, 9.0 g/kg/day from 9 to 12 weeks, and 9.5 g/kg/day from 13 to 24 weeks. At the same time, the rats were treated with 100 mg/kg/day silymarin (GIII) as positive control, 20 mg/kg/day fraxetin (GIV) and 50 mg/kg/day fraxetin (GV) through gavage. At the end of 24 weeks, all rats were killed and blood samples were collected. Liver samples were obtained from all groups and divided into two parts. One part was stored immediately at −80 °C for future analysis, whereas the other part was fixed in 10% formalin for histopathological examination.

2.3. Estimation of AST and ALT activities

Serum levels of ALT and AST were measured using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions.

2.4. Determination of plasma alcohol concentration

The blood alcohol level was determined using a commercial kit (Changzhou SEO Biotechnologies Inc. Changzhou, China). This enzymatic test for alcohol utilizes the coenzyme nicotinamide adenine dinucleotide (NAD) and alcohol dehydrogenase (ADH). The formation of NADH can be measured quantitatively by the increase in absorbance at 340 nm.

2.5. Estimation of hepatic alcohol metabolizing enzyme activities

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were measured in liver homogenate according to the protocol established in previous study [21].

2.6. Measurement of liver lipid peroxidation level and antioxidant capacity

The quantitative measurement of lipid peroxidation was performed by measuring the concentration of TBARS in the liver according to the method reported by Tsaiet et al. [22]. The liver TBARS level was expressed as nmole MDA equivalent (eq.)/mg protein. In brief, samples were mixed with a TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 N hydrochloric acid. The supernatant was collected, and its absorbance was measured at 535 nm with an ELISA plate reader. Hepatic GSH-Px activity was measured by taking the extinction coefficient of NADPH to be $6.22 \times 10^6\,nM^{-1}\,cm^{-1}$ at 340 nm and expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Hepatic SOD was detected by the inhibitory effect of SOD on purpurogallin of pyrogallol oxidation product, was recorded at 420 nm, and was expressed as milliunits per milligram of protein.

2.7. Liver histopathology

Liver samples were fixed in 10% buffered formaldehyde solution, processed by the paraffin slice technique. Sections about 4 μm thick were stained with hematoxylin and eosin (HE) and Masson's trichrome staining to investigate liver histological and fibrotic changes. The degree of liver damage was examined blindly by a special pathologist under a light Olympus microscope (Olympus, Hamburg, Germany).

2.8. Determination of hepatic TNF-α and IL-1β levels

Liver TNF-αand IL-1β concentrations were assayed using ELISA kits based on anti-mouse TNF-α and IL-1β monoclonal antibodies (BD Pharmin-gen, San Diego, CA, USA) and converted to the TNF-α and IL-1β levels expressed as picograms per milligrams of protein using standard curves.

2.9. Western blot analysis

The liver CYP2E1 and Hemeoxygenase-1 (HO-1) protein expressions in each group were determined by Western blot. Total protein was extracted from liver tissue and analyzed with a bicinchoninic acid (BCA) protein concentration assay kit (Shanghai Haoran Bio Technologies Co., Ltd., China). One hundred micrograms of the protein was subjected to a 12% SDS polyacrylamide electrophoresis and then electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with the primary antibody for CYP2E1, Hemeoxygenase-1 (HO-1) and anti-β-actin (Cell Signaling Technology, Beverly, MA, USA) for 18 h at 4 °C, followed by detection using horseradish peroxidase labeled anti-mouse IgG (Medical Biological Laboratory Co., Nagoya, Japan) for 2 h. Immunoreactive protein bands were visualized by ECL Western blotting kit (Amersham Biosciences Co., Piscataway, NJ, USA). The band was then scanned and the intensity of the protein was measured using densitometry software. β-actin was used as the internal control. All values were normalized to β-actin and expressed as arbitrary units relative to the control.

2.10. Statistical analysis

All the experimental values were expressed as mean ± SEM (n = 10). Statistical significance of the data was determined using SPSS 17.0 using one-way analysis of variance (ANOVA) and the group means were compared by applying Dunnett's Multiple Comparison Test. A difference was considered significant at the $p < 0.05$ level.

3. Results

3.1. Effects of fraxetin on serum ALT and AST activities

Compared with the normal control, a significant increase in the activities of serum AST (Fig. 1A) and ALT (Fig. 1B) was observed in the model group. However, the activities of serum AST and ALT were decreased by fraxetin administration in a dose-dependent manner ($p < 0.05$).

3.2. . Effects of fraxetin on alcohol metabolism in livers

CYP2E1 is the most important enzyme of the liver cytochrome P450 system related to alcohol metabolism. Activity of ADH and ALDH directly reflects the ability to eliminate alcohol. The alcohol group had higher CYP2E1 expression (Fig. 2C, D) ($p < 0.05$) and lower ADH