兽医内科与临床诊疗学研究

（2012 — 2014）

中国畜牧兽医学会兽医内科与临床诊疗学分会　组编

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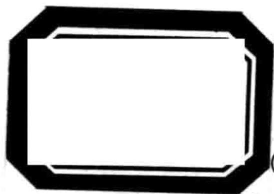

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前　言

光阴荏苒，中国畜牧兽医学会家畜内科学分会上一次在兰州举行学术研讨会至今已快两年了，现在我们又将迎来家畜内科学分会的新一次学术盛会（2014 年 7 月 19-21 日，云南昆明）。两年来，我国的兽医内科与临床诊疗学科有了长足的发展，许多研究工作进入了国际前沿，研究水平和深度有了极大地提高，取得了一些重要研究成果，临床诊疗技术也取得了不少突破。

本次会议共收到 147 篇研究论文，其中论文全文 45 篇、论文摘要 102 篇。经初步统计，2012 年 8 月 1 日至 2014 年 6 月 30 日，我国兽医内科诊断学培养已毕业的硕士研究生 381 名，培养已毕业的博士研究生 50 名，培养已出站的博士后 11 名。中国畜牧兽医学会家畜内科学分会代表获得省部级科技成果三等以上奖励 5 项，授权发明、实用新型专利 49 项，发表 SCI 收录论文 250 篇。主编兽医内科学与诊断学教材 11 部，主编兽医内科学与诊断学方面的专著 19 部，主译兽医内科学与动物诊断学著作 3 部。

经分会研究，本次会议将授予中国农业大学动物医学院王志教授、东北农业大学动物医学学院康世良教授和石发庆教授“中国畜牧兽医学会家畜内科学分会第四届终身成就奖”。同时，将授予 SCI 收录论文影响因子大于 5 的通讯作者：山西农业大学王俊东教授，扬州大学刘宗平教授，山东农业大学刘建柱博士、副教授，东北农业大学张志刚博士、副教授“优秀论文一等奖”；将授予 SCI 收录论文影响因子在 4 和 5 之间的通讯作者：山西农业大学王俊东教授，吉林大学动物医学学院张乃生教授，华中农业大学动物医学院郭定宗教授，东北农业大学动物医学学院徐世文教授，中国农业大学动物医学院韩博教授和吉林大学动物医学学院杨正涛博士、副教授“优秀论文二等奖”。

为了提高执业兽医，尤其是我国西南边陲从业人员的兽医临床诊疗水平，促进和加强执业兽医继续教育，中国畜牧兽医学会家畜内科学分会在本次会议上探索性地举办执业兽医继续教育项目培训班。经向中国兽医协会请示，同意本次培训可授予执业兽医人员继续教育学分。

随着我国兽医科技的发展，特别是兽医内科学、兽医临床诊断学和兽医临床治疗学以及小动物临床诊疗的迅速发展，家畜内科学这一名称已无法涵盖分会的内涵，况且中国畜牧兽医学会下设的兽医相关分会大都以兽医冠名，这也符合国际惯例。因此，在原有的兽医内科学基础上将兽医临床诊断学和兽医临床治疗学纳入分会，对提高分会的阵容和影响力，提升我国兽医临床诊疗的科教水平和动物疾病的临床诊疗水平，必将起到积极地推动作用。经中国畜牧兽医学会家畜内科学分会理事会研究讨论，建议将分会的名称变更为：中国畜牧兽医学会兽医内科与临床诊疗

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学分会。这项工作已经获得中国畜牧兽医学会的批复。

总之，上述的相关工作，展示了中国畜牧兽医学会兽医内科与临床诊疗学分会代表取得的丰硕成果，体现了中国畜牧兽医学会兽医内科与临床诊疗学分会代表的凝聚力和战斗力，表明了中国畜牧兽医学会兽医内科与临床诊疗学分会的生机和活力。希望大家在各自的岗位上继续努力奋斗，为学会的发展做出更大的贡献。

陈 越

中国畜牧兽医学会兽医内科与临床诊疗学分会 理事长

2014年6月30日于北京

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一、中国畜牧兽医学会兽医内科与临床诊疗学分会论文全文

Betulinic acid prevents alcohol-induced liver damage by improving the antioxidant system in mice

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Abstract: Betulinic acid (BA), a pentacyclic lupane-type triterpene, has a wide range of bioactivities. The main objective of this work was to evaluate the hepatoprotective activity of BA and the potential mechanism underlying the ability of this compound to prevent liver damage induced by alcohol *in vivo*. Mice were given oral doses of BA (0.25, 0.5, and 1.0 mg/kg) daily for 14 days, and induced liver injury by feeding 50% alcohol orally at the dosage of 10 ml/kg after 1 h last administration of BA. BA pretreatment significantly reduced the serum levels of alanine transaminase, aspartate transaminase, total cholesterol, and triacylglycerides in a dose-dependent manner in the mice administered alcohol. Hepatic levels of glutathione, superoxide dismutase, glutathione peroxidase, and catalase were remarkably increased, while malondialdehyde contents and microvesicular steatosis in the liver were decreased by BA in a dose-dependent manner after alcohol-induced liver injury. These findings suggest that the mechanism underlying the hepatoprotective effects of BA might be due to increased antioxidant capacity, mainly through improvement of the tissue redox system, maintenance of the antioxidant system, and decreased lipid peroxidation in the liver.

Keywords: alcohol, antioxidant capacity, betulinic acid, lipid peroxidation, liver damage

1 Introduction

Alcohol abuse and its associated social consequences are a major health problem in many areas of the world. Alcohol abuse is a brain-centered addictive behavioral disorder that develops regardless of gender, race, age, or economic standing, and can lead to alcoholic liver disease (ALD) in many patients[5,13]. ALD presents a broad spectrum of disorders ranging from simple fatty liver to more severe forms of liver injury that include alcoholic hepatitis (AH), cirrhosis, and superimposed hepatocellular carcinoma[12]. In the United States, the Centers for Disease Control and Prevention estimate that 50% of people aged 18 or older drink alcohol[3]. Among these, 5% are classified as heavy drinkers and 15% are binge drink. The National Institute on Alcohol Abuse and Alcoholism (USA) has reported that liver cirrhosis is the 12th leading cause of death with a total of 29 925 deaths in the United States in 2007 (48% of which were alcohol-related) and is also a major contributor to liver disease-related mortality in other countries[12].

The abuse of alcohol significantly increases morbidity and mortality from infectious diseases along with the risk of cardiovascular, brain, pancreatic, renal, cerebral, and oncological diseases[13]. The overall socioeconomic cost of alcohol abuse including direct (drug and hospitalization expenses) and indirect (due to loss of work productivity, crime, and reduction in health-related quality of life) costs has been estimated to be more than $185 million annually in the United States[20,25]. Despite the profound economic and health impact of ALD, many patients continue to consume alcohol. The primary treatment recommendation for this disease is abstinence from alcohol. Other treatment options, such as pharmacological and nutritional therapies, are also available[17]. Interest in potential

antioxidant therapy for treating AH has increased as a growing body of evidence indicates that oxidative stress is a key mechanism underlying alcohol-mediated. Alcohol intoxication leads to increased generation of reactive oxygen species (ROS) while suppressing the antioxidant defense system[28,31]. Antioxidants have the ability to scavenge/deactivate ROS and inhibit the oxidation of various cellular substances. Several antioxidants such as S-adenosylmethionine, N-acetylcysteine, beta-carotene, vitamin C, vitamin E, and selenium have been systematically evaluated[12,18]. It was reported that AH patients treated with vitamin E, selenium, and zinc supplements had an in-hospital mortality rate of 6.5% compared to a mortality rate of 40% for the placebo group[17]. Plants produce an extensive variety of antioxidant compounds such as phenolics, terpenoids, alkaloids, and bioactive protein or peptides. These reagents can be used as ingredients in nutritional supplements/nutraceuticals that combat oxidative stress with minimal side effects[35].

Betulinic acid (BA; 3β-hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic lupane-type triterpene (Fig. 1A) that exists widely in food, medicinal herbs, and plants, especially birch bark[9]. As an oxidation product of betulin (see Fig. 1B), BA has also been detected in extracts of white birch bark[9,32]. These reagents are of particular interest due to their wide spectrum of biological activities such as antioxidant, anti-inflammatory, anti-tumor, anti-angiogenesis, anti-viral, anti-HIV, anti-neoplastic, and anti-plasmodial properties[10,11,22,24,32]. BA was not found to be toxic at a concentration of 500 mg/kg in mice[22].

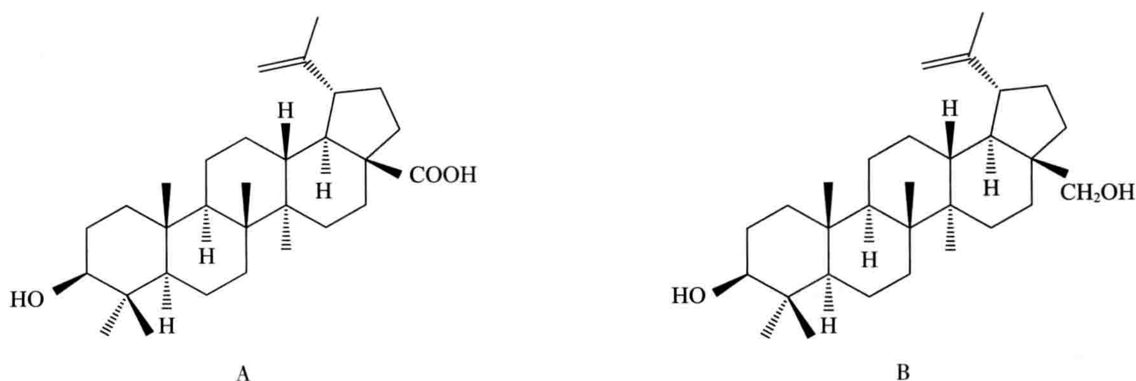

Fig. 1 Chemical structures of betulinic acid (A) and betulin (B)

Several studies have demonstrated that natural triterpenes such as lupeol, betulin, ursolic acid, and oleanolic acid effectively reduce hepatotoxicity induced by carbon tetrachloride, acetaminophen, ethanol, and cadmium *in vivo* and *in vitro* [29,38]. The protective effect of betulin, BA, and oleanolic acid against ethanol-induced cytotoxicity in HepG2 cells has also been reported[28]. The mechanisms underlying this effect include the suppression of enzymes activities that play roles in liver damage such as cytochrome P450 (CYP450), cytochrome B5 (CYB5), and cytochrome P4501A (CYP1A), or increased production of antioxidant substances including catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and metallothioneins that help protect liver mitochondria[28]. *In vitro*, BA inhibits ethanol-induced activation of hepatic stellate cells, as an antioxidant, suppressing of ROS, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) production as well as nuclear factor-kappa B/inhibitory protein of NF-κB (NF-κB/IκB) transduction signaling[28,29]. However, ability of BA to confer *in vivo* protection against liver injury induced by alcohol has not been previously reported. The objective of this study was to investigate the possible hepatoprotective effects of BA against alcohol induced acute liver damage in mice and elucidate the underlying mechanism.

2 Materials and Methods

2.1 Extraction, Synthesis, and Identification of BA from White Birch Bark

White birch bark samples were collected during the spring of 2009 in Wroclaw, Poland. All the collected bark samples were immediately dried at 60℃ and stored in a dry, dark place. To extract betulin, about 15 g of dried bark were subjected to reflux with 200 ml methanol for 3 h at 70℃. The methanol extract was dried under negative gauge pressure and dissolved in 150 ml dichloromethane. After adding 150 ml 2 mol/L sodium hydroxide and mixing, the lower liquid layer was collected and then filtered under negative gauge pressure. The residue was dissolved in 200 ml ether. Next, 200 ml water was added and the solution was thoroughly mixed. The upper liquid layer was subsequently collected, dried, and subjected to silica gel column chromatography (Flash column; Aisimo Corporation, China)[32]. Betulin was fractionated with hexane and ethyl acetate (6 : 1, V : V). BA was synthesized in two steps: 1) the betulin was oxidized with Jones reagent (1 : 6, molar ratio) at 20℃ for 3 h to obtain betulonic acid, and 2) betulonic acid was reduced by sodium borohydride (1 : 1, molar ratio, Sigma-Aldrich, USA) in 10 ml tetrahydrofuran (Sigma-Aldrich) to produce a mixture of 3α- and 3β-hydroxyl products (5 : 95) as previously described[9,32]. Crystallization of the product from methanol resulted in the 3β-hydroxyl product (BA) with a yield of 75%.

The semi-synthetic compound was a white powder, and characterized using Fourier transform infrared spectroscopy (FI-IR), high performance liquid chromatography-mass spectrometery (HPLC-MS), and nuclear magnetic resonance (NMR) as previously described[32]. Purity of the BA was measured with HPLC using a Zorbax Eclipse XDB-C8 column (4.6 mm × 50 mm, 5 μm; Agilent Technologies, USA). The injection volume was 20 μl and the column was eluted with an ethanol: water solution (86 : 14, *V* : *V*, 4 g/L ammonium formate in water) as the mobile phase at a flow rate of 1 ml/min, and UV detection was performed at 210 nm at room temperature. The Purity of the BA was 96.5%.

2.2 Animals and Experimental Design

A total of 50 male Kunming mice weighing 18 – 22 g (6 weeks old) were purchased from Hunan Silaikejingda Laboratory Animal Co., Ltd. (China). The mice were acclimated to the laboratory environment for 1 week before the experiment was initiated, and fed an M02 standard mouse diet from Hunan Silaikejingda Laboratory Animal (China). The mice had access to food and water *ad libitum*, and were maintained at a constant temperature (23 ± 1)℃ and humidity (60% ± 10%) with a 12-h light/dark cycle. The present study complied with the Animal Care and Use Guidelines of China and was approved by the Animal Care Committee of Hunan Agricultural University (China)[15].

The dose of BA and duration of treatment were established based on a previous study[37]. The mice were randomly divided into five groups: group A served as the control (no alcohol, no BA), group B served as the alcoholic liver injury model (alcohol, no BA), group C received a low dose of BA (0.25 mg/kg) and alcohol, group D received a moderate dose of BA (0.5 mg/kg) and alcohol, and group E consumed a high dose of BA (1 mg/kg) and alcohol. BA was administered orally to most mice with 1% starch jelly (Sinopharm Chemical Reagent, China) every day for 14 days while animals in groups A and B were given an equivalent amount of 1% starch jelly only. The mice were fasted overnight (16 – 18 h) before receiving a single dose of 50% ethanol (Sinopharm Chemical Reagent) at the dosage of 10 ml/kg on the 14th day of the study 1 h after the last administration of BA. Group A received an equal volume of sterile saline.

9 h after alcohol administration, blood samples were collected in tubes (Eppendorf, Germany) without

anticoagulant by venous puncture with the mice under light anesthesia induced by diethyl ether (Sinopharm Chemical Reagent). The animals were then sacrificed by cervical dislocation and liver samples were collected. Serum was collected by centrifugation (Z383K Universal High Speed Centrifuge; Hermle labortechnik, Germany) at 2 000 × g for 10 min at 4℃ and frozen at −70℃ until analysis.

Livers were quickly removed, weighed, and the liver indices expressed in milligrams of liver per 10 g of body weight were calculated. The liver tissues were washed in a chilled 0.9% NaCl solution and minced into small pieces on ice. A 10% (*W/V*) homogenate (Tenbroeck tissue grinders; Wheaton, USA) was prepared in 10 mmol/L phosphate buffer (pH 7.4) and centrifuged (Z383K Universal High Speed Centrifuge; Hermle labortechnik) at 2 500 × g for 15 min at 4℃. The resulting supernatant was collected and stored at −70℃ until analysis.

2.3 Evaluation of Serum Biomarkers of Liver Injury

Serum alanine transaminase (ALT), aspartate transaminase (AST), total cholesterols (TC), and triacylglycerides (TG) levels were measured using Mindray commercial kits and a Mindray BS-200 automatic biochemistry analyzer (Shenzhen Mindray Bio-Medical Electronics, China). ALT and AST levels were expressed as units per liter of serum (U/L) while TC and TG concentrations were expressed as nmol per liter of serum (nmol/L).

2.4 Measurement of SOD, GSH-Px, and CAT Activities Along with Hepatic MDA and GSH Contents

The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) were measured along with malondialdehyde (MDA) and glutathione (GSH) levels using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols. The levels of SOD, GSH-Px, and CAT activities were expressed as unit per mg of protein (U/mg protein). The MDA and GSH levels were expressed as nmol per mg protein (nmol/mg protein) and mg per g of protein (mg/g protein), respectively. Liver protein concentrations were determined with a Bradford assay using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

2.5 Histologic Analysis of Liver Tissues

Liver tissues from the mice were removed and fixed in 10% neutral buffered formalin. The tissues were then rinsed with water, dehydrated with ethanol, subsequently embedded in paraffin, and sectioned into slices (4-μm-thick) using a Leica RM2235 rotary microtome (Leica Microsystems, Germany). The sections were mounted onto glass slides (Beyotime Institute of Biotechnology, China), dewaxed in xylene and ethanol, and stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, China) for histological evaluation using a Motic BA410 microscope (Motic Incorporation, China). Image Pro-Plus Motic Med 6.0 software (Motic Incorporation) was used for image processing.

2.6 Statistical Analysis

The results are presented as the mean ± standard deviation (SD). The data were analyzed with a one-way analysis of variance (ANOVA) followed by the Student-Newman- Keuls test for multiple comparisons. All statistical analyses were performed using the SPSS 16.0 statistical package (SPSS, USA). A P value <0.05 was considered statistically significant and a P value <0.01 was considered highly significant.

3 Results

3.1 Effects of BA on Liver Indices and Serum Biomarker Expression in Mice

The effects of BA on alcohol-affected liver indices along with serum levels of ALT, AST, TC, and TG in mice

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