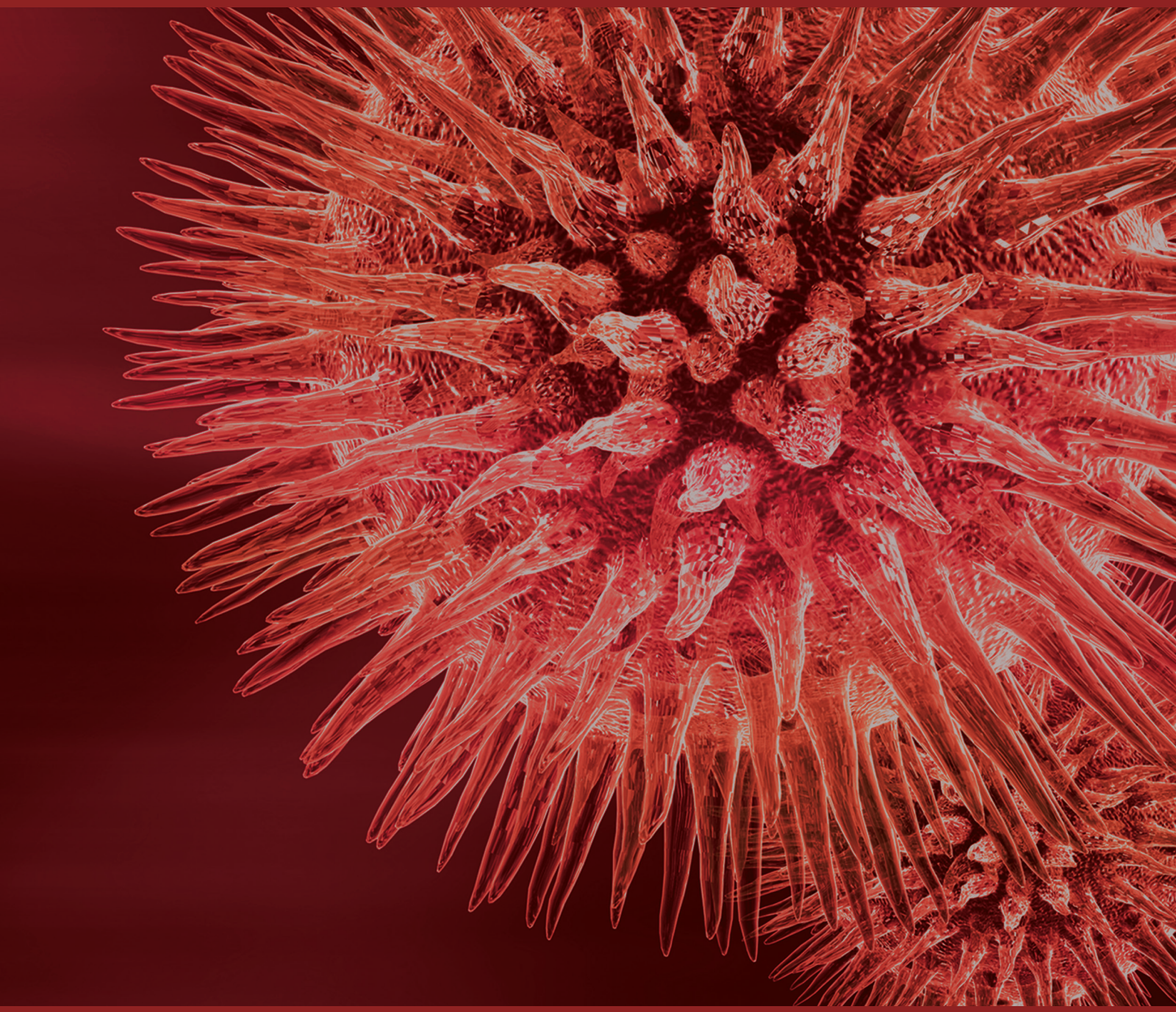
BioMed Research International

Chinese Medicine for Treating Endocrinology-Related Disease

Guest Editors: Chunchao Han, Jianyou Guo, Thomas G. Mitchell, and John E. Smith



Chinese Medicine for Treating Endocrinology-Related Disease

BioMed Research International

Chinese Medicine for Treating Endocrinology-Related Disease

Guest Editors: Chunchao Han, Jianyou Guo, Thomas G. Mitchell, and John E. Smith

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 680290, 2 pages
http://dx.doi.org/10.1155/2015/680290

Editorial

Chinese Medicine for Treating Endocrinology-Related Disease

Yizhou Xin,[1] Ying Zhang,[2] and Chunchao Han[3]

[1]*The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, China*
[2]*Shandong Eye Hospital, Jinan 250021, China*
[3]*School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*

Correspondence should be addressed to Chunchao Han; chunchaoh@126.com

Received 2 March 2015; Accepted 2 March 2015

According to new research accepted for publication in the Endocrine Society's Journal of Clinical Endocrinology & Metabolism, Chinese herbal medicines are well recognized for their medicinal properties and have been used in traditional medicine for millennia. The medicinal effects attributed to herbal medicines, based mainly on uncharacterized substances or extracts, include depression management, cerebrovascular disease management, Alzheimer's disease management, and antianxiety, immunomodulatory, antioxidant, radical scavenging, anti-inflammatory, antihyperlipidemic or antihypercholesterolemic, hepatoprotective, and antidiabetic effects.

4-Hydroxychalcone (4HCH) is an alpha, beta-unsaturated ketone with the core structure of chalcone and one hydroxyl-substituent on the 4-position of the A ring. It belongs to the largest class of plant secondary metabolites and is considered to be precursor of flavonoids and isoflavonoids serving plant defense mechanisms to counteract reactive oxygen species in order to survive and prevent molecular damage and damage by microorganisms, insects, and animals. F. Zhang investigated the preventive effects of 4-hydroxychalcone (4HCH) on resistant hypertension. The cryptochrome-null mice, which received high-salt treatment, were treated orally with 4HCH 10 mg/kg, 4HCH 20 mg/kg, and 4HCH 40 mg/kg, respectively. The salt administration in cryptochrome-null mice is able to induce an increase in systolic pressure which is associated with hyperaldosteronism, inflammation, and kidney injury. Treatment with 40 mg/kg 4HCH reduced systolic hypertension, serum IL-1β, and TNF-α levels and suppressed the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and renal injury. The impact of 4HCH on the hyperaldosteronism, inflammation, and kidney injury provides new insights for future development of therapeutic strategies in resistant hypertension.

Agaricus blazei Murrill (ABM), an edible mushroom is native to Brazil, which is widely used for nonprescribed and medicinal purposes. C. Han investigated the medium composition of ABM that was optimized using response surface methodology for maximum mycelial biomass and extracellular polysaccharide (EPS) production. The model predicts to gain a maximal mycelial biomass and extracellular polysaccharide at 1.047 g/100 mL and 0.367 g/100 mL, respectively, when the potato is 29.88 g/100 mL, the glucose is 1.01 g/100 mL, and the bran is 1.02 g/100 mL. The verified experiments showed that the model was significantly consistent with the model prediction and that the trends of mycelial biomass and extracellular polysaccharide were predicted by artificial neural network. After that, the optimized medium was used for the submerged culture of ABM. Then, alcohol-induced liver injury in mice model was used to examine the protective effect of ABM cultured using the optimized medium on the liver. And the hepatic histopathological observations showed that ABM had a relatively significant role in mice model, which had alcoholic liver damage. Y. Gao investigated the effect of *Agaricus brasiliensis* extract (ABE) on phenylhydrazine-induced neonatal jaundice in rats. Administration of ABE dose-dependently reduced the elevated bilirubin level induced by phenylhydrazine. It can be somewhat supported from the results of *in vitro* bilirubin degradation experiment. ABE treatment also reduced the total antioxidant status (TAOS), cascade O_2^-/SOD, level of NF-κB protein, and adrenomedullin (AM). Overall, the results of this study demonstrated that *Agaricus brasiliensis* extract may be beneficial to reducing bilirubin level without causing hepatotoxicity in neonatal jaundice.

Crocetin ($C_{20}H_{24}O_4$) is one of the major active constituents of saffron, which is derived from the dried stigma of *Crocus sativus* L., belonging to the Iridaceae family. C.-D. Li investigated the effect of crocetin ($C_{20}H_{24}O_4$) on methylcholanthrene- (MCA-) induced uterine cervical cancer in mice. After the mice were treated orally with crocetin, maleic dialdehyde (MDA), polymorphonuclear cells (PMN), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) were examined by ELISA or immunohistochemistry. The inducible nitric oxide synthase (iNOS) activation in *Hela* cells was analyzed using fluorescence microscopy for light microscopic examination. The MCA-mice showed a significant increase in plasma MDA, PMN, IL-1β, TNF-α, and nitrates levels. At the same time, the mRNA level of COX-2 in *Hela* cells was also significantly increased. These changes were attenuated by crocetin supplementation in the MCA mice. Crocetin supplementation in the MCA mice also showed the protection against cervical cancer. These results suggest that crocetin may act as a chemopreventive and an anti-inflammatory agent.

Fructus Ligustri Lucidi (FLL) is a well-known invigorator in Chinese materia medica with the effects of hepatoprotective effect, anticancer activity, antioxidant activity, and so on. C. Han reviewed the pharmacological effects of *Fructus Ligustri Lucidi*. It contains a number of bioactive components. Moreover, FLL has been known to have hepatoprotective effect, anticancer activity, antioxidant activity, immunomodulating effect, antiviral activity, and antiosteoporosis activity and other pharmacodynamic effects have been demonstrated as well, such as the treatment of coronary heart disease. The chemical constituents of FLL include polysaccharides, triterpenes, secoiridoids, and flavonoids, which maybe contributed to the pharmacological activities of FLL. Therefore, the pharmacological effects and health function of *Fructus Ligustri Lucidi* are more and more focused on in the world.

Breviscapine is an active ingredient of flavonoids extracted from dried *Erigeron breviscapus* (Vant.) Hand-Mazz. Z. Zhou reviewed combined therapy of diabetic peripheral neuropathy with breviscapine and mecobalamine. A meta-analysis on combined therapy of diabetic peripheral neuropathy (DPN) with breviscapine and mecobalamine was performed to evaluate the efficacy of this therapy. A total of 17 articles including 1398 DPN patients were identified. Homogeneity was observed among different studies ($P = 0.74$). The efficacy of combined therapy with breviscapine and mecobalamine was significantly better than that in control group ($P < 0.0001$ (OR = 5.01, 95% CI: 3.70–6.78)). Available findings suggest that the therapeutic efficacy of breviscapine combined with mecobalamine is superior to mecobalamine alone, and this strategy is required to be popularized in clinical practice.

Cordyceps is a genus of the family Clavicipitaceae that has been used in traditional oriental medicine for centuries. Recent studies have demonstrated that the bioactive components isolated from this genus have various pharmacological actions. Among them, cordycepin, also known as 3-deoxyadenosine, has been shown to possess multiple pharmacological activities such as inhibition of tumour growth, modulation of the immune response, and suppression of reactive oxygen species. G.-Y. Zhao reported that cordycepin can act as anti-inflammatory agent in magnesium silicate-induced inflammation in osteoporosis. The beneficial effects of cordycepin on improvement of osteoporosis in rats were attributable mainly to decrease ALP activity, TRAP activity, and CTX level. At the same time, cordycepin also increases the OC level in ovariectomized osteopenic rats. The histological examination clearly showed that dietary cordycepin can prevent bone loss caused by estrogen deficiency. These experimental results suggest that complement cordycepin is protective after ovariectomized osteopenic in specific way.

Yizhou Xin
Ying Zhang
Chunchao Han

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 651218, 6 pages
http://dx.doi.org/10.1155/2015/651218

Research Article

Therapeutic Effect of *Agaricus brasiliensis* on Phenylhydrazine-Induced Neonatal Jaundice in Rats

Lan Zhang,[1] Bo Yuan,[2] HuiPing Wang,[1] and Ya Gao[1]

[1]*Department of Pediatrics, Second Hospital of Xi'an Jiaotong University, Xi'an 710004, China*
[2]*Xi'an Central Hospital, Xi'an 710003, China*

Correspondence should be addressed to Ya Gao; yagao757@sina.cn

Received 7 November 2014; Accepted 2 December 2014

Academic Editor: John E. Smith

The present study was designed to investigate the effect of *Agaricus brasiliensis* extract (ABE) on phenylhydrazine-induced neonatal jaundice in rats. Administration of ABE dose-dependently reduced the elevated bilirubin level induced by phenylhydrazine. It can be somewhat supported from the results of *in vitro* bilirubin degradation experiment. ABE treatment also reduced the total antioxidant status (TAOS), cascade O_2^-/SOD, level of NF-κB protein, and adrenomedullin (AM). Overall, the results of this study demonstrated that *Agaricus brasiliensis* extract may be beneficial to reducing bilirubin level without causing hepatotoxicity in neonatal jaundice.

1. Introduction

Neonatal jaundice (NJ) occurs in more than 60% of normal newborns during their first week of life [1, 2]. The condition results from the abrupt cessation of bilirubin (BR) clearance by the placenta and transient deficiency in hepatic BR uptake, intracellular transport, and glucuronyltransferase conjugation [3]. It is a leading, yet preventable, cause of newborn rehospitalizations, deaths, and disabilities globally [4, 5]. Phototherapy (PT), which involves exposing a newborn's skin to electric lamp-generated blue light, is the standard treatment for removing excessive bilirubin, except in extreme cases when exchange transfusion becomes necessary [6]. Its efficacy is dependent on the color (wavelength) and intensity (irradiance) of the light emitted during phototherapy, the exposed body surface area, and the duration of exposure [7]. Unfortunately, PT may not be available in many countries because of the lack of devices and/or of reliable electrical power. And the conventionally used phototherapy may be increasing the risk of bilirubin-induced neurotoxicity [8, 9].

Presently, the plant kingdom is a wide field to search for natural effective hepatoprotective agent that has no side effects. More than 400 plants with glucose lowering effect are known [10]. Mushrooms and primarily basidiomycetous fungi are popular and valuable foods that are low in calories and high in minerals, essential amino acids, vitamins, and fibers [11]. Some of them produce substances with potential medical effects and are called medicinal mushrooms [12]. *Agaricus brasiliensis* is native to Brazil and is widely grown in Japan and China because of its medicinal properties. It has traditionally been used for the prevention of a range of diseases, including hepatitis [13]. A few studies have researched that *A. brasiliensis* extract could ameliorate or abrogate CCL_4-induced liver injury in rats [13, 14]. Hsu et al. [15] performed a 1-year open-label pilot study to observe whether *A. brasiliensis* extract improves liver function in patients with hepatitis B. However, the role of *A. brasiliensis* in neonatal jaundice has not been investigated. The aim of the present investigation was to evaluate the therapeutic effect of *Agaricus brasiliensis* in phenylhydrazine-induced neonatal jaundice rat model in order to propose a more effective and safer treatment for neonatal jaundice.

2. Materials and Methods

2.1. Animals. Healthy male adult Wistar rats (1 week old) were used in the study. The study was approved by Xi'an Jiaotong University Ethics Committee, and all procedures complied with the guidance set out in the Guidelines for Caring for Experimental Animals published by the Ministry of

Science and Technology of the People's Republic of China. Every care was taken to minimize discomfort, distress, and pain.

2.2. Agaricus brasiliensis Extract (ABE). The fermented mushroom of *Agaricus brasiliensis* was produced by the way introduced by Wang et al. [16]. The aqueous extraction was performed by adding 100 mL boiling water to 10 g air-dried mycelium. The infusion stood at room temperature for 30 minutes. After cooling and filtration, the extract was frozen and concentrated by lyophilization for five days overnight, in order to obtain the ABE (0.68 g).

2.3. Experimental Design. Animals were fasted for 12 h and were then injected (i.v.) with phenylhydrazine hydrochloride (75 mg/kg) solution that was made with physiological saline once daily for two consecutive days [17]. Forty NJ rats were selected and allocated equally into 4 groups. From then on, the 4 groups of rats of NJ were administered (i.g.) ABE 20 mg/kg/d, ABE 50 mg/kg/d, ABE 100 mg/kg/d, and saline, respectively. The other 10 normal rats were injected (i.v.) with the normal saline and used as the control group. All the treatments were done 4 h after phenylhydrazine administration on the second day. At the end of the experimental period (14 days later), the rats were fasted overnight and sacrificed by cervical dislocation. Blood samples drawn from the orbital sinus of the rats before killing were collected in plain and heparin vials for measuring the bilirubin level and liver function enzyme activity as well as O_2^- and superoxide dismutase (SOD).

2.4. Measurement of Serum Bilirubin. The serum total bilirubin (STB), conjugated bilirubin (SCB), and unconjugated bilirubin (SUB) levels were measured using commercial kits (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instructions.

2.5. Measurement of Serum ALT and AST. Serum ALT and AST activity were measured colorimetrically using a diagnostic kit (Shanghai Jinma Biological Technology, Inc., China) according to the instructions provided.

2.6. Histopathological Studies. Liver samples were collected and fixed in formalin for histology study. The formalin-fixed paraffin tissue sections were processed for staining with hematoxylin and eosin and then studied by light microscopy.

2.7. Measurement of Total Antioxidant Status. The total antioxidant status (TAOS) of liver was determined as previously described by Han [18]. The increase of absorbance at 405 nm was measured by a microplate reader (Shanghai Xunda Medical Technology, Inc., China).

2.8. Measurement of Cascade O_2^-/SOD. The level of O_2^- was measured by NBT (nitro blue tetrazolium) reaction in TRIS buffer in the plasma and spectrophotometrically read at 530 nm. The activity of SOD was measured according to the method of Misra and Fridovich [19]. Catalase activity was measured by the method of Beutler [20].

2.9. Measurement of NF-κB Activity. We used 10 μg of the nuclear extract from each liver. Activated NF-κB was quantified in liver tissue extracts via ELISA technique using the PathScan Phospho-NFκB p65 (Ser536) Sandwich ELISA Antibody Pair (Shanghai Xunda Medical Technology, Inc., China), following the manufacturer's instructions. The protein expression levels of NF-κB were measured by Western blot analysis.

2.10. Measurement of AM Levels. Serum AM levels were measured with ELISA method (Shanghai Xunda Medical Technology, Inc., China). Results were given as ng/mL.

2.11. In Vitro Bilirubin Degradation Experiment. Bilirubin (Sigma Chemical Co., Shanghai, China) was dissolved in a buffer solution (18.5% 0.1 N NaOH, 44.5% human albumin, and 37% 0.055 M Na_2HPO_4), with the final concentration and pH adjusted to 20 mg/dL and 7.4, respectively. In each group, ten microhematocrit tubes containing 100 L of the bilirubin solution per tube with or without different concentrations of ABE were for 5 h at room temperature. Bilirubin concentration was measured with Bilirubin Stat-Analyzer (Advanced Instruments Inc., USA).

2.12. Statistical Analysis. The data were analyzed using SPSS and expressed as means ± standard deviation (SD). Differences were considered statistically significant when $P < 0.05$ by one-way analysis of variance (ANOVA).

3. Results and Discussions

Neonatal jaundice occurs in newborns as a result of excessive bilirubin formation and transient inability of the neonatal liver to clear bilirubin rapidly enough from the blood. Severe hyperbilirubinemia is toxic to the developing central nervous system [21]. Prolonged and uncontrolled high levels of bilirubin lead to bilirubin encephalopathy and subsequently kernicterus [22]. We used phenylhydrazine to induce nonhepatic neonatal hyperbilirubinemia in rats because it increased unconjugated bilirubin level. The bilirubin level was measured to evaluate the role of ABE in NJ. The serum STB levels in control group were found to be 0.4 ± 0.07 mg/dL. A significant increase in the serum STB in serum was observed in phenylhydrazine group, as compared to the control group ($P < 0.01$), whereas ABE decreased the STB and SUB levels significantly in a dose-dependent manner (Table 1). It can be somewhat supported from the results of *in vitro* bilirubin degradation experiment (Figure 1). Figure 1 shows the action of the ABE in bilirubin solution. Treatment of ABE shows a significantly higher efficacy of bilirubin gradation than the control group in *in vitro* experiment in a dose dependent manner. This result indicated that the effects of ABE on bilirubin gradation may be one of its important mechanisms in fighting neonatal jaundice.

Table 1: Effect of ABE on serum bilirubin level.

Different groups	STB (mg/DL)	SUB (mg/DL)	SCB (mg/DL)
Control group	0.4 ± 0.07**	0.14 ± 0.03**	0.33 ± 0.05**
ABE-100	2.4 ± 0.50**	1.4 ± 0.51**	0.8 ± 0.50
ABE-50	4.2 ± 0.50*	3.2 ± 0.50*	0.6 ± 0.50
ABE-20	5.1 ± 0.60	4.1 ± 0.60	0.88 ± 0.60
Phenylhydrazine	6.0 ± 0.70	5.1 ± 0.70	0.6 ± 0.71

Values are shown as means ± SEM. $^{*}P < 0.05$ versus phenylhydrazine; $^{**}P < 0.01$ versus phenylhydrazine group.

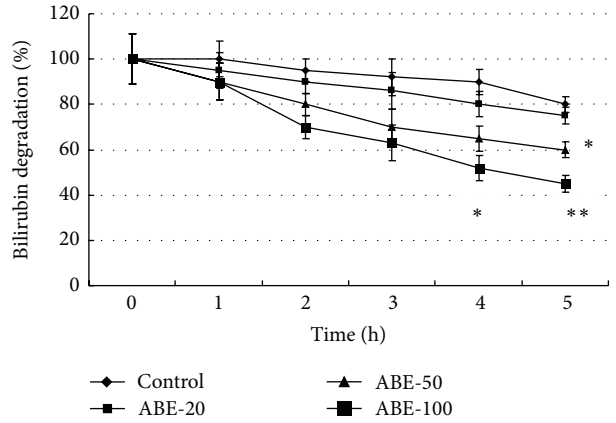


Figure 1: Comparison of *in vitro* efficacy of bilirubin degradation between different concentrations of ABE. Values are shown as means ± SEM. $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group.

Table 2: Effect of ABE on ALT and AST.

Different groups	ALT (U/L)	AST (U/L)
Control group	31.0 ± 3.2	36.0 ± 6.2
ABE-100	30.2 ± 1.0	31.5 ± 1.3
ABE-50	40.2 ± 1.5	34.5 ± 1.5
ABE-20	35.1 ± 8.0	32.6 ± 8.1
Phenylhydrazine	40.2 ± 1.0	40.5 ± 1.3

Phenylhydrazine induces neonatal jaundice conditions because it increases unconjugated bilirubin level without any significant change in the liver function. Liver function was evaluated by assessing serum ALT and AST, since AST and ALT are sensitive indicators of liver cell injury [23]. In the present study, we have confirmed that phenylhydrazine did not increase AST and ALT levels in serum of rats (Table 2). Consistent with the result, the liver histopathological observations also did not show any hepatic damage due to phenylhydrazine administration. As hypothesized, normal activity of the liver function enzymes and absence of any liver damage after ABE administration indicated the safety profile of ABE (Figure 2).

Several studies have shown that the antioxidant defense system is altered during pregnancy. Exposure to oxidative stress may result in excessive bilirubin production that, when combined with diminished conjugation capacity, severely exacerbates the potential for extreme hyperbilirubinemia [24]. Neonatal erythrocytes are prone to oxidative damage due to their unsaturated membrane lipids [25]. In this study, phenylhydrazine administration increased TAOS activity (Table 3). We measured TAOS activity as an indirect indication of the formation of O_2^- and other oxidant species. Those in the ABE-50- and ABE-100-treated groups were significantly lower than those in the phenylhydrazine-treated group ($P < 0.05$ and $P < 0.01$, resp.). Moreover, significantly higher cascade of O_2^-/SOD values was measured in the phenylhydrazine group ($P < 0.01$) compared to the control group. ABE-100 suppressed phenylhydrazine and induced the higher cascade of O_2^-/SOD values. Taken together, these results clearly indicated that oxidative stress was generated in erythrocyte of neonatal jaundiced mice. ABE treatment reversed these adverse effects (Figure 3).

Table 3: Effect of ABE on TAOS activity (μM L-ascorbate).

Different groups	TAOS activity (μM L-ascorbate)
Control group	28.41 ± 3.17
ABE-100	48.35 ± 3.33**
ABE-50	56.30 ± 4.00*
ABE-20	66.22 ± 2.11
Phenylhydrazine	80.33 ± 9.32

Values are shown as means ± SEM. $^{*}P < 0.05$ versus phenylhydrazine; $^{**}P < 0.01$ versus phenylhydrazine group.

An extreme high level of SUB elicits the release of proinflammatory cytokines, such as TNF-α and IL-1β, through the activation of NF-κB signaling pathways at the intracellular level [26, 27]. Substantially, hyperbilirubinemia induced a predominant increase in nuclear translocation of NF-κB (Figure 4(a)). As expected, level of NF-κB protein decreased in the nucleus of liver cells of ABE-100 group (Figure 4(b)). Data obtained from the treatment of rats with phenylhydrazine indicated that NF-κB played an important role in hyperbilirubinemia. The increased NF-κB protein expression was attenuated by ABE-100. It indicated that ABE performs its neonatal jaundice protective effect, at least partly, due to the regulation on NF-κB, which can further influence the release of proinflammatory cytokines.

Adrenomedullin (AM) is a peptide with 52 amino acids and has tyrosine amino acid at carboxy terminal. It plays a significant role in adverse effects and neuronal injury steps of significant hyperbilirubinemia [28]. In this study, there were statistically significant differences between the phenylhydrazine and control groups regarding AM levels ($P < 0.001$). Compared to phenylhydrazine group, ABE administration reduced AM levels in a dose-dependent manner (Table 4). In further analysis, there was a significant positive correlation between serum bilirubin levels and simultaneously measured serum AM levels (Figure 5).

In conclusion, our findings showed that *Agaricus brasiliensis* extract prevented the progression of phenylhydrazine-induced neonatal jaundice in rats. The therapeutic mechanism of *Agaricus brasiliensis* extract not only included

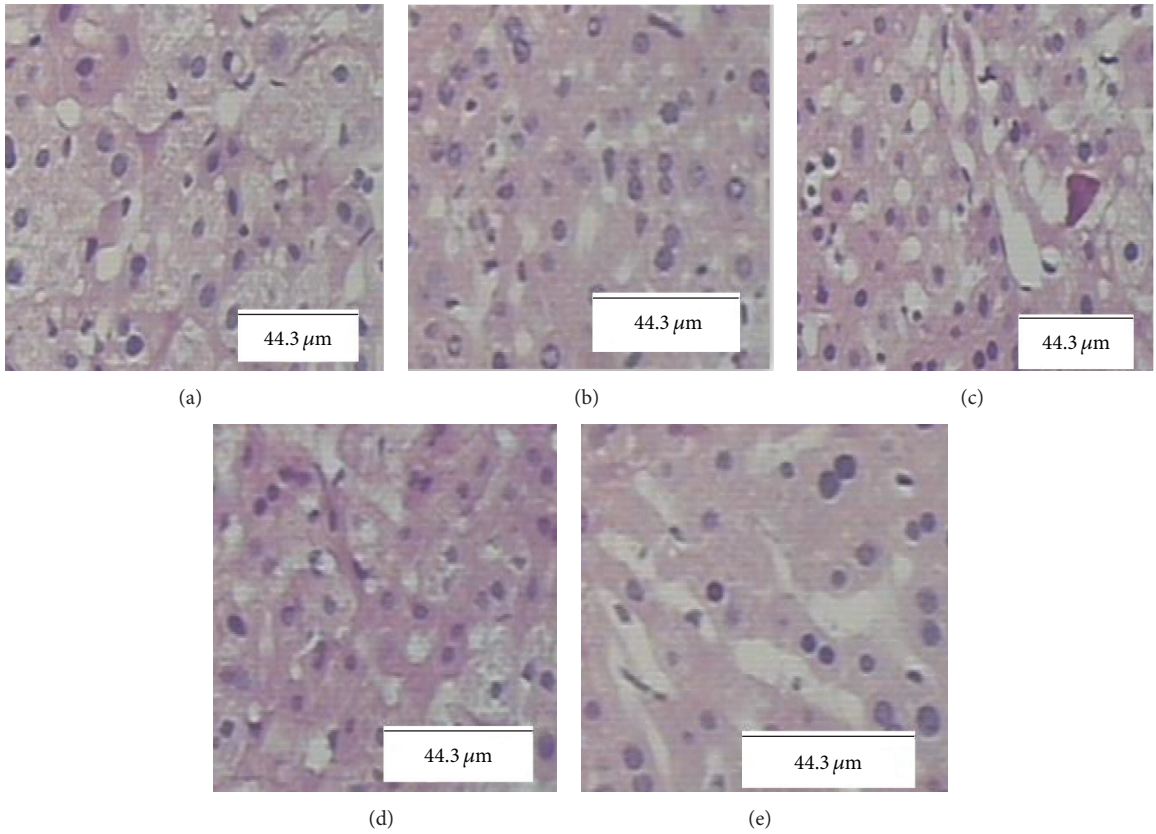


Figure 2: Histopathological analysis of rat liver sections using hematoxylin and eosin staining. (a) Section from a normal control rat liver. (b) Section from ABE-100 rat liver. (c) Section from ABE-50 rat liver. (d) Section from ABE-20 rat liver. (e) Section from a phenylhydrazine rat liver.

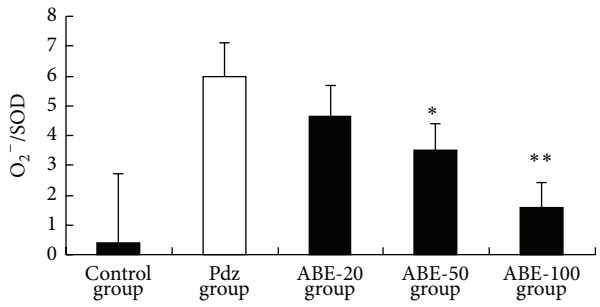


Figure 3: Effect of ABE on cascade of O_2^-/SOD values. Values are shown as means ± SEM. $^*P < 0.05$ versus phenylhydrazine, $^{**}P < 0.01$ versus phenylhydrazine group (Pdz: phenylhydrazine).

efficient bilirubin clearance potential but also reduced oxidative stress and regulation on NF-κB without causing hepatotoxicity. *In vitro* bilirubin degradation experiment showed that effects of ABE on bilirubin gradation may be one of its important mechanisms in fighting neonatal jaundice. From this study, we could conclude that *Agaricus brasiliensis* extract may be used as a protective food or medicine for neonatal jaundice. The potential application of *Agaricus brasiliensis* extract needs to be further studied.

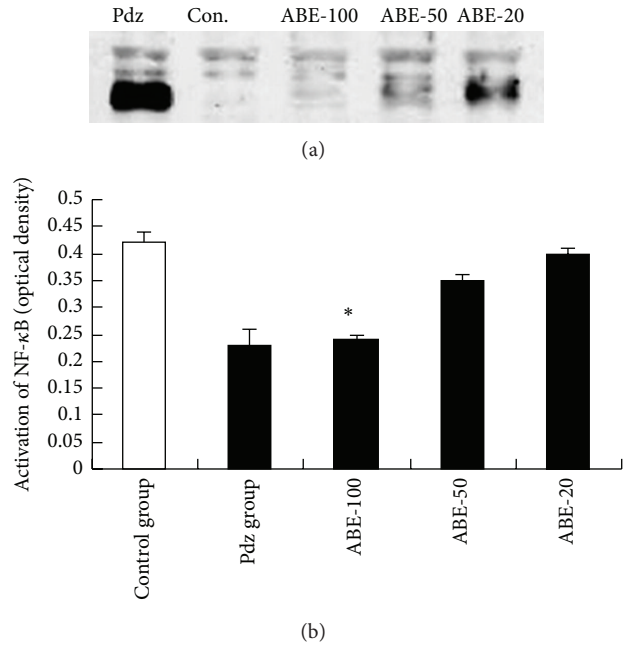


Figure 4: Effect of ABE on protein expression of NF-κB. Values represent the mean ± SEM. $^*P < 0.05$ versus phenylhydrazine group (Pdz: phenylhydrazine).

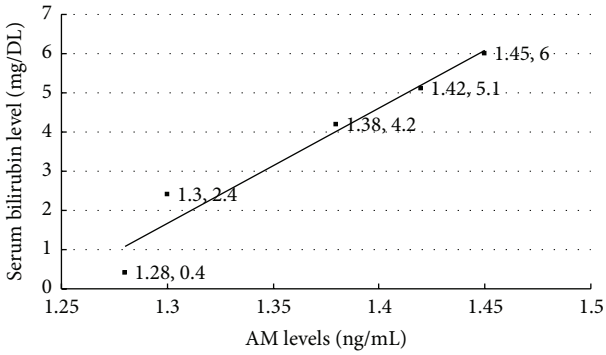


Figure 5: Correlation between serum AM levels and serum bilirubin levels.

Table 4: Effect of ABE on AM levels (ng/mL).

Different groups	AM levels
Control group	1.28 ± 0.07
ABE-100	1.30 ± 0.03***
ABE-50	1.38 ± 0.04*
ABE-20	1.42 ± 0.11
Phenylhydrazine	1.45 ± 0.06

Values are shown as means ± SEM. $^{*}P < 0.05$ versus phenylhydrazine; $^{***}P < 0.001$ versus phenylhydrazine group.

Conflict of Interests

The authors declare that there is no conflict of interests.

References

[1] V. K. Bhutani and L. Johnson, "Kernicterus in the 21st century: frequently asked questions," *Journal of Perinatology*, vol. 29, pp. S20–S24, 2009.

[2] T. Karen, H. U. Bucher, and J.-C. Fauchère, "Comparison of a new transcutaneous bilirubinometer (Bilimed) with serum bilirubin measurements in preterm and full-term infants," *BMC Pediatrics*, vol. 9, article 70, 2009.

[3] M. J. Maisels and A. F. McDonagh, "Phototherapy for neonatal jaundice," *The New England Journal of Medicine*, vol. 358, no. 9, pp. 920–928, 2008.

[4] T. M. Slusher and B. O. Olusanya, "Neonatal jaundice in low- and middle-income countries," in *Care of the Jaundiced Neonate*, chapter 13, pp. 263–273, 2012.

[5] M. K. Mwaniki, M. Atieno, J. E. Lawn, and C. R. Newton, "Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review," *The Lancet*, vol. 379, no. 9814, pp. 445–452, 2012.

[6] B. K. Cline, H. J. Vreman, K. Faber et al., "Phototherapy device effectiveness in nigeria: irradiance assessment and potential for improvement," *Journal of Tropical Pediatrics*, vol. 59, no. 4, pp. 321–325, 2013.

[7] M. J. Maisels, "Phototherapy—traditional and nontraditional," *Journal of Perinatology*, vol. 21, supplement 1, pp. S93–S97, 2001.

[8] K. L. Tan, G. C. Lim, and K. W. Boey, "Efficacy of "high-intensity" blue-light and "standard" daylight phototherapy for non-haemolytic hyperbilirubinaemia," *Acta Paediatrica*, vol. 81, no. 11, pp. 870–874, 1992.

[9] M. J. Maisels, "Why use homeopathic doses of phototherapy?" *Pediatrics*, vol. 98, no. 2, pp. 283–287, 1996.

[10] N. Manabe, Y. Azuma, M. Sugimoto et al., "Effects of the mycelial extract of cultured *Cordyceps sinensis* on in vivo hepatic energy metabolism and blood flow in dietary hypoferric anaemic mice," *British Journal of Nutrition*, vol. 83, no. 2, pp. 197–204, 2000.

[11] F. Firenzuoli, L. Gori, and G. Lombardo, "The medicinal mushroom *Agaricus blazei* murrill: review of literature and pharmaco-toxicological problems," *Evidence-based Complementary and Alternative Medicine*, vol. 5, no. 1, pp. 3–15, 2008.

[12] J.-Y. Guo, C.-C. Han, and Y.-M. Liu, "A contemporary treatment approach to both diabetes and depression by cordyceps sinensis, Rich in Vanadium," *Evidence-Based Complementary and Alternative Medicine*, vol. 7, no. 3, pp. 387–389, 2010.

[13] M.-F. Wu, Y.-M. Hsu, M.-C. Tang et al., "*Agaricus blazei* Murill extract abrogates CCl4-induced liver injury in rats," *In Vivo*, vol. 25, no. 1, pp. 35–40, 2011.

[14] J.-B. Chang, M.-F. Wu, Y.-Y. Yang et al., "Carbon tetrachloride-induced hepatotoxicity and its amelioration by *Agaricus blazei* Murrill extract in a mouse model," *In Vivo*, vol. 25, no. 6, pp. 971–976, 2011.

[15] C.-H. Hsu, K.-C. Hwang, Y.-H. Chiang, and P. Chou, "The mushroom Agaricus blazei Murill extract normalizes liver function in patients with chronic hepatitis B," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 3, pp. 299–301, 2008.

[16] H. Wang, G. Li, W. Zhang, and C. Han, "The protective effect of *Agaricus blazei* Murrill, submerged culture using the optimized medium composition, on alcohol-induced liver injury," *BioMed Research International*, vol. 2014, Article ID 573978, 9 pages, 2014.

[17] G. B. Mejia, C. R. Sanz, M. M. Avila et al., "Experimental hemolysis model to study bilirubin encephalopathy in rat brain," *Journal of Neuroscience Methods*, vol. 168, no. 1, pp. 35–41, 2008.

[18] C. Han, "Antinociceptive activity of agaricoglycerides extracted from mycelium of Ling Zhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Aphyllophoromycetideae)," *International Journal of Medicinal Mushrooms*, vol. 12, no. 3, pp. 273–278, 2010.

[19] H. P. Misra and I. Fridovich, "The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase.," *Journal of Biological Chemistry*, vol. 247, no. 10, pp. 3170–3175, 1972.

[20] E. Beutler, "Catalase," in *Red Cell Metabolism, A Manual of Biochemical Methods*, E. Beutler, Ed., pp. 105–106, Grune and Stratton, New York, NY, USA, 1982.

[21] M. K. Mwaniki, M. Atieno, J. E. Lawn, and C. R. J. C. Newton, "Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review," *The Lancet*, vol. 379, no. 9814, pp. 445–452, 2012.

[22] D. Pashankar and R. A. Schreiber, "Jaundice in older children and adolescents," *Pediatrics in Review*, vol. 22, no. 7, pp. 219–226, 2001.

[23] D. S. Pratt and M. M. Kaplan, "Evaluation of abnormal liver-enzyme results in asymptomatic patients," *The New England Journal of Medicine*, vol. 342, no. 17, pp. 1266–1271, 2000.

[24] M. Kaplan and C. Hammerman, "Glucose-6-phosphate dehydrogenase deficiency and severe neonatal hyperbilirubinemia: a complexity of interactions between genes and environment," *Seminars in Fetal and Neonatal Medicine*, vol. 15, no. 3, pp. 148–156, 2010.

[25] S. K. Jain, "The neonatal erythrocyte and its oxidative susceptibility," *Seminars in Hematology*, vol. 26, no. 4, pp. 286–300, 1989.

[26] D. Brites, A. Fernandes, A. S. Falcão, A. C. Gordo, R. F. M. Silva, and M. A. Brito, "Biological risks for neurological abnormalities associated with hyperbilirubinemia," *Journal of Perinatology*, vol. 29, supplement 1, pp. S8–S13, 2009.

[27] A. Fernandes, A. R. Vaz, A. S. Falcão, R. F. M. Silva, M. A. Brito, and D. Brites, "Glycoursodeoxycholic acid and interleukin-10 modulate the reactivity of rat cortical astrocytes to unconjugated bilirubin," *Journal of Neuropathology and Experimental Neurology*, vol. 66, no. 9, pp. 789–798, 2007.

[28] C.-F. Xia, H. Yin, C. V. Borlongan, J. Chao, and L. Chao, "Adrenomedullin gene delivery protects against cerebral ischemic injury by promoting astrocyte migration and survival," *Human Gene Therapy*, vol. 15, no. 12, pp. 1243–1254, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 281873, 5 pages
http://dx.doi.org/10.1155/2015/281873

Review Article

The Advances in Research on the Pharmacological Effects of *Fructus Ligustri Lucidi*

Zunting Pang,[1] Zhou Zhi-yan,[2] Wei Wang,[1] Yanni Ma,[1] Niu Feng-ju,[3] Xuelan Zhang,[1] and Chunchao Han[1]

[1] *School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[2] *School of Stomatology, Jilin University, Changchun 130021, China*
[3] *Jinan Fifth People's Hospital, Jinan 250022, China*

Correspondence should be addressed to Chunchao Han; chunchaoh@126.com

Received 10 September 2014; Accepted 17 November 2014

Academic Editor: John E. Smith

Fructus Ligustri Lucidi is a well-known invigorator in Chinese materia medica with hepatoprotective effect, anticancer activity, antioxidant activity, and so on. And oleanolic acids are the major pharmacologically active components in *Fructus Ligustri Lucidi*. So it has great value in medical health, and may be developed to a complementary and alternative medicine through further research. In this paper, the advances in research on pharmacological effects of *Fructus Ligustri Lucidi* were summarized by reviewing the recent related literature.

1. Introduction

Fructus Ligustri Lucidi (FLL, Nuzhenzi in Chinese) is the dried ripen fruit of *Ligustrum lucidum* Ait and it is a widely used herbal medicine for the prevention and treatment of a variety of pathologies. In the theory of Chinese traditional medicine, it has the effects of tonifying middle, calming five zang-organs, cultivating spirit, and nourishing the kidneys and liver. Besides, it can be applied to improve eyesight, replenish the liver and kidney, and promote the growth of black hair.

In recent decades, great progress about *Fructus Ligustri Lucidi* has been achieved by scholars inside and outside, and they have found a variety of active ingredients in *Fructus Ligustri Lucidi*, such as triterpenes, secoiridoid glucosides, volatile components, flavonoids, and phenolic compounds [1, 2]. In addition, modern pharmacological and chemical researches have indicated that *Fructus Ligustri Lucidi* has many pharmacological effects including hepatoprotective effect [3–5], anticancer activity [6, 7], antioxidant activity [8–10], immunomodulating effect [11], antiviral activity [12, 13], and antiosteoporosis activity [14–23] (Table 1). Although the clinical researches about pharmacological effects of *Fructus Ligustri Lucidi* are less, *Fructus Ligustri Lucidi* as the alternative chemotherapeutic and chemopreventive agents have recently received more and more attention.

2. The Pharmacological Effects of *Fructus Ligustri Lucidi*

2.1. Hepatoprotective Effect. Oleanolic acid (OA, Figure 1) is a triterpenoid compound that exists widely in *Fructus Ligustri Lucidi*, and the hepatoprotective effect of OA was first reported in China and it has been used to treat liver disease in humans [3, 4]. Yim et al. have demonstrated that the fractions of chloroform and butanol derived from *Fructus Ligustri Lucidi*, which were enriched with oleanolic acid (OA), presented a dose-dependent protection against CCl_4-induced hepatic injury *in vivo*. The promising hepatoprotective action may be associated with the enhancement of hepatic glutathione regeneration capacity (GRC), particularly under conditions of CCl_4-induced oxidative stress [5].

2.2. Anticancer Activity. Liver cancer remains the fifth most common cancer in men and the seventh in women

Table 1: The pharmacological effects and active compounds of *Fructus Ligustri Lucidi*.

The pharmacological effects	Active compounds	References
Hepatoprotective effect	Oleanolic acid	[3–5]
Anticancer activity	Aqueous extracts Methanol extracts	[6, 7]
Antioxidant activity	Secoiridoid glucosides Hydroxytyrosol and salidroside Ethanol extract	[8–10]
Immunomodulating effect	Oleanolic acid	[11]
Antiviral activity	Oleanolic acid Ursolic acid	[12, 13]
Antiosteoporosis activity	Ethanol extract Water extracts	[14–23]

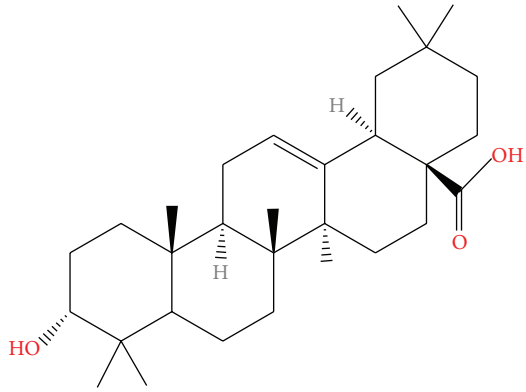


Figure 1: The structure of oleanolic acid (OA).

worldwide [24]. In China, traditional Chinese medicine (TCM) has a long history to treat liver cancer, and *Fructus Ligustri Lucidi* is known as the liver/kidney Yin tonifying herbs for liver cancer treatment [25]. Hu et al. have evaluated the effect of aqueous extracts of FFL on hepatocarcinoma cells, and significant apoptosis and cell senescence of hepatocellular carcinoma Bel-7402 cells by upregulating of p21 were observed [6].

On the other hand, because little information is available regarding the effect of FLL on glioma cell growth, Jeong et al. performed an experiment to investigate whether FLL extracts (extracted with methyl alcohol) affect glioma tumor growth. The results showed that glioma cell death could be caused by regulating the Akt/mTOR/survivin pathway *in vitro* [7].

2.3. Antioxidant Activity. Based on earlier reports, the human metabolic processes can produce harmful free radicals inevitably, which have been shown to possibly result in aging and other diseases [26, 27]. So, it is necessary to find effective radical scavengers in order to relieve the damaging effects of harmful free radicals.

Zhen-Dan he et al. have isolated ten secoiridoid glucosides from FLL by bioassay-guided analysis, and their effects on free radical induced by hemolysis of RBC were tested. And five of them ((1) oleoside dimethyl ester, (2) oleuropein, (3) neonuezhenide, (4) lucidumoside B, and (5) lucidumoside C, Figure 2) showed significant inhibitory effects on the hemolysis of red blood cells induced by free radicals [8]. Ju et al. extracted FLL five times with 50% ethanol, and the crude extract was partitioned with four-times-volume amounts of n-butanol, chloroform, and ethyl acetate. Then they performed a series of antioxidant experiments *in vitro* to evaluate the antioxidant and protective properties of the different fractions against H_2O_2-induced oxidative damage in SH-SY5Y cells. They demonstrated that the phenolic-enriched ethyl acetate (EtOAc) fraction, whose major components are hydroxytyrosol and salidroside, was the most active part in scavenging free radicals and increasing the levels of antioxidant enzymes [9].

In addition, Lin et al. examined the antioxidant activities of ethanol extract of *Ligustrum lucidum fruits* (ELL) and its pharmacological effects on BHT-induced oxidative stress in rats. In their study, compared to the BHT-treated group (1000 mg/kg), the significant decrease in the levels of sGOT, sGPT, BUN, sALP, Cr, TG, LDH, BALF LDH, and lipid peroxides in liver and lung and the enhancement in the levels of antioxidant enzymes in these organs in BHT-treated rats were observed in the ELL-treated groups (250, 500, and 1000 mg/kg), which supported the protective effect of ELL against BHT-induced oxidative stress [10].

2.4. Immunomodulating Effect. As mentioned above, OA is the main effective constituent of FLL. Because OA can stimulate Th1 cells leading to the secretion of Th1 cytokines and then upregulates the Th1/Th2 arms resulting in raising the percentage of $CD4^+CD8^-$cells and promoting lymphocyte proliferation, OA has potential immunomodulatory effects. Wang et al. selected LLE as an immunoregulator, and they employed supercritical CO_2 extraction technology to extract OA from FLL. Furthermore, immunomodulatory effect of OA on the immune cells of piglets was investigated through a series of experiments *in vitro*. Their results showed that OA, as an immunoadjuvant, has a beneficial and promising influence on the immune of piglets [11].

2.5. Antiviral Activity. It is well-known that hepatitis C virus (HCV) infection is a serious worldwide problem, which causes significant mortality and morbidity. Some reports have showed that HCV affects about 3% of the global population and approximately 20–30% have developed liver disease, such as liver cirrhosis and chronic hepatitis. To date, current therapy with adverse side effects has curative powers in about 50% of patients infected with HCV genotype 1 and many chronically infected patients remain untreated [28–30]. So the progress of searching for effective antiviral drugs against HCV continues to be needed. And more and more researchers have studied the HCV inhibitors from FFL. Fortunately, accumulating evidence indicates that some active compounds of FLL were claimed to have potent antiviral activities against HCV. Lingbao Kong et al. found that oleanolic acid and ursolic acid (Figure 3) extracted from FLL are two antiviral components that possess anti-HCV activity based on their isomeric structures. They could

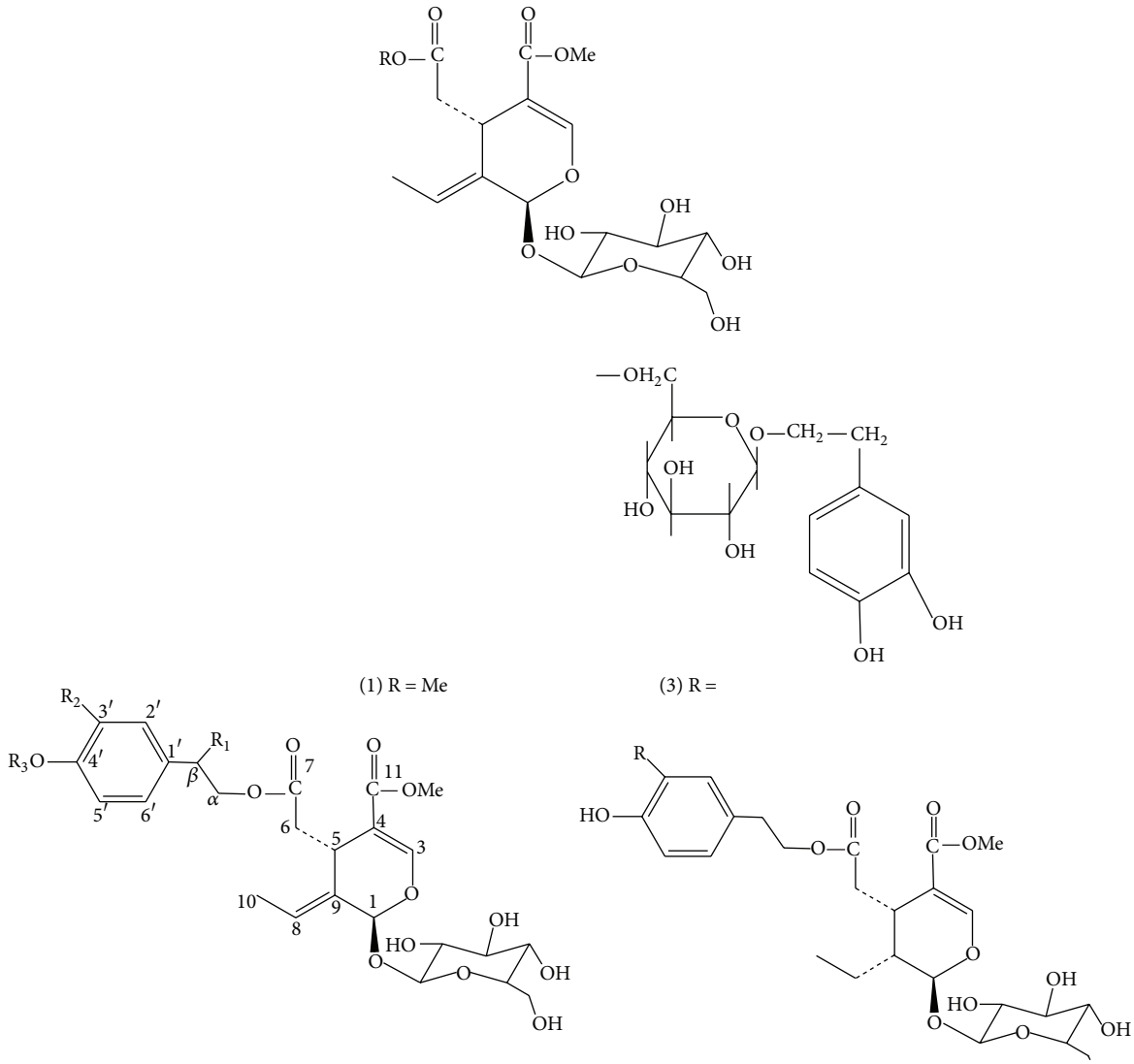


Figure 2: The structure of oleoside dimethyl ester, oleuropein, neonuezhenide, lucidumoside B, and lucidumoside C.

suppress intracellular HCV NS5B RdRp activity. Moreover, they significantly inhibited the replication of HCV genotype 1b replicon and HCV genotype 2a JFH1 virus. By contrast, the results of their studies showed that oleanolic acid has one CH_3 branched to last ring at C-20 position instead of C-19 position, so oleanolic acid had the better antiviral effect. In this way, the antiviral activity could be improved by modifying some compounds [12, 13].

2.6. Antiosteoporosis Activity. Fruit *Ligustrum lucidum* has long been used for the treatment of osteoporosis in China. Modern research in ovariectomized rats has demonstrated that the crude FLL extract could be useful to modulate the calcium balance and the turnover of bone [14–16]. Some reports showed that the ethanol extract of FLL (EFLL) could directly enhance the mineralization process resulting in improving bone properties and calcium balance in aged female rats [17–19]. These studies have provided evidence for the prevention and treatment of postmenopausal osteoporosis.

Ying Lyu et al. observed that FLL ethanol significantly increased bone mineral density and exerted beneficial improvement of bone mechanical properties in a dose-dependent manner by increasing Ca absorption and Ca retention, as well as reducing the RANKL/OPG ratio in growing female rats [20].

Estrogen deficiency and oxidative stress are considered as two major factors that cause the occurrence of osteoporosis [21, 22]. Thus, Chen et al. investigated the osteogenic

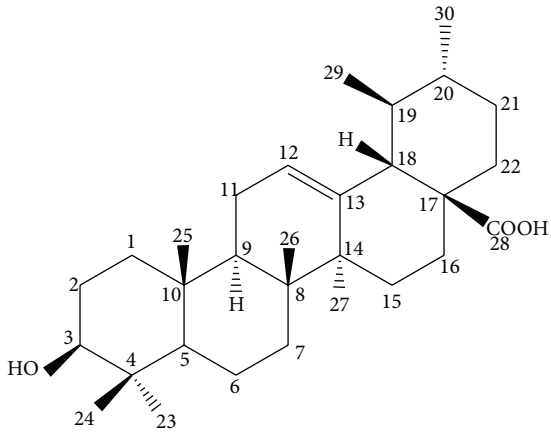


Figure 3: The structure of ursolic acid.

constituents of FLL. They isolated and identified eight compounds from the aqueous extract of FLL (AFLL), namely, tyrosol (1), tyrosyl acetate (2), salidroside (3), hydroxytyrosol (4), oleoside dimethyl ester (5), nuzhenide (6), oleoside-7-ethyl-11-methyl ester (7), and G13 (8). Further studies showed that all eight compounds exhibited the antiosteoporotic effect with different mechanisms such as antioxidative effects and ER-dependent or independent pathways [23].

3. Conclusion

Fructus Ligustri Lucidi (FLL, Chinese name, Nuzhenzi) has been used in traditional Chinese medicine for over 1000 years. It contains a number of bioactive components. Moreover, FLL has been known to have hepatoprotective effect, anticancer activity, antioxidant activity, immunomodulating effect, antiviral activity, and antiosteoporosis activity, and other pharmacodynamic effects have been demonstrated as well, such as the treatment of coronary heart disease [31]. The chemical constituents of FLL include polysaccharides, triterpenes, secoiridoids, and flavonoids, which may be contributed to the pharmacological activities of FLL [32–34]. Therefore, the pharmacological effects and health function of *Fructus Ligustri Lucidi* are more and more focused on in the world.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This work was supported by the Foundation of Jinan Science and Technology Development Program (201303055). This project was supported by Project of Shandong Province Higher Educational Science and Technology Program (J14LK61). This project was also supported by National Natural Science Foundation of China (81373968).

References

[1] Z.-D. He, H. Dong, H.-X. Xu, W.-C. Ye, H.-D. Sun, and P. P.-H. But, "Secoiridoid constituents from the fruits of Ligustrum lucidum," *Phytochemistry*, vol. 56, no. 4, pp. 327–330, 2001.

[2] Z.-D. He, S. Ueda, M. Akaji, T. Fujita, K. Inoue, and C.-R. Yang, "Monoterpenoid and phenylethanoid glycosides from Ligustrum pedunculare," *Phytochemistry*, vol. 36, no. 3, pp. 709–716, 1994.

[3] B. Wang and Z. Jiang, "Studies on oleanolic acid," *Chinese Pharmaceutical Journal*, vol. 17, no. 27, pp. 393–397, 1992.

[4] W. F. Pak, "Therepeutic efficacy of oleanolic acid on acute jaundice hepatitis and histology of experiment animals," *Bulletin Chinese Traditional Herb Drugs*, vol. 17, no. 4, pp. 32–36, 1977.

[5] T. K. Yim, W. K. Wu, W. F. Pak, and K. M. Ko, "Hepatoprotective action of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruits is mediated through an enhancement on hepatic glutathione regeneration capacity in mice," *Phytotherapy Research*, vol. 15, no. 7, pp. 589–592, 2001.

[6] B. Hu, Q. Du, S. Deng et al., "Ligustrum lucidum Ait. fruit extract induces apoptosis and cell senescence in human hepatocellular carcinoma cells through upregulation of p21," *Oncology Reports*, vol. 32, no. 3, pp. 1037–1042, 2014.

[7] J. C. Jeong, J. W. Kim, C. H. Kwon, T. H. Kim, and Y. K. Kim, "*Fructus ligustri lucidi* extracts induce human glioma cell death through regulation of Akt/mTOR pathway in vitro and reduce glioma tumor growth in U87MG xenograft mouse mode," *Phytotherapy Research*, vol. 25, no. 3, pp. 429–434, 2011.

[8] Z.-D. He, P. P.-H. But, T.-W. D. Chan et al., "Antioxidative glucosides from the fruits of Ligustrum lucidum," *Chemical and Pharmaceutical Bulletin*, vol. 49, no. 6, pp. 780–784, 2001.

[9] H.-Y. Ju, S. C. Chen, K.-J. Wu et al., "Antioxidant phenolic profile from ethyl acetate fraction of Fructus Ligustri Lucidi with protection against hydrogen peroxide-induced oxidative damage in SH-SY5Y cells," *Food and Chemical Toxicology*, vol. 50, no. 3-4, pp. 492–502, 2012.

[10] H. M. Lin, F. L. Yen, L. T. Ng, and C. C. Lin, "Protective effects of Ligustrum lucidum fruit extract on acute butylated hydroxytoluene-induced oxidative stress in rats," *Journal of Ethnopharmacology*, vol. 111, no. 1, pp. 129–136, 2007.

[11] J. Wang, A. Shan, T. Liu, C. Zhang, and Z. Zhang, "In vitro immunomodulatory effects of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruit (*Ligustrum lucidum* supercritical CO_2 extract) on piglet immunocytes," *International Immunopharmacology*, vol. 14, no. 4, pp. 758–763, 2012.

[12] L. Kong, S. Li, X. Han et al., "Inhibition of HCV RNA-dependent RNA polymerase activity by aqueous extract from *Fructus Ligustri Lucidi*," *Virus Research*, vol. 128, no. 1-2, pp. 9–17, 2007.

[13] L. Kong, S. Li, Q. Liao et al., "Oleanolic acid and ursolic acid: novel hepatitis C virus antivirals that inhibit NS5B activity," *Antiviral Research*, vol. 98, no. 1, pp. 44–53, 2013.

[14] Y. Zhang, W.-P. Lai, P.-C. Leung, C.-F. Wu, X.-S. Yao, and M.-S. Wong, "Effects of *Fructus Ligustri Lucidi* extract on bone turnover and calcium balance in ovariectomized rats," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 2, pp. 291–296, 2006.

[15] Y. Zhang, W.-P. Lai, P.-C. Leung, C.-T. Che, and M.-S. Wong, "Improvement of Ca balance by *Fructus Ligustri Lucidi* extract in aged female rats," *Osteoporosis International*, vol. 19, no. 2, pp. 235–242, 2008.

[16] X.-L. Dong, M. Zhao, K.-K. Wong, C.-T. Che, and M.-S. Wong, "Improvement of calcium balance by *Fructus Ligustri*

Lucidi extract in mature female rats was associated with the induction of serum parathyroid hormone levels," *British Journal of Nutrition*, vol. 108, no. 1, pp. 92–101, 2012.

[17] Y. Zhang, P.-C. Leung, C.-T. Che, H.-K. Chow, C.-F. Wu, and M.-S. Wong, "Improvement of bone properties and enhancement of mineralization by ethanol extract of *Fructus Ligustri Lucidi*," *British Journal of Nutrition*, vol. 99, no. 3, pp. 494–502, 2008.

[18] C. H. Ko, W. S. Siu, C. P. Lau, C. B. S. Lau, K. P. Fung, and P. C. Leung, "Osteoprotective effects of *Fructus Ligustri Lucidi* aqueous extract in aged ovariectomized rats," *Chinese Medicine*, vol. 5, article 39, 2010.

[19] G. Li, X.-A. Zhang, J.-F. Zhang et al., "Ethanol extract of *Fructus Ligustri Lucidi* promotes osteogenesis of mesenchymal stem cells," *Phytotherapy Research*, vol. 24, no. 4, pp. 571–576, 2010.

[20] Y. Lyu, X. Feng, P. Zhao et al., "*Fructus Ligustri Lucidi* (FLL) ethanol extract increases bone mineral density and improves bone properties in growing female rats," *Journal of Bone and Mineral Metabolism*, vol. 32, no. 6, pp. 616–626, 2014.

[21] Q. Chen, L. Yang, G. Zhang, and F. Wang, "Bioactivity-guided isolation of antiosteoporotic compounds from *Ligustrum lucidum*," *Phytotherapy Research*, vol. 27, no. 7, pp. 973–979, 2013.

[22] R. Pacifici, "Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis," *Journal of Bone and Mineral Research*, vol. 11, no. 8, pp. 1043–1051, 1996.

[23] M. Abdollahi, B. Larijani, R. Rahimi, and P. Salari, "Role of oxidative stress in osteoporosis," *Therapy*, vol. 2, no. 5, pp. 787–796, 2005.

[24] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[25] X. Bo, X. M. Zhao, H. T. Liu, W. G. Ma, and H. Li, "A study on 4 kinds of Chinese medicines reducing arrest of bone marrow by cyclophosphomidum," *Journal of the Beijing Union University (Natural Science)*, vol. 17, no. 8, pp. 58–61, 1994.

[26] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, 1999.

[27] M. E. J. Lean, M. Noroozi, I. Kelly et al., "Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA," *Diabetes*, vol. 48, no. 1, pp. 176–181, 1999.

[28] P. P. Ip, H. W. Nijman, J. Wilschut, and T. Daemen, "Therapeutic vaccination against chronic hepatitis C virus infection," *Antiviral Research*, vol. 96, no. 1, pp. 36–50, 2012.

[29] T. J. Liang, B. Rehermann, L. B. Seeff, and J. H. Hoofnagle, "Pathogenesis, natural history, treatment, and prevention of hepatitis C," *Annals of Internal Medicine*, vol. 132, no. 4, pp. 296–305, 2000.

[30] V. de Re, "Interferon-based therapy for chronic hepatitis C: current and future perspectives," *Hepatitis Monthly*, vol. 10, no. 3, pp. 231–232, 2010.

[31] M. L. Li, "Progress in the study on *Fructus Ligustri Lucidi*," *Zhongguo Zhong and Yao Za Zhi*, vol. 19, no. 8, pp. 504–506, 1994 (Chinese).

[32] X. H. Xu, N. Y. Yang, S. H. Qian, N. Xie, M. Y. Yu, and J. A. Duan, "Study on flavonoids in *Ligustrum lucidum*," *Journal of Chinese Medicinal Materials*, vol. 30, no. 2, pp. 538–540, 2007.

[33] X.-H. Xu, N.-Y. Yang, S.-H. Qian, N. Xie, and J.-A. Duan, "Dammarane triterpenes from *Ligustrum lucidum*," *Journal of Asian Natural Products Research*, vol. 10, no. 1, pp. 33–37, 2008.

[34] S. Aoki, Y. Honda, T. Kikuchi et al., "Six new secoiridoids from the dried fruits of *Ligustrum lucidum*," *Chemical and Pharmaceutical Bulletin*, vol. 60, no. 2, pp. 251–256, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 423869, 6 pages
http://dx.doi.org/10.1155/2015/423869

Research Article

Osteoprotective Effect of Cordycepin on Estrogen Deficiency-Induced Osteoporosis *In Vitro* and *In Vivo*

Da-wei Zhang,[1] Hualiang Deng,[2] Wei Qi,[1,3] Guang-yue Zhao,[1] and Xiao-rui Cao[1]

[1]*Department of Orthopedics, Xi Jing Hospital, The Fourth Military Medical University, Xi'an 710032, China*
[2]*Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[3]*The Surgery Department of 520th Hospital of PLA, Mianyang 621000, China*

Correspondence should be addressed to Guang-yue Zhao; guangyuezfmmu@yeah.net
and Xiao-rui Cao; xiaoruicao69@163.com

Received 16 December 2014; Accepted 24 January 2015

Academic Editor: Thomas G. Mitchell

The purpose of this study was to verify the effect of cordycepin on ovariectomized osteopenic rats. Fifty Wistar female rats used were divided into 5 groups: (1) sham-operation rats (control), (2) ovariectomized (OVX) rats with osteopenia, and (3) OVX'd rats with osteopenia treated with cordycepin (5 mg, 10 mg, and 20 mg) for 8 weeks. After the rats were treated orally with cordycepin, serum alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP), serum osteocalcin (OC), homocysteine (HCY) , C-terminal crosslinked telopeptides of collagen type I (CTX) level, and oxidative stress were examined, respectively. The femoral neck was used for mechanical compression testing. At the same time, we further investigated the effect of cordycepin *in vitro* assay. The beneficial effects of cordycepin on improvement of osteoporosis in rats were attributable mainly to decrease ALP activity, TRAP activity, and CTX level. At the same time, cordycepin also increases the OC level in ovariectomized osteopenic rats. The histological examination clearly showed that dietary cordycepin can prevent bone loss caused by estrogen deficiency. These experimental results suggest that complement cordycepin is protective after ovariectomized osteopenic in specific way.

1. Introduction

Osteoporosis is a major concern in public health care and the disease has severe consequences if untreated [1, 2]. It is characterized by low bone mineral density (BMD) and loss of the structural and biomechanical properties that are required to maintain bone homeostasis. Bone is a metabolically active tissue that undergoes remodeling throughout life, with roughly 5% remodeled at any time [3]. Over several weeks, a bone remodeling unit (BMU) will develop that incorporates several cell types, including osteoclasts, osteoblasts, and osteocytes. The loss of sclerostin and alterations in other secreted cytokines and chemotactic factors promote BMU's formation. Despite current treatment options that include vitamin D, hormone, and bisphosphonates therapy, osteoporosis results in significant morbidity and mortality. Development of novel therapies is vital for therapy against osteolytic bone diseases.

The herbal kingdom is a wide field to search for natural effective osteoporosis protective agent that has no side effects. As potential alternative treatments for osteoporosis, the preventive and therapeutic effects of natural products derived from plants have been reported [4–6]. *Cordyceps* is a genus of the family Clavicipitaceae that has been used in traditional Oriental medicine for centuries. Recent studies have demonstrated that the bioactive components isolated from this genus have various pharmacological actions [7–9]. Among them, cordycepin, also known as 3-deoxyadenosine, has been shown to possess multiple pharmacological activities such as inhibition of tumour growth, modulation of the immune response, and suppression of reactive oxygen species [10]. In the former paper, we have reported that cordycepin can act as anti-inflammatory agent in magnesium silicate-induced inflammation in osteoporosis [11]. However, the role of cordycepin in estrogen deficiency-induced osteoporosis in ovariectomized rats has not been investigated. The aim of the

present investigation was to discover cordycepin for effective osteoporosis treatment *in vivo* and *in vitro*.

2. Materials and Methods

2.1. Animals. Wistar rats (weighing 225 ± 25 g) were used in the study. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals. The study was submitted to, and approved by, the Fourth Military Medical University institutional ethics committee.

2.2. Drugs. Cordycepin with 98% purification was obtained following the extraction and separation using a column chromatographic method [12].

2.3. Experimental Design. Fifty rats were randomly divided into five groups of animals, four ovariectomized (OVX) and another given a sham-operation (control). Then groups 1 (sham) and 2 (OVX) were treated orally with 10 mL of saline; group 3, group 4, and group 5 were treated orally with cordycepin (5 mg, 10 mg, and 20 mg) for 8 weeks, respectively. Cordycepin was dissolved in distilled water and administrated orally twice daily using a feeding needle for 21 days, and control group received double distilled water instead of cordycepin. Body weight of the animals was recorded weekly.

On the last day of treatment and necropsy, blood was collected from dorsal aorta under ether anesthesia. After centrifugation, serum was harvested and kept at −20°C until analysis. The femoral neck was processed for mechanical testing. The entire fifth lumbar vertebrae and one tibia were processed for histology.

2.4. Mechanical Testing. The mechanical strength of the femoral neck was measured by applying a vertical load to the femoral head using a Shimadzu EZ-1 pressure system. The fracture load was recorded at the peak force as Newton (N) at the point that the femoral neck fractured [13].

2.5. Histomorphometry of Osteoblast Surface. The tibia and the lumbar vertebrae were decalcified in formic acid, embedded in paraffin, and longitudinally sectioned. Histomorphometric analyses were made by tracing the section image onto a digitizing platen with the aid of a camera lucida attachment on the microscope and Osteomeasure bone analysis software. To reveal osteoclasts, sections were stained for immunoreactivity to cathepsin K, an osteoclast marker [14]. Osteoblast perimeter was determined by scoring osteoblasts in direct contact with cancellous bone surfaces.

2.6. Plasma Enzyme Measurements. ALP and TRAP activity were determined by nitrophenol based method as described by Bessy et al. [15] and Godkar [16], respectively.

2.7. Plasma Proteins Measurements. Serum osteocalcin (OC) content was determined using an Osteocalcin EIA kit (Xinyubio-Technology, Inc., China) as described in the manufacturer's directions. Homocysteine (HCY) was measured by use of an enzymatic fluorescence polarization immunoassay on an Axsym analyzer (Abbott, Wiesbaden, Germany). C-terminal crosslinked telopeptides of collagen type I (CTX) were quantified by ELISA (Sunbio, Inc., China).

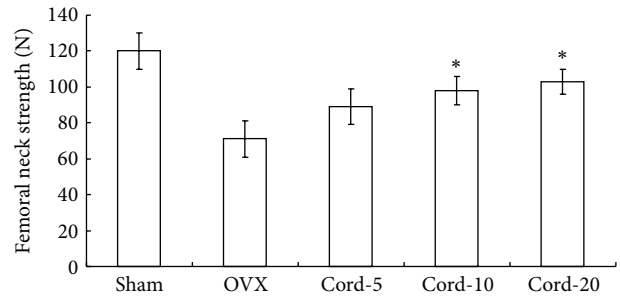


Figure 1: Effects of cordycepin on mechanical strength of the femoral neck. The data are presented as mean ± SD ($n = 10$ per group). $^{*}P < 0.05$ as compared with corresponding values in saline-treated OVX. Bone strength of the femoral neck was significantly lower in the saline-treated OVX compared with the sham group and cordycepin treatment group.

2.8. In Vitro Assay and Alkaline Phosphatase (ALP) and Tartrate Resistant Acid Phosphatase (TRAP). The murine mesenchymal stem cell line was purchased from the Beijing Lihao Inc., China, and grown in a DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cultured cells were incubated in a humidified atmosphere at 37°C and at 5% CO_2. The study used cells with passages 5–10 (after purchase) for all experiments in cell lines. Cells (3×10^3 cells/well) were incubated in a 96-well plate overnight and cotreated with different concentrations of cordycepin in the medium for 48 h. ALP activity was measured in total cell lysates after homogenization in a buffer containing 1 mmol/L Tris–HCl (pH 8.8), 0.5% Triton X-100, 10 mmol/L Mg2+, and 5 mmol/L p-nitrophenylphosphate as substrates. The absorbance was read at 405 nm. The differentiated osteoclast cells from monocytes were measured by a TRAP activity assay and staining using the Acid-Phosphatase Kit (Shanghai Jinma Biological Technology, Inc., China).

2.9. Oxidative Stress Assay. In serum, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na^+K^+ATPase activity, and glutathione S transferase (GST) activity were quantified by ELISA (Sunbio, Inc., China).

2.10. Statistical Analysis. Data were expressed as the mean ± S.E.M. and the results analyzed by ANOVA followed by Dunnett's t-test. A P value of <0.05 was considered significant.

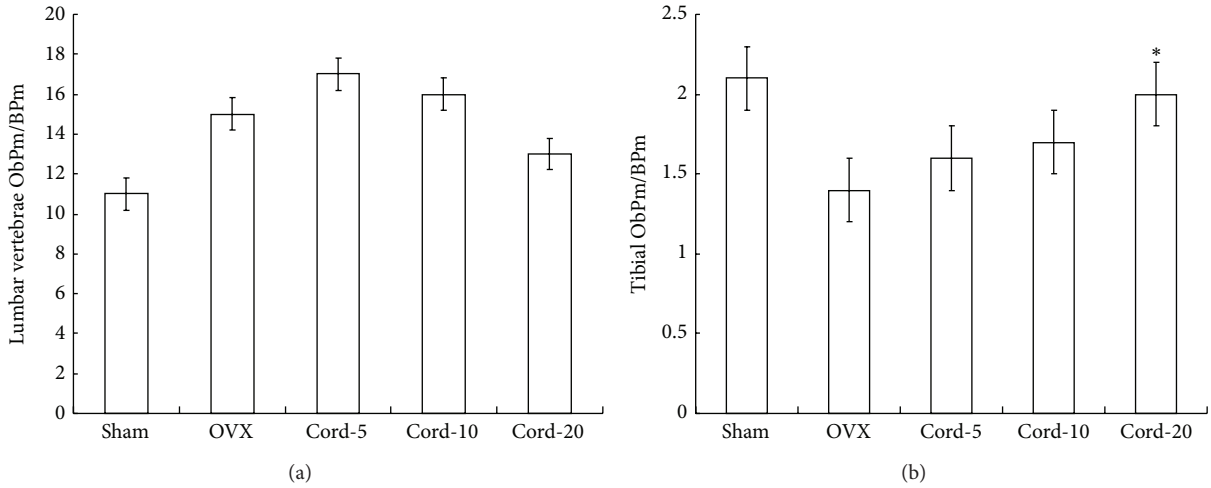


Figure 2: Effects of cordycepin on bone ObPm/BPm. Data are expressed as the mean ± SEM ($n = 10$ per group). $^*P < 0.05$, significant difference from vehicle-treated OVX rats.

3. Results and Discussion

Ovariectomized (OVX) animal models, in a variety of species, have been used to evaluate the mechanism of or to assess the effect of drugs on osteoporosis. Mechanical strength of bones is the most important parameter related to fracture risk. Therefore, this study first investigated the effect of cordycepin on mechanical strength in OVX osteopenic rats. The average maximum fracture loading to the femoral necks was lower in the OVX group compared with the sham group (Figure 1). Mechanical strength was significantly increased by treatment with cordycepin. It indicates that cordycepin had the positive effect on ovariectomized osteopenic rats.

Based on the results of mechanical strength, the trabecular number and thickness were studied. In the current study, we found that treatment of osteopenic OVX rats with cordycepin significantly increased maximal load compared to OVX animals. At the end of the 8-week treatment period, osteoblast surface in the lumbar vertebrae was not affected by OVX or any treatment (Figure 2(a)). A different cellular response was observed in the proximal tibial metaphysis. Cordycepin treatment caused a 3-fold increase in osteoblast surface compared with that in OVX rats ($P < 0.05$) (Figure 2(b)). It indicates that cordycepin administration improved bone strength mainly by increasing trabecular thickness. It agrees with the report of Ulrich [17].

Estrogen deficiency induces increased body weight in ovariectomized rats. The body weight gain pattern is shown in Figure 3. By the end of the fifth week, the ovariectomized rats gained significant weight compared to all other groups. From 5 weeks after the treatment was initiated, cordycepin-20 significantly increased body weights compared to sham group (Figure 3). The increase in rats' body weight in the cordycepin groups in the present study could be due to increased food intake as a result of lower leptin secretion though the impact is less severe compared to OVX rats [18].

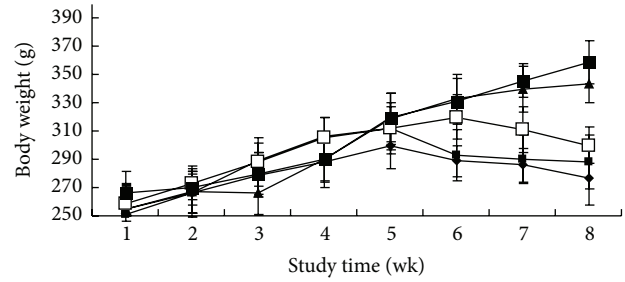
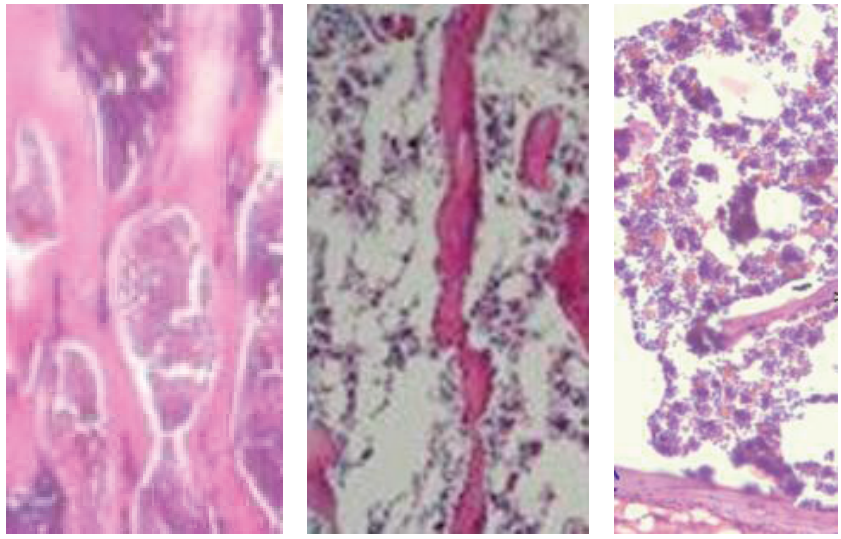


Figure 3: Effects of cordycepin on body weight changes for the study (◆sham group, ■OVX group, □cordycepin-5 group, ▲cordycepin-20 group, and ●cordycepin-10 group). Body weights were higher in the ovariectomized (OVX) animals than in sham-operated ones. Cordycepin-20 had similar body weights with the OVX animals in the former 5 weeks. From 5 weeks after the treatment was initiated, cordycepin-20 significantly increased body weights compared to sham group.

Bone histomorphometry was also performed to determine the effects of cordycepin treatment on cancellous bone mass and levels of bone formation and resorption. A sample photomicrograph is presented in Figure 4. It is quite clear that trabecular bone loss is much higher in the vertebrae of rat with OVX (Figure 4(b)), whereas the vertebrae of cordycepin-fed OVX rat (Figure 4(a)) appear to be near normal (Figure 4(c)).

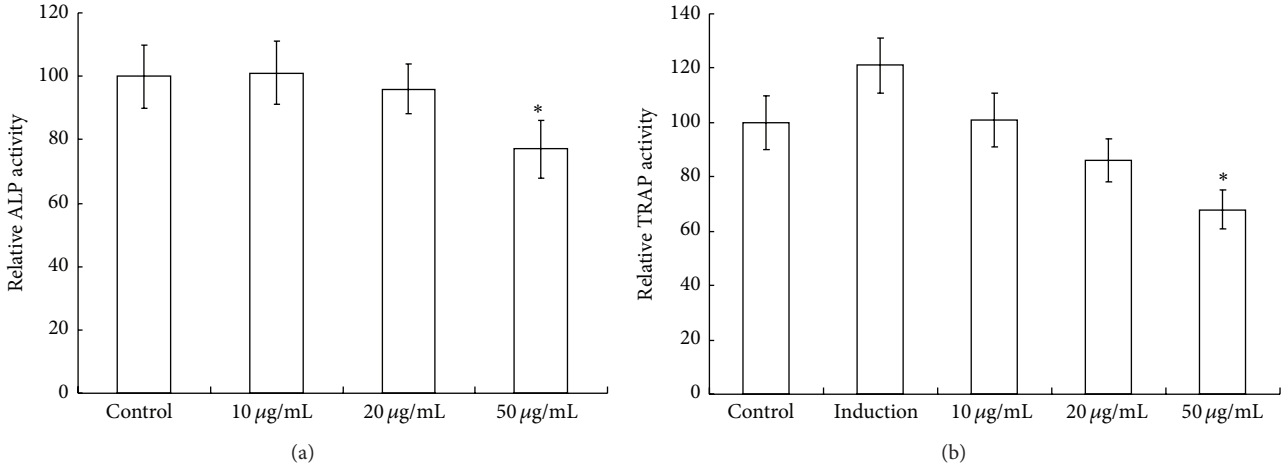
The current studies demonstrated that systemic treatment with cordycepin has a strong bone anabolic effect in OVX rats. The mechanism of it also needs to be studied in this paper.

ALP is a noncollagenous protein secreted by osteoblast, which is essential for bone mineralization [19]. Increased ALP level in serum has been observed in conditions such as rapid bone loss [20] and fracture risk [21, 22]. TRAP is secreted by osteoclasts during bone resorption, and serum TRAP activity correlates with resorptive activity in disorders of bone



(a) (b) (c)

Figure 4: Histology of lumbar vertebrae. The bone structure was photographed under a light microscope. It shows that there was a significant trabecular bone loss in the OVX rat (b), whereas the cordycepin-20 treatment rat section (c) seems near normal compared with sham-operated animals (a).



(a) (b)

Figure 5: *In vitro* assay cordycepin on ALP activity and TRAP activity. Treatment of 50 μg/mL of cordycepin showed significantly decreased ALP activity and TRAP activity. Values are mean ± SEM. $n = 10$. $^{*}P < 0.05$ compared to the control group at the same timepoint.

metabolism. In the present study significant increase in ALP and TRAP levels was observed in OVX control (Table 1). On the contrary, cordycepin significantly decreased ALP and TRAP levels ($P < 0.01$). Based on the above results, we further investigated the effect of cordycepin *in vitro* assay. Treatment of 50 μg/mL of cordycepin showed significantly decreased ALP activity (Figure 5(a)) and TRAP activity (Figure 5(b)). It suggested that the potency of cordycepin is due to decrease ALP activity, TRAP activity in OVX rats.

Osteocalcin (OC), homocysteine (HCY), and collagen type I (CTX) are known as serum markers reflecting osteoblast activities including bone formation and turnover [23–25]. The effects of treatment with cordycepin on OC, HCY, and CTX level were shown in Table 2. Treatment of 50 μg/mL of cordycepin increased OC level ($P < 0.01$). It also significantly decreased CTX level ($P < 0.01$). However,

Table 1: Effects of cordycepin on plasma enzymes in ovariectomized rats.

Groups	TRAP level (uM)	ALP level (mM)
Sham	0.22 ± 0.11**	3.25 ± 0.12**
OVX	0.82 ± 0.11	7.21 ± 0.10
Cordycepin-20	0.33 ± 0.02**	4.11 ± 0.04*
Cordycepin-10	0.61 ± 0.02	4.82 ± 0.06*
Cordycepin-5	0.70 ± 0.03	7.22 ± 0.01

Values are mean ± SEM. $n = 10$. $^{*}P < 0.05$ versus OVX control; $^{**}P < 0.01$ versus OVX control.

Table 2: Effects of cordycepin on plasma proteins.

Groups	Serum OC (ng/mL)	Serum HCY (μmol/L)	Serum CTX (ng/mL)
Sham	81.0 ± 5.0	7.7 ± 1.1	75.5 ± 4.2
OVX	61.4 ± 5.1	9.2 ± 2.0	100.3 ± 5.0
Cordycepin-20	84.1 ± 5.1**	8.0 ± 1.0	79.0 ± 8.1**
Cordycepin-10	73.6 ± 4.0*	9.2 ± 3.3	85.1 ± 9.0
Cordycepin-5	70.6 ± 3.2	8.0 ± 2.1	90.3 ± 4.4

Values are mean ± SEM. $n = 10$. * $P < 0.05$ versus OVX control; ** $P < 0.01$ versus OVX control.

Table 3: Effect of cordycepin on the activity of various enzymes.

Different groups	GPx	GR	GST	CAT	Na^+K^+ATPase
Sham	15.98 ± 1.23***	35.55 ± 2.51***	17.00 ± 1.22**	7.11 ± 0.33*	4.52 ± 0.32**
OVX	8.01 ± 0.42	20.88 ± 2.11	10.07 ± 1.11	4.22 ± 0.13	2.00 ± 0.13
Cordycepin-20	13.16 ± 1.32**	29.01 ± 2.21***	13.66 ± 0.90*	5.78 ± 0.20*	4.11 ± 0.22*
Cordycepin-10	9.10 ± 1.02	26.00 ± 2.22	13.00 ± 0.91	4.70 ± 0.21	3.51 ± 0.23
Cordycepin-5	8.16 ± 1.31	21.21 ± 2.20	10.10 ± 0.92	4.28 ± 0.33*	2.10 ± 0.11

Values are shown as means ± SEM. * $P < 0.05$ versus OVX group, ** $P < 0.01$ versus OVX group, and *** $P < 0.001$ versus OVX group.

compared with OVX control, there were no significant differences in the increase of HCY content in cordycepin groups (Table 2). These results suggested that the treatment with cordycepin induces the secretion of OC as well as decreased secretion of CTX after oral administration.

Oxidative stress and free radicals have been implicated in the pathogenesis of osteoporosis. Therefore, antioxidant compounds have the potential to be used in the prevention and treatment of the disease. Reduced glutathione (GSH) is one of the primary endogenous antioxidant defense systems, which removes hydrogen peroxide and lipid peroxides. Decline in GSH levels could either increase or reflect oxidative status [26, 27]. Therefore, the measurement of endogenous antioxidants enzymes, that is, GPx, GR, CAT, and GST, as well as Na^+K^+ATPase, has been performed to estimate the amount of oxidative stress. Activities of various antioxidant enzymes and Na^+K^+ATPase of different groups have been listed in Table 3. The activity of endogenous antioxidant enzymes was decreased significantly ($P < 0.01$) in the OVX group, as compared to the sham group, whereas in the cordycepin group, cordycepin treatment showed a significant ($P < 0.05$–-0.01) restoration in the level of various enzyme as compared with OVX group.

In conclusion, our findings first showed that oral administration of cordycepin can counteract the bone loss in an experimental model of established osteoporosis. These findings suggested that the mechanism of cordycepin is due to decrease ALP activity and TRAP activity both *in vitro* and *in vivo.* At the same time, oral administration of cordycepin can increase the OC level and decrease CTX and CTX level as well as restoring the oxidative stress in OVX animals. This suggests that cordycepin may be a good natural herbal medicine candidate for the treatment of osteoporosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This work was supported by Natural Science Foundation of Shanxi Province of China (2012JQ4020). This work was also supported by grants from the National High Technology Research and Development Program of China (863 Program) (no. 2012AA02A603) and Xijing Hospital Department Support Grant (no. XJZT13Z07).

References

[1] O. Johnell and J. Kanis, "Epidemiology of osteoporotic fractures," *Osteoporosis International*, vol. 16, no. 2, supplement, pp. S3–S7, 2005.

[2] S. Das and J. C. Crockett, "Osteoporosis—a current view of pharmacological prevention and treatment," *Drug Design, Development and Therapy*, vol. 7, pp. 435–448, 2013.

[3] T. J. Martin, N. A. Sims, and K. W. Ng, "Regulatory pathways revealing new approaches to the development of anabolic drugs for osteoporosis," *Osteoporosis International*, vol. 19, no. 8, pp. 1125–1138, 2008.

[4] S.-J. Suh, W.-S. Yun, K.-S. Kim et al., "Stimulative effects of *Ulmus davidiana* Planch (Ulmaceae) on osteoblastic MC3T3-E1 cells," *Journal of Ethnopharmacology*, vol. 109, no. 3, pp. 480–485, 2007.

[5] K. W. Kim, S. J. Suh, T. K. Lee et al., "Effect of safflower seeds supplementation on stimulation of the proliferation, differentiation and mineralization of osteoblastic MC3T3-E1 cells," *Journal of Ethnopharmacology*, vol. 115, no. 1, pp. 42–49, 2008.

[6] W. Caichompoo, Q.-Y. Zhang, T.-T. Hou, H.-J. Gao, L.-P. Qin, and X.-J. Zhou, "Optimization of extraction and purification of active fractions from Schisandra chinensis (Turcz.) and its osteoblastic proliferation stimulating activity," *Phytotherapy Research*, vol. 23, no. 2, pp. 289–292, 2009.

[7] K. Yue, M. Ye, Z. Zhou, W. Sun, and X. Lin, "The genus *Cordyceps*: a chemical and pharmacological review," *Journal of Pharmacy and Pharmacology*, vol. 65, no. 4, pp. 474–493, 2013.

[8] R. R. M. Paterson, "*Cordyceps*—a traditional Chinese medicine and another fungal therapeutic biofactory?" *Phytochemistry*, vol. 69, no. 7, pp. 1469–1495, 2008.

[9] X. Zhou, Z. Gong, Y. Su, J. Lin, and K. Tang, "Cordyceps fungi: natural products, pharmacological functions and developmental products," *Journal of Pharmacy and Pharmacology*, vol. 61, no. 3, pp. 279–291, 2009.

[10] S. Takahashi, M. Tamai, S. Nakajima et al., "Blockade of adipocyte differentiation by cordycepin," *British Journal of Pharmacology*, vol. 167, no. 3, pp. 561–575, 2012.

[11] D.-W. Zhang, Z.-L. Wang, W. Qi, W. Lei, and G.-Y. Zhao, "Cordycepin (3′-deoxyadenosine) down-regulates the proinflammatory cytokines in inflammation-induced osteoporosis model," *Inflammation*, vol. 37, no. 4, pp. 1044–1049, 2014.

[12] H. Ni, X.-H. Zhou, H.-H. Li, and W.-F. Huang, "Column chromatographic extraction and preparation of cordycepin from *Cordyceps militaris* waster medium," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, no. 22, pp. 2135–2141, 2009.

[13] C. M. Bagj, P. Ammann, R. Rizzoli, and S. C. Miller, "Effect of estrogen deficiency on cancellous and cortical bone structure and strength of the femoral neck in rats," *Calcified Tissue International*, vol. 61, no. 4, pp. 336–344, 1997.

[14] L. Xia, J. Kilb, H. Wex et al., "Localization of rat cathepsin K in osteoclasts and resorption pits: inhibition of bone resorption and cathepsin K-activity by peptidyl vinyl sulfones," *Biological Chemistry*, vol. 380, no. 6, pp. 679–687, 1999.

[15] O. A. Bessy, O. H. Lowry, and M. J. Brock, "A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum," *The Journal of Biological Chemistry*, vol. 164, pp. 321–329, 1946.

[16] P. Godkar, "Enzymes," in *Textbook of Medical Laboratory Techniques*, pp. 149–167, Bhalani Publishing House, Mumbai, India, 1994.

[17] D. Ulrich, B. van Rietbergen, A. Laib, and P. Rüegsegger, "The ability of three-dimensional structural indices to reflect mechanical aspects of trabecular bone," *Bone*, vol. 25, no. 1, pp. 55–60, 1999.

[18] R. Torto, S. Boghossian, M. G. Dube, P. S. Kalra, and S. P. Kalra, "Central leptin gene therapy blocks ovariectomy-induced adiposity," *Obesity*, vol. 14, no. 8, pp. 1312–1319, 2006.

[19] L. M. Havill, L. G. Hale, D. E. Newman, S. M. Witte, and M. C. Mahaney, "Bone ALP and OC reference standards in adult baboons (*Papio hamadryas*) by sex and age," *Journal of Medical Primatology*, vol. 35, no. 2, pp. 97–105, 2006.

[20] P. D. Ross and W. Knowlton, "Rapid bone loss is associated with increased levels of biochemical markers," *Journal of Bone and Mineral Research*, vol. 13, no. 2, pp. 297–302, 1998.

[21] G. Patrick, E. Sornay-Rendu, B. Claustrat, and P. D. Delmas, "Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: the OFELY study," *Journal of Bone and Mineral Research*, vol. 15, no. 8, pp. 1526–1536, 2000.

[22] P. D. Ross, B. C. Kress, R. E. Parson, R. D. Wasnich, K. A. Armour, and I. A. Mizrahi, "Serum bone alkaline phosphatase and calcaneus bone density predict fractures: a prospective study," *Osteoporosis International*, vol. 11, no. 1, pp. 76–82, 2000.

[23] D. Polak-Jonkisz and D. Zwolinska, "Osteocalcin as a biochemical marker of bone turnover," *Nephrology*, vol. 4, no. 5-6, pp. 339–346, 1998.

[24] J.-M. Koh, Y.-S. Lee, Y. S. Kim et al., "Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation," *Journal of Bone and Mineral Research*, vol. 21, no. 7, pp. 1003–1011, 2006.

[25] M. Herrmann, N. Umanskaya, B. Wildemann et al., "Stimulation of osteoblast activity by homocysteine," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 4, pp. 1205–1210, 2008.

[26] J. T. Coyle and P. O. Puttfarcken, "Oxidative stress, glutamate, and neurodegenerative disorders," *Science*, vol. 262, no. 5134, pp. 689–695, 1993.

[27] J. S. Bains and C. A. Shaw, "Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death," *Brain Research Reviews*, vol. 25, no. 3, pp. 335–358, 1997.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 829513, 5 pages
http://dx.doi.org/10.1155/2015/829513

Research Article

Crocetin Downregulates the Proinflammatory Cytokines in Methylcholanthrene-Induced Rodent Tumor Model and Inhibits COX-2 Expression in Cervical Cancer Cells

Bing Chen, Zhao-Hui Hou, Zhe Dong, and Chun-Dong Li

Department of Obstetrics and Gynaecology, Air Force General Hospital, PLA, Beijing 100142, China

Correspondence should be addressed to Chun-Dong Li; chundongli@tom.com

Received 9 August 2014; Accepted 11 September 2014

Academic Editor: John E. Smith

The effect of crocetin ($C_{20}H_{24}O_4$) on methylcholanthrene- (MCA-) induced uterine cervical cancer in mice was studied in this paper. After the mice were treated orally with crocetin, maleic dialdehyde (MDA), polymorphonuclear cells (PMN), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) were examined by ELISA or immunohistochemistry. The inducible nitric oxide synthase (iNOS) activation in *HeLa* cells was analyzed using fluorescence microscopy for light microscopic examination. The MCA mice showed a significant increase in plasma MDA, PMN, IL-1β, TNF-α, and nitrates levels. At the same time, the mRNA level of COX-2 in *HeLa* cells was also significantly increased. These changes were attenuated by crocetin supplementation in the MCA mice. Crocetin supplementation in the MCA mice also showed protection against cervical cancer. These results suggest that crocetin may act as a chemopreventive and an anti-inflammatory agent.

1. Introduction

Cervical cancer is the second most common cancer in women worldwide [1]. Despite improved knowledge of the etiology of cervical cancer, aggressive cytoreductive surgery, and modern combination chemotherapy, there has been little change in the mortality statistics over the last 30 years. Compelling evidence has shown that the majority of cancers arise from sites of chronic irritation, infection, and inflammation, solidifying the concept that chronic unabated inflammation is critical for tumour progression [2]. The microenvironment of the tumor highly resembles an inflammation site which results from enhancement of the levels of cytokines, chemokines, neutrophils, eosinophils, mast cells, lymphocytes, and macrophages both in the surrounding stroma and within the neoplasm itself [3]. Recent clinical trials have shown that long-term anti-inflammatory treatment can be beneficial in colorectal cancer [4]. So the pharmacological agents effective for the treatment of inflammatory diseases may also be employed in cervical cancer.

Crocetin ($C_{20}H_{24}O_4$) is one of the major active constituents of saffron, which is derived from the dried stigma of *Crocus sativus* L., and belongs to the Iridaceae family. Previous studies have demonstrated various pharmacological effects of this active constituent including its antioxidant, anti-inflammatory, and antitumor effects on some cell lines and animal models of cancer [4–8]. However, nothing is known about effects of crocetin in the uterine cervix tumorigenesis. In the present study, we investigated its anti-inflammatory effect on the interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and PMNs activity in a methylcholanthrene- (MCA-) induced uterine cervix tumorigenesis murine model system. Further, the effects of crocetin on expression of cyclooxygenase-2 (COX-2) in HeLa cells were also evaluated.

2. Material and Methods

2.1. Drugs. Methylcholanthrene (MCA) was purchased from Sigma Co., Ltd. Crocetin was purchased from Yiji Natural Products Co., Ltd., China.

2.2. Animals. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care

was taken to minimize discomfort, distress, and pain of the animals.

2.3. Experimental Design. Female Kunming strain mice weighing 20–22 g were maintained at room temperature under alternating natural light/dark photoperiod and had access to standard laboratory food and fresh water *ad libitum*. Murphy's string method [9] was followed for the induction of tumors in the uterine cervix of mice. Briefly, sterile double cotton thread impregnated with beeswax containing 600 μg of MCA was inserted into the canal of the uterine cervix by means of laparotomy under mild ether anaesthesia. Forty-eight of these mice were allocated equally into 4 groups: MCA-induced group, MCA and Crocetin-10 group, MCA and Crocetin-20 group, and MCA and Crocetin-40 group. The other 12 normal mice were used as the control group. From then on, the 5 groups of mice were administered orally saline, Crocetin 10 mg/kg, Crocetin 20 mg/kg, Crocetin 40 mg/kg, and saline, respectively. Crocetin was dissolved in distilled water and administrated orally twice daily using a feeding needle for 35 days, and control group received double distilled water instead of crocetin.

At the end of the experimental period, the animals were fasted overnight (18 h) and then sacrificed by decapitation, the blood was collected to be centrifuged at 3000 rpm for 20 min, and the clear serum was separated for the measurement of inflammatory cells and inflammatory mediators. The tumor volumes and iNOS protein histochemistry were determined.

2.4. Measurement of Maleic Dialdehyde (MDA). MDA was determined with thiobarbituric acid (TBA) using the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Total protein content of the samples was analyzed using Coomassie blue assay (Nanjing Jiancheng Bioengineering Institute).

2.5. Measurement of Infiltration of PMN. Myeloperoxidase (MPO) activity was measured to assess the extent of PMN infiltration. The method of assaying MPO activity was according to the guide of the assay kit (Nanjing Jiancheng Bioengineering Co. Ltd., China).

2.6. Measurement of IL-1β and TNF-α Level. The concentration of IL-1β and TNF-α was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instruction.

2.7. Assessment of iNOS Protein Histochemistry. The tumors were dissected free from soft tissues at the end of the study and collected on chrome gel subbed slides. Tissue sections were fixed in 50% acetone in phosphate-buffered saline for 3 minutes at room temperature and endogenous peroxidase activity was blocked by 1.5% H_2O_2 in methanol for 15 minutes. The iNOS protein was determined using a commercial ELISA kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacturer's instruction.

2.8. Cell Culture. HeLa cells (human cervical cancer HeLa cell line was obtained from Cell Culture Center of Shanghai Science Academy, China) were maintained in Petri dishes at 37°C in a Dulbecco-modified Eagle culture medium supplemented with 10% fetal calf serum and 1% antibiotic mixture, under a humidified atmosphere containing 5% carbon dioxide. The culture medium was changed twice per week. After they had reached their growth plateau, the cells were used for inoculation by mechanical harvest and transfer to fresh culture medium.

2.9. Crocetin Inhibited COX-2 Production in HeLa Cells. Analysis of COX-2 production was performed using an ELISA kit according to manufacturer's instructions (Shanghai Jinma Biological Technology, Inc., China). HeLa cells were treated with various concentrations of crocetin (1, 10, 50, and 100 μmol/L) for 24 h. Cell culture supernatants were collected to measure the concentration of COX-2. Production of COX-2 was normalized to protein concentrations.

Total RNA was extracted from crocetin-treated and untreated HeLa cells using GenElute Mammalian Genomic Total RNA Isolation Kit (Sigma) as per the manufacturer's protocol. The extracted RNA samples were then subjected to reverse transcription- (RT-) PCR for analysis of expression of COX-2.

2.10. Statistical Analysis. The data were expressed as mean ± SEM and results were analyzed by ANOVA followed by Dunnett's t test. $P < 0.05$ was considered significant.

3. Results and Discussion

A considerable body of evidence has supported the concept that tumors can originate at the sites of infection or chronic inflammation [10–12]. Many pathological disorders or diseases, including cervical cancer, are characterised by the exacerbated activation and maintenance of inflammatory pathways [13, 14]. In the current study, we found that MCA-induced carcinogenesis in the mouse uterine cervix in animals results in significantly increased circulating concentrations of inflammatory cytokines and tumor necrosis factor-α (TNF-α), when compared with controls. It is in agreement with previous data [10–14].

Peroxidation damage plays an important role in the progression of inflammation mediated diseases in particular cervical cancer. The central dogma in chronic inflammation hypothesis emphasizes the role of ROS generated by phagocytes, which cause cytotoxicity and mutagenesis [15–17]. Therefore, the antioxidant effects of crocetin were investigated by measuring MDA levels. The control animals showed low MDA levels; however, the MDA levels in the MCA group were significantly higher ($P < 0.05$). As shown in Figure 1, MDA levels in the crocetin (40 mg/kg) and crocetin (20 mg/kg) groups were significantly lower than those in the MCA group ($P < 0.01$ and $P < 0.05$, resp.). This result is in agreement with previous reports [18, 19]. It suggests that crocetin might exert a profound effect on inhibition of lipid peroxidation and free radical generation.

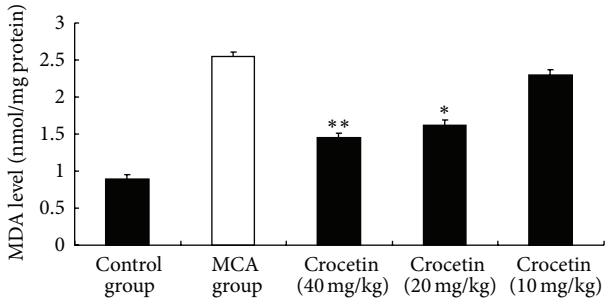


Figure 1: Effect of crocetin on MDA level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus MCA group. $^{**}P < 0.01$ versus MCA group.

Table 1: Effects of crocetin on PMNs activities.

Different groups	(μmol·g^{-1})
Control	0.99 ± 0.20
MCA	2.00 ± 0.20
Crocetin (40 mg/kg)	1.00 ± 0.10*
Crocetin (20 mg/kg)	1.30 ± 0.25
Crocetin (10 mg/kg)	1.80 ± 0.22

Values are shown as means ± SEM. $^{*}P < 0.05$ versus MCA group.

The association between tumor cells and polymorphonuclear cells (PMNs) has been demonstrated in several types of cancer [20, 21]. However, the role of PMNs in cervical cancer progression has not been well studied in vivo. In this study, the PMNs activity was relatively low in control group and significantly increased in the MCA group. Treatment with crocetin of 40 mg/kg significantly reduced PMNs activity (Table 1). Treatment with crocetin of 20 mg/kg reduced PMNs activity also. However, it is not significant. PMNs may contribute to secondary injury by causing microvessel occlusion and releasing oxygen radicals, cytolytic proteases, and proinflammatory cytokines, which may induce the neuronal damage [22, 23]. In the present study, we observed a dose-dependent inhibitory effect of crocetin on PMNs activity, indicating less neutrophil infiltration into the lesion site.

Key features of cancer-related inflammation include the infiltration of white blood cells and cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) [24]. IL-1β has been shown to be upregulated in many cancers and confers chemoresistance in pancreatic carcinoma [25]. Our study is consistent with these studies. Figure 2 shows that MCA significantly increased protein concentration of IL-1β. Crocetin (40 mg/kg) and crocetin (20 mg/kg) treatment decreased the level of IL-1β by 34% and 56% as compared to the MCA group, respectively ($P < 0.05$). TNF-α is an inflammatory cytokine which may play important roles in the progression of cervical lesions [26]. TNF-α was measured in blood to evaluate whether the concentration of this cytokine was systemically influenced by crocetin. Furthermore, this helps elucidate whether or not TNF-α can be implicated in the effect that was seen on neutrophil migration. As shown in Figure 3, the levels of TNF-α elevated significantly after induction of tumors with MCA. Crocetin (40 mg/kg)

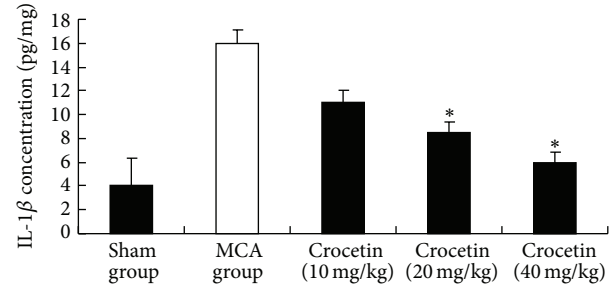


Figure 2: Effect of crocetin on IL-1β concentration. Values represent the mean ± SEM. $^{*}P < 0.05$ versus MCA group.

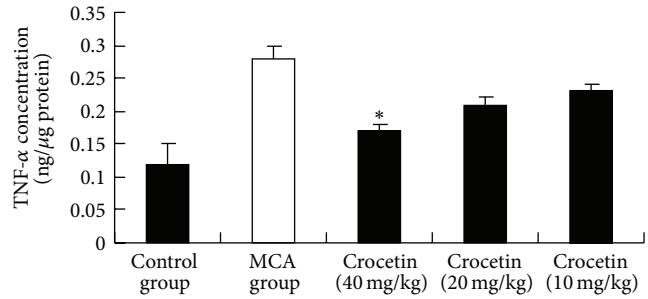


Figure 3: Effect of crocetin on TNF-α concentration. Values represent the mean ± SEM. $^{*}P < 0.05$ versus MCA group.

Table 2: Effects of crocetin on tumor volumes.

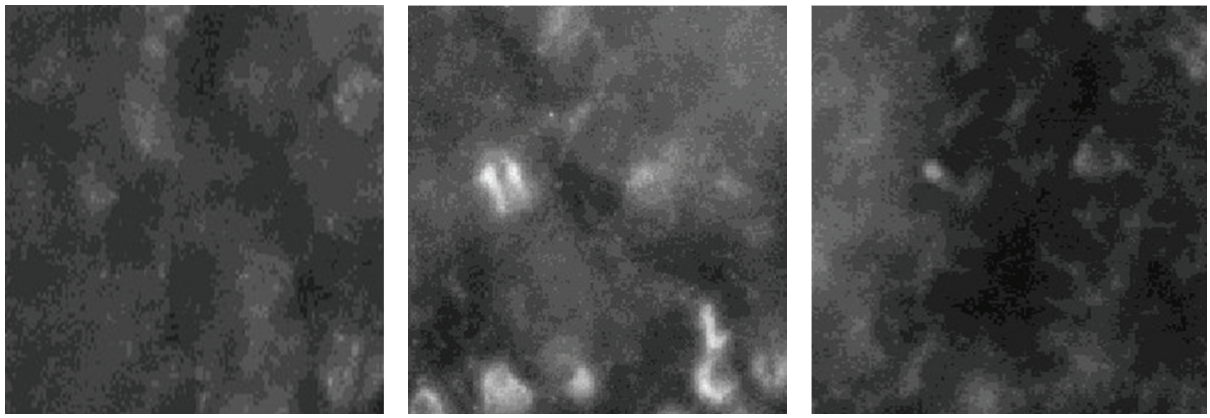
Different groups	Tumor volume (mm^3)
Control	0
MCA	10.4 ± 0.30
Crocetin (40 mg/kg)	6.00 ± 0.10*
Crocetin (20 mg/kg)	7.32 ± 0.22
Crocetin (10 mg/kg)	8.80 ± 0.33

Values are shown as means ± SEM. $^{*}P < 0.05$ versus MCA group.

suppressed this response ($P < 0.05$). We demonstrated in this study that administration of crocetin decreased serum levels of IL-1β and TNF-α that are known to be produced by induction of tumors with MCA. Results from studies on cytokines have given us some insight into the mechanisms involved in the protection of crocetin against cervical cancer (Table 2).

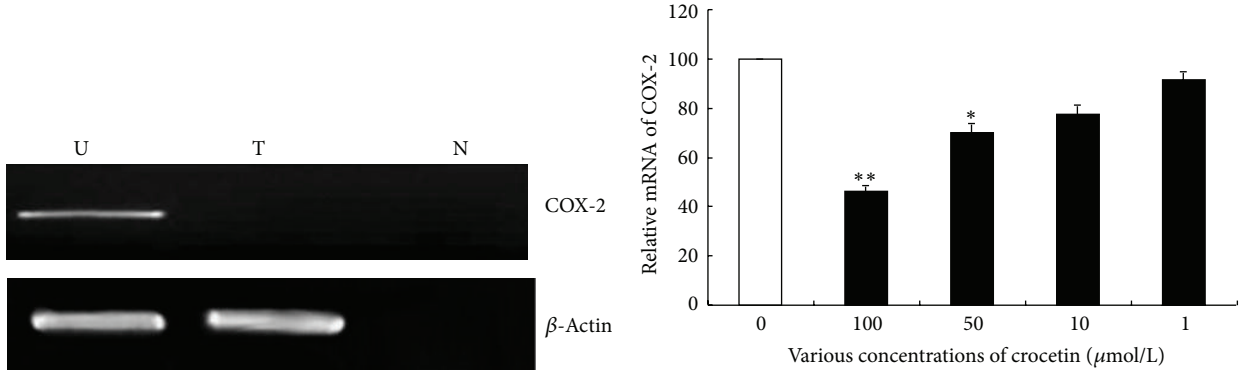
Inducible NOS is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that predominate during inflammation [27]. The excessive or inappropriate production of NO can damage tissue through the superoxide anion (O_2^-) [28]. The fluorescence microscopy analyses showed evidence of widespread iNOS expression in tumor cells from animals with MCA but no evidence of iNOS immunoreactivity in controls. Treatment with crocetin (40 mg/kg) also inhibited the iNOS expression in bone marrow cells (Figure 4).

According to a previous research by Subbaramaiah and Dannenberg, COX-2 is involved in inflammation and its upregulation has been reported in various cancers [29]. In the



(a) (b) (c)

Figure 4: Demonstration of iNOS protein in tumor. All photographs were taken at an exposure time of 1 s. Magnification ×400. (a) iNOS in cytoplasm of tumor cells of control rat. (b) iNOS in cytoplasm of tumor cells of IMO rat. (c) iNOS in cytoplasm of tumor cells of crocetin (40 mg/kg) rat.



(a) (b)

Figure 5: Crocetin inhibited COX-2 production and expression in HeLa cells. (a) Expression analysis of COX-2 in HeLa cells. Lane U shows untreated HeLa cells; lane T shows crocetin-treated HeLa cells; lane N shows negative control for RT-PCR. β-Actin was used as an internal control. Representative gels from one of the three experiments were used. (b) The mRNA level of COX-2 was measured in HeLa cells treated with various concentrations of crocetin for 24 h. Values represent the mean ± SEM. $^{*}P < 0.05$ versus control group.

present study, HeLa cells were shown to express high levels of COX-2, which was subsequently downregulated on treatment with crocetin (Figure 5(a)). Relative to nontreated controls, crocetin dose-dependently decreased COX-2 production in cervical cancer cells (Figure 5(b)). It has been suggested that COX-2 is an important target for the chemopreventive effects of these agents. The present study is consistent with this study [30]. Our observations prompt us to suggest that inhibition of COX-2 production by crocetin may suppress growth and invasiveness of cervical carcinomas.

4. Conclusion

This study suggested for the first time that crocetin has an anti-inflammatory effect by suppressing the level of IL-1β and TNF-α as well as PMNs activity in a MCA-induced uterine cervix tumorigenesis murine model system. Further, crocetin dose-dependently decreased COX-2 production in cervical cancer cells. Overall, crocetin demonstrated potent in vitro and in vivo anti-inflammatory activities. It provided support for the potential of using crocetin as a chemopreventive and an anti-inflammatory agent.

Conflict of Interests

The authors declare that there is no conflict of interests.

References

[1] M. Arbyn, X. Castellsagué, S. de sanjosé et al., "Worldwide burden of cervical cancer in 2008," *Annals of Oncology*, vol. 22, no. 12, pp. 2675–2686, 2011.

[2] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[3] C. Caruso, D. Lio, L. Cavallone, and C. Franceschi, "Aging, longevity, inflammation, and cancer," *Annals of the New York Academy of Sciences*, vol. 1028, pp. 1–13, 2004.

[4] T.-H. Tseng, C.-Y. Chu, J.-M. Huang, S.-J. Shiow, and C.-J. Wang, "Crocetin protects against oxidative damage in rat

primary hepatocytes," *Cancer Letters*, vol. 97, no. 1, pp. 61–67, 1995.

[5] A. Poma, G. Fontecchio, G. Carlucci, and G. Chichiriccò, "Anti-inflammatory properties of drugs from saffron crocus," *Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry*, vol. 11, no. 1, pp. 37–51, 2012.

[6] A. Dhar, S. Mehta, G. Dhar et al., "Crocetin inhibits pancreatic cancer cell proliferation and tumor progression in a xenograft mouse model," *Molecular Cancer Therapeutics*, vol. 8, no. 2, pp. 315–323, 2009.

[7] V. Magesh, J. P. Vijeya Singh, K. Selvendiran, G. Ekambaram, and D. Sakthisekaran, "Antitumour activity of crocetin in accordance to tumor incidence, antioxidant status, drug metabolizing enzymes and histopathological studies," *Molecular and Cellular Biochemistry*, vol. 287, no. 1-2, pp. 127–135, 2006.

[8] F. I. Abdullaev, "Inhibitory effects of crocetin on intracellular nucleic acid and protein synthesis in malignant cells," *Toxicology Letters*, vol. 70, no. 2, pp. 243–251, 1994.

[9] E. D. Murphy, "Studies in carcinogen-induced carcinoma of the cervix in mice," *American Journal of Pathology*, vol. 29, p. 608, 1953.

[10] M. M. Mueller and N. E. Fusenig, "Friends or foes—bipolar effects of the tumour stroma in cancer," *Nature Reviews Cancer*, vol. 4, no. 11, pp. 839–849, 2004.

[11] A. Hara and I. Okayasu, "Cyclooxygenase-2 and inducible nitric oxide synthase expression in human astrocytic gliomas: correlation with angiogenesis and prognostic significance," *Acta Neuropathologica*, vol. 108, no. 1, pp. 43–48, 2004.

[12] L. F. Hu, P. T. H. Wong, P. K. Moore, and J. S. Bian, "Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia," *Journal of Neurochemistry*, vol. 100, no. 4, pp. 1121–1128, 2007.

[13] K. J. Sales, A. A. Katz, B. Howard et al., "Cyclooxygenase-1 is up-regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin e receptors, and angiogenic factors by cyclooxygenase-1," *Cancer Research*, vol. 62, pp. 424–432, 2002.

[14] A. D. Santin, F. Zhan, E. Bignotti et al., "Gene expression profiles of primary HPV16- and HPV18-infected early stage cervical cancers and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy," *Virology*, vol. 331, no. 2, pp. 269–291, 2005.

[15] S. Tamir and S. R. Tannenbaum, "The role of nitric oxide ($NO^{\bullet}$) in the carcinogenic process," *Biochimica et Biophysica Acta (BBA)—Reviews on Cancer*, vol. 1288, no. 2, pp. F31–F36, 1996.

[16] V. Darley-Usmar, H. Wiseman, and B. Halliwell, "Nitric oxide and oxygen radicals: a question of balance," *FEBS Letters*, vol. 369, no. 2-3, pp. 131–135, 1995.

[17] S. S. S. Beevi, A. M. H. Rasheed, and A. Geetha, "Evaluation of oxidative stress and nitric oxide levels in patients with oral cavity cancer," *Japanese Journal of Clinical Oncology*, vol. 34, no. 7, pp. 379–385, 2004.

[18] V. Bhuvarahamurthy, N. Balasubramanian, and S. Govindasamy, "Effect of radiotherapy and chemoradiotherapy on circulating antioxidant system of human uterine cervical carcinoma," *Molecular and Cellular Biochemistry*, vol. 158, no. 1, pp. 17–23, 1996.

[19] V. Manju, J. K. Sailaja, and N. Nalini, "Circulating lipid peroxidation and antioxidant status in cervical cancer patients: a case-control study," *Clinical Biochemistry*, vol. 35, no. 8, pp. 621–625, 2002.

[20] T. Yamanaka, S. Matsumoto, S. Teramukai, R. Ishiwata, Y. Nagai, and M. Fukushima, "The baseline ratio of neutrophils to lymphocytes is associated with patient prognosis in advanced gastric cancer," *Oncology*, vol. 73, no. 3-4, pp. 215–220, 2008.

[21] Q. D. I. Wu, J. H. Wang, C. Condron, D. Bouchier-Hayes, and H. P. Redmond, "Human neutrophils facilitate tumor cell transendothelial migration," *The American Journal of Physiology: Cell Physiology*, vol. 280, no. 4, pp. 814–822, 2001.

[22] D. Widomski, D. J. Fretland, A. F. Gasiecki, and P. W. Collins, "The prostaglandin analogs, misoprostol and SC-46275, potently inhibit cytokine release from activated human monocytes," *Immunopharmacology and Immunotoxicology*, vol. 19, no. 2, pp. 165–174, 1997.

[23] G. J. del Zoppo, G. W. Schmid-Schönbein, E. Mori, B. R. Copeland, and C.-M. Chang, "Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons," *Stroke*, vol. 22, no. 10, pp. 1276–1283, 1991.

[24] J. K. Kundu and Y.-J. Surh, "Inflammation: gearing the journey to cancer," *Mutation Research—Reviews in Mutation Research*, vol. 659, no. 1-2, pp. 15–30, 2008.

[25] A. G. Pantschenko, I. Pushkar, K. H. Anderson et al., "The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression," *International Journal of Oncology*, vol. 23, pp. 269–284, 2003.

[26] F. Balkwill, "Tumour necrosis factor and cancer," *Nature Reviews Cancer*, vol. 9, no. 5, pp. 361–371, 2009.

[27] R. M. Clancy, A. R. Amin, and S. B. Abramson, "The role of nitric oxide in inflammation and immunity," *Arthritis and Rheumatism*, vol. 41, pp. 1141–1151, 1998.

[28] H. Yaren, H. Mollaoglu, B. Kurt et al., "Lung toxicity of nitrogen mustard may be mediated by nitric oxide and peroxynitrite in rats," *Research in Veterinary Science*, vol. 83, no. 1, pp. 116–122, 2007.

[29] K. Subbaramaiah and A. J. Dannenberg, "Cyclooxygenase 2: a molecular target for cancer prevention and treatment," *Trends in Pharmacological Sciences*, vol. 24, no. 2, pp. 96–102, 2003.

[30] R. N. DuBois and W. E. Smalley, "Cyclooxygenase, NSAIDs, and colorectal cancer," *Journal of Gastroenterology*, vol. 31, no. 6, pp. 898–906, 1996.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 680756, 11 pages
http://dx.doi.org/10.1155/2015/680756

Review Article

Combined Therapy of Diabetic Peripheral Neuropathy with Breviscapine and Mecobalamin: A Systematic Review and a Meta-Analysis of Chinese Studies

Chanjiao Zheng, Weilin Ou, Huanyu Shen, Zhiheng Zhou, and Jiaji Wang

School of Public Health, Guangzhou Medical University, Guangzhou 510182, China

Correspondence should be addressed to Zhiheng Zhou; zhihengz@163.com

Received 20 September 2014; Revised 13 January 2015; Accepted 16 January 2015

Academic Editor: Konstantinos Papatheodorou

Objective. A meta-analysis on combined therapy of diabetic peripheral neuropathy (DPN) with breviscapine and mecobalamin was performed to evaluate the efficacy of this therapy. *Methods*. Six English databases (Medline, Cochrane Library, PubMed, EMBASE, Web of Science, and CINAHL) and four Chinese databases (China National Knowledge Infrastructure, VIP Journals Database, CBM, and Wanfang database) were searched for studies on the clinical trials in which DPN was treated with breviscapine and mecobalamin, and RevMan 5.1 package was employed for analyzing pooled trials and publication bias. *Results*. A total of 17 articles including 1398 DPN patients were identified. Homogeneity was observed among different studies ($P = 0.74$). The efficacy of combined therapy with breviscapine and mecobalamin was significantly better than that in control group [$P < 0.0001$ (OR = 5.01, 95% CI: 3.70–6.78)]. *Conclusion*. Available findings suggest that the therapeutic efficacy of breviscapine combining mecobalamin is superior to mecobalamin alone, and this strategy is required to be popularized in clinical practice.

1. Introduction

Diabetic peripheral neuropathy (DPN) is a diabetes mellitus (DM) induced disorder of the peripheral nervous system [1] and is characterized by the pain and loss of sensation due to symmetrical degeneration of distal peripheral nerves. The symptoms will deteriorate with the progression, which may result in diabetic ulcers or even nontraumatic amputation. Statistics revealed that the incidence of DPN was as high as 30%, 60%, and 90% at 5, 10, and 20 years after diagnosis of DM, and foot injury had occurred in 50% of DPN patients when they were asymptomatic [2]. The incidence of neuropathy is now estimated to be about 8% in new cases of DM, and neuropathy will be a lifelong disease in more than 50% of DM patients, which is about 4 times the figure (12.3%) in DM patients in 2001 [1, 3, 4]. Thus, DPN has been an important economic burden of the medical system [5] and significantly influenced the quality of life of DM patients. The pathogenesis of DPN is complicated and still poorly understood [6, 7]. Studies have found that DPN was closely associated with metabolic disorder, vascular diseases, and oxidative stress [8–10]. Currently, pharmacotherapy of DPN is mainly to relieve pain with tricyclic antidepressants, anticonvulsants (gabapentin, phenytoin, lamotrigine, opioids, and tramadol), focal analgesics (capsaicin), and nonsteroidal anti-inflammatory drugs [11]. In addition, studies also revealed that vitamin B12 was also beneficial for the improvement of symptoms of DPN patients [12].

Breviscapine is an active ingredient of flavonoids extracted from dried *Erigeron breviscapus* (Vant.) Hand. Mazz. [13, 14]. In clinical practice, breviscapine tablets and breviscapine injections are mainly used in the therapy of various diseases. There were evidences showing that breviscapine was able to dilate blood vessels, reduce vascular resistance, increase blood flow, improve microcirculation, inhibit angiogenesis, and suppress aggregation of platelets [14–18]. Breviscapine may act as an antioxidant [19–21] and has been used in the therapy of DPN in China. Mecobalamin is an endogenous coenzyme B12 and can be used alone or in combination with other drugs. It is also widely used in the therapy of DPN [22–25]. Previous Chinese studies revealed that combined therapy of breviscapine and mecobalamin

had better efficacy and safety for DPN when compared with mecobalamin alone [26, 27]. Although several randomized controlled trials have been conducted in the combined therapy of DPN with breviscapine and mecobalamin, the sample size was small and the test potency was low, resulting in low reliability of these studies. In this meta-analysis, studies on the combined therapy of DPN with breviscapine and mecobalamin were selected for pooled analysis, aiming to evaluate the therapeutic efficacy of this strategy in DPN patients.

2. Materials and Methods

2.1. Literature Searching and Data Extraction. A comprehensive literature search was performed for the randomized, controlled studies on the combined therapy of DPN with breviscapine and mecobalamin published before September 2014 by using Medline database (1989 to September 2014), Cochrane Library (1993 to September 2014), PubMed (1966 to September 2014), EMBASE (1980 to September 2014), Web of Science (1945 to September 2014), CINAHL (1982 to September 2014), CNKI database (1979 to September 2014), Chinese Biomedical Literature database (1990 to September 2014), Wanfang database (1982 to September 2014), and VIP database (1989 to September 2014). The following terms were used: (DPN OR diabetic peripheral neuropathy OR diabetic neuropathy) AND (breviscapine OR erigeron breviscapus). Searching was done by two authors (Weilin Ou and Huanyu Shen) independently.

2.2. Inclusion and Exclusion Criteria

2.2.1. Inclusion Criteria Included. (1) The internationally accepted diagnostic criteria were used in these studies: DM was diagnosed according to the WHO criteria for diabetes mellitus in 1999 [27], patients had related symptoms of motor and sensory nerves, and other causes of peripheral neuropathy were excluded (such as hypothyroidism, genetics, alcoholism, and drugs); (2) studies were clinically randomized controlled trials; (3) patients were treated with mecobalamin alone in control group; patients were treated with mecobalamin and breviscapine in intervention group; (4) studies investigated the therapeutic efficacy or the changes in the conduction velocity of motor or sensory nerves.

2.2.2. Exclusion Criteria Included. (1) There was no control group, or patients in control group were not treated with mecobalamin; (2) other parameters were used for evaluation of therapeutic efficacy; (3) studies were descriptive trials; (4) mecobalamin or breviscapine in combination with other drugs was used to treat DPN.

2.3. Data Extraction. Relevant data was systematically collected from each included study by two authors (Weilin Ou and Huanyu Shen) using a standardized form. The following information was extracted: number of patients in different groups, age, gender, course of DM, course of DPN, study duration, daily dose of breviscapine, and endpoints. This information was collected independently by two authors and inconsistence was resolved after consultation with a third investigator (Chanjiao Zheng).

2.4. Outcomes. There is no unified curative effect evaluation standard. So evaluation index was chosen according to the selected trials to evaluate curative effect. The authors in the 17 articles were observing two groups of patients before and after treatment about the change of subjective symptom and lower limb nerve reflex and using the electromyography tested the motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) of total nerve and median nerve before and after treatments. Therapeutic effect criteria include the following [28]: (1) excellence: self-conscious symptoms were markedly improved, obvious tendon reflexes improved or recovered, and the MNCV/SNCV increased by more than 5 m/s or back to normal; (2) effectiveness: self-conscious symptoms were improved, tendon reflexes improved or recovered, and the MNCV/SNCV increased by less than 5 m/s or increased slightly; (3) invalidism: self-conscious symptoms were not improved and tendon reflexes and MCV/SC did not change. Total efficiency was equal to the excellence and effectiveness. The primary outcomes included therapeutic efficacy and absolute values of or changes in median MNCV, peroneal MNCV, median SNCV, and peroneal SNCV. The secondary outcomes included the improvement of clinical symptoms (overall effectiveness) and side effects. The effectiveness was defined as the improvement of clinical symptoms, tendon reflexes, and nerve conduction velocity.

2.5. Quality Assessment. The quality assessment of studies was done with the Cochrane Collaboration's tool (The Cochrane Library) which contained random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other potential sources of bias. The outcome of each item was classified as low risk of bias, unclear risk of bias, and high risk of bias. The quality assessment was performed independently by two investigators (Weilin Ou and Chanjiao Zheng) and inconsistence was resolved after consultation with a third investigator (Jiaji Wang).

2.6. Data Synthesis and Analysis. Review Manager 5.1 software was used for the analysis of data. The odds ratio (OR) and 95% confidence interval (CI) were calculated for dichotomic data, and quantitative data were expressed as weighted mean difference (WMD) and 95% CI. Heterogeneity analysis was done with q test. $P > 0.1$ and $I^2 < 50\%$ suggested homogeneity among studies. For data without significant heterogeneity, fixed effects model was employed for pooled analysis. When significant heterogeneity ($P \leq 0.1$ and $I^2 \geq 50\%$) was present, random effects model was employed for pooled analysis. The significance of pooled data was further tested, and a value of $P < 0.05$ was considered statistically significant. When enough studies were included, funnel plot was delineated and the publication bias was evaluated.

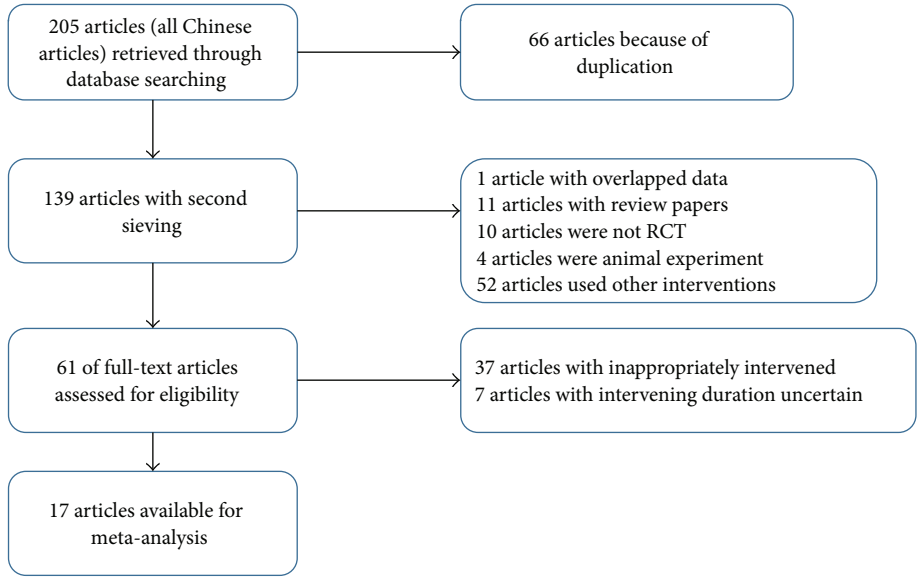


Figure 1: Flowchart of study selection.

3. Results

3.1. Study Selection. A total of 205 literatures were identified after searching. Among them, 66 were identical in different databases, 1 was duplicated, 11 were retrospective reviews, 10 were noncontrolled studies, 4 were studies on animals, and other 52 literatures were excluded due to other reasons. The remaining 61 literatures were included for the evaluation of full text. Of these 61 literatures, the interventions were inappropriate in 37 and the duration of therapy was indefinite in 7 literatures. Thus, 17 articles were finally eligible for meta-analysis [25, 26, 28–42]. The flowchart of study selection is shown in Figure 1.

3.2. Evaluation of Quality of Included Studies. Among 17 studies, there were 1398 patients including 718 treated with breviscapine and mecobalamin and 680 treated with mecobalamin alone. In these studies, patients received diet control, excising, and glucose-lowering therapy before interventions. In most of the studies, patients were treated continuously for 2–6 weeks, except for intervals of 2-3 days in 2 studies [32, 40] and an interval of 2 weeks in 1 study [25]. The detailed information of included studies is shown in Table 1. Among 17 studies, randomized grouping was addressed in 1 study [39], random number method was used in 1 study [25], and only randomization was addressed in remaining studies but the specific method for randomization was not described. Of these studies, only 1 was classified as high risk of bias, and the quality of included studies is shown in Figures 2 and 3.

3.3. Meta-Analysis

3.3.1. Overall Effectiveness on the Basis of Improvement of Clinical Symptoms and Signs. A total of 17 studies were conducted to evaluate the therapeutic efficacy of breviscapine and mecobalamin as compared to that of mecobalamin alone. There were 718 patients treated with breviscapine and mecobalamin and 680 patients treated with mecobalamin alone in meta-analysis. There was no heterogeneity among groups ($P = 0.74$, $I^2 = 0\%$), and fixed effects model was employed for pooled analysis which showed that OR was 5.01, 95% CI was 3.70–6.78, Z was 10.44 ($P < 0.0001$), and the diamond on the right side of the vertical line was complete, suggesting that the overall effectiveness of breviscapine and mecobalamin was significantly superior to that of mecobalamin alone (Figure 4).

3.3.2. Meta-Analysis of Nerve Conduction Velocity after Therapy

(1) Median MNCV. The median MNCV was compared between groups in 7 studies [25, 28, 29, 31, 33, 36, 37]. There were 311 patients treated with breviscapine and mecobalamin and 287 patients treated with mecobalamin alone. There was significant heterogeneity among groups ($P < 0.0001$, $I^2 = 96\%$), and random effects model was employed for pooled analysis. The pooled WMD was 7.53, 95% CI was 4.65–10.42, Z was 5.11 ($P < 0.0001$), and the diamond on the right side of the vertical line was complete, suggesting that the median MNCV of patients treated with breviscapine and mecobalamin was significantly higher than that of patients treated with mecobalamin alone (Figure 5).

(2) Median SNCV. The median SNCV was compared between groups in 7 studies [25, 28, 29, 31, 33, 36, 37]. There were 311 patients treated with breviscapine and mecobalamin and 287 patients treated with mecobalamin alone. There was significant heterogeneity among groups ($P < 0.0001$, $I^2 = 97\%$), and random effects model was employed for pooled analysis. The pooled WMD was 4.98, 95% CI was 1.75–8.21,

Table 1: Characteristics of included studies.

Trial	Number	Age (Y)	Duration of diabetes (Y)	Duration of DPN (Y)	Treatment drugs/day			Main outcome measures	Course of treatment	Adverse events
					B + M		M			
	(B + M/M)	(B + M/M)	(B + M/M)	(B + M/M)	B + M	M				
Shi et al. (2013) [25]	90 (45/45)	58.4 ± 11.2/57.9 ± 10.8	11.2 ± 2.3/10.7 ± 2.6	3.01 ± 0.93/3.15 ± 0.87	50 mg iv.	1.5 mg n.r.	1.5 mg n.r.	TER, SNCV, MNCV	6 w	0
Lan and Wu (2010) [26]	82 (43/39)	53.2 ± 3.7/53.6 ± 3.6	9.3 ± 8.3/9.54 ± 3.1	4.3 ± 1.3/4.2 ± 1.2	150 mg ivgtt.	0.5 mg im.	0.5 mg im.	TER, SNCV	4 w	n.r.
Chang (2007) [29]	86 (43/43)	69.4/63.7	12.0/11.0	4/3.5	40 mL ivgtt.	0.5 mg po.	0.5 mg po.	TER, SNCV, MNCV	30 d	5/0
Chen and Shao (2008) [30]	132 (68/64)	72 ± 12.3/69 ± 11.9	n.r.	n.r.	30 mL ivgtt.	1.5 mg po.	1.5 mg po.	TER	2 w	2/0
Duan and Yan (2004) [31]	64 (36/28)	55.5 ± 5.2	5.5 ± 4.2/n.r.	3.6 ± 2.5/n.r.	30 mL ivgtt.	0.5 mg im.	0.5 mg im.	TER, SNCV, MNCV	6 w	n.r.
Feng et al. (2009) [32]	91 (46/45)	n.r.	n.r.	n.r.	20 mL ivgtt.	1.0 mg ivgtt.	1.0 mg ivgtt.	TER	4 w	n.r.
Jin et al. (2010) [33]	70 (35/35)	57 ± 10/56 ± 10	8.5 ± 3.4/8.5 ± 3.2	n.r.	20 mL ivgtt.	0.5 mg im.	0.5 mg im.	TER, SNCV, MNCV	4 w	n.r.
Lan et al. (2007) [34]	82 (43/39)	53.2 ± 3.7/53.6 ± 3.6	9.3 ± 8.3/9.54 ± 8.1	4.3 ± 1.3/4.2 ± 1.2	150 mg ivgtt.	0.5 mg im.	0.5 mg im.	TER, SNCV	4 w	n.r.
Li et al. (2009) [28]	120 (64/56)	60 ± 6.52/60.5 ± 8.33	5.5 ± 4.2	2.6 ± 0.4/2.8 ± 0.5	20 mL ivgtt.	0.5 mg im.	0.5 mg im.	TER, SNCV, MNCV	4 w	0
Li et al. (2006) [35]	56 (29/27)	55 ± 3/56 ± 4	6.75 ± 1.2/6.8 ± 1.2	2.15 ± 0.21/1.98 ± 0.23	135 mg ivgtt.	1.5 mg po.	1.5 mg po.	TER	4 w	2/0
Luo and Tang (2013) [36]	80 (40/40)	61.3 ± 12.08/62 ± 11.87	3.02 ± 1.75/3.04 ± 1.93	5.57 ± 2.03/5.42 ± 2.2	70 mg ivgtt.	1.0 mg iv.	1.0 mg iv.	TER, SNCV, MNCV	2 w	n.r.
Peng et al. (2012) [37]	88 (40/48)	61.56 ± 12.18/62.36 ± 6.25	11.83 ± 2.3/12.04 ± 4.52	4.9/5.5	75 mg ivgtt.	1.0 mg ivgtt.	1.0 mg ivgtt.	TER, SNCV, MNCV	2 w	n.r.
Sun (2014) [38]	60 (30/30)	53.2 ± 3.6/51.7 ± 2.8	10.6 ± 4.2/9.5 ± 3.4	n.r.	20 mL ivgtt.	1.0 mg ivgtt.	1.0 mg ivgtt.	TER, SNCV, MNCV	2 w	n.r.
Wang (2013) [39]	68 (34/34)	62.4 ± 8.4	n.r.	n.r.	200 mg ivgtt.	1.5 mg po.	1.5 mg po.	TER	4 w	2.0/3.0
Wu and Zhang (2007) [40]	74 (38/36)	48/47	7.34/7.28	2.32/2.24	40 mL ivgtt.	500 mL im.	500 mL im.	TER	2–6 w	n.r.
Zhang (2012) [41]	60 (30/30)	59.06 ± 7.82	7.15 ± 1.32	2.06 ± 0.59	50 mg ivgtt.	1.5 mg po.	1.5 mg po.	TER	4 w	0
Zhou (2009) [42]	65 (33/32)	n.r.	n.r.	n.r.	40 mL ivgtt.	1.5 mg po.	1.5 mg po.	TER	4 w	0

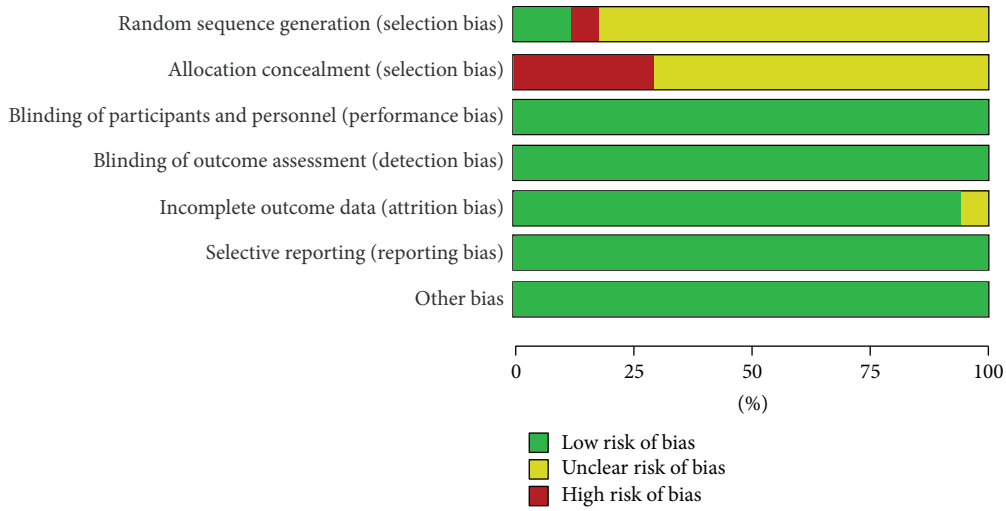


Figure 2: Risk of bias across studies assessed using the Cochrane risk of bias tool.

Z was 3.02 ($P = 0.003$), and the diamond on the right side of the vertical line was complete, suggesting that the median SNCV of patients treated with breviscapine and mecobalamin was significantly higher than that of patients treated with mecobalamin alone (Figure 6).

(3) Peroneal MNCV. The peroneal MNCV was compared between groups in 9 studies [25, 26, 28, 29, 31, 33, 34, 36, 37]. There were 410 patients treated with breviscapine and mecobalamin and 382 patients treated with mecobalamin alone. There was significant heterogeneity among groups ($P < 0.00001$, $I^2 = 92\%$), and random effects model was employed for pooled analysis. The pooled OR was 6.20, 95% CI was 4.69–7.72, Z was 8.02 ($P < 0.0001$), and the diamond on the right side of the vertical line was complete, suggesting that the peroneal MNCV of patients treated with breviscapine and mecobalamin was significantly higher than that of patients treated with mecobalamin alone (Figure 7).

(4) Peroneal SNCV. The peroneal SNCV was compared between groups in 9 studies [25, 26, 28, 29, 31, 33, 34, 36, 37]. There were 410 patients treated with breviscapine and mecobalamin and 382 patients treated with mecobalamin alone. There was significant heterogeneity among groups ($P < 0.00001$, $I^2 = 89\%$), and random effects model was employed for pooled analysis. The pooled OR was 4.06, 95% CI was 2.80–5.32, Z was 6.33 ($P < 0.0001$), and the diamond on the right side of the vertical line was complete, suggesting that the peroneal SNCV of DPN patients treated with breviscapine and mecobalamin was significantly higher than that of patients treated with mecobalamin alone (Figure 8).

3.4. Adverse Events. After therapy for 2–6 weeks, patients were tolerant to both therapies and there were no severe side effects related to therapies. However, there were mild side effects: 1 with mild headache, 2 with mild nausea [29], 5 with itching [29, 30, 35], and 2 with palpitation at injection [35]. However, these side effects resolved soon after discontinuation of therapy.

3.5. Publication Bias. Funnel plot was used for the evaluation of publication bias of studies included in this meta-analysis. Results showed that the funnel plot was nearly symmetrical, suggesting no publication bias in these studies (Figure 9).

4. Discussion

DM is one of the most common diseases, and its incidence is increasing worldwide with the acceleration of economic developments and the pace of living. It is estimated that the prevalence of DM in 2030 will be 50.7% higher than that in 2011, and about 48% of new DM cases will be found in China and India [43]. DPN is one the most common complications of DM. Dyck et al. [44] found that the incidence of DPN was 66% and 59% in patients with type 1 and type 2 DM, respectively. DPN may cause damage to the motor, sensory, and autonomic nerves and even cause limb gangrene and amputation.

The pathogenesis of DPN is very complicated and is currently regarded as a result of interaction among multiple factors under a hyperglycemic state including suppressions of glycation end products generation, changes in protein kinase C signaling pathway [45], activation of polyol pathway [46], and increases in cytokines due to ischemia and/or hypoxia [47]. Modern pharmacological study [48] showed that breviscapine was able to dilate blood vessels, reduce blood viscosity, inhibit platelet aggregation, increase activities of plasma endothelin, renin, and angiotensin, dilate arterioles, improve microcirculation, increase blood supplies of nerves, improve ischemia and hypoxia, and elevate nerve conduction velocity, which were helpful to improve the symptoms and signs of DPN and increase the sensory nerve conduction velocity of limbs. Mecobalamin is a derivative of coenzyme vitamin B12 and may act to repair myelin to improve DPN.

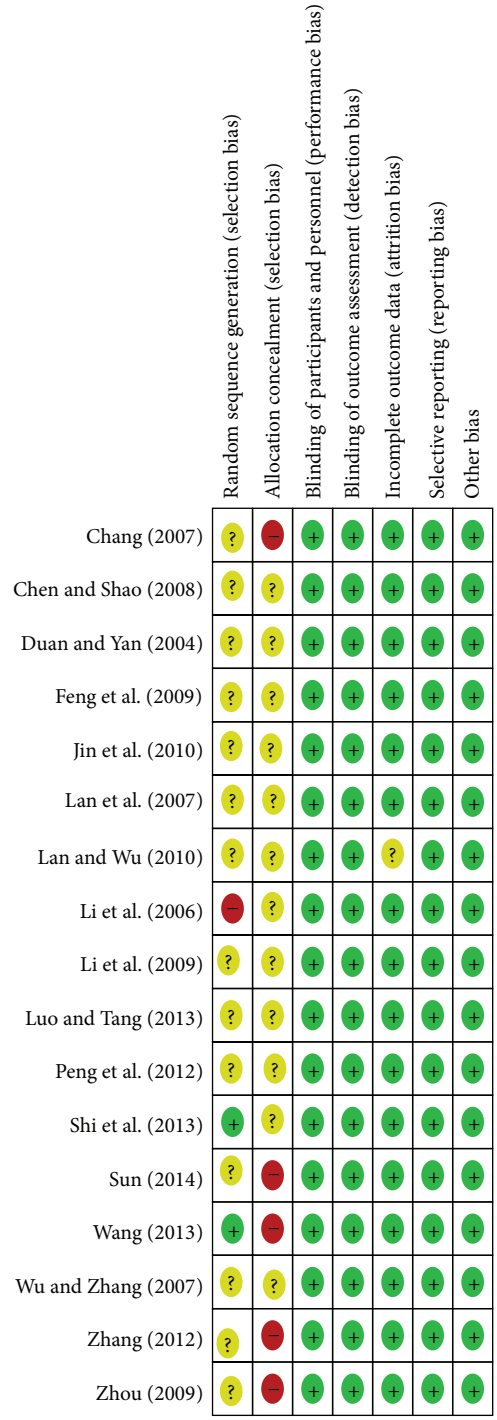


Figure 3: Risk of bias in individual studies using Cochrane risk of bias.

Breviscapine and mecobalamin may exert synergistic effects on DPN in different mechanisms and significantly increase the therapeutic efficacy [49].

Liu [50] found that breviscapine could improve the therapeutic efficacy in DPN patients in a systematic review, but the safety was not good. Our results showed that the therapeutic efficacy of breviscapine in combination with mecobalamin in DPN patients was remarkably superior to that of mecobalamin alone. Among all the studies included in this meta-analysis, the combined therapy group was significantly different from control group. Of these studies, 7 confirmed that breviscapine could improve or even cure the symptoms of motor and sensory nerves in DPN patients [25, 28, 29, 31, 33, 36, 37]. In addition, therapy with breviscapine and mecobalamin had no severe side effects. Hu et al. [51] found that the inner wall of blood vessels of lower limbs

Study or subgroup	B + M Events	B + M Total	M Events	M Total	Weight	Odds ratio M-H, fixed, 95% CI
Chang, 2007	40	43	26	43	4.4%	8.72 [2.32, 32.73]
Chen and Shao, 2008	64	68	53	64	7.7%	3.32 [1.00, 11.03]
Duan and Yan, 2004	34	36	16	28	2.4%	12.75 [2.55, 63.82]
Feng et al., 2009	42	46	30	45	6.3%	5.25 [1.58, 17.40]
Jin et al., 2010	29	35	19	35	7.8%	4.07 [1.35, 12.26]
Lan et al., 2007	39	43	26	39	6.1%	4.88 [1.43, 16.61]
Lan and Wu, 2010	52	56	33	56	5.7%	9.06 [2.87, 28.56]
Li et al., 2009	58	64	31	56	7.5%	7.80 [2.89, 21.02]
Li et al., 2006	21	29	13	27	8.9%	2.83 [0.93, 8.58]
Luo and Tang, 2013	37	40	31	40	5.6%	3.58 [0.89, 14.39]
Peng et al., 2012	45	48	32	40	5.2%	3.75 [0.92, 15.24]
Shi et al., 2013	41	45	32	45	6.8%	4.16 [1.24, 14.00]
Sun, 2014	27	30	27	30	6.5%	1.00 [0.19, 5.40]
Wang, 2013	29	34	23	34	8.1%	2.77 [0.84, 9.12]
Wu and Zhang, 2007	36	38	20	36	2.6%	14.40 [3.00, 69.10]
Zhang, 2012	28	30	21	30	3.4%	6.00 [1.17, 30.72]
Zhou, 2009	30	33	22	32	4.9%	4.55 [1.12, 18.48]
Total (95% CI)		**718**		**680**	**100.0%**	**5.01 [3.70, 6.78]**
Total events	652		455			

Heterogeneity: $\chi^2 = 12.10$, df = 16 ($P = 0.74$); $I^2 = 0\%$
Test for overall effect: $Z = 10.44$ ($P < 0.00001$)

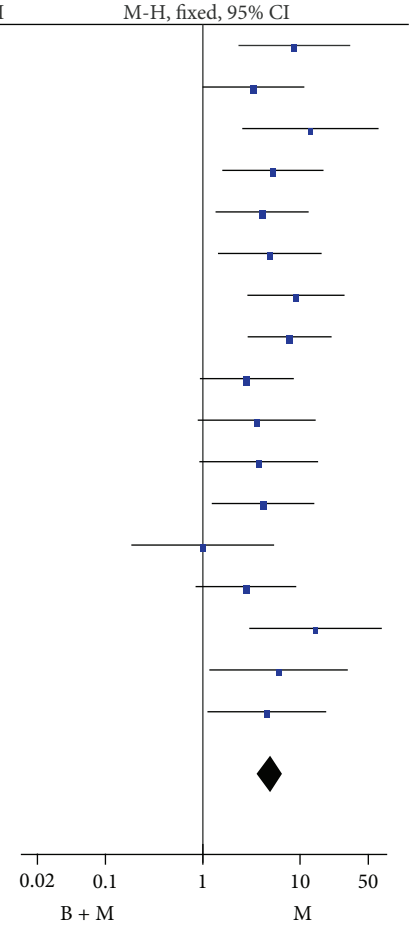


Figure 4: Overall effectiveness of therapy with breviscapine and mecobalamin versus mecobalamin.

Study or subgroup	B + M Mean	B + M SD	B + M Total	M Mean	M SD	M Total	Weight	Mean difference IV, random, 95% CI
Chang, 2007	53.46	5.23	43	41.42	3.51	43	14.2%	12.04 [10.16, 13.92]
Duan and Yan, 2004	54.7	3.9	36	51.7	8	28	12.7%	3.00 [−0.23, 6.23]
Jin et al., 2010	47.8	4.2	35	42.5	3.5	35	14.3%	5.30 [3.49, 7.11]
Li et al., 2009	55.83	3.93	64	46.59	4.13	56	14.5%	9.24 [7.79, 10.69]
Luo and Tang, 2013	52.6	2.7	40	42.2	2	40	14.8%	10.40 [9.36, 11.44]
Peng et al., 2012	53.8	2.9	48	43.4	2	40	14.8%	10.40 [9.37, 11.43]
Shi et al., 2013	48.6	2.93	45	46.8	3.24	45	14.7%	1.80 [0.52, 3.08]
Total (95% CI)			**311**			**287**	**100.0%**	**7.53 [4.65, 10.42]**

Heterogeneity: $\tau^2 = 14.40$; $\chi^2 = 168.97$, df = 6 ($P < 0.00001$); $I^2 = 96\%$
Test for overall effect: $Z = 5.11$ ($P < 0.00001$)

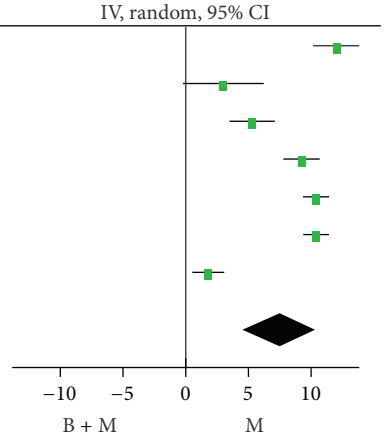


Figure 5: Median MNCV of two groups after therapy.

Study or subgroup	B + M Mean	B + M SD	B + M Total	M Mean	M SD	M Total	Weight	Mean difference IV, random, 95% CI
Chang, 2007	45.11	5.31	43	45.73	5.1	43	13.9%	−0.62 [−2.82, 1.58]
Duan and Yan, 2004	41.6	1	36	37.6	6.5	28	13.7%	4.00 [1.57, 6.43]
Jin et al., 2010	40.9	3.5	35	37.4	2.5	35	14.4%	3.50 [2.08, 4.92]
Li et al., 2009	46.75	5.19	64	41.13	3.96	56	14.3%	5.62 [3.98, 7.26]
Luo and Tang, 2013	54.9	3.7	40	44.8	2.2	40	14.5%	10.10 [8.77, 11.43]
Peng et al., 2012	57.2	4	48	46.7	2.2	40	14.5%	10.50 [9.18, 11.82]
Shi et al., 2013	39.4	2.46	45	37.9	2.48	45	14.6%	1.50 [0.48, 2.52]
Total (95% CI)			**311**			**287**	**100.0%**	**4.98 [1.75, 8.21]**

Heterogeneity: $\tau^2 = 18.28$; $\chi^2 = 197.64$, df = 6 ($P < 0.00001$); $I^2 = 97\%$
Test for overall effect: $Z = 3.02$ ($P = 0.003$)

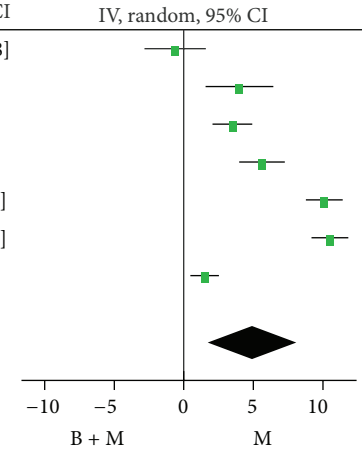


Figure 6: Median MNCV of patients in two groups.

Study or subgroup	B + M Mean	B + M SD	B + M Total	M Mean	M SD	M Total	Weight	Mean difference IV, random, 95% CI
Chang, 2007	46.72	5.2	43	38.3	4.12	43	11.0%	8.42 [6.44, 10.40]
Duan and Yan, 2004	45.1	3.8	36	40.3	6	28	9.8%	4.80 [2.25, 7.35]
Jin et al., 2010	44	3.5	35	38.5	2.9	35	11.9%	5.50 [3.99, 7.01]
Lan et al., 2007	48	9	43	40	7	39	7.9%	8.00 [4.53, 11.47]
Lan and Wu, 2010	48	9	56	40	7	56	8.9%	8.00 [5.01, 10.99]
Li et al., 2009	47.65	5.13	64	41.18	4.51	56	11.5%	6.47 [4.75, 8.19]
Luo and Tang, 2013	47.6	1.3	40	40.4	1.4	40	13.2%	7.20 [6.61, 7.79]
Peng et al., 2012	49.9	1.6	48	42.7	1.5	40	13.2%	7.20 [6.55, 7.85]
Shi et al., 2013	40.5	2.8	45	39.2	2.68	45	12.6%	1.30 [0.17, 2.43]
Total (95% CI)			**410**			**382**	**100.0%**	**6.20 [4.69, 7.72]**

Heterogeneity: $\tau^2 = 4.42$; $\chi^2 = 99.48$, df = 8 ($P < 0.00001$); $I^2 = 92\%$
Test for overall effect: $Z = 8.02$ ($P < 0.00001$)

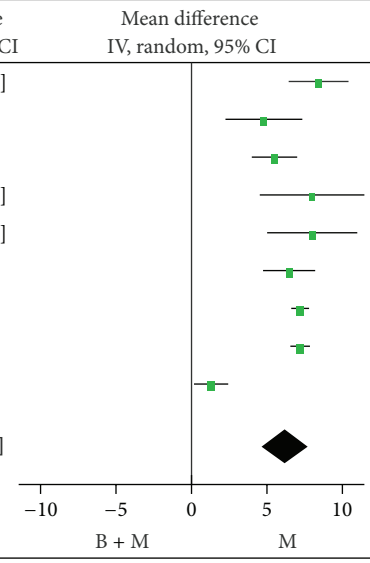


Figure 7: Peroneal MNCV of DPN patients in two groups.

was thickened and became rough, and vascular stenosis and atheromatous plaques were observed, suggesting that thrombosis, vascular narrowing, and increased blood viscosity were closely associated with DPN. In clinical practice, DPN is treated with mecobalamin, which is effective in relieving the pain, numbness, and hypoesthesia, but the therapeutic efficacy is still unsatisfactory [52]. Thus, some researchers [25, 26, 28–42] attempted to apply breviscapine in combination with mecobalamin in the therapy of DPN. Results showed that breviscapine was able to significantly increase the therapeutic efficacy of DPN, reduce the blood viscosity, and elevate the MNCV and SNCV, and other effects related to elevated therapeutic efficacy were also superior to those after monotherapy with mecobalamin. Similar findings were also obtained in the present study.

However, meta-analysis still has limitations. In a majority of studies, the method used for randomization was not addressed, whether there was allocation concealment of randomization is unclear, the blinding method was not described in these studies, and whether intention to treat analysis was performed was still unclear. In addition, patients in these studies were not followed up after interventions, and thus the recurrence of DPN after combined therapy with breviscapine and mecobalamin was not able to be determined.

5. Conclusion

Meta-analysis shows that combined therapy with breviscapine and mecobalamin is safe and effective for DPN patients. The number of studies included in this meta-analysis is small, there is still heterogeneity in the nerve conduction velocity among studies, and the methodology in these studies has defects. Thus, more high-quality, controlled, randomized clinical trials are needed to further confirm the therapeutic

Study or subgroup	B + M Mean	B + M SD	B + M Total	M Mean	M SD	M Total	Weight	Mean difference IV, random, 95% CI
Chang, 2007	41.58	4.23	43	42.89	4.63	43	10.4%	−1.31 [−3.18, 0.56]
Duan and Yan, 2004	40.5	6.3	36	36.7	2.5	28	9.4%	3.80 [1.54, 6.06]
Jin et al., 2010	40.2	2.4	35	37.2	2.6	35	12.1%	3.00 [1.83, 4.17]
Lan et al., 2007	46	6	43	41	6	39	8.6%	5.00 [2.40, 7.60]
Lan and Wu, 2010	46	6	56	41	6	56	9.5%	5.00 [2.78, 7.22]
Li et al., 2009	42.15	4.36	64	35.19	3.17	56	11.7%	6.96 [5.61, 8.31]
Luo and Tang, 2013	45.5	1.4	40	40.5	1.7	40	13.1%	5.00 [4.32, 5.68]
Peng et al., 2012	47.8	1.7	48	42.3	2	40	12.9%	5.50 [4.72, 6.28]
Shi et al., 2013	38.1	2.82	45	34.9	2.71	45	12.2%	3.20 [2.06, 4.34]
Total (95% CI)			**410**			**382**	**100.0%**	**4.06 [2.80, 5.32]**

Heterogeneity: $\tau^2 = 3.03$; $\chi^2 = 69.81$, df = 8 ($P < 0.00001$); $I^2 = 89\%$
Test for overall effect: $Z = 6.33$ ($P < 0.00001$)

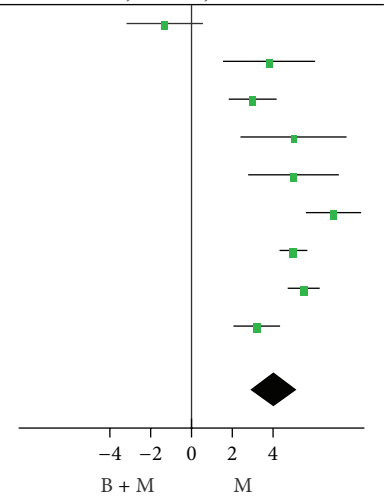


Figure 8: Peroneal SNCV of DPN patients in two groups.

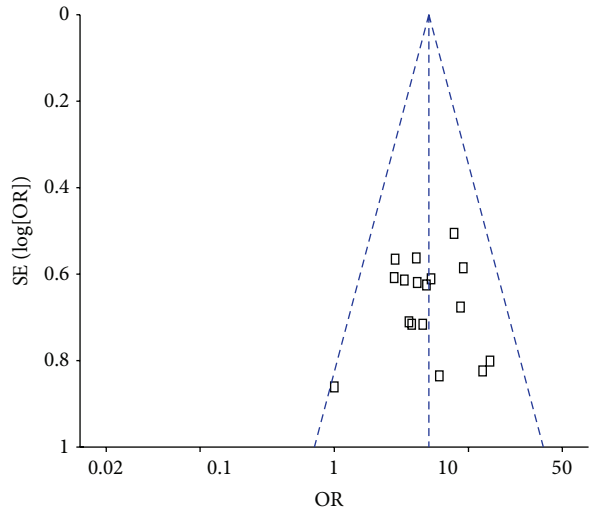


Figure 9: Funnel plot for B-M group versus M alone group for DPN.

efficacy of breviscapine in combination with mecobalamin in DPN patients.

Conflict of Interests

The authors declared that no conflict of interests existed.

Authors' Contribution

Chanjiao Zheng and Zhiheng Zhou conceived and designed the research. Weilin Ou, Chanjiao Zheng, and Huanyu Shen were responsible for literature searching and data extraction. Weilin Ou and Jiaji Wang were responsible for the quality assessment of studies. Chanjiao Zheng and Zhiheng Zhou wrote the paper. Chanjiao Zheng, Weilin Ou, and Huanyu Shen contributed equally to this paper.

Acknowledgments

The authors thank Dr. Qianglin Duan for his helpful suggestions and advice on this paper. This work was supported by the Social Development Research Project of Guangdong Science and Technology Commission, China (no. 2011B032200019), and the Training Project of Outstanding Young College Teachers in Guangdong Province, China (no. Yq2013138).

References

[1] G. Deli, E. Bosnyak, G. Pusch, S. Komoly, and G. Feher, "Diabetic neuropathies: diagnosis and management," *Neuroendocrinology*, vol. 98, no. 4, pp. 267–280, 2014.

[2] A. J. M. Boulton, A. I. Vinik, J. C. Arezzo et al., "Diabetic neuropathies: a statement by the American Diabetes Association," *Diabetes Care*, vol. 28, no. 4, pp. 956–962, 2005.

[3] M. C. Spruce, J. Potter, and D. V. Coppini, "The pathogenesis and management of painful diabetic neuropathy: a review," *Diabetic Medicine*, vol. 20, no. 2, pp. 88–98, 2003.

[4] S. Tesfaye and D. Selvarajah, "Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy," *Journal of Endocrinological Investigation*, vol. 35, no. 4, pp. 434–448, 2012.

[5] A. Gordois, P. Scuffham, A. Shearer, A. Oglesby, and J. A. Tobian, "The health care costs of diabetic peripheral neuropathy in the U.S.," *Diabetes Care*, vol. 26, no. 6, pp. 1790–1795, 2003.

[6] J. L. Edwards, A. M. Vincent, H. T. Cheng, and E. L. Feldman, "Diabetic neuropathy: mechanisms to management," *Pharmacology and Therapeutics*, vol. 120, no. 1, pp. 1–34, 2008.

[7] A. M. Vincent, B. C. Callaghan, A. L. Smith, and E. L. Feldman, "Diabetic neuropathy: cellular mechanisms as therapeutic targets," *Nature Reviews Neurology*, vol. 7, no. 10, pp. 573–583, 2011.

[8] P. Fernyhough, S. K. R. Chowdhury, and R. E. Schmidt, "Mitochondrial stress and the pathogenesis of diabetic neuropathy," *Expert Review of Endocrinology & Metabolism*, vol. 5, no. 1, pp. 39–49, 2010.

[9] P. A. Low, K. K. Nickander, and H. J. Tritschler, "The roles of oxidative stress and antioxidant. Treatment in experimental diabetic neuropathy," *Diabetes*, vol. 46, supplement 2, pp. S38–S42, 1997.

[10] N. E. Cameron, S. E. M. Eaton, M. A. Cotter, and S. Tesfaye, "Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy," *Diabetologia*, vol. 44, no. 11, pp. 1973–1988, 2001.

[11] C.-Z. Hao, F. Wu, L. Lu et al., "Chinese herbal medicine for diabetic peripheral neuropathy: an updated meta-analysis of 10 high-quality randomized controlled studies," *PLoS ONE*, vol. 8, no. 10, Article ID e76113, 2013.

[12] Y. Sun, M.-S. Lai, and C.-J. Lu, "Effectiveness of vitamin B12 on diabetic neuropathy: systematic review of clinical controlled trials," *Acta Neurologica Taiwanica*, vol. 14, no. 2, pp. 48–54, 2005.

[13] S. Wang, H. Wang, H. Guo, L. Kang, X. Gao, and L. Hu, "Neuroprotection of Scutellarin is mediated by inhibition of microglial inflammatory activation," *Neuroscience*, vol. 185, pp. 150–160, 2011.

[14] M. Wang, W.-B. Zhang, J.-H. Zhu, G.-S. Fu, and B.-Q. Zhou, "Breviscapine ameliorates cardiac dysfunction and regulates the myocardial Ca^{2+}-cycling proteins in streptozotocin-induced diabetic rats," *Acta Diabetologica*, vol. 47, no. 1, supplement, pp. S209–S218, 2010.

[15] C. Guo, Y. Zhu, Y. Weng et al., "Therapeutic time window and underlying therapeutic mechanism of breviscapine injection against cerebral ischemia/reperfusion injury in rats," *Journal of Ethnopharmacology*, vol. 151, no. 1, pp. 660–666, 2014.

[16] J. Zhang, X. S. Li, and W. D. Zhang, "The advance in studies on chemical component and pharmacology of *Erigeron breviscapus*," *Journal of Pharmaceutical Practice*, vol. 20, no. 2, pp. 103–107, 2002.

[17] J. M. Cui and S. Wu, "The advance on the research of breviscapine," *Natural Product Research and Development*, vol. 15, no. 3, pp. 255–258, 2003.

[18] M. Li, Y. Zheng, F.-Y. Shan, J. Zhou, T. Gong, and Z.-R. Zhang, "Development of ionic-complex-based nanostructured lipid carriers to improve the pharmacokinetic profiles of breviscapine," *Acta Pharmacologica Sinica*, vol. 34, no. 8, pp. 1108–1115, 2013.

[19] H. Hong and G.-Q. Liu, "Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin," *Life Sciences*, vol. 74, no. 24, pp. 2959–2973, 2004.

[20] H. Hong and G.-Q. Liu, "Scutellarin attenuates oxidative glutamate toxicity in PC12 cells," *Planta Medica*, vol. 70, no. 5, pp. 427–431, 2004.

[21] K. A. Head, "Peripheral neuropathy: pathogenic mechanisms and alternative therapies," *Alternative Medicine Review*, vol. 11, no. 4, pp. 294–329, 2006.

[22] V. A. Fonseca, L. A. Lavery, T. K. Thethi et al., "Metanx in type 2 diabetes with peripheral neuropathy: a randomized trial," *The American Journal of Medicine*, vol. 126, no. 2, pp. 141–149, 2013.

[23] B. A. Yaqub, A. Siddique, and R. Sulimani, "Effects of methylcobalamin on diabetic neuropathy," *Clinical Neurology and Neurosurgery*, vol. 94, no. 2, pp. 105–111, 1992.

[24] H. Ide, S. Fujiya, Y. Asanuma, M. Tsuji, H. Sakai, and Y. Agishi, "Clinical usefulness of intrathecal injection of methylcobalamin in patients with diabetic neuropathy," *Clinical Therapeutics*, vol. 9, no. 2, pp. 183–192, 1987.

[25] Y. S. Shi, Y. Yuan, P. Zhang et al., "Breviscapine injection the treatment of diabetic peripheral neuropathy of 45 cases," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 19, no. 23, pp. 274–277, 2013.

[26] H. P. Lan and F. Q. Wu, "Therapeutic efficacy of Breviscapine in combination with mecobalamin in Diabetic Peripheral Neuropathy," *Chinese Journal of Rural Medicine and Pharmacy*, vol. 17, no. 1, pp. 34–35, 2010.

[27] World Health Organization, *Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications: Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus*, World Health Organization, Geneva, Switzerland, 1999.

[28] H. Li, Z. B. Li, and G. T. Liang, "Clinical efficacy of combined therapy of diabetic peripheral neuropathy with fleabane injection and mecobalam: an analysis of 64 cases," *Hebei Journal of Traditional Chinese Medicine*, vol. 31, no. 4, pp. 605–606, 2009.

[29] L. J. Chang, "Therapeutic efficacy of fleabane in combination with mecobalamin in diabetic peripheral neuropathy," *Chinese Journal of Practical Nervous Diseases*, vol. 10, no. 9, pp. 120–122, 2007.

[30] G. Chen and K. Shao, "Therapy of in Diabetic Peripheral Neuropathy with fleabane injection and mecobalamin: an analysis of 68 cases," *Forum on Traditional Chinese Medicine*, vol. 23, no. 6, article 40, 2008.

[31] Y. F. Duan and X. H. Yan, "Therapy of diabetic peripheral neuropathy with fleabane injection and methycobal injection: an analysis of 36 cases," *Central Plains Medical Journal*, vol. 31, no. 2, p. 46, 2004.

[32] B. Feng, F. L. Sun, W. H. Feng et al., "Clinical study of combined treatment with breviscapine and mecobalamin on diabetic peripheral neuropathy in the elderly with DM," *Chinese Journal of Natural Medicines*, vol. 209, no. 5, pp. 372–374, 2009.

[33] L. Jin, H. S. Xu, L. S. Tian et al., "Effects of therapy of breviscapine combined with mecobalamin on diabetic peripheral neuropathy," *China Practical Medicine*, vol. 5, no. 17, pp. 26–27, 2010.

[34] H. P. Lan, F. Q. Wu, L. L. Tian et al., "Clinical efficacy of combined therapy of diabetic peripheral neuropathy with breviscapine and mecobalam," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 16, no. 31, pp. 4641–4642, 2007.

[35] W. G. Li, J. Z. Ma, X. H. Tian et al., "Clinical efficacy of combined therapy of diabetic peripheral neuropathy with fleabane and mecobalamin," *Xinjiang Medical Journal*, vol. 36, no. 4, pp. 21–22, 2006.

[36] C. S. Luo and Y. Tang, "Clinical application of breviscapine combined with mecobalamin in the treatment of diabetic peripheral neuropathy," *Journal of Clinical Research*, vol. 30, no. 3, pp. 530–532, 2013.

[37] Y. H. Peng, H. L. Zeng, Y. Cai et al., "Clinical effect analysis of breviscapine and methylcobalam on diabetic peripheral neuropathy," *Journal of Clinical and Experimental Medicine*, vol. 11, no. 13, pp. 1003–1005, 2012.

[38] L. P. Sun, "Therapeutic efficacy of breviscapine in combination with methycobal in diabetic peripheral neuropathy," *Journal of Practical Diabetology*, no. 3, pp. 32–33, 2014.

[39] F. Q. Wang, "Therapeutic efficacy of breviscapine injection in combination with methycobal in 34 cases of diabetic peripheral neuropathy," *Shaanxi Journal of Traditional Chinese Medicine*, vol. 34, no. 7, pp. 825–826, 2013.

[40] H. X. Wu and X. Zhang, "Therapeutic efficacy of fleabane in combination with methycobal in 38 cases of diabetic peripheral neuropathy," *Chinese Journal of Practical Nervous Diseases*, vol. 10, no. 52, pp. 103–104, 2007.

[41] H. M. Zhang, "Therapeutic efficacy of breviscapine and mecobalamin in diabetic peripheral neuropathy: a clinical analysis," *China Foreign Medical Treatment*, vol. 31, no. 19, p. 97, 2012.

[42] D. L. Zhou, "Combined therapy of diabetic peripheral neuropathy with breviscapine and mecobalamin," *China Modern Doctor*, vol. 47, no. 4, pp. 79–80, 2009.

[43] D. R. Whiting, L. Guariguata, C. Weil, and J. Shaw, "IDF Diabetes Atlas: global estimates of the prevalence of diabetes for 2011 and 2030," *Diabetes Research and Clinical Practice*, vol. 94, no. 3, pp. 311–321, 2011.

[44] P. J. Dyck, K. M. Kratz, J. L. Karnes et al., "The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study," *Neurology*, vol. 43, no. 4, pp. 817–824, 1993.

[45] J. Eichberg, "Protein kinase C changes in diabetes: is the concept relevant to neuropathy?" *International Review of Neurobiology*, vol. 50, pp. 61–82, 2002.

[46] N. E. Cameron and M. A. Cotter, "Metabolic and vascular factors in the pathogenesis of diabetic neuropathy," *Diabetes*, vol. 46, no. 2, pp. S31–S37, 1997.

[47] P. A. Low, T. D. Lagerlund, and P. G. McManis, "Nerve blood flow and oxygen delivery in normal, diabetic, and ischemic neuropathy," *International Review of Neurobiology*, vol. 31, pp. 355–438, 1989.

[48] D. H. Xie, "Advances in the clinical application of breviscapine," *New Medicine*, vol. 38, no. 6, pp. 408–410, 2007.

[49] J. Li, L. Zhang, and S. Zhao, "Meta analysia of alpha-lipoic acid and mecobalamin in the treatment of diabetic peripheral neuropathy," *China Medical Herald*, vol. 10, no. 3, pp. 88–90, 2013.

[50] X. D. Liu, *Dreviscapine for Diabetic Peripheral Neuropathy: A Systematic Review*, Guangxi Medical University, 2010.

[51] H. Hu, H. Li, F.-P. Zheng, Y. Cheng, J. Miao, and W. Zhang, "A comparison of clinical effectiveness of different neuropathy scoring systems in screening asymptomatic diabetic peripheral neuropathy," *Chinese Journal of Internal Medicine*, vol. 1, no. 51, pp. 13–17, 2012.

[52] Cooperative Group of Multi-center Clinical Study in Shanghai, "A pilot study on the therapy of diabetic peripheral neuropathy with mecobalamin," *Chinese Journal of Endocrinology and Metabolism*, vol. 13, no. 4, pp. 197–200, 1997.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 573978, 9 pages
http://dx.doi.org/10.1155/2014/573978

Research Article

The Protective Effect of *Agaricus blazei* Murrill, Submerged Culture Using the Optimized Medium Composition, on Alcohol-Induced Liver Injury

Hang Wang,[1] Gang Li,[2] Wenyu Zhang,[1] Chunchao Han,[1] Xin Xu,[3] and Yong-Ping Li[3]

[1] *School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[2] *Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, Xi'an 710032, China*
[3] *Department of Vascular and Endocrine Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China*

Correspondence should be addressed to Chunchao Han; chunchaoh@126.com, Xin Xu; xinxu22@163.com and Yong-Ping Li; yongpingli11@163.com

Received 15 May 2014; Accepted 5 June 2014; Published 10 July 2014

Academic Editor: Jianyou Guo

Agaricus blazei Murrill (ABM), an edible mushroom native to Brazil, is widely used for nonprescript and medicinal purposes. Alcohol liver disease (ALD) is considered as a leading cause for a liver injury in modern dietary life, which can be developed by a prolonged or large intake of alcohol. In this study, the medium composition of ABM was optimized using response surface methodology for maximum mycelial biomass and extracellular polysaccharide (EPS) production. The model predicts to gain a maximal mycelial biomass and extracellular polysaccharide at 1.047 g/100 mL, and 0.367 g/100 mL, respectively, when the potato is 29.88 g/100 mL, the glucose is 1.01 g/100 mL, and the bran is 1.02 g/100 mL. The verified experiments showed that the model was significantly consistent with the model prediction and that the trends of mycelial biomass and extracellular polysaccharide were predicted by artificial neural network. After that, the optimized medium was used for the submerged culture of ABM. Then, alcohol-induced liver injury in mice model was used to examine the protective effect of ABM cultured using the optimized medium on the liver. And the hepatic histopathological observations showed that ABM had a relatively significant role in mice model, which had alcoholic liver damage.

1. Introduction

Alcoholic liver disease (ALD) has become a common global healthcare problem, which is due to excessive alcohol intake for a long duration. Liver is the leading organ for synthesizing vitals, metabolizing ingesta, and detoxifying noxious substances. People who drink a lot are more likely to suffer from ALD including steatohepatitis, hepatic fibrosis, and cirrhosis [1, 2]. Hepatic tissue can be damaged by dangerous byproducts of alcohol breakdown like acetaldehydes, which can react with cellular proteins to generate adducts. Therefore, it is particularly important to find a kind of natural and low-toxic medicine in treatment of alcoholic liver disease.

Mushrooms are defined as macrofungi with distinctive and visible fruiting bodies that may grow above or below ground [3]. Nutritional value of edible mushrooms is due to high protein, fiber, vitamin and mineral contents, and a low-fat level [4–7]. Owing to their attractive taste, aroma, and nutritional values, edible mushrooms were used as food and medicine for centuries [8, 9], including biologically active polysaccharides in the fruiting bodies and bioactive compounds in submerged broth. And some mushrooms popular in the Far East have been reported to have medicinal value, including antitumor, antiviral, and hypolipidemic effects [3, 10, 11].

Agaricus blazei Murrill (ABM), a mushroom native to Brazil, is a basidiomycete brown fungus, which is widely used for nonprescript, medicinal purposes, both as an edible mushroom and in the form of extracts. Due to alleged health effects, ABM was brought to Japan and is widely used

Table 1: Variables and experimental design levels for response surface.

Variables (g/100 mL)	Coded symbols	Coded levels		
		−1	0	1
Potato	X_1	15	20	25
Glucose	X_2	1	2	3
Wheat bran	X_3	1	2	3

today in oriental countries both as an edible mushroom, considered a functional food, and as a natural therapy in the form of a medicinal extract mostly for prevention and treatment of cancer [12]. And many bioactive components of ABM have been studied showing that it has relatively notable pharmacological effects, especially the polysaccharides. To reduce the cost and improve the productivity, numerous researchers have studied the production of the mycelium and polysaccharide by submerged fermentation of ABM [13–17]. There are a good number of advantages of submerged culture including higher polysaccharide and mycelium production in a more compact space and shorter time [18] and as an alternative for efficient production of polysaccharide with similar biological activity [19].

Consequently, in the present paper, the most optimum conditions for maximum mycelia biomass and extracellular polysaccharide production were investigated in ABM using response surface methodology and the artificial neural network (ANN). What is more, this study investigated protective effects of oral administration of ABM on alcoholic liver damage.

2. Materials and Methods

2.1. Microorganism. ABM was obtained from the Center for Culture Collection of Pharmaceutical Microorganisms, China. The culture was maintained in potato dextrose agar (PDA) slant containing (per liter) 200.0 g potato juice, 20.0 g glucose, and 20.0 g agar and incubated at 25°C for 7 days. The slants were subcultured every three months and then stored at 4°C.

2.2. Flask Culture. Potato dextrose broth (PDB) was prepared as follows: 200.0 g potatoes were cut into 1 cm^3 pieces and boiled in 500 mL of water for 30 min. Simultaneously, 20.0 g wheat bran was also boiled in 500 mL of water for 30 min and then the extracts of potatoes and bran were collected by filtration through gauze. Next, 20.0 g glucose, 1.0 g KH_2PO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, and water were added to the extracts to 1 L total volume. Then the medium was autoclaved at 121°C for 30 min. The flask culture experiments were performed in 250 mL flasks containing 150 mL of fermentation medium, which was inoculated with 10% (v/v) of the seed culture. Finally, the flasks were inoculated and incubated at 25°C for 6 days in a static condition.

2.3. Response Surface Methodology for Optimizing Medium Components. Response surface methodology (RSM) was used to investigate the optimum concentration of the variables (concentration of glucose, potato, and bran extract) on the maximum yield of mycelial biomass growth and EPS production from ABM. The software Design-Expert 7.1 Trial was applied in the experimental design, data analysis, and quadratic equation construction. The experimental design was a Box-Behnken design with three key factors and three levels (Table 1). Triplicates at the center (−1, 0, and 1) of the design were intended to allow the estimation of the pure error sum of squares. For statistical calculation, the independent variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad i = 1, 2, 3, \tag{1}$$

where X_i is the real value of an independent variable, X_0 is the real value of the independent variable on the center point and ΔX_i is the step change value, and x_i represents the coded values for X_i. As shown in Table 1, these three independent variables were coded as X_1, X_2, and X_3, respectively.

The behavior of the system is explained by the following second-degree polynomial equation:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j, \tag{2}$$

where Y is the predicted response value, b_0 is the intercept term, b_i is the linear term, b_{ii} is the squared term, b_{ij} is the interaction term, and X_i and X_j are the coded level of independent variables.

Statistical analysis of the model was performed to evaluate ANOVA. The goodness of fit of the polynomial model equation was expressed by Fisher's F-test, the coefficient of determination R^2. And the surface and contour plots express the fitted polynomial equation, which can visualize the relationship between experimental levels of each factor and the response to deduce the optimum conditions [20].

2.4. Estimation of the Mycelium Dry Weight and Extraction of EPS. The mycelial biomass of ABM was measured by filtering the fungal culture through the gauze until a clear filtrate was obtained. The obtained mycelium was washed three times with distilled water, dried to constant weight at 60°C, and weighed. Then EPS was precipitated from the culture filtrate, mixed with five volumes of 95% (v/v) ethanol, and left to stand overnight at 4°C to precipitate crude EPS. The precipitated EPS was centrifuged by centrifugation at 4500 rpm for 5 min and the supernatant was concentrated so that ethanol could be recovered. The centrifugated precipitate was resuspended in an equal volume of 75% ethanol [21] to remove oligosaccharides and centrifuged again. The precipitated EPS was dried to constant weight at 60°C to remove residual ethanol and weighed.

2.5. Assay of Extracellular Polysaccharide from ABM. The dry EPS was ground into fine power and estimated by the phenol-sulfuric acid colorimetric assay [22]. In brief, 20 mg EPS was fully dissolved in 100 mL of double distilled water and mixed evenly. 0.4 mL of the above solution was added in 1.6 mL double distilled water to 2 mL total volume, followed by addition of 1 mL of phenol and 5 mL of sulfuric

Table 2: Design and experimental results of the four-factor Box-Behnken design.

Standard	Run order	X_1	X_2	X_3	The yield of mycelial biomass (g/100 mL)		The yield of extracellular polysaccharide (g/100 mL)	
					Experimental	Predicted	Experimental	Predicted
4	1	25.00	3.00	2.00	1.033	1.038	0.268	0.264
9	2	20.00	1.00	1.00	0.761	0.761	0.367	0.367
2	3	25.00	1.00	2.00	0.882	0.877	0.285	0.289
3	4	15.00	3.00	2.00	0.852	0.857	0.230	0.226
8	5	25.00	2.00	3.00	0.936	0.931	0.243	0.247
5	6	15.00	2.00	1.00	0.764	0.769	0.320	0.316
6	7	25.00	2.00	1.00	0.945	0.950	0.358	0.354
7	8	15.00	2.00	3.00	0.755	0.750	0.205	0.209
11	9	20.00	1.00	3.00	0.710	0.720	0.252	0.244
16	10	20.00	2.00	2.00	0.841	0.841	0.264	0.264
14	11	20.00	2.00	2.00	0.841	0.841	0.264	0.264
13	12	20.00	2.00	2.00	0.841	0.841	0.264	0.264
1	13	15.00	1.00	2.00	0.701	0.696	0.246	0.246
9	14	20.00	2.00	2.00	0.841	0.841	0.264	0.264
10	15	20.00	3.00	1.00	0.912	0.901	0.318	0.326
15	16	20.00	2.00	2.00	0.841	0.841	0.264	0.264
12	17	20.00	3.00	3.00	0.902	0.902	0.235	0.235

acid, and then the polysaccharide content of the mixture was determined spectrophotometrically at 490 nm by using glucose as standard.

2.6. Optimization of Media Components by Artificial Neural Network. The artificial neural network (ANN) is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables. Application of ANN has been considered as a promising tool because of their simplicity towards simulation, prediction, and modeling. A back-propagation algorithm is a multilayer feed-forward ANN, which can train and then evaluate the system performance using an adaptive gradient learning rule [23–25]. In this paper, experimental values of three variables, mycelial dry weight, and extracellular polysaccharide in Table 2 were normalized in the range of 0 to 1. Then three neurons in the input layer, three in a hidden layer, and one in the output layer using the tanh transfer function were used to predict the yield of mycelial biomass and EPS, respectively (Matlab 7.1 software).

2.7. Evaluation of ABM against Alcohol-Induced Liver Injury in Mice

2.7.1. Preparation of ABM. The optimized medium was used for the submerged culture of ABM. After the completion of the fermentation culture, ABM was filtered with gauze after submerged culture. Then the filtrate was collected as ABM fermentation broth (ABM-fb). Fermentation mycelia were watered twice with distilled water and homogenized with organization pounding machine (ABM-fm). ABM fermentation product (ABM-fp), including fermentation broth and mycelia, was homogenized with organization pounding machine. The above samples were stored at 4°C in bottles and kept sterile until instilled intragastrically in mice.

2.7.2. Animals Model and Liver Histology. Male Kunming mice were obtained from Lukang Animal Pharmaceutical Co., Ltd. (Shandong, China) and acclimated for 1 week in the animal experimental research laboratory. Animals were randomly divided into 5 groups with 10 mice per each. The normal control group was fed with normal diet and water. The ethanol control group was fed with Chinese white liquor except the normal diet. The other three experimental groups were also fed with Chinese white liquor and were simultaneously fed with ABM fermentation broth (ABM-fb), ABM fermentation mycelia (ABM-fm), and ABM fermentation products (ABM-fp), respectively. All animals were maintained on the treatments for a total of 4 weeks. Liver samples were collected and fixed in formalin for histology study. And formalin-fixed paraffin tissue sections were processed for staining with hematoxylin and eosin and then studied by light microscopy.

2.8. Statistical Analysis. The data were analyzed using SPSS and expressed as means ± standard deviation (SD). Differences were considered statistically significant when $P < 0.05$ by one-way analysis of variance (ANOVA).

3. Results and Discussions

The Box-Behnken design matrix was used to determine the effects of the three independent variables including concentration of potato extract (X_1), concentration of glucose (X_2), and concentration of wheat bran extract (X_3) on the yield of mycelial biomass and extracellular polysaccharide. The

experimental and predictive values of responses (yield of the extracellular polysaccharides and mycelial dry weight) under different treatment conditions are presented in Table 2.

3.1. Optimization of the Yield of Mycelial Biomass by RSM. The predicted response Y_{biomass} for the mycelial biomass in terms of coded unit was obtained as follows:

$$Y_{\text{biomass}} = 0.84 + 0.091X_1 + 0.081X_2 - 0.010X_3 + 0.010X_2X_3 + 0.027X_1^2 - 0.0014X_2^2 - 0.018X_3^2, \quad (3)$$

where Y_{biomass} is the response in terms of g/100 mL of mycelial biomass, where X_1, X_2, and X_3 are independent variables in coded units containing concentration of potato extract, concentration of glucose, and concentration of wheat bran extract, respectively. Statistical testing of the regression model was done in the form of an F-test and the analysis of variance (ANOVA), which is required to test the significance and adequacy of the model (Table 3).

In this study, ANOVA of the regression model demonstrated that the model was highly significant for the mycelial biomass yield, as was evident from the calculated F-value (model = 216.98) with a very low probability value ($P > F$) < 0.0001 [26]. Values of "$P > F$" less than 0.05 indicate that model terms are significant. In this case, the P values were much less than 0.05, indicating that all these variables were more significant. The P value was used as a tool to check the significance of each of the coefficients, which are necessary to understand the pattern of mutual interactions between the test variables. The correlation measure for testing the goodness of the model was the coefficient of determination (R^2), which should be closer to 1. In the present study, the R^2 was 0.9964, much closer to 1. The predictive R^2 (Pred-R^2) of 0.9429 was in reasonable agreement with the adjustable R^2 (adj-R^2) of 0.9918. The adequate precision was also used to measure the ratio of signal to noise and it was generally desired to be greater than 4 [27]. The value of adequate precision was 56.205 suggesting that the model was of an adequate signal and could be used to navigate the design space. Hence, the regression model given in (3) is a good prediction of the experimental results and the factor effects are real.

The graphical representations of the regression equation (3), called the response surfaces and the contour plots, are presented in Figures 1(a), 1(b), and 1(c). The three-dimensional response surface (3D-surface) plot and two-dimensional response projection (2D-projection) were able to visually show the response over a region of interesting factor levels, the relationship between experimental levels of each variable, and the response and the type of interactions between test variables so that the optimum conditions for mycelium production could be deduced [27]. Each plot shows the effect of two independent variables varying within the experimental range of mycelial biomass. Figure 1(a) shows the effect of potato and glucose concentration on mycelial dry weight. A quadratic effect of potato and glucose concentration on the response was observed. The 3D-plot showed evidence that the yield of mycelial biomass increased upon increasing potato from 15 g/100 mL to 25 g/100 mL and glucose from 1 g/100 mL to 3 g/100 mL, respectively. Figure 1(b) shows the effect of potato and bran concentration on mycelial dry weight. Unlike Figure 1(a), the yield of biomass decreased with the increase of bran. Figure 1(c) shows the effect of glucose and bran concentration on mycelial dry weight. A quadratic effect of glucose and bran in the response was observed. And a similar trend of Figure 1(b) was also observed.

3.2. Optimization of the Yield of EPS by RSM. Using the designed experimental data (Table 2), the polynomial model for EPS yield Y_{EPS} in terms of coded unit was expressed as follows:

$$Y_{\text{EPS}} = 0.26 + 0.019X_1 - 0.012X_2 - 0.054X_3 - 0.00025X_1X_2 + 0.008X_2X_3 - 0.0091X_1^2 + 0.0024X_2^2 + 0.027X_3^2, \quad (4)$$

where Y_{EPS} is the response in terms of g/100 mL of the yield of extracellular polysaccharide, where X_1, X_2, and X_3 are independent variables in coded units containing concentration of potato extract (g/100 mL), concentration of glucose (g/100 mL), and concentration of wheat bran extract (g/100 mL), respectively.

Table 3 showed the analysis of variance (ANOVA) for response surface quadratic model and the statistical significance of (4) that was checked by F-test. The ANOVA of the regression model demonstrated that the model was highly significant for the extracellular polysaccharide, as was evident from the calculated F-value (model = 90.04) with a very low probability value ($P > F$) < 0.0001 [26]. And a high degree of precision, reliability, and high degree of correlation between the observed and predicted values were demonstrated by the values of correlation coefficient R^2 (0.9914), adj-R^2 (0.9804), and Pred-R^2 (0.8630) [28]. In addition, the value of adequate precision was 33.529 suggesting that the model was of an adequate signal and could be used to navigate the design space. Hence, the regression model given in (4) is a good prediction of the experimental results, and the factor effects are real.

The graphical representations of the regression equation (4), called the response surfaces and the contour plots, are presented in Figures 2(a), 2(b), and 2(c). The 3D-surface plot and 2D-projection could visually show the interaction between the potato (X_1), glucose (X_2), and wheat bran (X_3). The yield of EPS increased sharply with the increase of potato and glucose in Figure 2(a). However, Figure 2(b) showed that the yield of extracellular polysaccharide increased with the increase of potato and the decrease of bran. The maximum yield of EPS was presented when the potato was 25 g/100 mL and the bran was 1 g/100 mL in Figure 2(b). Figure 2(c) shows the effect of glucose and bran concentration on the extracellular polysaccharide. And the 3D-surface plot and 2D-projection showed the same trend as that in Figure 2(a).

3.3. Optimization of the Mycelial Dry Weight and Polysaccharide Production by ANN. The artificial neural network with back-propagation algorithm was used to model the

Table 3: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of biomass production and optimization of extracellular glucan production.

Source		Model	Lack of fit	Pure error	Corrected total
Sum of squares	Biomass	0.12	$4.412E-004$	0.000	0.12
	EPS	0.031	$2.643E-004$	0.000	0.031
D.f.	Biomass	9	3	4	16
	EPS	9	3	4	16
Mean square	Biomass	0.014	$1.471E-004$	0.000	
	EPS	$3.399E-003$	$8.808E-005$	0.000	
F-value	Biomass	216.98	6.37		
	EPS	90.04	5.84		
Probability ($P > F$)	Biomass	<0.0001	0.0753 not significant		
	EPS	<0.0001	0.0528 not significant		

R^2: 0.9964, 0.9914; adj-R^2: 0.9918, 0.9804.

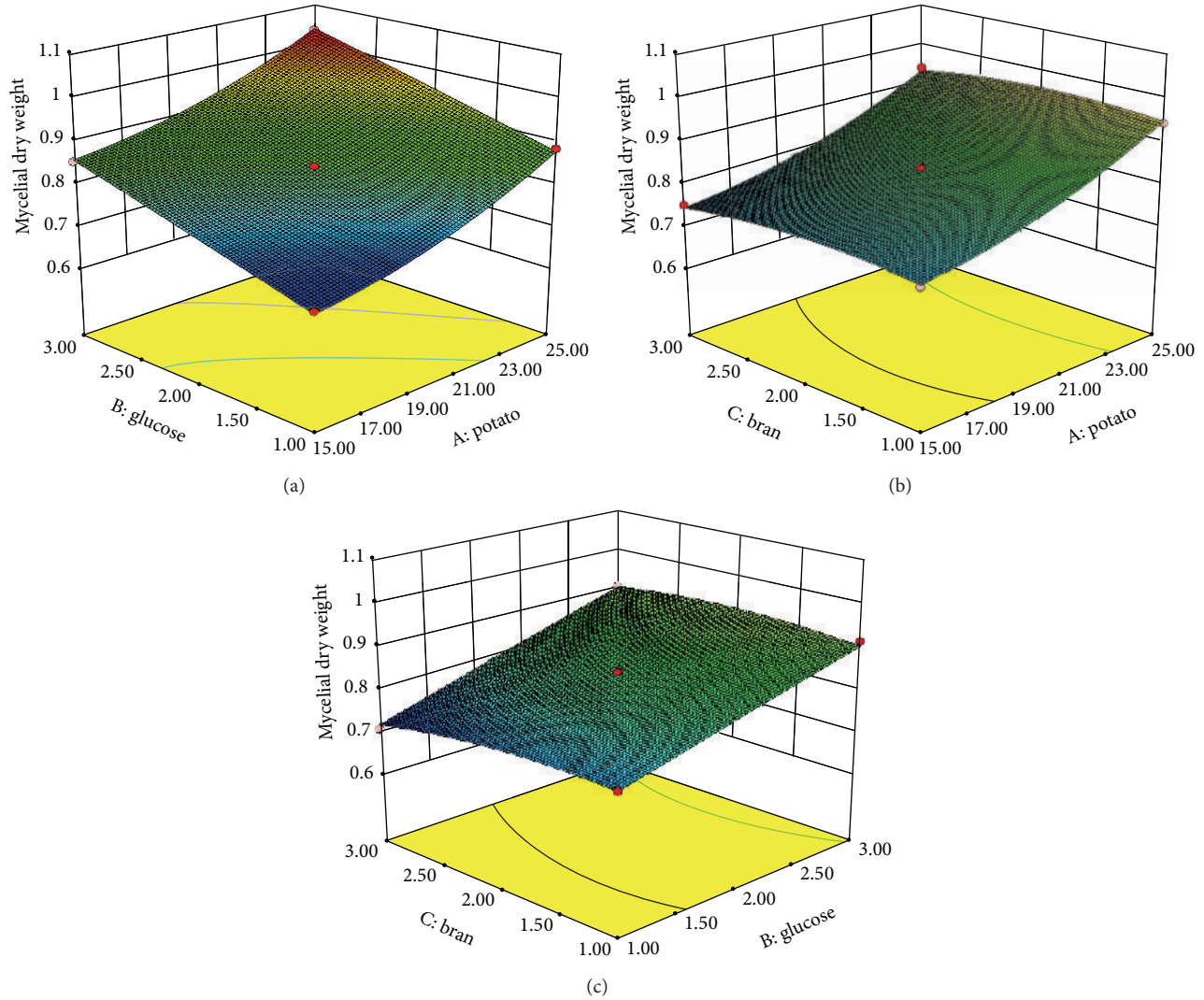


Figure 1: The 3D-plot and 2D-projection of response surface represent the interaction between two factors in the yield of mycelial biomass (g/100 mL) by keeping the other two factors constant: (a) potato and glucose (g/100 mL), (b) potato and bran (g/100 mL), and (c) glucose and bran (g/100 mL).

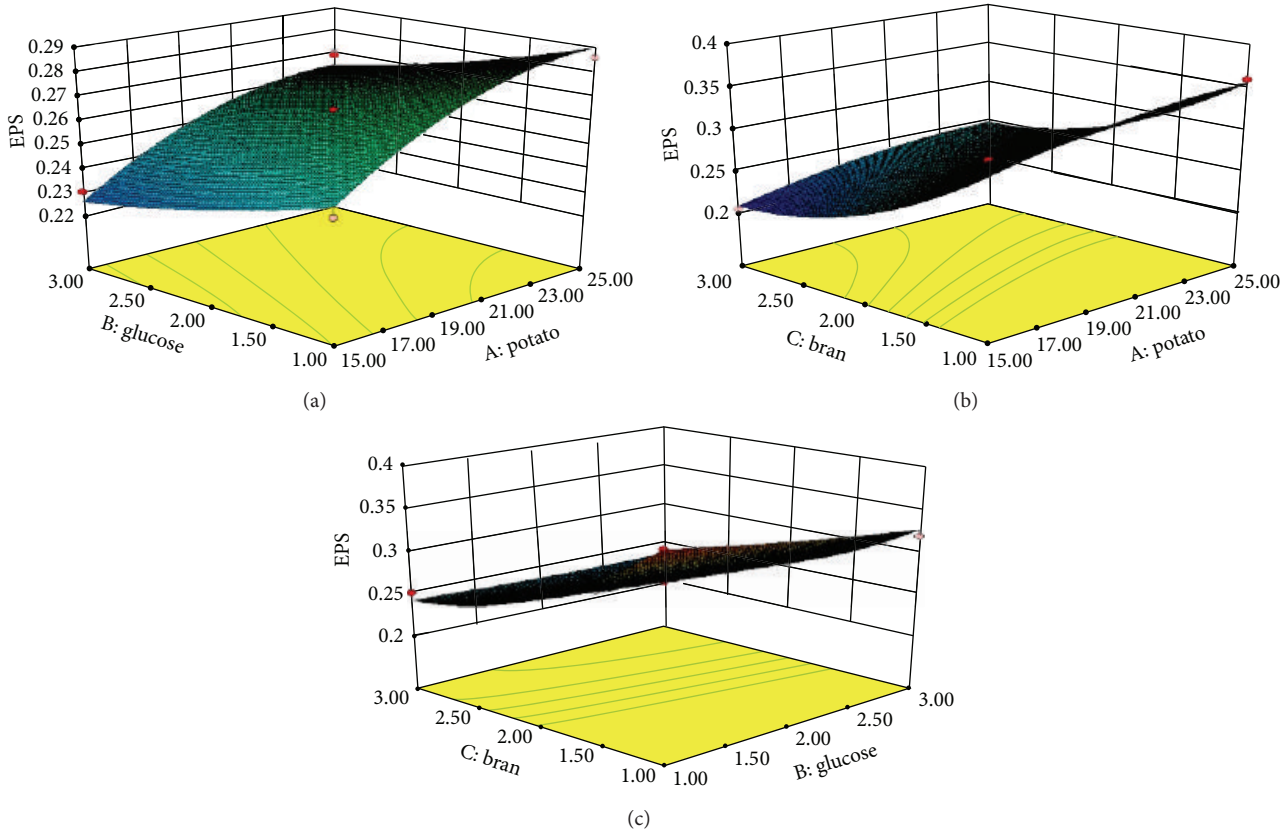


Figure 2: The 3D-plot and 2D-projection of response surface represent the interaction between two factors in the yield of extracellular polysaccharide (EPS) (g/100 mL) by keeping the other two factors constant: (a) potato and glucose (g/100 mL), (b) potato and bran (g/100 mL), and (c) glucose and bran (g/100 mL).

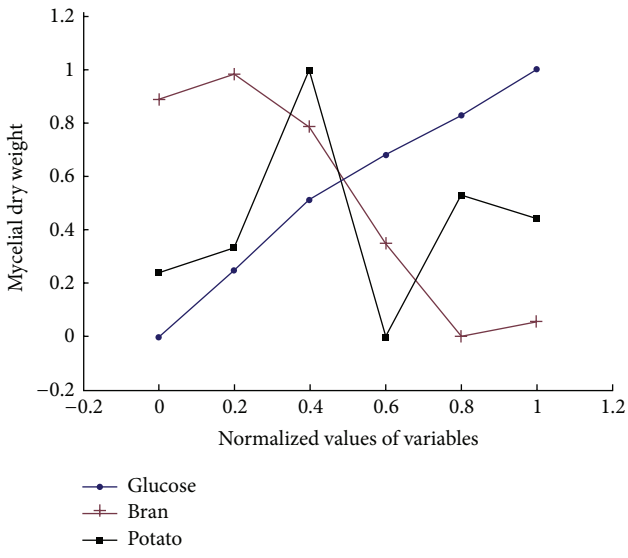


Figure 3: Predicted trend of mycelial dry weight by ANN.

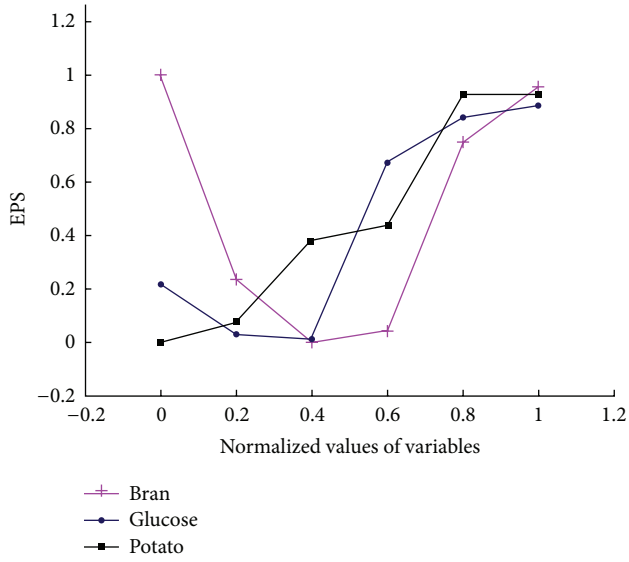


Figure 4: Predicted trend of EPS by ANN.

effect of media components such as potato, glucose, and bran on mycelial dry weight and EPS yield. The predictive trend of mycelial biomass and EPS was showed in Figures 3 and 4, respectively. The yield of mycelial dry weight with the increase of potato showed an increased-after-decreased trend in Figure 3 (black line). Meanwhile, the yield increased

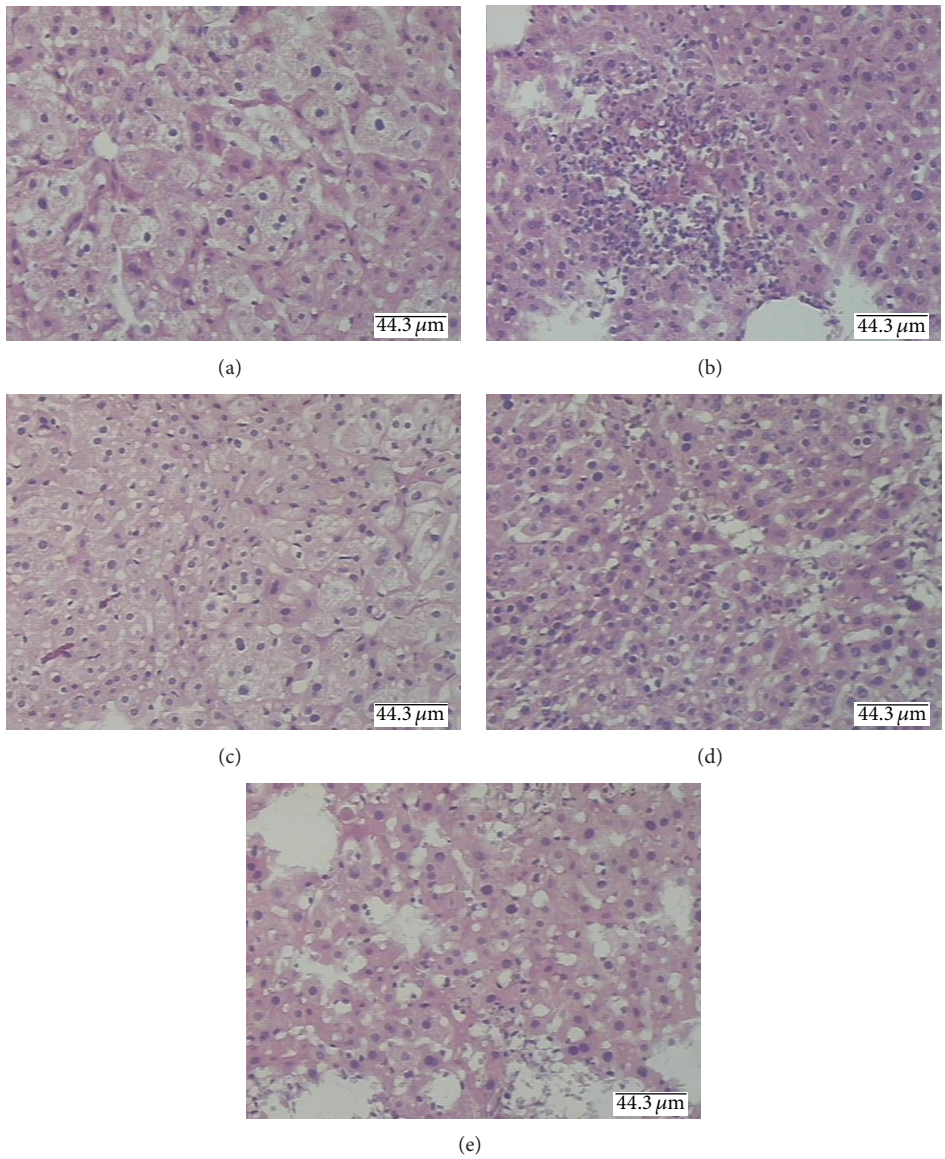


Figure 5: Histopathological analysis of mouse liver sections using hematoxylin and eosin staining. (a) Section from a normal control mouse liver. (b) The liver section that was obtained from alcohol-induced mice showed a variety of cavitation and necrosis in hepatocytes. (c) Liver tissue section prepared from the ABM-fp-treated group showed less cavitation and necrosis compared to (b). (d) Liver tissue section prepared from the ABM-fb-treated group showed less cavitation and necrosis. (e) Liver tissue section prepared from the ABM-fm-treated group showed less cavitation and necrosis compared to (b), but more than (c) and (d).

sharply with the increase of glucose (blue line). On the contrary, the yield of mycelial biomass decreased when the bran increased (red line). And Figure 4 showed the different predictive trends of EPS. The yield of EPS showed a trend of rising with both the increase of potato (black line) and that of glucose (blue line) in Figure 4. However, the yield showed a decreased-after-increased trend when the bran increased in the range of experiment (red line).

3.4. Verification of Predictive Model. According to the regression equation, the predicted maximum value of mycelial dry weight and EPS extraction was 1.047 g/100 mL and 0.367 g/100 mL, respectively, when the potato is 29.88 g/100 mL, the glucose is 1.01 g/100 mL, and the bran is 1.02 g/100 mL. To ensure the suitability of the model equation for predicting the optimum response values, experimental rechecking was performed using the recommended optimal conditions. It was found that the experimental value (0.914 ± 0.207 g/100 mL, 0.280 ± 0.123 g/100 mL, $n = 3$, resp.) was in agreement with the predicted one, indicating that the response surface model was suitable for optimizing the mycelial biomass and polysaccharide production.

3.5. Effect of ABM on Alcohol-Induced Liver Injury. Ethanol-induced hepatic injury was indicated by liver pathological changes characterised by lymphocytes and neutrophils infiltration around the veins of hepatic tissues. In the blank control group (Figure 5(a)), there was no cavitation, necrosis,

or fibrosis. The hepatocytes and plate from hepatic tissue sample had an intact structure, and the boundary between hepatocytes was clear. In contrast, the hepatocytes showed the hepatocytes' morphological damage in veins and the collection of lymphocyte and neutrophils in the ethanol control group (Figure 5(b)). This section displayed apparent cavitations in broad areas. However, the broad cavitations and the collection of lymphocyte and neutrophils in liver were somewhat attenuated in mice treated with ABM-fp (Figure 5(c)), ABM-fb (Figure 5(d)), and ABM-fm (Figure 5(e)). Compared with the ethanol control group, Figures 5(c) and 5(d) showed markedly fewer cavitations and less fibrosis in the liver. Yet, Figure 5(e) displayed few cavitations and moderate inflammatory changes. These experimental phenomena indicated that ABM could weaken or treat the liver injury caused by alcohol.

4. Conclusion

ABM as an edible and medicine mushroom is widely used in the world. And a variety of biological activities have been reported by several groups [29–33]. Several studies have actually found that the ABM extract could recover/repair the liver injury induced by CCl_4 [34–36]. From this study, the maximal yield of mycelial biomass and extracellular polysaccharide was predicted at 1.047 g/100 mL and 0.367 g/100 mL, respectively, when the potato is 29.88 g/100 mL, the glucose is 1.01 g/100 mL, and the bran is 1.02 g/100 mL, using the response surface methodology. Simultaneously, the variation tendency was predicted by artificial neural network. Therefore, it is evident that the use of statistical method not only helped in locating the optimum levels of the most significant factors but also proved to be useful and satisfactory in this process-optimization. What is more, the recovery/reparative effects of ABM by submerged culture were observed through the hepatic histological sections of mice induced by alcohol. It indicated that ABM has a significant protective effect on alcohol-induced liver injury. And despite all this, some other experimental conditions are not included in the investigation and the application of ANN is not skilled. In addition, some related indicators of liver injury need to be further determined. Hence, further studies are still needed.

Disclosure

Hang Wang and Gang Li are co-first authors.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the Foundation of Jinan Science and Technology Development Program (201303055). This project was supported by Project of Shandong Province Higher Educational Science and Technology Program (J14LK61).

References

[1] S. Pincock, "Binge drinking on rise in UK and elsewhere. Government report shows increases in alcohol consumption, cirrhosis, and premature deaths," *The Lancet*, vol. 362, pp. 1126–1127, 2003.

[2] J. Rehm, C. Mathers, S. Popova, M. Thavorncharoensap, Y. Teerawattananon, and J. Patra, "Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders," *The Lancet*, vol. 373, no. 9682, pp. 2223–2233, 2009.

[3] P. G. Miles and S. T. Chang, *Mushroom Biology: Concise Basics and Current Developments*, World Scientific Publishing, Singapore, 1997.

[4] Z. Bano and S. Rajarathnam, "Pleurotus mushrooms. Part II. Chemical composition, nutritional value, post-harvest physiology, preservation, and role as human food," *Critical Reviews in Food and Science Nutrition*, vol. 27, pp. 87–158, 1988.

[5] P. Manzi, L. Grambelli, S. Marconi, V. Vivanti, and L. Pizzoferrato, "Study of the embryofoetotoxicity of alpha-terpinene in the rat," *Food Chemistry*, vol. 65, pp. 477–482, 1999.

[6] P. Mattilda, K. Könkö, M. Eurola et al., "Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 5, pp. 2343–2348, 2001.

[7] L. Barros, T. Cruz, P. Baptista, L. M. Estevinho, and I. C. Ferreira, "Wild and commercial mushrooms as source of nutrients and nutraceuticals," *Food and Chemical Toxicology*, vol. 46, no. 8, pp. 2742–2747, 2008.

[8] J. P. Park, S. W. Kim, H. J. Hwang, and J. W. Yun, "Optimization of submerged culture conditions for the mycelial growth and exo-biopolymer production by *Cordyceps militaris*," *Applied Microbiology and Biotechnology*, vol. 33, pp. 76–81, 2001.

[9] S. P. Wasser, "Review of medicinal mushroom advances: good news from old allies," *HerbalGram*, vol. 56, pp. 28–33, 2002.

[10] P. P. Johl, H. S. Sodhi, S. Dhanda, and S. Kapoor, "Mushrooms as medicine—a review," *Journal of Plant Science Research*, vol. 11-12, p. 73, 1995.

[11] W. M. Breene, "Nutritional and medicinal value of specialty mushrooms," *Journal of Food Protection*, vol. 53, p. 883, 1990.

[12] F. Firenzuoli, L. Gori, and G. Lombardo, "The medicinal mushroom *Agaricus blazei* murrill: review of literature and pharmaco-toxicological problems," *Evidence-Based Complementary and Alternative Medicine*, vol. 5, no. 1, pp. 3–15, 2008.

[13] M. Yang, L. Lin, and J. C. Chang, "Submerged fermentation and amino acid analysis of Agaricus blazei mycelium," *Edible Fungi of China*, vol. 16, pp. 41–42, 1996.

[14] M. Yoneyama, S. Meguro, and S. Kawachi, "Liquid culture of *Agaricus blazei*," *Journal of the Japan Wood Research Society*, vol. 43, pp. 349–355, 1997.

[15] M. Yoneyama, S. Meguro, and S. Kawachi, "Liquid culture of Agaricus blazei II—affinity for dissolved oxygen," *Journal of the Japan Wood Research Society*, vol. 43, pp. 948–55, 1997.

[16] S. Y. Liu and C. H. Shu, *Effect of temperature on submerged fermentation of Agaricus blazei [M.S. thesis]*, Granduate Institute of Chemical Engineering, National Central University, Taoyuan, Taiwan, 2001.

[17] C. H. Shu and B. J. Wen, "Enhanced shear protection and increased production of an anti-tumor polysaccharide by Agaricus blazei in xanthan-supplemented cultures," *Biotechnology Letters*, vol. 25, pp. 873–876, 2003.

[18] C. P. Pokhrel and S. Ohga, "Submerged culture conditions for mycelial yield and polysaccharides production by *Lyophyllum decastes*," *Food Chemistry*, vol. 105, no. 2, pp. 641–646, 2007.

[19] D. H. Kim, B. K. Yang, S. C. Jeong et al., "Production of a hypoglycemic, extracellular polysaccharide from the submerged culture of the mushroom, phellinus linteus," *Biotechnology Letters*, vol. 23, pp. 513–517, 2001.

[20] C. H. Lu, N. J. Engelmann, M. A. Lila, and J. W. Erdman, "Optimization of lycopene extraction from tomato cell suspension culture by response surface methodology," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 17, pp. 7710–7714, 2008.

[21] E. S. Lin and Y. H. Chen, "Factors affecting mycelial biomass and exopolysaccharide production in submerged cultivation of Antrodia cinnamomea using complex media," *Bioresource Technology*, vol. 98, pp. 2511–2517, 2007.

[22] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, pp. 350–356, 1956.

[23] J. H. Holland, *Adaptation in Natural and Artificial Systems*, University of Michigan Press, Ann Arbor, Mich, USA, 1975.

[24] A. Ebrahimpour, R. Rahman, D. Ean Chng, M. Basri, and A. Salleh, "A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus sp.* strain ARM," *BMC Biotechnology*, vol. 8, article 96, 2008.

[25] G. Baskar and S. Renganathan, "Design of experiments and artificial neural network linked genetic algorithm for modeling and optimization of L-asparaginase production by Aspergillus terreus MTCC 1782," *Biotechnology and Bioprocess Engineering*, vol. 16, pp. 50–58, 2011.

[26] M. S. R. C. Murthy, T. Swaminathan, S. K. Rakshit, and Y. Kosugi, "Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology," *Bioprocess Engineering*, vol. 22, pp. 35–39, 2000.

[27] Y. L. Feng, W. Q. Li, X. Q. Wu, J. W. Cheng, and S. Y. Ma, "Statistical optimization of media for mycelial growth and exopolysaccharide production by *Lentinus edodes* and a kinetic model study of two growth morphologies," *Biochemical Engineering Journal*, vol. 49, pp. 104–112, 2010.

[28] H. Xu, L. P. Sun, Y. Z. Shi et al., "Optimization of cultivation conditions for extracellular polysaccharide and mycelium biomass by Morchella esculenta As51620," *Biochemical Engineering Journal*, vol. 39, pp. 66–73, 2008.

[29] T. W. Oh, Y. A. Kim, W. J. Jang et al., "Semipurified fractions from the submerged-culture broth of *Agaricus blazei* Murill reduce blood glucose levels in streptozotocin-induced diabetic rats," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 7, pp. 4113–4119, 2010.

[30] M. M. Padiha, A. A. Avila, P. J. Sousa, L. G. Cardoso, F. F. Perazzo, and J. C. Carvalho, "Anti-inflammatory activity of aqueous and alkaline extracts from mushrooms (*Agaricus blazei* Murrill)," *Journal of Medicinal Food*, vol. 12, no. 2, pp. 359–364, 2009.

[31] L. K. Ellertsen and G. Hetland, "An extract of the medicinal mushroom Agaricus blazei Murrill can protect against allergy," *Clinical Molecular Allergy*, vol. 7, no. 6, 2009.

[32] G. Hetland, E. Johnson, and T. Lyberg, "Effects of the medicinal mushroom Agaricus blazei Murrill on immunity, infection and cancer," *Scandinavian Journal of Immunology*, vol. 68, no. 4, pp. 363–370, 2008.

[33] C. H. Hsu, K. C. Hwang, Y. H. Chiang, and P. Chou, "The mushroom Agaricus blazei Murrill extract normalizes liver function in patients with chronic hepatitis B," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 3, pp. 299–301, 2008.

[34] A. M. Al-Dbass, S. K. Al-Daihan, and R. Shafi Bhat, "Agaricus blazei Murill as an efficient hepatoprotective and antioxidant agent against CCl4-induced liver injury in rats," *Saudi Journal of Biological Sciences*, vol. 19, no. 3, pp. 303–309, 2012.

[35] J.-B. Chang, M.-F. Wu, Y.-Y. Yang et al., "Carbon tetrachloride-induced hepatotoxicity and its amelioration by Agaricus blazei Murrill extract in a mouse model," *In Vivo*, vol. 25, pp. 971–976, 2011.

[36] M.-F. Wu, Y.-M. Hsu, M.-C. Tang, H.-C. Chen, and J.-G. Chung, "Agaricus blazei Murill extract abrogates CCl4-induced liver injury in rats," *In Vivo*, vol. 25, no. 1, pp. 35–40, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 603415, 5 pages
http://dx.doi.org/10.1155/2014/603415

Research Article

4-Hydroxychalcone Attenuates Hyperaldosteronism, Inflammation, and Renal Injury in Cryptochrome-Null Mice

Qi Qu,[1] Bingguang Dai,[1] Bo Yang,[1] Xuelian Li,[1] Yimin Liu,[2] and Fuling Zhang[1]

[1] *Department of Cardiovascular Surgery, The Fourth People's Hospital of Jinan City, Jinan 250031, China*
[2] *Department of Cardiovascular Surgery, Qilu Children's Hospital of Shandong University, Jinan 250031, China*

Correspondence should be addressed to Yimin Liu; yiminliujinan@126.com and Fuling Zhang; zhangfulingjinan@126.com

Received 7 May 2014; Accepted 16 May 2014; Published 9 June 2014

Academic Editor: Chuncho Han

In the present study, we aimed to investigate the preventive effects of 4-hydroxychalcone (4HCH) on resistant hypertension. We used cryptochrome-null mice, which characteristically show high plasma aldosterone levels, inflammation, and renal injury. The cryptochrome-null mice received high-salt treatment and were treated orally with 4HCH 10 mg/kg, 4HCH 20 mg/kg, and 4HCH 40 mg/kg, respectively. The salt administration in cryptochrome-null mice is able to induce an increase in systolic pressure which is associated with hyperaldosteronism, inflammation, and kidney injury. Treatment with 40 mg/kg 4HCH reduced systolic hypertension, serum IL-1β, and TNF-α levels and suppressed the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and renal injury. The impact of 4HCH on the hyperaldosteronism, inflammation, and kidney injury provides new insights for future development of therapeutic strategies in resistant hypertension.

1. Introduction

Resistant hypertension (RH) refers broadly to high blood pressure that is resistant to pharmacologic therapy. Although the etiology of resistant hypertension is almost always multifactorial, aldosterone excess has been shown to contribute importantly to the development of RH [1]. There is evidence that aldosterone can promote endothelial dysfunction and induce vascular inflammation, vascular and myocardial fibrosis, and myocardial ischemia [2–4]. Consistent with the high degree of aldosterone excess demonstrable in patients with resistant hypertension, blockade of aldosterone has been demonstrated to provide particular benefit to RH [5].

Chalcones (1,3-diaryl-2-propen-1-ones) belong to the largest class of plant secondary metabolites and are considered to be precursors of flavonoids and isoflavonoids serving in plant defense mechanisms to counteract reactive oxygen species in order to survive and prevent molecular damage and damage by microorganisms, insects, and animals [6]. Chalcones and their derivatives exerted a lot of biological properties [7, 8], but few previous reports referred to the ability of these classes of compounds to lower blood pressure via the blockade of aldosterone. 4-Hydroxychalcone (4HCH) is an alpha, beta-unsaturated ketone with the core structure of chalcone and one hydroxyl substituent on the 4 positions of the A ring. We present for the first time the evidence that 4HCH inhibits RH by attenuating hyperaldosteronism, inflammation, and renal injury in cryptochrome-null mice (CNM).

Cryptochrome-null mice show increased mRNA expression and protein levels of 3β hydroxysteroid dehydrogenase [9]. The enzyme is expressed particularly in the zona glomerulosa, where aldosterone production is known to exclusively take place. In the present study, we used cryptochrome-null mice, which characteristically show high plasma aldosterone levels, to evaluate the efficacy of 4HCH to lower blood pressure and prevent progressive hyperaldosteronism, inflammation, and renal injury.

2. Materials and Methods

2.1. Drugs. 4HCH with 98% purification was obtained following the extraction and separation using a column chromatographic method [10].

2.2. Animals. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals. The CNM were developed by the way introduced by Vitaterna et al. [11]. CNM received high-salt treatment. The normal salt chow had a 0.2% NaCl content, whereas the high-salt chow had a 3.15% NaCl content, and the drinking water contained 1% NaCl and 0.2% KCl.

2.3. Experimental Design. Forty-eight of these mice were allocated equally into 4 groups: CNM group, CNM and 4HCH-10 group, CNM and 4HCH-20 group, and CNM and 4HCH-40 group. The other 12 wild-type (WT) littermates mice were used as the control group receiving normal salt treatment. From then on, the 5 groups of mice were orally administered saline, 10 mg/kg 4HCH, 20 mg/kg 4HCH, 40 mg/kg 4HCH, and saline, respectively. 4HCH was dissolved in distilled water and administrated orally twice daily using a feeding needle for 35 days, and control group received double distilled water instead of 4HCH.

2.4. Blood Pressure Measurement. Blood pressure was determined in conscious, trained mice using a noninvasive computer-automated tail-cuff system (BP-98A, Softron, Shanghai, China). The average value of 12 measurements was used for data analysis.

2.5. Blood Biochemical Analysis. After 32 weeks of treatment, blood was obtained for biochemical analysis by cardiac puncture before the mice were killed. Serum IL-1β, TNF-α, and aldosterone were measured using a commercially available enzyme immunoassay kit (Shanghai Jinma Biological Technology, Inc., China) according to the protocol described by the manufacturer.

2.6. Quantification of NF-κB Activity. At the end of the 32-week experimental period, kidney tissue samples were collected. Activated NF-κB was quantified in kidney tissue extracts via ELISA technique using the PathScan Phospho-NFκB p65 (Ser536) Sandwich ELISA Antibody Pair (Shanghai Yubo Biological Technology, Inc., China), following the manufacture's instruction. The protein expression levels of NF-κB were measured by Western blot analysis.

2.7. Histological Examination of Kidney Sections. Kidney samples were fixed in 4% buffered formalin (pH 7.2), processed, and embedded in paraffin wax. Sections of 5 mm thickness were then generated and stained with Periodic acid–Schiff reagent for subsequent light microscope examination. Histological evaluation was performed in a blinded manner. A minimum of two slides per rat were read.

2.8. Statistical Analysis. The data were expressed as mean ± SEM and results were analyzed by ANOVA followed by Dunnett's t test. $P < 0.05$ was considered significant.

3. Results

3.1. The Effect of 4HCH on Blood Pressure of Mice. CNM have elevated blood pressure compared with their WT littermate controls (Figure 1). WT mice ($n = 12$) exhibited a systolic pressure of 103.1 ± 5.5. In contrast, CNM ($n = 10$) were hypertensive (118.0 ± 8.6). At 10 and 20 mg/kg, 4HCH did not produce a significant change from blood pressure baseline. At the higher dosage of 40 mg/kg, 4HCH lowered blood pressure in CNM ($P < 0.05$) (Figure 1).

3.2. The Effect of 4HCH on Serum Aldosterone Levels. Figure 2 shows plasma aldosterone concentrations in CNM and WT mice, respectively. Serum aldosterone levels were significantly greater in CNM compared with WT mice ($P < 0.001$). However, aldosterone levels in the 40 mg/kg 4HCH and 20 mg/kg 4HCH groups were significantly lower than those in the CNM group ($P < 0.01$ and $P < 0.05$, resp.).

3.3. The Effect of 4HCH on Serum IL-1β and TNF-α Level. In comparison to CNM group (Figure 3), treatment with 10 and 20 mg/kg 4HCH resulted in a marked decrease in IL-1β levels compared with those in CNM group ($P < 0.05$ and $P < 0.01$, resp.). In addition, the levels of TNF-α were significantly increased in CNM group (Figure 4). 4HCH (40 mg/kg) suppressed CNM-induced TNF-α production ($P < 0.05$).

3.4. The Effect of 4HCH on Protein Expression of NF-κB. The protein expression of NF-κB represents NF-κB activation. As shown in Figure 5(a), protein expression of NF-κB was significantly increased in the CNM group, suggesting CNM induced a predominant increase in nuclear translocation of NF-κB substantially. Conversely, level of NF-κB protein decreased in the nucleus of kidney cells of 4HCH (40 mg/kg) group (Figure 5(b)).

3.5. The Effect of 4HCH on Renal Injury. On histologic analysis, CNM with high-salt treatment developed severe glomerulosclerosis and tubulointerstitial injury and moderate tubulointerstitial infiltration with inflammatory cells (Figure 6(b)). It was dramatically reduced and became more normal in 4HCH (40 mg/kg) treated mice (Figure 6(c)). Figure 6(a) showed that there were no marked renal abnormalities in WT mice.

4. Discussion

In the present study, we demonstrated that 4HCH reduces hypertension in CNM mice. This antihypertensive property of 4HCH may be explained by the attenuation of hyperaldosteronism and anti-inflammatory activities and recovery of renal injury.

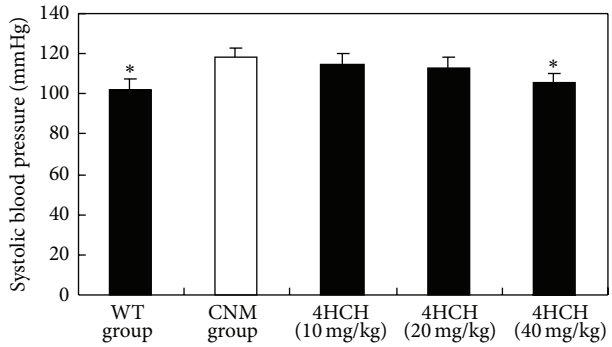


Figure 1: Effect of 4HCH on systolic blood pressure. Data are presented as the SEM. $^{*}P < 0.05$ as compared with CNM group.

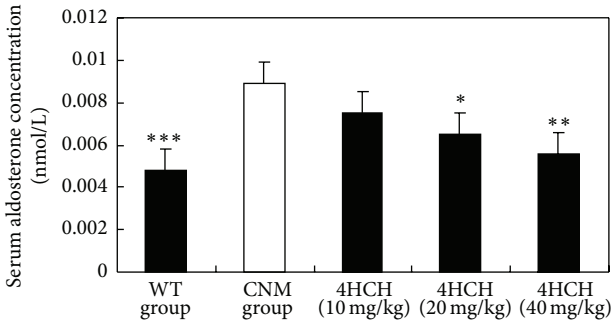


Figure 2: Effect of 4HCH on serum aldosterone levels. Data are presented as the SEM. $^{*}P < 0.05$ as compared with CNM group, $^{**}P < 0.01$ as compared with CNM group, and $^{***}P < 0.001$ as compared with CNM group.

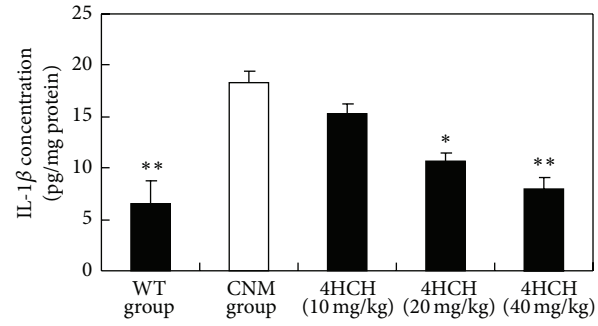


Figure 3: Effect of 4HCH on IL-1β level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus CNM group. $^{**}P < 0.01$ versus CNM group.

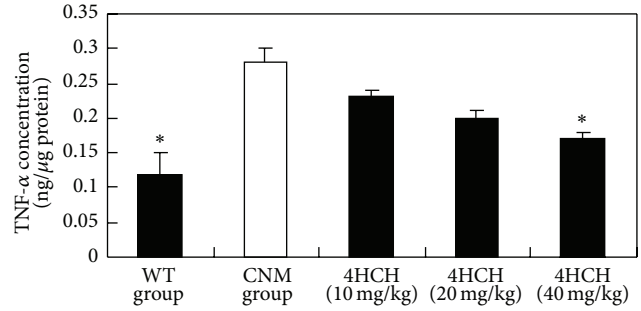


Figure 4: Effect of 4HCH on TNF-α level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus CNM group.

A now very sizable body of literature implicates aldosterone as an important mediator of incident hypertension and severity of hypertension, and, in particular, a common cause of resistance to antihypertensive treatment [12–14]. CNM show an adrenal disorder characterized by chronic overproduction of aldosterone that persists even in the reduced plasma renin activity [9]. The levels of aldosterone measured in the current study relate well to previously published studies [11]. The studies suggest that hypertension in high-salt-treated CNM was caused by mineralocorticoid receptor activation owing to both hyperaldosteronism and high-salt exposure. Here we have clearly demonstrated 4HCH lower level of aldosterone and blood pressure. It indicated that blockade of the aldosterone pathway by 4HCH prevented the increase in systolic pressure (Figure 1).

The importance of inflammation as being central to the development of atherosclerosis has been a fundamental tenet of cardiovascular medicine [15–17]. The low-grade inflammation occurs in the vasculature in various conditions that predispose to cardiovascular disease, including hypertension [18]. We noted that the CNM in the present study developed inflammation following high-salt treatment and in the absence of elevated blood pressure. It is consistent with previous findings that renal proinflammatory response plays an important role in mediating hypertension [19–22]. In our study, we show in CNM that salt-sensitive hypertension increased serum IL-1β and TNF-α level, which are normalized by 4HCH treatment.

NF-κB is a key transcription factor in the activation of genes related to proinflammatory response. It is one of the important renal mechanisms linking proinflammatory response to SS hypertension [23]. The present study demonstrates that a high-salt-induced renal activation of NF-κB correlates with a significant upregulation of IL-1β and TNF-α level in CNM. Our results indicate that 32 wk of treatment with 4HCH suppressed high-salt-induced renal activation of NF-κB (Figure 6). Such a mechanism contributes probably to the beneficial effect of 4HCH on hypertension in CNM.

It is known that salt-sensitive hypertension in human and experimental animal model has been associated with progressive kidney damage leading to end-stage renal disease caused by elevated inflammation [24, 25]. Consequently, in the present study, we hypothesized that the 4HCH ameliorates hypertension and the associated kidney injury. The renal protection is evident from histopathologic observations including markedly reduced tubular cast formation and fibrosis in the kidney (Figure 6). This is the first study to demonstrate that 4HCH has a direct effect on protection against salt-induced kidney injury dependent on its ability to lower blood pressure.

In conclusion, this study demonstrates that salt administration in CNM is able to induce an increase in systolic pressure which is associated with hyperaldosteronism, inflammation, and kidney injury. These arterial modifications

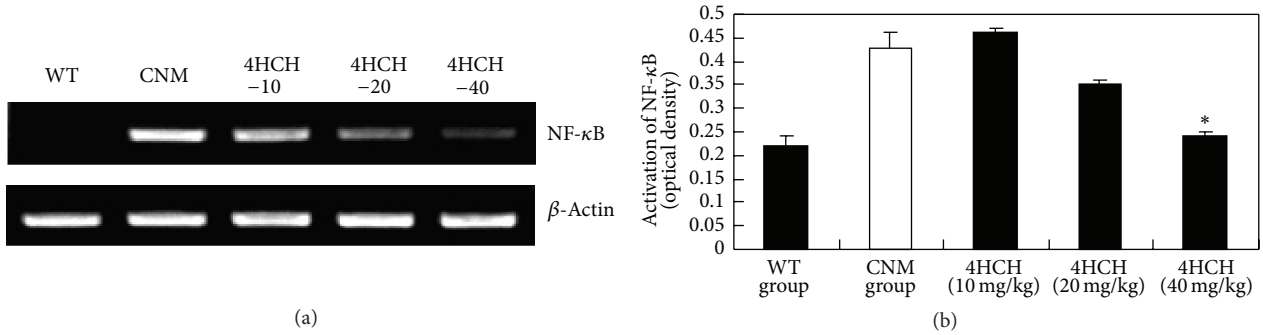


Figure 5: Effect of 4HCH on protein expression of NF-κB. Values represent the mean ± SEM. $^{*}P < 0.05$ versus CNM group.

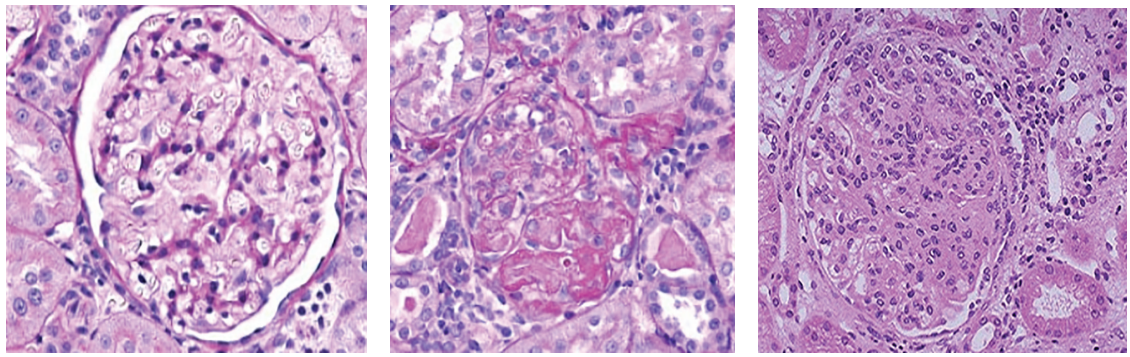


Figure 6: Effects of 4HCH on salt-induced kidney damage were evaluated by morphological analysis of (a) WT mice, (b) CNM, and (c) 4HCH-40 mice. Magnification ×200.

could represent an early step in the development of resistant hypertension. These changes were all reversed by orally administrated 4HCH. The impact of 4HCH on the hyperaldosteronism, inflammation, and kidney injury provides new insights for future development of therapeutic strategies in resistant hypertension.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] D. A. Calhoun, D. Jones, S. Textor et al., "Resistant hypertension: siagnosis, evaluation, and treatment a scientific statement from the american heart association professional education committee of the council for high blood pressure research," *Hypertension*, vol. 51, no. 6, pp. 1403–1419, 2008.

[2] J. N. Cohn and W. Colucci, "Cardiovascular effects of aldosterone and post-acute myocardial infarction pathophysiology," *American Journal of Cardiology*, vol. 97, no. 10, pp. 4–12, 2006.

[3] J. W. Funder, "The nongenomic actions of aldosterone," *Endocrine Reviews*, vol. 26, no. 3, pp. 313–321, 2005.

[4] E. L. Schiffrin, "Effects of aldosterone on the vasculature," *Hypertension*, vol. 47, no. 3, pp. 312–318, 2006.

[5] N. Chapman, J. Dobson, S. Wilson et al., "Effect of spironolactone on blood pressure in subjects with resistant hypertension," *Hypertension*, vol. 49, no. 4, pp. 839–845, 2007.

[6] S. Hirai, Y.-I. Kim, T. Goto et al., "Inhibitory effect of naringenin chalcone on inflammatory changes in the interaction between adipocytes and macrophages," *Life Sciences*, vol. 81, no. 16, pp. 1272–1279, 2007.

[7] N. J. Lawrence, R. P. Patterson, L.-L. Ooi, D. Cook, and S. Ducki, "Effects of α-substitutions on structure and biological activity of anticancer chalcones," *Bioorganic and Medicinal Chemistry Letters*, vol. 16, no. 22, pp. 5844–5848, 2006.

[8] Y. Kimura, M. Taniguchi, and K. Baba, "Antitumor and antimetastatic activities of 4-hydroxyderricin isolated from Angelica keiskei roots," *Planta Medica*, vol. 70, no. 3, pp. 211–219, 2004.

[9] M. Doi, Y. Takahashi, R. Komatsu et al., "Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6," *Nature Medicine*, vol. 16, no. 1, pp. 67–74, 2010.

[10] H. Ni, X.-H. Zhou, H.-H. Li, and W.-F. Huang, "Column chromatographic extraction and preparation of cordycepin from Cordyceps militaris waster medium," *Journal of Chromatography B*, vol. 877, no. 22, pp. 2135–2141, 2009.

[11] M. H. Vitaterna, C. P. Selby, T. Todo et al., "Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 21, pp. 12114–12119, 1999.

[12] M. Alajmi, A. T. Mulgrew, J. Fox et al., "Impact of continuous positive airway pressure therapy on blood pressure inpatientswithobstructive sleepapneahypopnea: ameta-analysis of randomizedcontrolled trials," *Lung*, vol. 185, pp. 67–72, 2007.

[13] L. Mo and Q. He, "Effect of long-term continuous positive airway pressure ventilation on blood pressure in patients with obstructive sleep apnea hypopnea syndrome: a meta-analysis of clinical trials," *Zhonghua Yi Xue Za Zhi*, vol. 87, no. 17, pp. 1177–1180, 2007.

[14] P. Haentjens, A. Van Meerhaeghe, A. Moscariello et al., "The impact of continuous positive airway pressure on blood pressure in patients with obstructive sleep apnea syndrome: evidence from a meta-analysis of placebo-controlled randomized trials," *Archives of Internal Medicine*, vol. 167, no. 8, pp. 757–765, 2007.

[15] F. Kim, M. Pham, I. Luttrell et al., "Toll-like receptor-4 mediates vascular inflammation and insulin resistance in diet-induced obesity," *Circulation Research*, vol. 100, no. 11, pp. 1589–1596, 2007.

[16] P. J. Boyle, "Diabetes mellitus and macrovascular disease: mechanisms and mediators," *American Journal of Medicine*, vol. 120, no. 9, pp. S12–S17, 2007.

[17] J. Nigro, N. Osman, A. M. Dart, and P. J. Little, "Insulin resistance and atherosclerosis," *Endocrine Reviews*, vol. 27, no. 3, pp. 242–259, 2006.

[18] S. A. Cooper, A. Whaley-Connell, J. Habibi et al., "Renin-angiotensin-aldosterone system and oxidative stress in cardiovascular insulin resistance," *American Journal of Physiology*, vol. 293, no. 4, pp. H2009–H2023, 2007.

[19] B. Rodríguez-Iturbe, J. Herrera-Acosta, and R. J. Johnson, "Interstitial inflammation, sodium retention, and the pathogenesis of nephrotic edema: a unifying hypothesis," *Kidney International*, vol. 62, no. 4, pp. 1379–1384, 2002.

[20] B. Rodríguez-Iturbe, H. Pons, Y. Quiroz et al., "Mycophenolate mofetil prevents salt-sensitive hypertension resulting from angiotensin II exposure," *Kidney International*, vol. 59, no. 6, pp. 2222–2232, 2001.

[21] K. Shen, F. A. DeLano, B. W. Zweifach, and G. W. Schmid-Schonbein, "Circulating leukocyte counts, activation, and degranulation in Dahl hypertensive rats," *Circulation Research*, vol. 76, no. 2, pp. 276–283, 1995.

[22] A.-K. Siegel, P. Kossmehl, M. Planert et al., "Genetic linkage of albuminuria and renal injury in Dahl salt-sensitive rats on a high-salt diet: comparison with spontaneously hypertensive rats," *Physiological Genomics*, vol. 18, pp. 218–225, 2004.

[23] V. Alvarez, Y. Quiroz, M. Nava, H. Pons, and B. Rodríguez-Iturbe, "Overload proteinuria is followed by salt-sensitive hypertension caused by renal infiltration of immune cells," *American Journal of Physiology*, vol. 283, no. 5, pp. F1132–F1141, 2002.

[24] N. Tian, K. D. Thrasher, P. D. Gundy, M. D. Hughson, and R. D. Manning Jr., "Antioxidant treatment prevents renal damage and dysfunction and reduces arterial pressure in salt-sensitive hypertension," *Hypertension*, vol. 45, no. 5, pp. 934–939, 2005.

[25] N. Tian, J.-W. Gu, S. Jordan, R. A. Rose, M. D. Hughson, and R. D. Manning Jr., "Immune suppression prevents renal damage and dysfunction and reduces arterial pressure in salt-sensitive hypertension," *American Journal of Physiology*, vol. 292, no. 2, pp. H1018–H1025, 2007.