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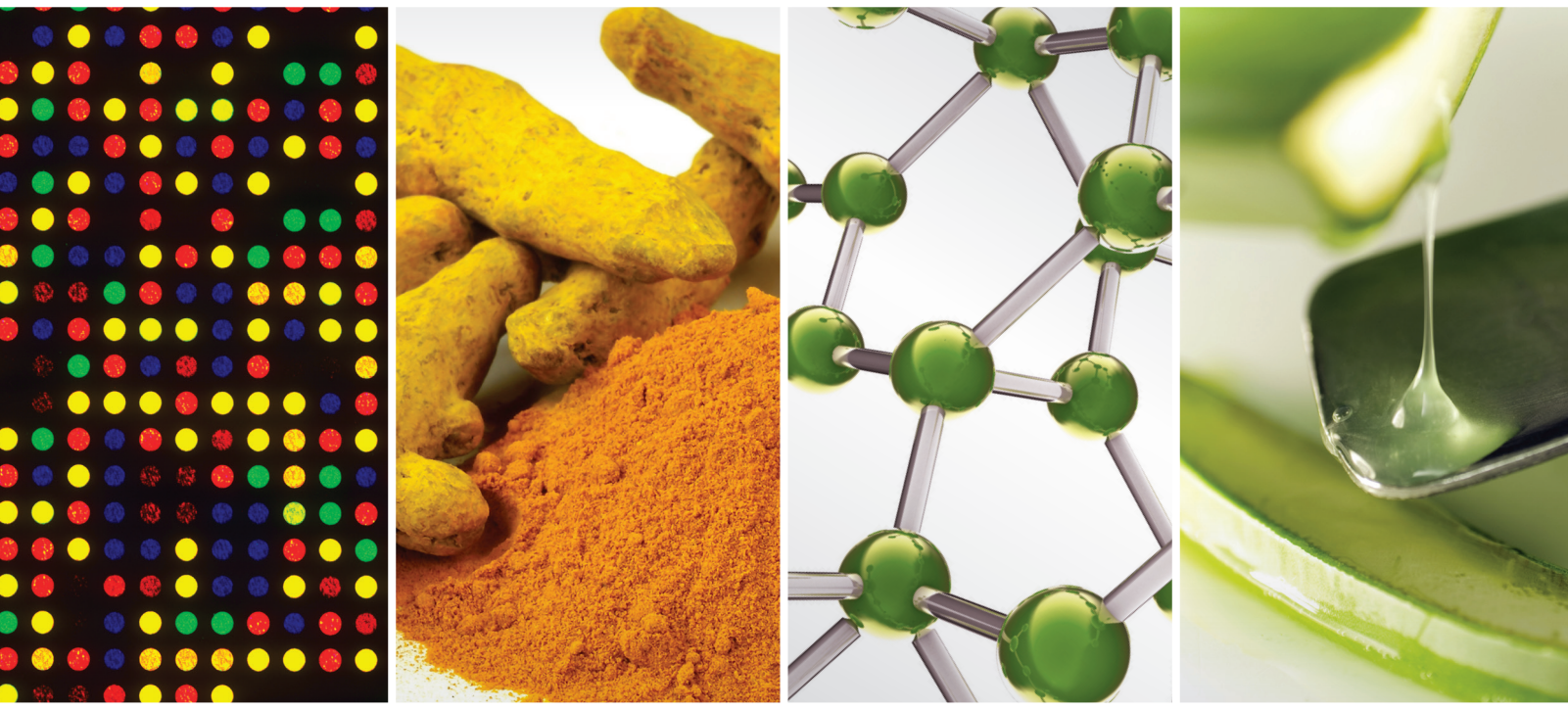
Medicinal Mushroom for Prevention of Disease of Modern Civilization

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



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Contents

Medicinal Mushroom for Prevention of Disease of Modern Civilization, Da-wei Qin, Zhengwei Gu, and Jian-you Guo
Volume 2015, Article ID 812725, 2 pages

Protective Effect of *Hericium erinaceus* on Alcohol Induced Hepatotoxicity in Mice, Lijun Hao, Yuxi Xie, Guikai Wu, Aibin Cheng, Xiaogang Liu, Rongjuan Zheng, Hong Huo, and Junwei Zhang
Volume 2015, Article ID 418023, 5 pages

Aqueous Extract of *Agaricus blazei* Murrill Prevents Age-Related Changes in the Myenteric Plexus of the Jejunum in Rats, Ana Paula de Santi-Rampazzo, João Paulo Ferreira Schoffen, Carla Possani Cirilo, Mariana Cristina Vicente Umada Zapater, Fernando Augusto Vicentini, Andréia Assunção Soares, Rosane Marina Peralta, Adelar Bracht, Nilza Cristina Buttow, and Maria Raquel Marçal Natali
Volume 2015, Article ID 287153, 13 pages

Protective Effect of Ethanol Extracts of *Hericium erinaceus* on Alloxan-Induced Diabetic Neuropathic Pain in Rats, Zhang Yi, Yang Shao-long, Wang Ai-hong, Sun Zhi-chun, Zhuo Ya-fen, Xu Ye-ting, and He Yu-ling
Volume 2015, Article ID 595480, 5 pages

The Chemical Constituents and Pharmacological Actions of *Cordyceps sinensis*, Yi Liu, Jihui Wang, Wei Wang, Hanyue Zhang, Xuelan Zhang, and Chunchao Han
Volume 2015, Article ID 575063, 12 pages

Agaricoglycerides Protect against Hepatic Ischemia/Reperfusion Injury by Attenuating Inflammatory Response, Oxidative Stress, and Expression of NF-*κ*B, Xiang-qian Zhao, Bin Liang, Yang Liu, and Xiao-qiang Huang
Volume 2015, Article ID 142736, 6 pages

Protective Effect of Polysaccharides from *Inonotus obliquus* on Streptozotocin-Induced Diabetic Symptoms and Their Potential Mechanisms in Rats, Bao-zhong Diao, Wei-rong Jin, and Xue-jing Yu
Volume 2014, Article ID 841496, 5 pages

Effects of Fermented Mushroom of *Cordyceps sinensis*, Rich in Selenium, on Uterine Cervix Cancer, Jing Ji, Juan Liu, Haijuan Liu, and Yueling Wang
Volume 2014, Article ID 173180, 7 pages

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Volume 2015, Article ID 812725, 2 pages
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Editorial

Medicinal Mushroom for Prevention of Disease of Modern Civilization

Da-wei Qin,[1] Zhengwei Gu,[2] and Jian-you Guo[3]

[1]*School of Chemistry and Pharmaceutical Engineering, Qilu University of Technology, Jinan 250353, China*
[2]*School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[3]*School of Pharmacy, Beijing University of Chinese Medicine 100029, China*

Correspondence should be addressed to Da-wei Qin; daweiqin@sina.cn

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Disease of modern civilization (DMC) such as type 2 diabetes and cardiovascular disease is caused by the pressure and tension, as well as nutritional imbalance, coupled with a lack of exercise, long-term cumulative a class of diseases. Medicinal mushrooms have diverse morphological, physiological, and ecological characteristics that support their diverse lifestyles. These specific interspecies interactions depend on the production of a wide range of bioactive substances. Medicinal mushrooms are well recognized for their medicinal properties and have been used in traditional medicine for millennia.

Cordyceps sinensis is an abundant resource in nature with various biological activities. Within the genus *Cordyceps*, over 400 species have been described so far, of which *Cordyceps sinensis*, also called "Winter Worm, Summer Grass," is recognized as the most famous tonic herb in traditional Chinese medicine (TCM) for centuries. Many studies have shown that *Cordyceps sinensis* modulates immune responses, decreases plasma cholesterol levels, enhances hepatic function, regulates insulin sensitivity, and improves hypotensive and vasorelaxant activity. C. Han et al. review the chemical constituents and pharmacological actions of *Cordyceps sinensis*. Many bioactive components of *Cordyceps sinensis* have been extracted including nucleoside, polysaccharide, sterol, protein, amino acid, polypeptide, and others. In addition, these constituents' corresponding pharmacological actions were also shown in the study, such as anti-inflammatory, antioxidant, antitumour, antiapoptosis, and immunomodulatory. So we can use different effects of *C. sinensis* against different diseases and provide reference for the study of *Cordyceps sinensis* in the future. Y. Wang et al. investigated the effect of fermented mushroom of *Cordyceps sinensis* (CS) rich in selenium (Se-CS) on uterine cervical cancer in mice. Se-CS treatment (MCA and Se-0.4 group) showed a significant ($P < 0.05$–0.01) restoration in the level of the glutathione content, lipid peroxidation, glutathione peroxidase activity, glutathione reductase activity, catalase activity, Na+/K+-ATPase activity, and glutathione S transferase activity in MCA-induced tumor model (MCA-induced group). It may have helped the tissues recover from MCA injury. At the same time, Se-CS treatment (MCA and Se-0.4 group) results in significant reduction in the occurrence of cervical carcinomas ($P < 0.05$) compared with the MCA-induced group. This finding suggested that the concomitant use of Se and CS could be a potential therapeutic approach to improve the efficacy of therapy for uterine cervical cancer.

H. erinaceum is a temperate mushroom that has been domesticated and is commercially grown in China. The previous study demonstrated that this mushroom exhibited cytoprotection activity against ethanol-induced gastric ulcers in rats. Y. Xie et al. investigated the effects of *Hericium erinaceum* (HEM) on liver injury induced by acute alcohol administration in mice. HEM administration markedly ($P < 0.05$) decreased sera ALT, AST, and MDA levels. The hepatic histopathological observations showed that HEM had a relatively significant role in mice model, which had alcoholic liver damage. H. Yu-ling et al. investigated the effects of *Hericium erinaceum* (HEE) on alloxan induced diabetic neuropathic

pain in laboratory rats. After 6 weeks of treatments, treatment with HEE 40 mg/kg in diabetic animals showed significant increase in pain threshold and paw withdrawal threshold and significant decrease in serum glucose and urine glucose. We also observed a significant increase in lactate dehydrogenase (LDH), lipid peroxidation (LPO), glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na+K+ATPase activity, and glutathione S transferase (GST) activity along with significant decreased levels of glutathione (GSH) content in diabetic rats. The total antioxidant statuses (TAOS) in the HEE-treated groups were significantly lower than those in the alloxan-treated group.

Inonotus obliquus (PIO) is a mushroom habiting the cold latitudes of Europe and Asia, which was used as traditional Chinese medicine for a long history. In the last decade, several studies have reported biological activities of PIO such as anticancer, antioxidation, anti-inflammatory, and antihyperglycemic activities and enhancement of immunity. X. Yu et al. investigated the therapeutic effects of polysaccharides from *Inonotus obliquus* (PIO) on streptozotocin- (STZ-) induced diabetic symptoms and their potential mechanisms. The results show that administration of PIO can restore abnormal oxidative indices near normal levels. The STZ-damaged pancreatic β-cells of the rats were partly recovered gradually after the mice were administered with PIO 6 weeks later.

Agaricus brasiliensis, a mushroom of Brazilian origin, is widely used for nonprescript, medicinal purposes, both as an edible mushroom and in the form of extracts, which has been used as a health care product for the prevention of a wide range of illnesses including cancer, tumor, chronic hepatitis, diabetes, atherosclerosis, and hypercholesterolemia. A. P. de Santi-Rampazzo et al. investigated the effects of the supplementation with aqueous extract of *Agaricus blazei* Murrill (ABM) on biometric and blood parameters and quantitative morphology of the myenteric plexus and jejunal wall in aging Wistar rats. Supplementation with the ABM extract preserved the myenteric plexus in old animals, in which no differences were detected in the density and cell body profile of neurons and glial cells in the CA12 and CA23 groups, compared with C7 group. The supplementation with the aqueous extract of ABM efficiently maintained myenteric plexus homeostasis, which positively influenced the physiology and prevented the death of the neurons and glial cells.

Grifola frondosa (GF) is a Basidiomycete fungus belonging to the order Aphyllophorales and family Polyporaceae. It has recently attracted considerable attention for its various physiological activities. The extracts from the basidiomas of GF exerted a highly significant hepatoprotective effect by reducing the paracetamol-induced acute elevation of the AST and ALT levels. The agaricoglycerides (AG) are a new class of fungal secondary metabolites that constitute esters of chlorinated 4-hydroxy benzoic acid and glycerol. Agaricoglycerides showed strong activities against neurolysin, a protease involved in the regulation of dynorphin and neurotensin metabolism, and even exhibited anti-inflammatory and antinociceptive properties.

By compiling these papers, we hope to enrich our readers and researchers with respect to medicinal mushroom for disease of modern civilization.

Da-wei Qin
Zhengwei Gu
Jian-you Guo

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Research Article

Protective Effect of *Hericium erinaceus* on Alcohol Induced Hepatotoxicity in Mice

Lijun Hao,[1] Yuxi Xie,[2] Guikai Wu,[1] Aibin Cheng,[2] Xiaogang Liu,[3] Rongjuan Zheng,[1] Hong Huo,[1] and Junwei Zhang[2]

[1]*Department of Gastroenterology, Tangshan Gongren Hospital, Tangshan 06300, China*
[2]*Department of Intensive Care Unit, Hebei United University Affiliated Hospital, Tangshan 06300, China*
[3]*Department of Hepatobiliary Surgery, Tangshan Gongren Hospital, Tangshan 06300, China*

Correspondence should be addressed to Yuxi Xie; yuxixiemm@sina.com

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We investigated the effects of *Hericium erinaceus* (HEM) on liver injury induced by acute alcohol administration in mice. Mice received ethanol (5 g/kg BW) by gavage every 12 hrs for a total of 3 doses. HEM (200 mg/kg BW) was gavage before ethanol administration. Subsequent serum alanine aminotransferase (ALT) level, aspartate aminotransaminase (AST) level, Maleic dialdehyde (MDA) level, hepatic total antioxidant status (TAOS), and activated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were determined by ELISA and immunohistochemistry, respectively. HEM administration markedly ($P < 0.05$) decreased serum ALT, AST, and MDA levels. The hepatic histopathological observations showed that HEM had a relatively significant role in mice model, which had alcoholic liver damage. In conclusion, we observed that HEM (200 mg/kg BW) supplementation could restrain the hepatic damage caused by acute alcohol exposure.

1. Introduction

Alcohol is the most abused substance worldwide and a significant source of liver injury [1]. Long-term alcohol consumption induces oxidative stress in the liver due to the imbalance between the prooxidant and the antioxidant systems [2]. Persistent oxidative stress results in fatty liver, which can lead to inflammation, fibrosis, cirrhosis, and even liver cancer [3]. Although important progress has been made in understanding the pathogenesis of alcoholic liver disease, the mechanisms involved in the development of the disease are not fully understood [4–6]. Thus, novel agents that correct the fundamental cellular disturbances resulting from excessive alcohol consumption are needed.

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 [7, 8]. Some of them produce substances with potential medical effects and are called medicinal mushrooms [9–12]. *H. erinaceus* is a temperate mushroom that has been domesticated and is commercially grown in China. The previous study demonstrated that this mushroom exhibited cytoprotection activity against ethanol-induced gastric ulcers in rats [13]. However, the roles of HEM on alcohol induced hepatotoxicity have not been reported. Thus, the present work aims to investigate the effects of *Hericium erinaceus* (HEM) on serum alanine aminotransferase (ALT) level, aspartate aminotransaminase (AST) level, Maleic dialdehyde (MDA) level, and hepatic total antioxidant status (TAOS) in mice liver injury induced by acute alcohol administration.

2. Material and Methods

2.1. Animals. Female Kunming strain mice weighing 20–22 g were purchased from the Experimental Animal Center, Hebei University of Traditional Chinese Medicine, China. The mice were maintained at room temperature under alternating natural light/dark photoperiod and had access to standard laboratory food and fresh water *ad libitum*. All animal experiments followed the Guidelines published by

the Ministry of Science and Technology of China. Care was taken to minimize discomfort, distress, and pain to the animals.

2.2. Preparation of Hericium erinaceus (HEM). Fermented mushroom of HEM was produced in Hebei United University, China. 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 HEM.

2.3. Experimental Design. The binge drinking mouse model developed by Wertheimer et al. [14] was utilized for ethanol challenge. Nine-week-old mice were allocated equally into three groups with 10 mice in each group: HEM (200 mg/kg BW)/ethanol, ethanol treatment, and control treatment. Mice received 5 g/kg BW ethanol by gavage every 12 hrs for a total of 3 doses. Control mice received an isocaloric maltose solution. In the AbM/ethanol group, AbM was dissolved in PBS and gavaged simultaneously with EtOH at a dose of 200 mg/kg BW. After the final ethanol dose, the mice were sacrificed and the blood and livers were collected. The serum was obtained by centrifugation using a serum separator tube and the stored immediately at −20°C to estimate serum alanine aminotransferase (ALT), serum aspartate aminotransaminase (AST), and liver was used to estimate inflammatory cells and inflammatory mediators.

2.4. Estimation of Serum ALT and AST. Serum ALT and AST activity was measured colorimetrically using a diagnostic kit (Procedure number 505, Sigma Chemical Co., St Louis, MO) according to the instructions provided.

2.5. Estimation 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).

2.6. Quantification of NF-κB Activity. Liver tissue extracts were obtained by homogenization of snap-frozen liver tissue in Cell Lysis Buffer, subsequent sonication, and centrifugation. Activated NF-κB was quantified in liver tissue extracts via ELISA-technique using the PathScan Phospho-NFκB p65 (Ser536) Sandwich ELISA Antibody Pair (Shanghai Yubo Biological Technology, Inc., China), following the manufacturer's instruction.

2.7. Measurement of Total Antioxidant Status. The total antioxidant status (TAOS) of liver was determined as previously described by Laight et al. [15]. The increase of absorbance at 405 nm was measured by a microplate reader (Shanghai Xunda Medical Technology, Inc., China).

Table 1: Effect of HEM on ALT and AST.

Different groups	ALT (U/L)	AST (U/L)
Ethanol group	111.0 ± 21.1	111.0 ± 11.0
Control group	31.6 ± 3.3*	36.6 ± 6.8*
HEM 500 group	66.6 ± 8.8*	50.6 ± 8.3*

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

Table 2: Effect of HEM on MDA level.

Different groups	MDA level (μmol/g)
Ethanol group	1.26 ± 0.05
Control group	0.431 ± 0.01**
HEM 500 group	0.86 ± 0.01*

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

2.8. Histological Examination of Liver. 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.

2.9. Statistical Analysis. All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data are shown as the mean ± SEM. The significant level of 5% ($P < 0.05$) was used as the minimum acceptable probability for the difference between the means.

3. Results

3.1. The Effect of HEM on Serum ALT and AST. The results of serum ALT and AST are shown in Table 1. A significant increase of ALT and AST levels was observed in the ethanol group, as compared to the control mice ($P < 0.05$). HEM (500 mg/kg) treated mice showed a significant decrease in ALT and AST levels as compared to the ethanol group (Table 1) ($P < 0.05$).

3.2. The Effect of HEM on MDA. The liver homogenates from control mice contained low MDA level. MDA in ethanol group was significantly higher than that of control group ($P < 0.01$). HEM (500 mg/kg) treated mice showed significantly ($P < 0.05$) decreased ethanol-induced MDA elevation in hepatic tissues (Table 2).

3.3. Effects of HEM on Total Antioxidant Status (TAOS). The results of hepatic TAOS are shown in Table 3. TAOS in the ethanol group was significantly ($P < 0.01$) higher than those in the normal group. Those in the HEM (500 mg/kg) treated group were significantly lower than those in the ethanol group ($P < 0.01$).

3.4. The Effect of HEM on NF-κB Activation. As shown in Figure 1(a), protein expression of NF-κB was significantly

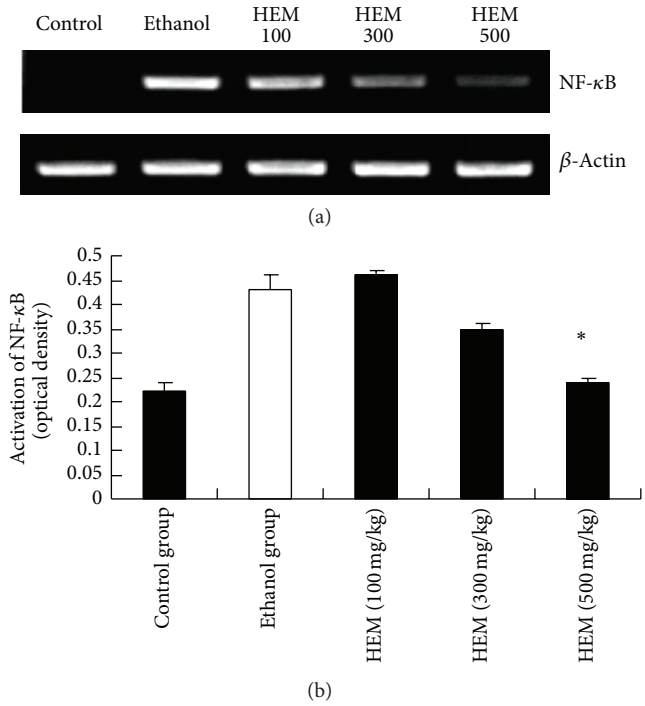


Figure 1: Effect of HEM on protein expression of NF-κB. Values represent the mean ± SEM. $^{*}P < 0.05$ versus ethanol group.

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

Different groups	TAOS activity (μM L-ascorbate)
Ethanol group	80.31 ± 9.30
Control group	26.46 ± 3.16**
HEM 500 group	66.46 ± 3.22*

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

increased in the ethanol group, suggesting that ethanol induced an increase in nuclear translocation of NF-κB. Conversely, protein expression of NF-κB in the HEM (500 mg/kg) treated group was significantly lower than those in the ethanol group (Figure 1(b)).

3.5. The Effect of HEM on Hepatic Injury. On histologic analysis, ethanol treatment developed severe lymphocytes and neutrophils infiltration around the veins of hepatic tissues (Figure 2(b)). It was dramatically reduced and became more normal in HEM treated mice (Figure 2(c)). Figure 2(a) showed that there were no cavitations, necrosis, or fibrosis in normal mice.

4. Discussion

In the current experiments, we used an animal model of acute binge drinking and acute ethanol toxicity showed that supplementation of HEM attenuated acute ethanol-induced liver injury. Liver damage in animals due to ingestion of ethanol is a well-known phenomenon. One sign of hepatic injury is the leakage of cellular enzymes into plasma [16]. In the present study, we have confirmed that HEM inhibited increased AST and ALT levels in serum of mice treated by ethanol. The magnitude of hepatic damage is assessed by measuring the level of released cytosolic transaminases including ALT and AST in circulation [17]. Liver function was evaluated by assessing serum ALT and AST, since AST and ALT are sensitive indicators of liver cell injury [18].

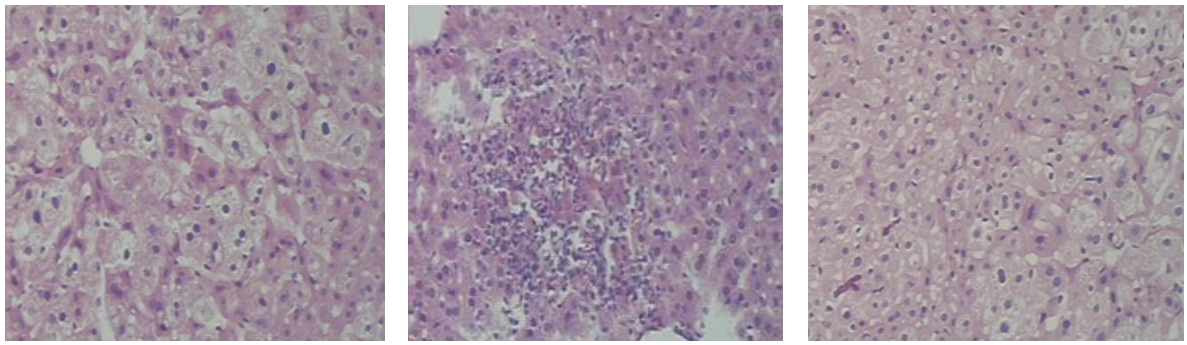
Hepatic MDA activity is commonly used as an indicator of liver tissue damage involving a series of chain reactions [19]. Induction of oxidative stress was identified as key element in the pathophysiology of liver injury induced by acute alcohol administration [20]. In this study, MDA in ethanol group was significantly higher than that of control group ($P < 0.01$). MDA level in HEM group was significantly lower than that of ethanol group ($P < 0.05$). It indicated that the free radicals being released in the liver were effectively scavenged by HEM. This result may account for the reason to inhibit ethanol toxicity.

Oxidative stress is a serious causative factor of hepatic dysfunction and plays an important role in the pathophysiology of several diseases, including atherosclerosis, diabetes, neuronal disorders, and ischaemia-reperfusion injury [21]. We measured TAOS activity as an indirect indication of oxidative stress. In the alcohol induced liver injury mouse model, we detected higher hepatic TAOS, which was decreased in the HEM-treated group. We hypothesized that HEM inhibit ethanol toxicity through decreasing the levels of TAOS activities.

NF-κB is a key transcription factor in the activation of genes related to proinflammatory response. It is one of the important mechanisms linking proinflammatory response to alcoholic liver disease [22]. The present study demonstrates that alcohol induced activation of NF-κB. Our results indicate that treatment with HEM suppressed alcohol induced hepatic activation of NF-κB (Figure 1). Such a mechanism contributes probably to the beneficial effect of HEM on alcohol induced hepatotoxicity in mice.

Ethanol-induced hepatic injury was indicated by liver pathological changes characterized by lymphocytes and neutrophils infiltration around the veins of hepatic tissues. In the blank control group (Figure 2(a)), the hepatocytes and plate from hepatic tissue sample had an intact structure, and the boundary between hepatocytes was clear. However, the hepatocytes showed the hepatocytes' morphological damages in vein and the collection of lymphocyte and neutrophils in the ethanol control group (Figure 2(b)). Compared with the ethanol control group, Figure 2(c) showed markedly fewer cavitations and less fibrosis in the liver. These experimental phenomena indicated that HEM could weaken the liver injury caused by alcohol.

In conclusion, this study demonstrates that HEM supplementation could restrain the hepatic damage caused by acute alcohol exposure. HEM reduced damage by the inhibition of NF-kB activation and decreased the levels of TAOS activities. Hence, the present results suggest for the first time



(a) (b) (c)

Figure 2: Effects of HEM on mouse liver sections using hematoxylin and eosin staining. (a) Section from a normal control mouse liver. (b) The liver section obtained from alcohol-induced mice showed a variety of cavitation and necrosis in hepatocytes. (c) Liver tissue section prepared from the HEM-treated group showed less cavitation and necrosis than (b).

hepatoprotective effects of HEM in a model of alcoholic liver disease.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Lijun Hao and Yuxi Xie equally contributed to the paper.

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Research Article

Aqueous Extract of *Agaricus blazei* Murrill Prevents Age-Related Changes in the Myenteric Plexus of the Jejunum in Rats

Ana Paula de Santi-Rampazzo,[1] João Paulo Ferreira Schoffen,[2] Carla Possani Cirilo,[1] Mariana Cristina Vicente Umada Zapater,[1] Fernando Augusto Vicentini,[1] Andréia Assunção Soares,[3] Rosane Marina Peralta,[3] Adelar Bracht,[3] Nilza Cristina Buttow,[1] and Maria Raquel Marçal Natali[1]

[1]*Department of Morphological Sciences, State University of Maringá (UEM), 87020-900 Maringá, PR, Brazil*
[2]*Center of Biological Sciences, State University of the North of Paraná (UENP), Bandeirantes, PR, Brazil*
[3]*Department of Biochemistry, State University of Maringá (UEM), 87020-900 Maringá, PR, Brazil*

Correspondence should be addressed to Ana Paula de Santi-Rampazzo; anapaulasanti@gmail.com

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This study evaluated the effects of the supplementation with aqueous extract of *Agaricus blazei* Murrill (ABM) on biometric and blood parameters and quantitative morphology of the myenteric plexus and jejunal wall in aging Wistar rats. The animals were euthanized at 7 (C7), 12 (C12 and CA12), and 23 months of age (C23 and CA23). The CA12 and CA23 groups received a daily dose of ABM extract (26 mg/animal) via gavage, beginning at 7 months of age. A reduction in food intake was observed with aging, with increases in the Lee index, retroperitoneal fat, intestinal length, and levels of total cholesterol and total proteins. Aging led to a reduction of the total wall thickness, mucosa tunic, villus height, crypt depth, and number of goblet cells. In the myenteric plexus, aging quantitatively decreased the population of HuC/D^+ neuronal and $S100^+$ glial cells, with maintenance of the $nNOS^+$ nitrergic subpopulation and increase in the cell body area of these populations. Supplementation with the ABM extract preserved the myenteric plexus in old animals, in which no differences were detected in the density and cell body profile of neurons and glial cells in the CA12 and CA23 groups, compared with C7 group. The supplementation with the aqueous extract of ABM efficiently maintained myenteric plexus homeostasis, which positively influenced the physiology and prevented the death of the neurons and glial cells.

1. Introduction

Aging is associated with a progressive decline in physiological function and metabolic processes [1]. The causes of this decline are linked to immune system dysfunction and disorders of energy metabolism that create oxidative stress [2]. Oxidative stress occurs in cell systems whenever the production of free radical molecules exceeds antioxidant capacity. If not removed, free radicals attack and damage proteins, lipids, and nucleic acids, diminishing their activity and leading to losses in energy metabolism, cell signaling, transport, and other important functions [3], in addition to their role in cellular death through necrosis or apoptosis [4]. To minimize the impact of an imbalance between reactive oxygen species and antioxidants, investigations of substances with possible antioxidant capacity have garnered significant scientific interest.

The benefits of ingesting traditional mushrooms are widely recognized, and these mushrooms are used worldwide as food supplements. To evaluate their effects, studies have been performed using basidiomycetes, particularly the species *Agaricus blazei* Murrill (ABM; known popularly as *cogumelo do sol*). These studies have focused on both nutritional and pharmacological objectives and assessed possible

antioxidant properties and the prevention of various diseases, including cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic types of hepatitis [5].

Variations occur in the gastrointestinal tract during development and senescence. These changes involve structural and functional changes, such as decreases in the frequency and amplitude of peristaltic movements, digestion, nutrient absorption, and cell immunity [6, 7]. Although some studies have suggested maintenance of the structure of the intestinal tunica during aging [8], other reports have indicated that aging can alter villus height, crypt depth, and muscle layer thickness [9, 10].

Functional impairment of the gastrointestinal tract is directly linked to changes in extrinsic nerve components (i.e., sympathetic and parasympathetic peripheral nerve fibers) and an intrinsic component (i.e., the enteric nervous system [ENS]). This system modulates complex functions, such as motility, secretion, and blood flow. It consists of a ganglionated plexus and two ganglionated plexi: the myenteric plexus (with ganglia located between the layers of smooth muscle of the muscular tunica) and the submucosal plexus (which has its ganglia in the submucosal tunica) [11].

Aging causes a reduction of the number of neurons in the ENS. This loss is associated with not only an increase in free radicals [12] but also a reduction of neurotrophic factors that originate in glial cells, which are essential for neuronal development and maintenance [13]. Moreover, previous studies have found significant age-related increases in cell body area [8, 14], which may be justified by rearrangement of the remaining neurons, demonstrating the neuroplasticity of fully differentiated tissue.

Considering the possible antioxidant potential of edible and medicinal mushrooms, the objective of the present study was to evaluate the effects of an aqueous extract of *Agaricus blazei* on the morphology and intrinsic innervation of the intestine and myenteric plexus in aging rats.

2. Materials and Methods

2.1. Obtaining Agaricus Blazei Murrill (ABM) Extract. The present study used dehydrated basidiomes of ABM produced in Ibema, PR, Brazil (25°6′50″ south, 53°0′53″ west). The basidiomes were milled to form a fine powder and then subjected to aqueous extraction, modified from the methodology of Soares et al. [15]. Distilled water (100 mL) was added to every 10 g of milled basidiome, which remained under agitation at 28°C for 3 h. The residual solids were removed by vacuum filtration using size-1 Whatman filter paper and again subjected to extraction, which was repeated three times. The filtrates were lyophilized and kept in a freezer at −20°C. The chemical characterization of aqueous extract of the *Agaricus blazei* is described by Soares [16].

2.2. Animals. Starting at 7 months of age, 25 male Wistar rats (*Rattus norvegicus*) were housed in polypropylene boxes (four individuals per box) in the Animal House in the Morphological Sciences Department under a 12 h/12 h light/dark cycle at 22 ± 2°C. The rats were assigned to five groups: 7 months of age (C7), 12 months of age (C12 and CA12), and 23 months of age (C23 and CA23). All of the procedures in this study that involved the use of animals were approved by the Committee for Ethics in Animal Experimentation of Maringá State University (procedure number 063/2010).

2.3. Treatment and Euthanasia. The animals were fed *ad libitum* with standard rodent chow (NUVILAB, NUVITAL). The rats in the CA12 and CA23 groups were supplemented daily via gavage with 1 mL of an aqueous solution that contained 26 mg of freeze-dried ABM beginning at 7 months of age.

The study evaluated body weight, food intake by offering 100 g daily per animal and calculating the remainder, and water intake by offering 300 mL per day and calculating the remainder throughout the experimental period. At 7, 12, and 23 months of age, the animals intravenously received vincristine sulfate (0.5 mg/kg body weight), a protein synthesis blocker, 2 h prior to euthanasia. The injections occurred at the same time each day (6:00 AM) in all of the experiments to avoid circadian variations.

Afterwards, the animals were intraperitoneally anesthetized with sodium thiopental (Thionembutal, Abbott Laboratories, North Chicago, IL, USA) at a dose of 40 mg/kg of body weight, and the nasoanal length was measured to determine the Lee index (body weight$^{1/3}$ (g)/nasoanal length (cm) × 1000). Following blood collection by cardiac puncture for biochemical analyses, the animals were euthanized by an overdose of anesthetic. Laparotomy was performed to remove and measure the length of the small intestine and weigh periepididymal and retroperitoneal adipose tissues. Jejunum samples were sent for histological processing and immunohistochemical techniques to study the myenteric plexus.

2.4. Biochemical Analysis of Blood Components. For the analysis of total proteins, albumin, globulins, triglycerides, and total cholesterol, blood was collected and placed in a test tube to obtain serum. To measure glucose levels, the blood was kept in a test tube that contained ethylenediaminetetraacetic acid (EDTA) fluorinated at a rate of 50 μL/3 mL to obtain plasma and nonfluorinated EDTA to analyze aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes and total plasma antioxidant capacity (TAC-ABTS [2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)]). The samples were centrifuged at 3000 rotations per minute for 15 min, and the levels were determined using Analisa kits (Gold Analisa Diagnóstica Ltda, Minas Gerais, Brazil). TAC-ABTS was assessed according to the methodology described by Erel [17].

2.5. Tissue Processing and Histological Analysis. Samples of the jejunum were opened at the mesenteric border, fixed in Bouin's solution (750 mL saturated picric acid solution, 250 mL formaldehyde, and 50 mL glacial acetic acid) for 6 h, stored in 70% alcohol, and subjected to the following procedures.

2.5.1. Paraffin Inclusion. The jejunum samples were dehydrated in a series of increasing alcohol concentrations, cleared

in xylol, and embedded in paraffin to obtain 7 μm-thick semiserial histological sections using a Leica RM 2145 microtome. These sections were then stained with hematoxylin-eosin (H&E) to evaluate the thickness of the mucosa tunic, muscular coat, and total intestinal wall.

Morphometric analyses were performed by sampling images captured using a 10x lens on an Olympus BX41 optical microscope coupled to a high-resolution Olympus Q Color 3 camera. Thicknesses were estimated by measuring 10 random points per section, for a total of 100 measurements per animal, using ImagePro Plus 4.5 image analysis software (Media Cybernetics). The results are expressed as micrometers.

2.5.2. Historesin Inclusion. The jejunum samples were dehydrated in 95% alcohol, 100% alcohol, and 100% alcohol + infiltration solution (resin activator) at a ratio of 1 : 1 and stored overnight at −4°C in infiltration solution. The samples were then placed inside specific containers with solution for inclusion (infiltration solution + hardener) and oven-dried at 37°C for approximately 10 days. The blocks were then subjected to microtomy to obtain 2.5 μm-thick semiserial sections, stained with H&E to morphometrically analyze the villi, intestinal crypts, and metaphase index (MetI), and then subjected to the periodic acid-Schiff (PAS) histochemical technique to identify globet cells.

(1) Measuring Villi and Intestinal Crypts. The heights of 90 villi and 90 crypts per animal were measured longitudinally from images of the mucosa captured using a 10x lens on an Olympus BX41 optical microscope coupled to a high-resolution Olympus Q Color 3 camera with ImagePro Plus 4.5 image analysis software (Media Cybernetics). The results are expressed as micrometers.

(2) Metaphase Index. The MetI is expressed as the percentage of metaphase nuclei divided by the total number of counted nuclei obtained in longitudinal crypts with visible lumen. A total of 2,500 cells per animal were quantified using an Olympus BX41 light microscope (Tokyo, Japan) with a 40x lens. The MetI was multiplied by Tannok's constant (Kt = 0.57) to correct tissue geometry and avoid overestimating the number of metaphases [18]. The following equation was used to calculate the MetI: MetI = number of cells in metaphase × 100 × Kt/total number of cells in crypts.

(3) Histochemical Analysis of Globet Cells. The semiserial sections were subjected to the PAS histochemical technique to quantify the population of goblet cells in 50 microscopic fields (0.352 mm^2/field) per animal. The quantitative analyses were performed with images obtained with an Olympus BX41 optical microscope coupled to a high-resolution Olympus Q Color 3 camera. The cells were counted with the aid of ImagePro Plus 4.5 software (Media Cybernetics).

2.6. Morphoquantitative Analysis of the Myenteric Plexus

2.6.1. Obtaining Membrane Preparations. Jejunum samples were washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) to remove any residue, filled with Zamboni's fixative, tied at the end, submerged in the same fixative, and kept in cool storage for 18 h. They were then opened and subjected to dehydration using increasing concentrations of alcohol (95% and 100%), cleared in xylol, and rehydrated in a decreasing series of alcohol concentrations (100%, 90%, 80%, and 50%). They were then stored in 0.1 M PBS (pH 7.4) with 0.08% sodium azide at 4°C. Total preparations of the muscle tunica were obtained by microdissection of the samples with a stereoscope with transillumination to remove the mucosa and submucosa tunics.

Table 1: Characteristics of primary and secondary antibodies used for immunohistochemical analysis.

Primary antibody	Supplier	Dilution	Secondary antibody*
Anti-HuC/HuD (mouse)	Invitrogen, USA	1 : 500	Alexa fluor 488 (anti-mouse)
Anti-nNOS (rabbit)	Santa Cruz Biotechnology, USA	1 : 500	Alexa fluor 546 (anti-rabbit)
Anti-S-100 (rabbit)	Sigma, USA	1 : 200	Alexa fluor 546 (anti-rabbit)

*The secondary antibodies were utilized with 1 : 500 dilution and supplied by Invitrogen, USA.

2.6.2. Double HuC/D-nNOS and HuC/D-S100 Immunolabeling. The total preparations of the jejunum muscle tunica were subjected to immunohistochemical techniques to detect HuC/D protein [19], neuronal nitric oxide synthase (nNOS) enzyme [20], and S100 protein [21]. The membranes were rinsed twice in 0.1 M PBS (pH 7.4) with 0.05% Triton X-100 for 10 min and immersed for 1 h in a solution that contained 0.1 M PBS (pH 7.4), 0.05% Triton X-100, 2% bovine serum albumin (BSA), and 10% goat serum to avoid nonspecific binding. The tissues were then incubated for 48 h in a solution that contained 0.1 M PBS (pH 7.4) with 0.05% Triton X-100, 2% BSA, 2% goat serum, and primary antibodies (Table 1). The membranes were washed three times in 0.1 M PBS (pH 7.4) with 0.05% Triton X-100 for 5 min, and incubated for 2 h at room temperature with secondary antibodies (Table 1). The preparations were washed with 0.1 M PBS (pH 7.4) and arranged between the slides with ultrapure glycerol.

2.6.3. Morphoquantitative Analysis. To quantify the HuC/D^+ myenteric neuron population, $nNOS^+$ subpopulation, and $S\text{-}100^+$ glial cells, all of which are immunofluorescent, an Olympus BX40 light microscope was used, fitted with specific immunofluorescence filters and coupled to a Moticam 2500 camera. The density (expressed as cells/cm^2) was calculated by counting neuronal and glial cell bodies in microscopic images (32 images/animal) captured from the middle region (60°–120°; 240°–300°) of the intestinal circumference, considering 0° as the mesenteric insertion [22], using a 20x lens. The area of each analyzed image was 0.093 mm^2. Neurons and glial cells were also counted in 50 ganglia per animal in the C7, C23, and CA23 groups. For the morphometric analysis, cell body areas (μm^2) were measured in 100 $HuC/D\ neurons^+$

Table 2: Body weight (BW), food intake (FI), water intake (WI), Lee index (ILee), weight of periepididymal (PER) and retroperitoneal (RET) fat, total fat (Σ), and small intestine length (SIL) in aging rats (C7, C12, and C23 groups) and aging rats supplemented with the aqueous extract of *A. blazei* (CA12 and CA23 groups). The results are expressed as mean ± standard error.

	C7	C12	CA12	C23	CA23
BW (g)	489.5 ± 8.72a*	492.9 ± 4.32a	483.7 ± 8.97a	510.3 ± 7.67a	513.5 ± 3.66a
FI (g)	28.26 ± 0.38a	21.34 ± 0.38b	21.92 ± 0.61b	22.66 ± 0.47b	24.06 ± 0.60b
WI (mL)	43.7 ± 1.16a	40.11 ± 2.00a	43.0 ± 4.19a	48.45 ± 1.55a	39.0 ± 2.17a
ILee	295.6 ± 2.72a	301.7 ± 3.83ab	292.5 ± 1.47a	307.3 ± 1.55b	308 ± 0.73b
PER (g/100 g)	1.54 ± 0.18a	1.84 ± 0.04a	1.66 ± 0.19a	1.72 ± 0.15a	1.67 ± 0.11a
RET (g/100 g)	1.56 ± 0.15a	1.98 ± 0.17a	1.61 ± 0.24a	2.54 ± 0.15b	2.26 ± 0.16ab
Σ (g/100 g)	3.10 ± 0.32a	3.82 ± 0.15a	3.28 ± 0.36a	4.26 ± 0.30a	3.94 ± 0.24a
SIL (cm)	112.8 ± 5.66a	103.2 ± 4.81a	97.0 ± 5.03a	125.8 ± 5.38b	113.5 ± 1.83ab

*Different letters in the same line indicate significant statistical difference ($P < 0.05$).

Table 3: Total cholesterol (TC), total protein (TP), globulin (GB), albumin (AL), triglycerides (TG), and glycemia (GL) in aging rats (C7, C12, and C23 groups) and aging rats supplemented with the aqueous extract of *A. blazei* (CA12 and CA23 groups). The results are expressed as mean ± standard error.

	C7	C12	CA12	C23	CA23
TC (mg/dL)	101.6 ± 10.51a*	109.7 ± 8.22ab	125.1 ± 13.37ab	166 ± 22.14b	140.3 ± 17.08ab
TP (g/dL)	6.04 ± 0.15a	7.03 ± 0.14b	6.7 ± 0.18b	6.9 ± 0.10b	6.85 ± 0.09b
GB (g/dL)	3.78 ± 0.06a	4.59 ± 0.11b	4.43 ± 0.17b	4.55 ± 0.08b	4.42 ± 0.14b
AL (g/dL)	2.27 ± 0.16a	2.43 ± 0.08a	2.27 ± 0.09a	2.35 ± 0.07a	2.42 ± 0.04a
TG (mg/dL)	135.4 ± 11.99a	147.4 ± 15.79a	146.2 ± 15.2a	166 ± 33.09a	145 ± 34.85a
GL (mg/dL)	124.5 ± 2.95a	111.8 ± 6.56a	120.1 ± 4.06a	132.3 ± 7.16a	150.8 ± 6.8b

*Different letters in the same line indicate significant statistical difference ($P < 0.05$).

and 100 S-100$^+$ glial cells per animal and 70 nNOS$^+$ cell bodies per animal using ImagePro Plus 4.5 software (Media Cybernetics).

2.7. Statistical Analysis. The data were analyzed for normality using the Kolmogorov-Smirnov test. The parametric data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test using Prism 5.0 software (GraphPad, San Diego, CA, USA). The nonparametric data were analyzed using a block design with Statistica software (StatSoft) followed by Tukey's *post hoc* test. The significance level was 5%, and the results are expressed as mean ± standard error.

3. Results

3.1. Biometric Parameters. Body weight, food and water intake, the Lee index, periepididymal and retroperitoneal fat weight, and small intestine length are presented in Table 2.

3.2. Biochemical Analysis of Blood Components. The blood levels of total cholesterol, total proteins, globulins, albumin, triglycerides, and glycemia are shown in Table 3. The plasma levels of the liver enzymes AST and ALT, which were evaluated at the beginning (C7) and end (C23 and CA23) of the experiment, were not significantly different between groups (Figure 1). The total antioxidant capacity of plasma (TAC-ABTS) was significantly reduced in 23-month-old animals. ABM supplementation did not significantly differ between the C23 and CA23 groups. A tendency ($P > 0.05$) toward an improvement in antioxidant capacity was observed in supplemented 12-month-old animals, but the difference between the C7 and C12 groups was not significant (Figure 2).

3.3. Histological Analysis. The intestinal morphometry results, MetI, and number of goblet cells are shown in Table 4.

3.4. Morphoquantitative Analysis of the Myenteric Plexus. Morphoquantitative changes were detected in HuC/HuD$^+$ myenteric neurons as a result of aging. Significant reductions of neuronal density and the number of neurons/ganglia (29.2% and 32.4%, resp.) were observed in the C23 group compared with the C7 group. Supplementation with the aqueous extract of ABM had a positive effect on HuC/HuD$^+$ myenteric neurons, in which the detected losses of density (8.5%) and number of neurons/ganglia (17%) in the C7 and CA23 groups were lower than in the 23-month-old control group. An increase in neuronal area ($P \leq 0.05$) was observed in the C12 and C23 groups. The stable neuronal area in supplemented animals (CA12 and CA23 groups) compared with the C7 group indicates a neuroprotective effect of the ABM aqueous extract (Figure 3, Table 5).

The density of the subpopulation of nitrergic neurons (nNOS$^+$) was preserved, regardless of age or supplementation. However, the neuronal profile of that population increased ($P \leq 0.05$) with regard to age, with a positive effect of supplementation with the ABM aqueous extract at 12 months of age, with the exception of the CA23 group, in which the nitrergic neuron profile exhibited a significant reduction compared with the C7 and C23 groups (Figure 3).

Table 4: Intestinal morphometry: total wall (TW), mucosa (MC), and muscle (MM) tunicae, villus height (VH), crypt depth (CD), metaphase index (MetI), and number of goblet cells (GLC) in the jejunum in aging rats (C7, C12, and C23 groups) and aging rats supplemented with the aqueous extract of *A. blazei* (CA12 and CA23 groups). The results are expressed as mean ± standard error.

	C7	C12	CA12	C23	CA23
TW (μm)	835.13 ± 1.97a*	760.65 ± 2.32b	805.95 ± 3.11c	802.91 ± 6.52c	806.29 ± 2.86c
MC (μm)	700.85 ± 2.20a	639.61 ± 2.24b	674.99 ± 3.24c	648.92 ± 5.56b	641.19 ± 2.19b
MM (μm)	100.8 ± 0.59a	101.1 ± 0.89a	98.61 ± 0.67a	103.2 ± 0.82a	113.9 ± 1.13b
VH (μm)	494.03 ± 1.75a	449.10 ± 2.52b	464.42 ± 2.36c	458.05 ± 3.23c	420.02 ± 2.91d
CD (μm)	274.20 ± 1.72a	218.61 ± 1.15b	212.31 ± 1.14c	244.00 ± 1.56d	235.50 ± 1.36e
MetI (%)	7.59 ± 0.35a	6.78 ± 0.57a	6.74 ± 0.34a	7.42 ± 0.52a	7.53 ± 0.32a
GLC#	72.09 ± 2.49a	75.34 ± 2.99a	74.48 ± 2.59a	53.07 ± 1.88b	60.98 ± 1.86b

*Different letters in the same line indicate significant statistical difference ($P < 0.05$). #Values expressed as goblet cells per field.

Table 5: Number of neurons (HuC/HuD$^+$) and glial cells (S100$^+$) per ganglion and glia/neuron ratio in rats at 7 months of age (C7 group) and 23 months of age (C23 group) and rats supplemented with the aqueous extract of *A. blazei* (CA23 group). The results are expressed as mean ± standard error.

	C7	C23	CA23
HuC/HuD$^+$/ganglion neurons	24.84 ± 0.74a*	16.77 ± 0.50b	20.44 ± 0.66c
S100$^+$/ganglion glias	34.17 ± 0.98a	24.81 ± 0.67b	29.37 ± 0.90c
glias/neurons proportion	1.37 ± 0.03a	1.50 ± 0.12a	1.44 ± 0.07a

*Different letters in same line indicate significant statistical difference ($P < 0.05$).

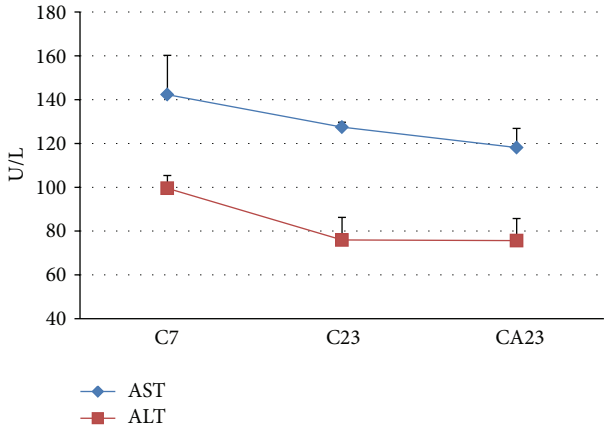


Figure 1: Plasma levels of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in rats at 7 months of age (C7 group) and 23 months of age (C23 group) and 23-month-old rats supplemented with the aqueous extract of *A. blazei* (CA23 group). The results are expressed as mean ± standard error.

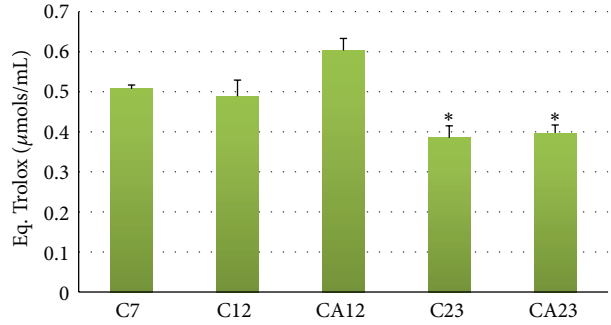


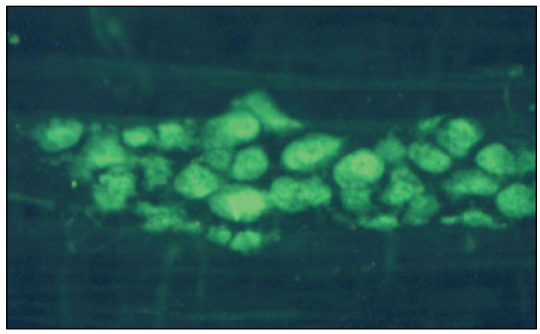
Figure 2: Total plasma antioxidant capacity (TAC-ABTS) in aging rats (C7, C12, and C23 groups) and aging rats supplemented with the aqueous extract of *A. blazei* (CA12 and CA23 groups). *$P < 0.05$, compared with C7 group. The results are expressed as mean ± standard error.

The behavior of glial cells when comparing animals at 7 and 23 months of age revealed significant reductions of the density and number of glia of 26.3% and 27.3%, respectively, and an increase in the glial cell profile ($P \leq 0.05$). A positive effect of the ABM aqueous extract was observed, with a 9% reduction of glial density, 14% reduction of the number of glia, and maintenance of a similar glial profile as the 7-month-old animals (Figure 4, Table 5). The glia/neuron ratio was maintained among the analyzed groups (Table 5).

4. Discussion

4.1. Biometric Parameters. The concentration of the ABM aqueous extract lyophilized used for animal supplementation during the aging process was 26 mg/animal/day (52 mg/kg). To standardize the supplementation dose, the commercial dosages ingested by humans were adopted as a reference, thus establishing a rate of intake of 4 g of dried mushrooms for a person who weighs approximately 60 kg. Additionally, the concentration corresponds to one-quarter of the dosage of mushroom extract used in acute treatments (200 mg/kg) described for rats [23].

Throughout the experimental period, a reduction of food intake ($P < 0.05$) was observed beginning at 12 months of age compared with the 7-month-old group, with maintenance of body weight and water intake. The reduced food intake in 12-month-old animals was 25%, whereas the reduction for 23-month-old animals was 21% compared with 7-month-old animals. Raul et al. [10] reported that food intake decreases by approximately 20% in rats during the aging process.



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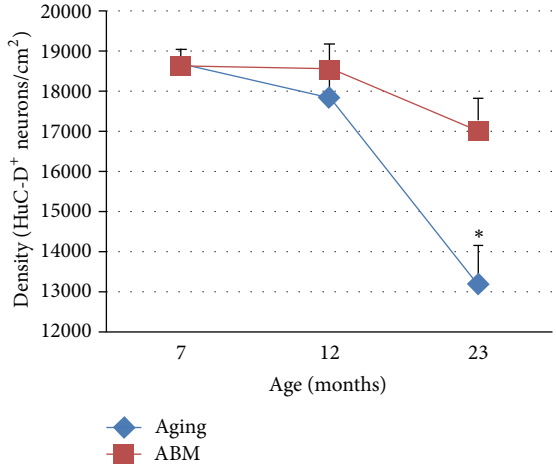

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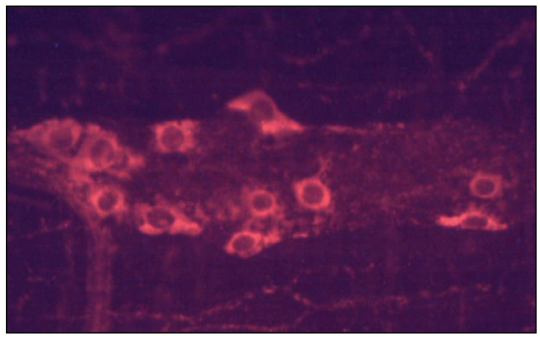

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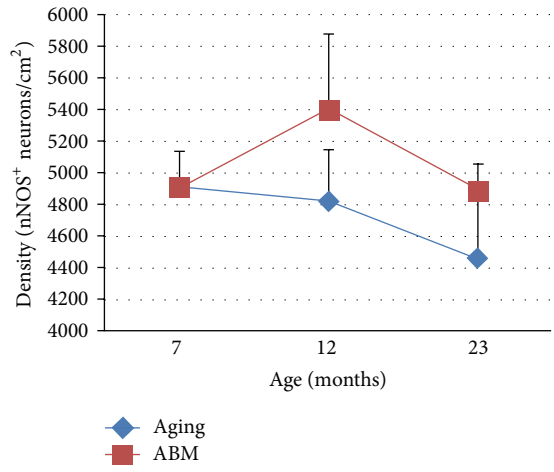

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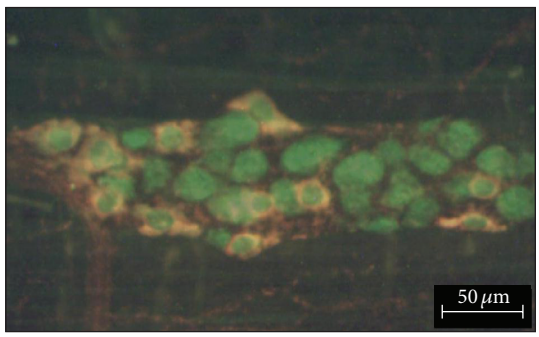

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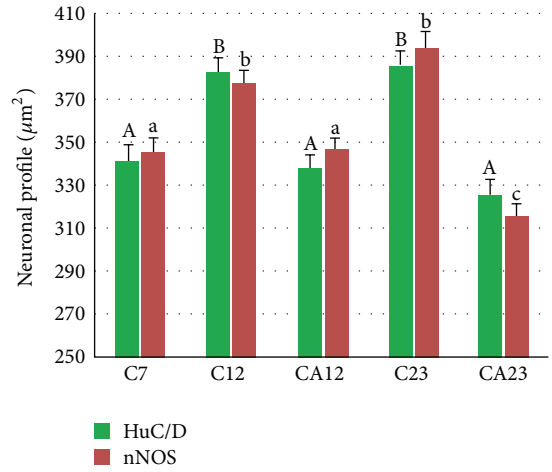

(f)

FIGURE 3: Myenteric ganglion of the jejunum in rats at 7 months of age: (a) HuC/D$^+$ neurons and (c) nNOS$^+$ neurons. (e) Overlay of images from (a) and (c). Density of (b) HuC/D $^+$ and (d) nNOS$^+$ myenteric neurons. Neuronal profile (μm^2) of HuC/D$^+$ (green) and nNOS$^+$ (red) neurons shown in (f). Different letters in same-colored columns differ statistically (f). $^*P < 0.05$, compared with C7 group. AGING, C7, C12, and C23 groups; ABM, C7, CA12, and CA23 groups. The results are expressed as mean ± standard error. Scale bar = 50 μm.

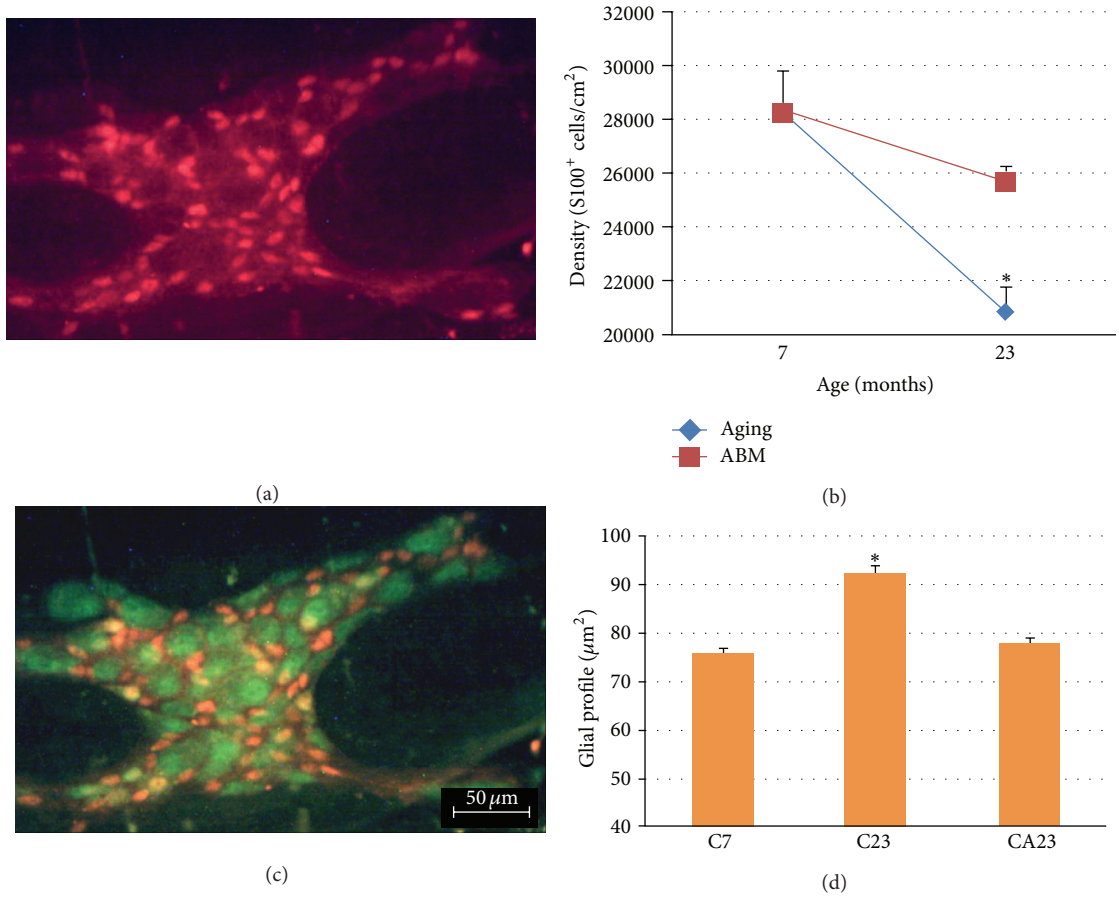

FIGURE 4: Myenteric ganglion of the jejunum in rats at 7 months of age: (a) $S100^+$ glial cell body and (b) density of $S100^+$ glial cells. HuC/HuD^+ and glial $S100^+$ glial neuron cell bodies (c) and glial profile presented in (d). $^*P < 0.05$, compared with C7 and CA23 groups. AGING, C7, and C23 groups; ABM, C7, and CA23 groups. The results are expressed as mean ± standard error. Scale bar = 50 μm.

The association between final body weight and nasoanal length allows the calculation of the Lee index, a parameter comparable to body mass index. Together with the increase in retroperitoneal adipose tissue, the Lee index significantly increased in the C23 and CA23 groups compared with the C7 group, with no significant differences in periepididymal fat or total fat. The aging process in rats is associated with an increase in body fat [24] and decrease in muscle mass [25]. These changes mainly occur because of a decrease in basal metabolic rate and diminished physical activity [26, 27]. We detected marked hypoactivity in the animals throughout the experiment and a significant reduction of food intake, which explain the stable body weight.

Age, regardless of supplementation, led to an increase in small intestine length in the C23 and CA23 groups. This increase may cause slower intestinal transit, with the possibility of constipation, a frequently reported symptom among the elderly [7]. Similar results were reported by Phillips et al. [28] and Johnson et al. [29], who observed a progressive increase in small intestine length at 24 months of age in Fischer 344 and Sprague Dawley rats, respectively. In Wistar rats, maintenance [10] and a reduction [8] of the size of the small intestine related to aging were found in 29- and 15-month-old animals, respectively.

Prolonged supplementation with the aqueous extract of ABM did not influence the analyzed biometric parameters, indicating that prolonged intake was responsible neither for the onset of being overweight or obesity nor for any stimulus toward greater feed or water intake. Notably, the *Agaricus blazei* mushroom is regarded as a highly nutritious food because of its carbohydrate, protein, and fiber content and low levels of fat [30].

4.2. Biochemical Analysis of Blood Components. Aging significantly increased ($P < 0.05$) total plasma cholesterol levels in 23-month-old animals. This has been reported both for rats [31] and humans [32] in old age and is related to a higher incidence of diseases such as atherosclerosis [33].

One hypothesis for aging-related hypercholesterolemia involves the natural deficiency of growth hormone (GH) with aging. Parini et al. [31] reported a reduction of cholesterol in 18-month-old rats treated with GH, which indicates the pleiotropic effects of this hormone on lipoprotein metabolism.

Considering that plasma low-density lipoprotein levels are determined by the balance between its synthesis and elimination, some authors have suggested an increase in the intestinal absorption of cholesterol with aging [34]. However,

Gälman et al. [35] found that aging-related hypercholesterolemia in rats was attributable to reduced excretion and not to the higher intestinal absorption of cholesterol.

Supplementation with the ABM extract showed a marked tendency to reduce total cholesterol in 23-month-old animals, but this result was not statistically significant. The beneficial effects of an ethanolic extract of the *Pleurotus ostreatus* mushroom on cholesterol metabolism were observed in rats supplemented by gavage at a dose of 300 mg/kg/day [36]. A similar effect was observed with supplementation with 200 mg/kg of *Agaricus bisporus* in rats for 3 weeks [37]. In humans supplemented with *Agaricus blazei* for 3 months using a hot aqueous extract at a daily dose of 3 g, Liu et al. [38] also reported a reduction of total cholesterol.

These results suggest a beneficial effect of mushrooms in reducing total cholesterol, but the form of extraction and daily dosage should be considered. We used a cold aqueous extract because it is safer with regard to the release of hepatotoxic substances previously described for extracts over 60°C [39]. We also supplemented animals with a low dosage administered over a prolonged period of time as a preventive measure and not as a treatment.

Aging also increased the serum levels of total proteins and globulins, but these levels are within the normal range for Wistar rats [40], with no influence of supplementation on these parameters. No effect of age or *A. blazei* supplementation on the blood levels of albumin or triglycerides was found.

Although glycemic levels are constant during the aging process, they were high ($P < 0.05$) in the CA23 group. The literature diverges with regard to glycemia in rodents, but we can discard the possible occurrence of a diabetic state because our values were less than 300 mg/dL, the threshold value that indicates diabetes in Wistar rats [41].

Aging did not alter the plasma levels of AST and ALT, indicating that aging did not lead to liver damage as described for rats [42] and humans [43]. Moreover, prolonged supplementation with the ABM aqueous extract did not influence these enzymes, which demonstrates the reliability of the concentration administered daily to the animals over a prolonged period of time.

Lee et al. [44] supplemented Fischer 344 rats for 2 years with *A. blazei* aqueous extract and observed no carcinogenic effects in several organs, such as the liver, brain, lungs, and intestine. Antimutagenic effects of the ABM aqueous extract were reported by Barbisan et al. [45] prior to the chemical induction of cancer in rats, demonstrating protection in the initial stage of liver carcinogenesis but no effect when administered in the postinduction period. Previous studies have also shown that *A. blazei* exerts a protective effect on liver function in rats following injury induced by carbon tetrachloride (CCl_4; [46]).

We observed a reduction of the total plasma antioxidant capacity (TAC-ABTS) in 23-month-old animals. The reduction of this capacity in animals in advanced age is frequently reported in the literature [47]. When analyzing the effect of ABM supplementation on this parameter, we observed a tendency toward improved plasma antioxidant capacity in 12-month-old supplemented animals, but no significant differences were found between the C23 and CA23 groups.

4.3. Histological Analysis. The classic histological organization of the jejunum was maintained in rats in all of the groups [48, 49]. Nevertheless, morphometric alterations were detected in the aging process and with daily supplementation with the aqueous extract of *Agaricus blazei*. When analyzing the total intestinal wall, we observed a significant reduction of thickness in the 23-month-old animals; the same was observed for the mucosa tunic, villus height, and crypt depth in the intestine compared with 7-month-old rats (Table 3).

Reductions of the thickness of the intestinal wall and mucosa related to aging have been observed in rats [9, 10] and humans [50] as a consequence of the reduced food intake seen with aging, in which the availability of nutrients in the intestinal lumen demonstrably acts as a trophic factor [51].

According to Raul et al. [10], villus atrophy in Wistar rats between 12 and 29 months of age may be related to a decrease in the renewal rate of the epithelium, reflected by a corresponding reduction of crypt size. Höhn et al. [9] found villus atrophy of approximately 20–25% in 30-month-old rats, followed by architectural irregularity compared with 4-month-old rats.

Despite the reduction of these parameters, the cellular proliferation, reflected by the MetI, remained constant (Table 3). Equilibrium between cellular synthesis, migration, and extrusion processes leads to the maintenance of villus size and consequently the preservation of digestive and intestinal absorption capacity. Pluske et al. [52] highlighted that in situations of maintenance or reduction in the cell proliferation rate (MetI) associated with increased cell extrusion, in the apex of the villi, results in a reduction of their size. Our results indicate that absorption capacity was minimized and that supplementation with the ABM aqueous extract did not reverse this condition. The number of goblet cells per field was reduced ($P < 0.05$) with aging compared with the C7 group (Table 3), which is consistent with the reductions of villi and crypts discussed previously. Valenkevich and Zhukova [50] reported similar results for goblet cells in the duodenum in advanced-age humans. Caliciform cells are involved in the production of mucus that protects and lubricates the surface of the intestinal epithelium; therefore, reduced mucus secretion in response to aging [53] can damage the intestine by reducing the protective barrier against pathogens, facilitating their transport toward the inside of the mucosa and increasing the susceptibility to infection [51]. Fasting or dietary changes can also result in a reduction of the protective mucus layer [51].

Supplementation with the aqueous extract of *A. blazei* showed a tendency toward alleviating this condition in the CA23 group, but no significant differences were detected compared with the C23 group. This improvement may be explained by considering that the aqueous extract provided protein supplementation in the diet.

The muscular coat did not exhibit significant differences in thickness during the aging process (Figure 1). This result is consistent with Marese et al. [8], who attributed this result to a normal developmental condition imposed on the animal by diets with a normal protein content, regardless of age, although a reduction of intestinal motility associated with age was reported [53].

Supplementation with the ABM aqueous extract increased the muscular coat in old supplemented animals (CA23 group) compared with nonsupplemented animals (C23). The amount of proteins and amino acids present in the ABM aqueous extract [54, 55] likely contributed to the increase in the muscular coat during the long period of supplementation. Low-protein diets have been shown to reduce muscular coat thickness in Wistar rats [56].

4.4. Morphoquantitative Analysis of the Myenteric Plexus. We found that the density and number of cells per ganglion in the myenteric neuron (HuC/D^+) and glial cell ($S100^+$) population were significantly reduced at 7 and 23 months of age, whereas the density of the nitrergic subpopulation ($nNOS^+$) remained stable.

Quantitative reductions of the enteric neuron population are frequently reported in the literature and may be linked to gastrointestinal problems, such as dysphagia, gastroesophageal reflux disorders, diarrhea, constipation, and fecal incontinence [57]. In addition to alterations in the ENS, damage to the sympathetic innervation of the plexi may be a possible mechanism for the decline in gastrointestinal motor function seen in old rats [58].

Reduction of the number of neurons has been observed in humans [59], guinea pigs [60], and rats [61, 62] in both the small intestine [8, 21, 28] and large intestine [21, 28]. The latter is seen as the most susceptible to aging-associated damage. In addition to effects in different species, organs, and segments, neuron populations and subpopulations can be affected differently.

Nitrergic neurons synthesize nitric oxide through nitric oxide synthase, and cholinergic neurons synthesize acetylcholine through choline acetyltransferase. These two neuronal subpopulations combined represent almost the entire myenteric neuron population in rats [63]. Phillips et al. [28] compared 3- and 24-month-old rats and found that neuronal loss in the small and large intestines occurred only with cholinergic neurons, thus corroborating the data obtained in the present study. Neurons marked by the pan neuronal marker anti-HuC/D in the cytoskeleton [64] were reduced by 29.2% in 23-month-old animals compared with the C7 group. The density of the nitrergic subpopulation was not significantly reduced, supporting the hypothesis that certain neuron classes may be more susceptible to aging than others [61, 65].

The cell profile analysis showed that the aging process significantly increased the cell body area for both HuC/HuD^+ and $nNOS^+$ neurons beginning at 12 months of age, although no quantitative alterations were detected during that period, which remained unchanged until 23 months of age. Age-related neuronal cell body hypertrophy was also described by Marese et al. [8] and Schoffen and Natali [14], who compared the duodenum in 3- and 14-month-old Wistar rats and the proximal colon in 3- and 12-month-old Wistar rats, respectively. This was considered a neuronal adaptation attributable to the reduced number of neurons.

Notably, despite maintaining their density, the changes in the cell profile of nitrergic neurons indicated that they were completely spared from the effects of aging. Phillips et al. [28] used the NADPH-diaphoresis technique and observed an increase in the cell body area of nitrergic neurons in the colon and rectum in 24-month-old Fischer 344 rats, with no alterations in the small intestine. These data differ from the results obtained in the present study.

The ENS changes that occur with aging may be related to a reduction of neurotrophic factors secreted by glial cells, which are important in neuronal development and maintenance [13]. In our work, glial loss was proportional to myenteric neuronal death. The glia:neuron ratio did not change over the different ages studied. The same pattern of cellular death was observed by Phillips et al. [21], who performed double-HuC/HuD-S100 immunostaining in Fischer 344 rats at 6 and 26 months of age, suggesting the interdependence of these two cell types.

The glial profile was also altered as a consequence of aging. An increase was observed in the mean cell body area in 23-month-old rats compared with 7-month-old rats. The progressive hypertrophy of astrocytes immunolabeled by the S-100 protein in the central nervous system also occurs frequently during the aging process in rats [66]. However, that population in the enteric nervous system has been seldom evaluated.

The quantitative reduction of cells present in enteric ganglia may be directly related to oxidative stress because of the higher production of free radicals and a decrease in the activity of antioxidant enzymes [1] that occurs during the aging process. Thrasivoulou et al. [12] performed *in vitro* analyses of myenteric neurons in Sprague-Dawley rats and found that the start of cellular death is linked to higher intraneuronal levels of reactive oxygen species (ROS).

Given that the total plasma antioxidant capacity (TAC) was reduced in 23-month-old rats and that the ABM aqueous extract has demonstrable antioxidant potential [55], we infer that the extract efficiently preserved neurons and glia in the myenteric plexus in old animals. No differences were detected in the number or profile of the cell bodies of glial cells and myenteric $HuC/D\ neurons^+$ in the supplemented groups (CA12 and CA23) compared with the 7-month-old group.

Despite the numeric maintenance of nitrergic neurons during aging, atrophy was detected in the middle area of cell bodies in that neuronal subpopulation in 23-month-old supplemented animals compared with the 7-month-old group.

The possible involvement of specific components of the ABM aqueous extract, such as polyphenols, might justify this reduction because of their reported ability to sequester nitric oxide *in vitro* [67] and *in vivo* [68]. van Acker et al. [67] reported the neuroprotective effects of polyphenols. Epigallocatechin, a polyphenol isolated from green tea administered intraperitoneally in rats, attenuated oxidative stress by reducing the expression of nNOS and NADPH-d in parasympathetic ganglion neurons that extrinsically innervate the digestive tube in rats following hypoxia.

The precise mechanism by which *A. blazei* prevents neuronal death is still unclear. Nevertheless, considering that neuronal death can be a consequence of glial death [69], we can infer that the functions of glial cells were preserved by

prolonged intake of the ABM aqueous extract during the aging process.

One hypothesis is that antioxidant compounds contained in the ABM aqueous extract have direct actions on free radicals generated in enteric glial cells. Given that they are similar to central nervous system (CNS) astrocytes with regard to their morphological and immunohistochemical characteristics [70, 71], this hypothesis can be supported by the results obtained by Sharma et al. [72], which indicate a positive effect of flavonoids on astrocytes through the modulation of glial fibrillary acidic protein (GFAP) and glutamine synthetase, with involvement in protective events, reducing apoptosis in a neuronal culture. Another hypothesis is that the chronic intake of glutamic acid (or glutamate) found in the aqueous extract of *A. blazei* has indirect antioxidant effects [54, 73]. Glutamic acid can be converted into glutamine, a precursor of glutathione, one of the most powerful cellular antioxidants. This substance is produced and released by enteric glial cells for neuronal preservation in cases of oxidative stress [74]. Moreover, Muyderman et al. [75] demonstrated that mitochondrial glutathione is essential for preserving the viability of astrocytes in the SNC during conditions of increased levels of free radicals, which supports our hypothesis.

A positive relationship between antioxidant compound supplementation and enteric neuroprotection in diabetic neuropathy has been reported by several authors. This diabetic condition, similar to aging, is associated with oxidative stress as one of its main degenerative factors [3]. Among the supplements studied in experimental models of diabetic neuropathy are ascorbic acid [76], α-tocopherol [77], L-glutamine [78], *Ginkgo biloba* [79], and quercetin [80].

In aging, a neuroprotective effect was detected in the ENS in the small intestine [81] and large intestine [82] in rats supplemented with *Ginkgo biloba* extract. Similarly, [83] found that supplementation with ascorbic acid had a neurotrophic effect on myenteric neurons in old rats, suggesting neuroprotection.

5. Conclusions

Aging modifies biometric, blood, and morphofunctional parameters in the jejunum and causes morphoquantitative changes in the enteric nervous system. Prolonged supplementation with the aqueous extract of *Agaricus blazei* efficiently maintained myenteric plexus homeostasis, which positively influenced the physiology and prevented the death of the neurons and glial cells.

Conflict of Interests

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

Acknowledgments

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Research Article

Protective Effect of Ethanol Extracts of *Hericium erinaceus* on Alloxan-Induced Diabetic Neuropathic Pain in Rats

Zhang Yi,[1] Yang Shao-long,[2] Wang Ai-hong,[3] Sun Zhi-chun,[1] Zhuo Ya-fen,[1] Xu Ye-ting,[4] and He Yu-ling[4]

[1] *Department of Endocrinology, Quanzhou First Hospital Affiliated to Fujian Medical University, Quanzhou, Fujian 362000, China*
[2] *Department of Pathology, Zhengzhou Railway Vocational and Technical College, Zhengzhou, Henan 450000, China*
[3] *Department of Endocrinology, 306 Hospital of PLA, Beijing 100101, China*
[4] *Endocrine and Metabolic Diseases Division, First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi 530021, China*

Correspondence should be addressed to Zhang Yi; zhangyi123543@yeah.net and He Yu-ling; heyulingnanning@163.com

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We investigated the effects of *Hericium erinaceus* (HEE) on alloxan induced diabetic neuropathic pain in laboratory rats. Alloxan induced diabetic rats were administered orally HEE. After 6 weeks of treatments, treatment with HEE 40 mg/kg in diabetic animals showed significant increase in pain threshold and paw withdrawal threshold and significant decrease in serum glucose and urine glucose. We also observed a significant increase in lactate dehydrogenase (LDH), Lipid peroxidation (LPO), glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na^+K^+ATPase activity, and glutathione S transferase (GST) activity along with significant decreased levels of glutathione (GSH) content in diabetic rats. The total antioxidant status (TAOS) in the HEE-treated groups was significantly lower than that in the alloxan-treated group. HEE can offer pain relief in diabetic neuropathic pain. The improvement in diabetic state after HEE treatment along with the antioxidant activity could be the probable way by which it had alleviated diabetic neuropathy.

1. Introduction

One of the most common chronic complications of diabetes mellitus is diabetic neuropathy, which is mainly characterized by spontaneous pain and abnormal sensations such as paresthesia, allodynia, and hyperalgesia [1]. A large number of neuroanatomical, neurophysiological, and neurochemical mechanisms are thought to contribute to the development and maintenance of diabetic neuropathic pain (DNP) [2]. Current treatment of PDN involves the use of tricyclic antidepressant, selective serotonin reuptake inhibitors [3], anticonvulsants, opioids and antioxidant protein kinase C inhibitors, COX-2 inhibitors [4] and nonsteroidal anti-inflammatory drugs as mild analgesics, and so on. However, these therapies provide relief only to a fraction of patients and their side effect profiles limit their use [5, 6]. Therefore, there is a need to identify an effective and safe clinical treatment for PDN. Complementary medicines have gained in popularity among clinicians in recent years. Many indigenous medicinal plants/herbs have been found to be useful to successfully manage pain in various chronic pain models.

Hericium erinaceus is a mushroom that grows on old or dead broadleaf trees. *H. erinaceus* is taken as a food in Japan and China without harmful effects. *H. erinaceus* extracts and compounds have been found with special central effects that could be of pharmacological interest [7, 8]. However, the role of *H. erinaceus* in diabetic complications has not been investigated. The aim of the present investigation was to evaluate the neuroprotective effect of *H. erinaceus* against STZ-induced neuropathic pain and to assess its mechanism of action in rats.

2. Material and Methods

2.1. Preparation of H. erinaceus Extracts (HEE). Fresh fruiting bodies of *H. erinaceus* were lyophilized and powdered. The dry powder (5 g) of mushrooms was extracted with 150 mL of

ethanol for 2 h at room temperature, and *H. erinaceus* ethanol extract (499 mg) was obtained.

The extracts were stored at −30°C before use.

2.2. Animals. Healthy male adult Wistar rats (2 months old and 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.

2.3. Chemicals. Alloxan was purchased from Sigma (USA) and was dissolved in 0.1 N citrate buffers.

2.4. Experimental Design. Experimental diabetes was induced by a single intraperitoneal (i.p.) injection with alloxan (75 mg/kg) solution that was made with saline [9]. Forty-eight hours later, blood samples were collected from the tail veins of the rats. The blood glucose was analyzed with a Glucometer-4 (Bayer). Forty hyperglycemic rats (the blood glucose level greater than 11.1 mmol/L) were selected and allocated equally into 4 groups. From then on, the 4 groups of hyperglycemic rats were administered orally saline, HEE 10 mg/kg/d, HEE 20 mg/kg/d, and HEE 40 mg/kg/d, respectively. HEE was dissolved in the same amount of saline. The other 10 normal rats were administered orally with the saline and used as the control group. The serum glucose was measured before and at the end of the experiment to see the effect of pharmacological interventions on these parameters.

2.5. Assessment of Thermal Hyperalgesia. The nociceptive threshold was tested according to the Hargreaves procedure [10]. In brief, each animal was placed in a clear plexiglass box and the hind paw was exposed to a constant beam of radiant heat through a plexiglass surface. The time in seconds from initial heat source activation until paw withdrawal was recorded. HEE was administered in diabetic animals for 6 weeks, and withdrawal latency was noted daily 30 min after administration of HEE.

2.6. Assessment of Mechanical Allodynia. Mechanical allodynia was assessed before diabetes induction and subsequent to alloxan injection. In brief, each animal was placed in a test cage with a wire mesh floor, and the tip of a von Frey type filament was applied to the middle of the plantar surface of the hind paw and began to exert an upwards force until the paw was withdrawn or the preset cutoff was reached (40 g). A brisk foot withdrawal in response to von Frey type filament stimulation was recorded. The force required to elicit a withdrawal responses was measured in grams.

2.7. Estimation of Blood Glucose and Urine Glucose. The blood glucose was analyzed with a Glucometer-4 (Bayer). Urine glucose was assessed in fresh urine using glucose indicator sticks (Boehringer Mannheim, Germany).

2.8. Estimation of Antioxidant Enzymes Level. In serum, lactate dehydrogenase (LDH), glutathione (GSH) content, Lipid peroxidation (LPO), glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na^+K^+ATPase activity, and glutathione S transferase (GST) activity were estimated using a method described by Lum et al. [11–17].

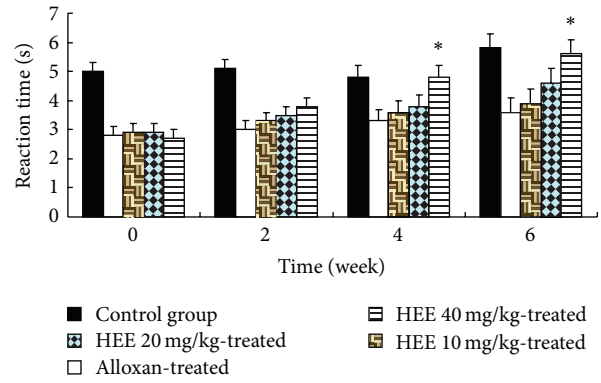

Figure 1: Effect of HEE treatment on tail withdrawal latency. Results are expressed as mean ± SEM ($n = 10$). The data was analysed using and one-way analysis of variance (ANOVA) followed by Dunnett's test. $^*P < 0.05$ versus diabetic group.

2.9. Estimation of Total Antioxidant Activity. The total antioxidant status (TAOS) of the supernatant of centrifuged plasma was determined by the way introduced by Laight et al. [18]. The increase in absorbance at 405 nm was measured by using a microplate reader (Shanghai Xunda Medical Technology, Inc., China).

2.10. Statistical Analysis. All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data are shown as the mean ± SEM. The significant level of 5% ($P < 0.05$) was used as the minimum acceptable probability for the difference between the means.

3. Results

3.1. Effect of HEE Treatment on Thermal Hyperalgesia. Marked thermal allodynia was observed in the alloxan rats as evidenced by a reduction in the pain thresholds compared to control rats (Figure 1). Treatment of diabetic rats with HEE 40 mg/kg induced a significant increase in pain threshold compared to alloxan-treated animals after four weeks of treatment.

3.2. Effect of HEE Treatment on Mechanical Hyperalgesia. There was a marked mechanical hyperalgesia as evidenced by a reduction in the paw pressure withdrawal thresholds in the alloxan-treated animals (68.22 ± 8.47 g) compared to control-treated rats (266.14 ± 15.95 g). In rats receiving treatment of HEE 40 mg/kg mean paw withdrawal threshold was significantly and dose dependently increased compared to diabetic control rats (Figure 2).

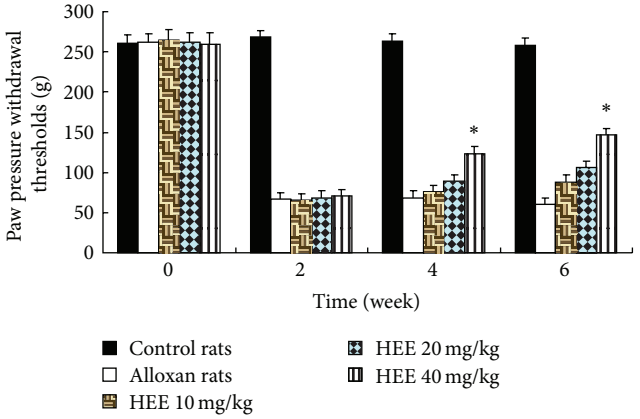

Figure 2: Effect of HEE treatment on paw withdrawal threshold. Results are expressed as mean ± SEM ($n = 10$). The data was analysed using and one-way analysis of variance (ANOVA) followed by Dunnett's test. $^{*}P < 0.05$ versus diabetic group.

Table 1: Effect of HEE on changes in the levels of blood glucose and urine sugar.

Groups	Blood glucose mmol/L	Urine sugar
Control group	5.04 ± 0.39	NIL
Alloxan group	8.08 ± 0.27	+++
HEE 10 mg/kg group	7.88 ± 0.20	+++
HEE 20 mg/kg group	7.03 ± 0.20	++
HEE 40 mg/kg group	5.26 ± 0.27*	NIL

Results are expressed as mean ± SEM ($n = 10$). The data was analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test. $^{*}P < 0.05$ versus diabetic group.
+++ indicates more than 2% sugar; NIL indicates 0 sugar; ++ and + indicate less than 2% sugar.

3.3. Effect of HEE Treatment on Blood Glucose and Urine Glucose. The results of blood glucose and urine sugar from hyperglycemic rats are presented in Table 1. A significant decrease in the level of blood glucose and urine sugar were observed in HEE 40 mg/kg treated groups.

3.4. Effect of HEE Treatment on Antioxidant Enzymes Level. The serum LDH levels in control-treated rats were found to be 86.322±2.663 IU/L. A significant increase in the activity of LDH in serum was observed in alloxan-treated animals. HEE 40 mg/kg significantly ($P < 0.05$) resulted in decreased serum LDH levels when compared with alloxan rats (Table 2). Also, HEE 40 mg/kg produced the increase in the level of GSH (Table 3). At the same time, a significant increase ($P < 0.001$) in the content of LPO was observed in the alloxan-treated animals. In the HEE group, a significant decrease ($P < 0.01$) was seen in the level of LPO when compared with the alloxan group (Table 4).

It has been proposed that antioxidant changes reflect an altered redox balance in several pathological states. 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 5. HEE treatment showed a significant ($P < 0.05$–0.01) restoration in the level of various enzyme as compared with alloxan group.

Table 2: Effect of HEE on serum LDH levels.

Different groups	LDH (IU/L)
Control group	80.322 ± 2.643**
Alloxan group	170.201 ± 3.441
HEE 10 mg/kg group	146.611 ± 2.221
HEE 20 mg/kg group	116.632 ± 3.221*
HEE 40 mg/kg group	130.230 ± 2.360*

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

Table 3: Effect of HEE on GSH.

Different groups	(nmol GSH/mg protein)
Control group	1.800 ± 0.016*
Alloxan group	1.011 ± 0.010
HEE 10 mg/kg group	1.090 ± 0.011
HEE 20 mg/kg group	1.500 ± 0.013*
HEE 40 mg/kg group	1.300 ± 0.011*

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

Table 4: Effect of HEE on LPO level.

Different groups	nmol
Control group	15.21 ± 0.66**
Alloxan group	21.20 ± 1.40
HEE 10 mg/kg group	20.00 ± 0.20
HEE 20 mg/kg group	17.36 ± 0.21*
HEE 40 mg/kg group	17.20 ± 0.20*

Values are shown as means ± SEM. $^{*}P < 0.01$ versus diabetic group and $^{**}P < 0.001$ versus diabetic group.

3.5. Effect of HEE Treatment Total Antioxidant Activity. The results of TAOS are shown in Table 6. TAOS in the alloxan group were significantly ($P < 0.01$) higher than those in the control group. Those in the HEE-treated groups were significantly lower than those in the alloxan-treated group ($P < 0.05$).

4. Discussion

Diabetic neuropathy is characterized by clinical features like allodynia, hyperalgesia due to elevated nociceptive response. The present study indicates that alloxan-induced diabetes lowers pain threshold as evident by the presence of thermal hyperalgesia and mechanical allodynia. Although evaluation of mechanisms causing these symptoms is complicated because of the overlap between the systemic effects of hyperglycemia and its toxic effects within the peripheral nervous system, direct functional toxicity of hyperglycemia in the peripheral nervous system [19], administration of HEE after

Table 5: Effect of HEE on the activity of various enzymes.

Different groups	GPx	GR	GST	CAT	Na^+K^+ATPase
Control	$15.90 \pm 1.20^{***}$	$35.44 \pm 2.50^{***}$	$17.44 \pm 1.00^{**}$	$7.00 \pm 0.55^{*}$	$4.88 \pm 0.31^{**}$
Alloxan	8.00 ± 0.41	20.55 ± 2.10	10.55 ± 1.44	4.33 ± 0.10	2.44 ± 0.11
HEE 10	9.11 ± 1.01	21.22 ± 2.00	10.66 ± 0.96	4.77 ± 0.21	2.00 ± 0.11
HEE 20	$13.15 \pm 1.33^{**}$	$26.11 \pm 2.11^{***}$	$13.66 \pm 0.90^{*}$	$5.66 \pm 0.33^{*}$	$3.78 \pm 0.10^{*}$
HEE 40	$11.11 \pm 1.10^{*}$	$25.55 \pm 2.35^{**}$	$12.66 \pm 0.77^{*}$	$6.88 \pm 0.38^{*}$	$4.66 \pm 0.11^{*}$

Values are shown as means ± SEM. $^{*}P < 0.05$ versus diabetic group, $^{**}P < 0.01$ versus diabetic group, and $^{***}P < 0.001$ versus diabetic group.

Table 6: Effect of HEE on TAOS activity (μM L-ascorbate).

Different groups	TAOS activity (μM L-ascorbate)
Control group	$28.41 \pm 3.17^{**}$
Alloxan group	80.33 ± 9.32
HEE 10 mg/kg group	$72.22 \pm 2.78^{*}$
HEE 20 mg/kg group	$64.30 \pm 3.38^{*}$
HEE 40 mg/kg group	$56.35 \pm 4.33^{**}$

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

the 4 weeks of alloxan reversed diabetes induced thermal hyperalgesia (Figure 1) and mechanical allodynia (Figure 2). At the same time, treatment with HEE in diabetic animals showed significant decrease in serum glucose and urine sugar (Table 1). It indicates that the improvement on serum glucose levels may be attributed to the improvement on metabolic dysfunction in diabetic rats.

Hyperglycaemia-induced oxidative stress is an important mechanism leading to both the development and progression of hyperalgesia and allodynia in rats [20]. We observed a significant increase in LDH and reduction in endogenous antioxidant enzymes like GPx, GR, CAT, and GST as well as Na^+K^+ATPase activity along with significant decreased levels of GSH in diabetic rats. Treatment with HEE for six weeks restored the abovementioned biochemical parameters in diabetic rats in dose dependent manner. The TAOS is an indication of O_2^- and other oxidant species. We also measured TAOS activity as an indirect indication of the formation of O_2^- and other oxidant species. A marked increase in TAOS in diabetic animals has been observed in the present investigation. We also observed that HEE treatments dose-dependently attenuated oxidative stress, measured in terms of TAOS level, which may have further assist in management of painful diabetic neuropathy.

Collectively, administration of HEE could attenuate the diabetic neuropathy in rats. The improvement in diabetic state after HEE treatment along with the antioxidant activity could be the probable way by which it had alleviated diabetic neuropathy.

Disclosure

Zhang Yi and Yang Shao-long are cofirst authors.

Conflict of Interests

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

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Review Article

The Chemical Constituents and Pharmacological Actions of *Cordyceps sinensis*

Yi Liu,[1] Jihui Wang,[2] Wei Wang,[1] Hanyue Zhang,[3] Xuelan Zhang,[1] and Chunchao Han[1]

[1]*School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[2]*Teaching Experiment Center, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*
[3]*College of Acumox and Tuina, Shandong University of Traditional Chinese Medicine, Jinan 250355, China*

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

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Cordyceps sinensis, also called DongChongXiaCao (winter worm, summer grass) in Chinese, is becoming increasingly popular and important in the public and scientific communities. This study summarizes the chemical constituents and their corresponding pharmacological actions of *Cordyceps sinensis*. Many bioactive components of *Cordyceps sinensis* have been extracted including nucleoside, polysaccharide, sterol, protein, amino acid, and polypeptide. In addition, these constituents' corresponding pharmacological actions were also shown in the study such as anti-inflammatory, antioxidant, antitumour, antiapoptosis, and immunomodulatory actions. Therefore can use different effects of *C. sinensis* against different diseases and provide reference for the study of *Cordyceps sinensis* in the future.

1. Introduction

The genus *Cordyceps* is an important kind of medicinal fungi belonging to the Ascomycota, Pyrenomycetes, Hypocreales, and Clavicipitaceae [1–5]. *Cordyceps* are specific macrofungi because of their characteristic parasitic habit on larvae and pupae of insects. As a pleomorphic fungus distributed worldwide, *Cordyceps* is particularly abundant in tropical forests and humid temperate [3–5]. Within the genus *Cordyceps*, over 400 species have been described so far [4, 5], of which *Cordyceps sinensis*, also called as "winter worm, summer grass," is recognized as the most famous tonic herb in traditional Chinese medicine (TCM) for centuries.

Cordyceps sinensis is an abundant resource in nature with various biological activity and has been used extensively as a tonic and health supplement for subhealth patients especially seniors in China and other Asian countries. Till now, numerous bioactive constituents have been extracted such as cordycepin, polysaccharides, ergosterol, mannitol, and adenosine [6, 7]. Meanwhile, various pharmacological actions of these chemical constituents have been reported, including antitumour effect, hepatoprotective and inflammatory effects, and antioxidant, nephroprotective, and antiapoptotic properties [8–14]. To sum up, the effect of *C. sinensis* may be caused by a single active ingredient or by the combined action of many active agents that existed in the extract.

Research is necessary to get an overview about the genus *Cordyceps sinensis* because of the increasing interest both for medicine and mycology [15, 16]. Therefore, our study has reviewed the chemical constituents and their corresponding pharmacological actions of the *Cordyceps sinensis* for its significant role in the development of new drugs and therapeutics for various diseases. Moreover, realizing the pharmacological action of the monomer composition could strengthen the drug efficacy through extracting a single ingredient in *Cordyceps sinensis*. Therefore, it is necessary to review the development on the research of *C. sinensis*.

2. Chemical Constituents and Their Corresponding Pharmacological Actions of *Cordyceps sinensis*

2.1. Nucleosides. Nucleosides, a major active component of *C. sinensis*, are used as a valuable chemical marker for quality control of *Cordyceps* [1]. Besides, nucleosides play

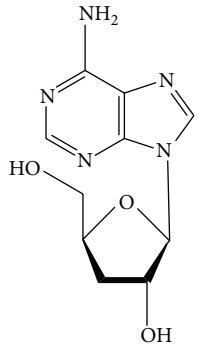


Figure 1: Chemical structure of cordycepin.

an important role in the drug development of cancer and infectious diseases, and nucleosides and their derivatives have been widely used in anticancer and antiviral therapies. Since 3′-deoxyadenosine, namely cordycepin, was isolated from cultured *Cordyceps militaris*, nucleosides in *Cordyceps* have become a focus [1]. In succession, more than ten nucleosides and their related compounds, including adenine, adenosine, inosine, cytidine, cytosine, guanine, uridine, thymidine, uracil, hypoxanthine, and guanosine, have been isolated from *Cordyceps sinensis*. Almost all of the nucleotides and nucleosides in *C. sinensis* can be transformed reciprocally [17]. Furthermore, many scholars began to study its pharmacological effects and had a lot of achievements [18, 19]. An UPLC method for fast simultaneous determination of several nucleosides was developed this year and was also applied for the determination of the analytes in cultured *Cordyceps sinensis* [19, 20]. Then, a series of researches about nucleosides was carried out quickly. For example, nucleosides can adjust and control the human body physiological activities through purinergic and/or pyrimidine receptors [17]. Therefore, determination of nucleosides and their related compounds is extremely important for the pharmacological study and quality control of *C. sinensis* and its products.

2.1.1. Cordycepin. Early in 1950, cordycepin was first isolated from *C. militaris* and its structural formula was confirmed as 3′-deoxyadenosine but it is only found in natural *C. sinensis* with very low content and cannot be detected in the cultured ones [17]. Cordycepin is the most considerable adenosine analogue from some *Cordyceps* [21], which is a derivative of the nucleoside adenosine differing from the latter by the absence of oxygen in the 3′ position of its ribose entity (Figure 1). Cordycepin was separated with a mixture of acetonitrile and water (5 : 95, v/v) at a flow rate of 1.0 mL/min, which is the commonly used method to extract the composition [22].

Cordycepin is a category of compounds that exhibits significant therapeutic potential and has many intracellular targets, including nucleic acid, apoptosis, and cell cycle. Tuli et al. researched the variety of molecular mechanisms that mediate the pharmacological effects of cordycepin. Besides, they deem that cordycepin can participate in various molecular processes in cells because of its similarity with adenosine [7]. Wang et al. investigated the effects of cordycepin in prevention of focal cerebral ischemic/reperfusion (IR) injury and suggested that cordycepin has a neuroprotective effect in the ischemic brain, which is due to the inhibition of inflammation and increase of antioxidants activity related to lesion pathogenesis [23, 24]. So cordycepin could be an attractive therapeutic candidate with oral activity against I/R-associated heart diseases such as myocardial infarction [25]. Besides, cordycepin showed the obvious analgesic effect through acetic acid-induced abdominal constrictions, hot-plate test, and neurolysin inhibition assay in mice [26]. Qian et al. considered that cordycepin is a potent anti-inflammatory and analgesic medicine. There are several studies demonstrating that *C. sinensis* stimulates steroidogenesis in primary mouse Leydig cell and activates apoptosis in MA-10 mouse Leydig tumor cells in dose- and time-dependent manners [27, 28]. The steroidogenic and apoptotic mechanism of cordycepin is also clear—cordycepin stimulated intracellular PLC/PKC and MAPK signal transduction pathways to induce steroidogenesis and cell death in MA-10 mouse Leydig tumor cells [29]. In addition, cordycepin stimulated the release of some cytokines of resting PBMCs and influenced proliferation of PBMCs and transcription factors in THP-1 cell line. Accordingly, cordycepin can intensively regulate the functions of human immune cells in vitro [30]. Besides, cordycepin is a broad spectrum biocidal compound possessing not only antitumor activity but also antibacteria, antivirus, and insecticidal activities [2]. To sum up, cordycepin was confirmed as a marker for *C. militaris* within the content profiles of nucleosides in *Cordyceps* product [1].

2.1.2. Adenosine. Adenosine (Figure 2), which plays an important role in biochemical process in the organism, is a major nucleoside in *Cordyceps* spp. [31]. The content of adenosine is much higher in cultured *Cordyceps sinensis* than in the natural one. Among them, cultured *C. sinensis* has a large number of adenosines, which are much higher than those in cultured *C. militaris* [20, 31]. Nucleotide named AMP can be degraded to adenosine and the source of inosine in natural *C. sinensis* may be the oxidative deamination of adenosine [17]. Many other adenosine analogues such as 2′-deoxyadenosine, 2′3′-dideoxyadenosine, cordycepin triphosphate, and 3′-amino-3′-deoxyadenosine have also been found in *Cordyceps sinensis* [1]. Yang and Li introduced three methods to extract adenosine: organic solvent pressurized liquid extraction, boiling water extraction, and ambient temperature water extraction. They found that the extraction ratio of adenosine is much affected by extracting time and natural *Cordyceps sinensis* may contain some enzymes which can decompose adenosine [32–34].

Also, adenosine is an energy transfer and signal transductant in cells and can still exert a wide spectrum of cytoprotection or prevent tissue damage such as treating chronic heart failure, anti-inflammatory properties, and anticonvulsant activity [35–38]. In addition, adenosine is reported to suppress cell growth via diverse extrinsic and intrinsic signaling pathways. In both pathways, adenosine activates caspases in a mitochondria-dependent and/or -independent manner [39–41]. For example, Ma et al. first observed that

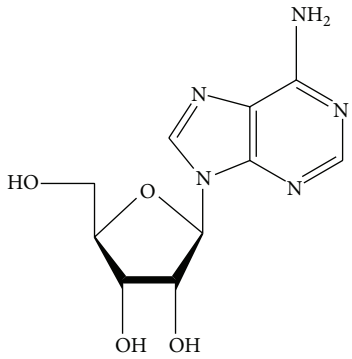


Figure 2: Chemical structure of adenosine.

adenosine increases ROS production in tumor cells and identified the positive feedback loop for ROS-mediated mitochondrial membrane dysfunction which amplifies the death signals in the cells [42]. However, Iannone et al. support the hypothesis that inhibition of adenosine production in tumors or inhibition of A2aR is a promising strategy to increase the effectiveness of melanoma immunotherapy, because they have done a lot of experiments proving that adenosine can limit the therapeutic effectiveness of anti-CTLA4 mAb in a mouse melanoma model [43]. In fact, adenosine mediates its effects through activation of a family of four G-protein coupled receptors, namely A_1, A_{2A}, A_{2B}, and A_3 [44]. This nucleoside plays an important role in immunity and inflammation, and the adenosine A_{2A} plays an important role in depression, locomotion, and anxiety [45]. In particular in skin cells this endogenous nucleoside, acting at one or more of its receptors, could participate in dermal tissue protection and repair. To sum up, adenosine and its analogues have received so much attention due to their various pharmacological effects.

2.1.3. Nucleobases. To date, six nucleobases (Figure 3), including cytosine, uracil, thymine, adenine, guanine, and hypoxanthine, were determined in natural and cultured *Cordyceps sinensis*. The overall content of nucleosides is much higher in cultured *Cordyceps sinensis* than in natural ones by comparison [32]. A method based on optimum acid hydrolysis followed by high-performance liquid chromatography (HPLC) with diode array detection was developed for quantitative determination of these bioavailable nucleosides by Fan et al. which is now the most recognized appraisal method [46]. As a result, the total purine and pyrimidine bases may be the reasonable marker for evaluation of the nutrition of the materials containing nucleosides [47]. However, the pharmacological effects on nucleobases alone have not been reported currently.

2.1.4. Nucleotides. Three nucleotides, namely, uridine-5′-monophosphate (UMP), adenosine-5′-monophosphate (AMP), and guanosine-5′-monophosphate (GMP), were separated by ion-pairing reversed-phase liquid chromatography-mass spectrometry (IP-RP-LC-MS) developed by Yang et al. [17]. In the pharmacological aspect, nucleotides were reported to

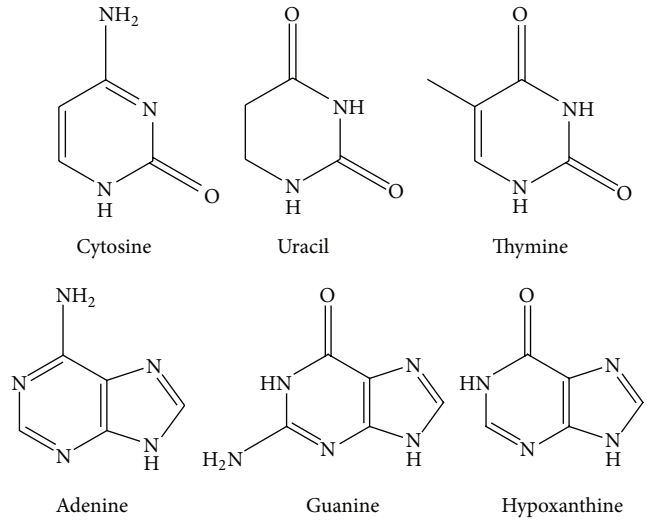


Figure 3: Chemical structure of six nucleosides.

enhance the immune response, influence metabolism of fatty acids, help the absorption of iron gut, and improve the gastrointestinal injury after repair [17, 48]. The nucleotides such as AMP, GMP, and UMP can be degraded to adenosine, guanosine, and uridine, respectively. Actually, nucleotides could be considered as an amphoteric molecule with base and phosphoric acid. Guanosine has the highest content of all in natural and artificial *Cordyceps sinensis* showed by many investigations [48]. Nucleotides can inhibit urethral inflammation, promote blood circulation, and improve brain function, and their most important effect is enhancing human immunity, which has been reported in previous studies [49].

2.2. Polysaccharides. *Cordyceps sinensis* contains a great deal of polysaccharides, which can be in the range of 3–8% of the total weight [50, 51]. *Cordyceps* polysaccharides mainly include extracellular polysaccharide and intracellular polysaccharide. A large amount of experimental evidence has shown that fungal polysaccharides have a wide range of bioactivities including antitumor [52], anti-influenza virus [53], immunopotentiation [54], hypoglycemic [55], hypocholesterolemic [56], and antioxidant effects [51]. Other studies have suggested that the pharmacological activity of the polysaccharide was correlated with its characteristics. For example, it is polysaccharides' high molecular weight that determines the antitumor activity [57]. In other words, Sasaki et al. confirmed that the fungi polysaccharide's antitumor activity is related to the molecular weight, and the fungi polysaccharide has antitumor activity if its molecular weight is greater than 16000. But beyond that, ten monosaccharides, namely, rhamnose, ribose, arabinose, xylose, mannose, glucose, galactose, mannitol, fructose, and sorbose in 13 samples of natural and cultured *C. sinensis*, were qualitatively and quantitatively analyzed [58]. However, *Cordyceps* polysaccharides were usually composed of these monosaccharides and they play a prominent role in the organism. Polysaccharides are the main contributor towards the pharmacological properties of *C. sinensis*. Nevertheless,

its application has been limited so far because of its limited supply. It is an endangered species due to the excessive harvest of the natural fungus [59, 60]. Meanwhile, cultured *C. sinensis* has shown as many pharmacological properties as natural *C. sinensis* [50].

2.2.1. EPSF. Exopolysaccharide fraction (EPSF), a heteropolysaccharide, was extracted from the cultured supernatant of *C. sinensis*. The cultured supernatant was collected and then treated with three times volume of 95% ethanol for precipitation. As a result, the sediment contains a large number of EPSF [60]. EPSF has a large number of pharmacological effects; two of the most important are immunomodulatory and antitumour effects [61]. Previous reports have shown that EPSF could scavenge free radical, induce differentiation of cancer cells, and enhance antitumor ability via activating different immune responses in the host [61]. Thus it can be seen that elevating immunity is much helpful in tumor therapy. In order to explore the effects of exopolysaccharide fraction (EPSF), ICR mice were treated with EPSF for 7 days at different doses after H_{22} tumor cells' injection. The data of these studies show that EPSF could elevate the immunocytes' activities in H_{22} tumor-bearing mice, which might be closely related to elevating peritoneal macrophages' and splenic lymphocytes' activity [60]. Studies have shown that mature DCs (dendritic cells) are important modulators of immune response and their ability to initiate cytotoxic T lymphocyte is very valuable in cancer immunotherapy, and maturation of DCs is a critical factor for the initiation of immune response [62]. Song et al. found that EPSF can promote DC's maturation and activation, which is probably related to the inhibition of STAT3 phosphorylation [63]. This is another mechanism of EPSF's antitumor effect. Yang et al. have investigated the effects of the EPSF on c-Myc, c-Fos, and vascular endothelial growth factor (VEGF) expression of tumor-bearing mice using Simple PCI image analysis software. The c-Myc, c-Fos, and VEGF levels in the lungs and livers of EPSF-treated mice were found to be significantly lower than those of untreated mice, which suggests that EPSF had inhibited tumor growth in the lungs and livers of mice. As a result, it might be a potential adjuvant in cancer therapy [64]. As mentioned above, the EPSF can inhibit a variety of cancer cells; moreover, it may enhance the antitumour ability of animals or humans by activating different immune responses in the host. EPS-1, which is an exopolysaccharide produced by the medicinal fungus *Cordyceps sinensis*, has been specifically named and widely concerned. A recent study has shown that the sulfated EPS-1 derivatives have remarkable antioxidant activities. So, sulfation was an effective and favorable strategy for improving the physicochemical properties and bioactivities of fungal polysaccharides [65], which is a good idea for the study of polysaccharides.

2.2.2. APS. An acid polysaccharide (APS) was isolated from cultivated *C. sinensis* mycelia by ion-exchange and sizing chromatography. APS is composed of mannose, glucose, and galactose in an approximate molar ratio of 3.3 : 2.3 : 1 [66]. In the present study, pretreatment of PC_{12} cells with APS could reduce H_2O_2-induced cell death, which was investigated by measuring cell viability, lactate dehydrogenase (LDH) release, antioxidant enzyme activity, malondialdehyde (MDA) levels, and intracellular accumulation of reactive oxygen species (ROS) and Ca^{2+} [66]. In conclusion, APS possesses protective effects in PC_{12} cells against H_2O_2-induced injury [67]. However, the antioxidant mechanism of APS remains unclear and needs further investigation. In view of the fact that acid polysaccharide fraction (APSF), extracted from *C. sinensis* fungus, has stimulating effects on macrophages [68], Chen et al. have proved that APSF may convert M_2 macrophages to M_1 phenotype by activating NF-κB pathway. So APSF also has immunomodulatory effects as many other polysaccharides [69].

2.2.3. CPS-1. A water-soluble polysaccharide named CPS-1 had been isolated from *C. sinensis* mycelium by hot water extraction, ethanol precipitation, anion-exchange, and gel permeation chromatography [70]. CPS-1 was a glucomannogalactan with the monosaccharide composition of glucose : mannose : galactose = 2.8 : 2.9 : 1. $(1\rightarrow)$ and $(1\rightarrow 3,6)$ linkage of glucose composed the backbone of CPS-1. Present studies have demonstrated that CSP-1 had strong antioxidation activities, which can be used to reduce the blood glucose level [71] and treat renal failure [70]. On one hand, CPS-1 can scavenge hydroxyl radicals and reduce power- and Fe^{2+}-chelating. That indicated a connection between antioxidant activity and reparation of renal failure. On the other hand, CPS-1 stimulates pancreatic release of insulin and/or reduces insulin metabolism, so the polysaccharide can treat diabetes. Especially, the reducing power of CPS-1 was very potent and nearly as effective as ascorbic acid [71].

2.2.4. CPS-2. CPS-2, a *Cordyceps sinensis* polysaccharide, was found to be mostly of α-$(1\rightarrow 4)$-D-glucose and α-$(1\rightarrow 3)$-D-mannose, branched with α-$(1\rightarrow 4,6)$-D-glucose every twelve residues on average (Figure 4). A monosaccharide analysis conducted by the PMP precolumn derivation method showed that CPS-2 was composed of mannose, glucose, and galactose with the ratio of 4 : 11 : 1 [72]. CPS-2, which appeared as white powder, has been demonstrated to have significant therapeutic activity against chronic renal failure. Recently, the underlying molecular mechanism has been explored by scientists. Wang et al. found that CPS-2 could reduce PDGF-BB-induced cell proliferation through the PDGF/ERK and TGF-b1/Smad pathways [73]. As a result, CPS-2 inhibits PDGF-BB-induced human mesangial cells (HMCs) proliferation in a dose-dependent manner.

2.2.5. Other Polysaccharides. A neutral mannoglucan with a molecular weight of 7.7×10^3 Da was obtained from the 0.05 M acetate buffer extract of *C. sinensis* mycelium. It is a branched polysaccharide with a backbone composed mainly of $(1\rightarrow 4)$- and $(1\rightarrow 3)$-linked D-glucosyl residues. Moreover, mannoglucan showed weak cytotoxicity activity against SPC-I cancer line and no obvious cytotoxicity activities against BCAP37 and SW480 cancer line [74]. Similarly, a water-soluble polysaccharide fraction, CME-1, with a molecular mass of 27.6 kDa, was prepared from *Cordyceps sinensis*

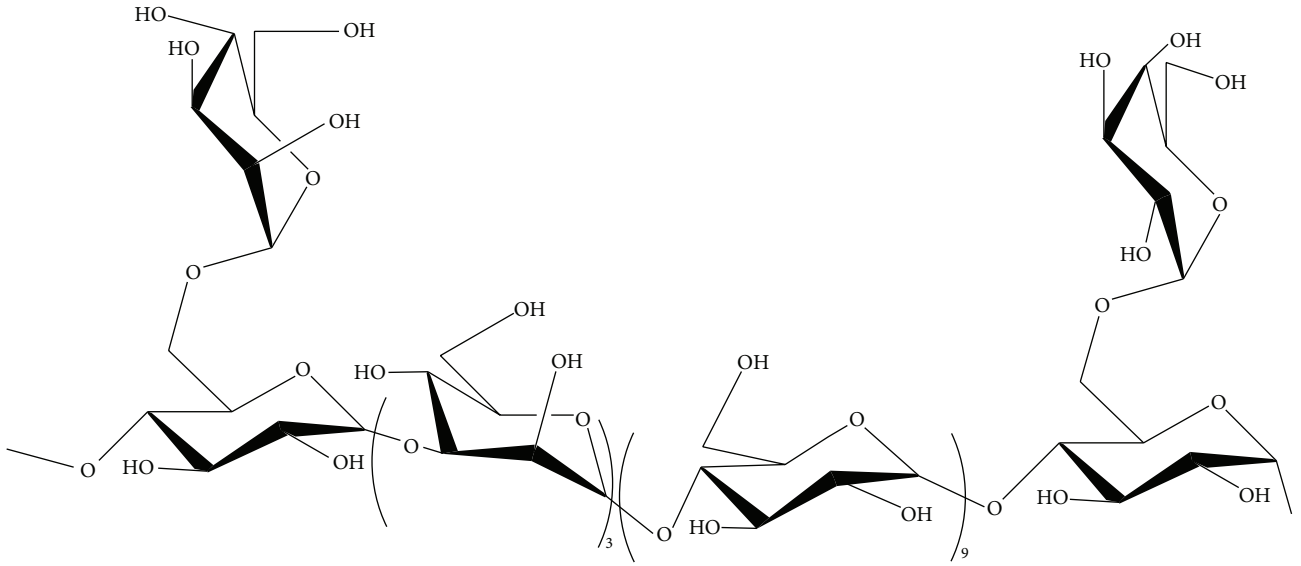


Figure 4: Predicted structure of CPS-2 isolated from the fruiting bodies of cultured *Cordyceps sinensis*.

mycelia and identified by NMR and GC-MS [75]. Wang et al. finally found that CME-1 can protect RAW264.7 cells against oxidative stress through inhibition of SMase activity and reduction of C_{16}- and C_{18}-ceramide levels [75]. In addition, a new component named cordyglucans was released by successive extractions with hot water and 0.05 M sodium hydroxide solutions. Cordyglucans were found to exhibit potent antitumor activity, which could be correlated to their $(1 \rightarrow 3)$-β-D-glucan linkages [76]. Besides, two other polysaccharides, named CS-F10 and cordysinocan, were extracted from the cultured mycelium of *Cordyceps sinensis*, respectively. The former has the hypoglycemic activity, which can lower the plasma glucose level and decrease protein content of facilitative glucose transporter isoform 2 from rat liver following i.p. administration [55] and the latter can not only induce the cell proliferation, but also increase the phagocytosis activity and the enzymatic activity of acid phosphatase [77].

2.3. Sterols. Sterols components of fungus have important physiological function; they also have a variety of biological activities at the same time. So studying sterols has important theoretical significance and application prospects.

2.3.1. Ergosterol. Ergosterol (Figure 5) is a characteristic of fungi sterol and an important source of vitamin D_2 [78]. Ergosterol did not get enough attention in the study of *C. sinensis* although it is a characteristic of fungi sterol [79]. Y. H. Li and X. L. Li determined the content of ergosterol in *Cordyceps sinensis* with HPLC method and obtained a high yield. They also presented that ergosterol existed in free and combined states [80]. It is important that ergosterol is a food, feed, and pharmaceutical raw material. In addition, it is an important raw material in the production of steroid hormone drugs [81]. Zheng et al. have proved the cytotoxicity and antimicrobial activity of ergosterol; it possesses weak

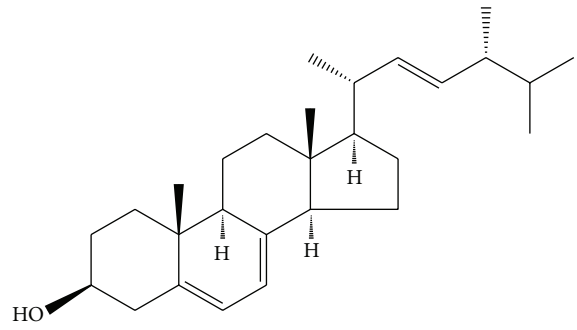
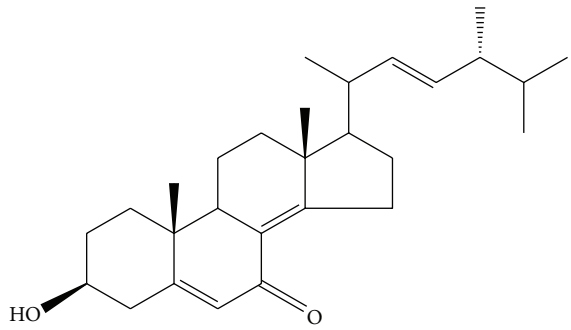


Figure 5: The structure of ergosterol.

cytotoxicity against HL-60 and BEL-7402 cell lines and moderate antimicrobial activity against the bacteria *E. aerogenes* and *P. aeruginosa* and the fungus *C. albicans* [82]. At present, the ergosterol biosynthesis pathway research has made great progress which will provide theoretical guidance to get high yield strains by genetic engineering [83].

2.3.2. H1-A. H1-A (Figure 6), a pure compound used in traditional Chinese medicine, has been isolated from *C. sinensis*. To clarify the pharmacologic properties of H1-A, a series of researches studied its effect on mesangial cell proliferation, cytotoxicity, cell cycles, and apoptosis. These findings suggest that H1-A modulates some subcellular signal transduction pathways and changes the balance between proliferation and apoptosis of mesangial cells in vitro or in vivo. H1-A may be effective in the management of autoimmune disorders, and the modulation of the signal transduction proteins Bcl-2 and Bcl-XL may represent a target for future pharmacologic interventions [84]. As early as four years ago, they have reported the effect of H1-A on inhibiting autoimmune disease in MRL lpr/lpr mice. Additionally, the structure has been



H1-A

Figure 6: Chemical structure of compound H1-A.

analyzed with NMR by Yang et al., as shown in Figure 6. It is like an ergosterol and has been proved to be without glucocorticosteroid receptor binding ability. From another point of view, H1-A is a kind of ergosterol and its structure looks like testosterone and dehydroepiandrosterone [85]. Meanwhile, H1-A can suppress the activated HMC and alleviate IgAN (Berger's disease) with clinical and histologic improvement. Lin et al. predicted that H1-A as a therapeutic regimen might be used in the future [86].

2.3.3. Other Sterols. Two identified compounds, ergosteryl-3-O-β-D-glucopyranoside (a) and 22,23-dihydroergosteryl-3-O-β-D-glucopyranoside (b), were isolated during the fractionation of the methanol extract of *C. sinensis* [87]. Besides, 5α,8α-epidioxy-24(R)-methylcholesta-6,22-dien-3β-D-glucopyranoside (c) and 5α,6α-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol (d) are two glycoside derivatives of a sterol and they also exist in the methanol extract of *C. sinensis* [87, 88]. These are the other four important sterol compounds (Figure 7), which are sterol derivatives, and their structure as shown in Figure 7. Moreover, Matsuda et al. have confirmed through a large number of experiments that the latter two have the anticancer activity, but the first two do not [88]. The glycosylated form of ergosterol peroxide was found to be a greater inhibitor to the proliferation of K562, Jurkat, WM-1341, HL-60, and RPMI-8226 tumor cell lines [88].

2.4. Protein. Most of the proteins in *C. sinensis* are enzymes, including the intracellular proteases and extracellular proteases.

2.4.1. CSDNase. A new acid deoxyribonuclease (DNase) that acted at an acidic pH without divalent ions was extracted from *C. sinensis* and designated CSDNase. Although acid DNase was first studied biochemically in the 1960s [89], its structure has not been elucidated. DNases may be broadly divided into two classes: DNase I and DNase II. CSDNase belongs to the latter. The protein was purified by $(NH_4)_2SO_4$ precipitation and a series of chromatographic separations. It was found to be single-chained with an apparent molecular mass of 34 kDa and act on both dsDNA and ssDNA as a deoxyribonuclease but act preferentially on dsDNA. The activity of CSDNase was primarily expressed during fungal mycelium growth and it was an endocellular enzyme [90]. Furthermore, CSDNase was an endonuclease, which was found to hydrolyze DNA and to generate 3-phosphate and 5-OH termini. These results indicated that the nucleolytic properties of CSDNase were essentially the same as those of other well characterized acid DNases.

2.4.2. CSP. A novel serine protease with fibrinolytic activity named CSP was purified from the culture supernatant of the fungus *Cordyceps sinensis*. CSP is a single polypeptide chain with an apparent molecular weight of 31 kD. It is a novel extracellular protease with a free cysteine residue near the active site. It also can hydrolyse bovine serum albumin (BSA) and human serum albumin (HSA) to a lesser extent. Li et al. have found that CSP was a plasmin-link protease but not a plasminogen activator, and it preferentially cleaved the Aα chain of fibrinogen and the α-chain of fibrin. In conclusion, the presence of CSP possibly linked *C. sinensis* to its pharmacological use for cardiovascular disease, which will provide a new insight into the protein engineering of new thrombolytic agents [91].

2.5. Amino Acid and Polypeptide. C. sinensis contains many amino acids and polypeptides, which played an important role in clinical trials. For example, some polypeptide macromolecule in *C. sinensis* could significantly reduce the mean arterial pressure of rats and induce a direct endothelium-dependent vasorelaxant effect by stimulating the production of nitric oxide and endothelium-derived hyperpolarizing factor [92]. Thus, it could be used for the treatment of hypertension. As a result, it is necessary to explore the pharmacological effects of amino acid and polypeptide in *C. sinensis*.

2.5.1. Cordymin. Cordymin is a peptide from the medicinal mushroom *Cordyceps sinensis* with the putative beneficial effect on diabetic osteopenia in diabetic rats. The relationship between diabetes and osteoporosis is widely studied [93, 94]. However, the mechanism of cordymin for the treatment of diabetic osteopenia is complicated. To sum up, the significant effect of cordymin on diabetic osteopenia might be directly through weakening ALP and TRAP activity and mediately through recovery of β cells and lowering the concentration of serum glucose, which subsequently triggered a lower extent of oxidative stress in diabetic rats [95]. All those findings indicated a major breakthrough for the treatment of diabetic osteopenia using monomer composition—cordymin.

2.5.2. Cordycedipeptide A. A new cyclodipeptide named cordycedipeptide A was isolated from the culture liquid of *Cordyceps sinensis* (Figure 8). Its structure was elucidated as 3-acetamino-6-isobutyl-2,5-dioxopiperazine. Jia et al. have reported the cytotoxic activities of the constituent to L-929, A375, and Hela and its better effect on several tumor cell lines [96]; another pharmacological action of cordycedipeptide A remains to be further researched however.

2.5.3. Cordyceamides A and B. Two new aurantiamides named as cordyceamides A and B were isolated from

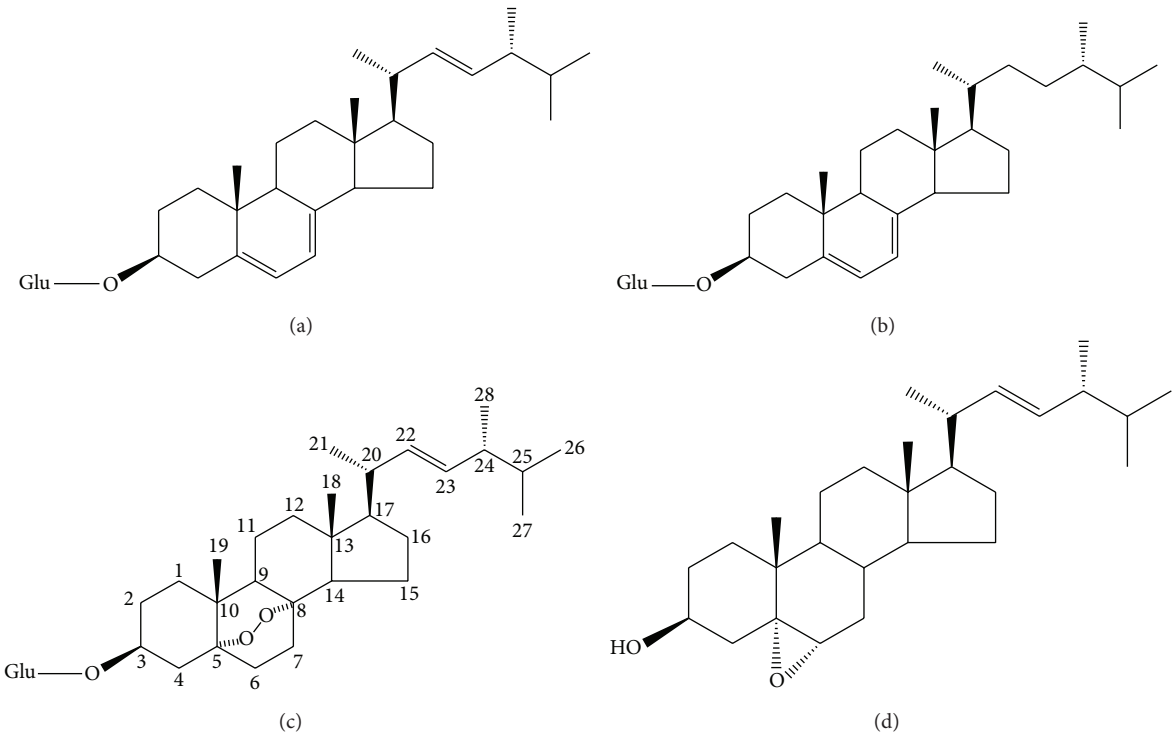


Figure 7: Four important sterol compounds.

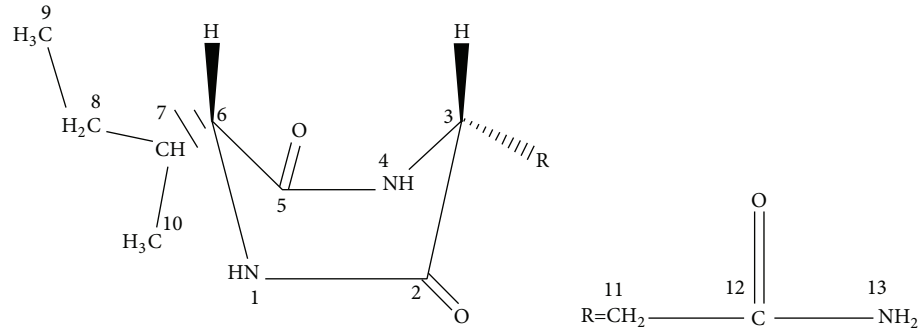
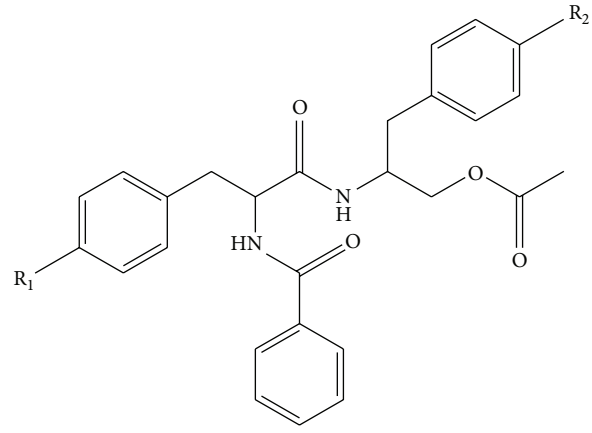


Figure 8: The structure of cordycedipeptide A.

the culture liquid of *Cordyceps sinensis* (Figure 9). Their structures were elucidated as N-benzoyl-L-tyrosinyl-L-phenylalaninol acetate and N-benzoyl-L-tyrosinyl-L-p-hydroxyphenylalaninol acetate by NMR techniques. Previous studies suggested that both Cordyceamides A and B had cytotoxic effects on L929, A375 and Hela cell lines. A showed better effect than B on L929 cell and A375 cell, but on Hela cell B showed better effect [97].

2.5.4. Tryptophan. There are 18 kinds of amino acids in *C. sinensis* and they mainly played a sedative hypnotic effect. Tryptophan is the most effective ingredient among them. Tryptophan is the precursor of serotonin material, which has close relationship with animals' insomnia [98]. Otherwise, glutamic acid has the effect of immune inhibition. Due to the performance of the combined effect being more complex, more research needs to be explored.



(a) R_1=OH, R_2=H (b) R_1=OH, R_2=OH

Figure 9: The structure of (a) and (b). (a) Cordyceamides A. (b) Cordyceamides B.

Table 1: Chemical constituents and their corresponding pharmacological actions of *C. sinensis*.

Chemical constituents of *C. sinensis*	Pharmacological effects	References
Cordycepin	Anti-inflammatory effect Analgesic effect Stimulates steroidogenesis Enhances immunity Antitumor activity Antibacteria, antivirus, and insecticidal activities	[17, 21–30]
Adenosine	Anticonvulsant activity Inhibits cancer cell growth Anti-inflammatory effect	[20, 31–46]
EPSF	Immunomodulatory effect Antitumour effect Antioxidant effect	[60–65]
APS	Antioxidant effect Immunomodulatory effects	[66–69]
CPS-1	Antioxidant effect	[70, 71]
CPS-2	Inhibits cell proliferation	[72, 73]
Mannoglucan	Cytotoxicity activity	[74]
CME-1	Antioxidant effect	[75]
Cordyglucans	Antitumour effect	[76]
CS-F10	Hypoglycemic activity	[55, 77]
Cordysinocan	Induces cell proliferation	[77]
Ergosterol	Cytotoxicity Antimicrobial activity	[78–83]
H1-A	Immunoregulation	[84–86]
CSDNase	Hydrolyzes DNA Nucleolytic properties	[89, 90]
CSP	Fibrinolytic activity	[91]
Cordymin	Antidiabetic	[93–95]
Tryptophan	Sedative hypnotic effect	[98]
Cordycepic acid	Treating liver fibrosis diuretic Improving the plasma osmotic pressure Anti-free radical	[99–103]
Monosaccharide saponins	Antitumor activity	[104]

2.6. Others. *C. sinensis* contains a lot of D-mannitol, also known as cordycepic acid, and the content in insect body is higher than that in stroma. Its structure has been determined as 1,3,4,5-tetrahydroxy-cyclohexanoic acid, isomeric with quinic acid. It differs mainly from the natural quinic acid in being dextrorotatory and not forming the lactone [99]. Cordycepic acid played a significant role in treating liver fibrosis of hepatic stellate cells. Liver fibrosis is a variety of factors involved in complex process [100]. Cordycepic acid ameliorates the LPs-induced inflammatory phenotype and TGFβ1-induced fibrogenic response of cultured HSCs, which are the drug's therapeutic mechanisms to inhibit and resolve liver fibrosis [101]. Additionally, cordycepic acid in *C. sinensis* has effects on diuretic, improving the plasma osmotic pressure and anti-free radical according to pharmacological research and clinical reports [102, 103]. So it is regarded as one of the active ingredients in *C. sinensis*.

A new monosaccharide saponin, whose structure is 3-O-glucopyranoside, was isolated and identified from the mycelia of *C. sinensis*. It displayed very good antitumor activity, but the content of the mycelia was very low [104].

Moreover, there are many other ingredients in the fungi *C. sinensis*, for example, alkane, polyamine, vitamin, and microelement. Each of these has its own pharmacological effects. More constituents need to be separated and studied in the future research work, however.

3. Conclusion

C. sinensis, a macro fungus of biomedical importance, contains a number of bioactive components (Table 1). Many of them are biological response modifiers which activate our immune systems for a multitude of defensive functions. The immunomodulating effects are associated with its antitumour

activity, which is the most proverbial effect of *C. sinensis*. Many ingredients in *C. sinensis* have the antitumour activity as shown above, such as cordycepin, adenosine, EPSF, cordyglucans, and monosaccharide saponins. As investigation into this fungus continues, more bioactive constituents with potential therapeutic value will be isolated. However, new methods and technologies need to be adopted to extract and analyse the components, requiring evaluation along the modern scientific line. Overall, so far, we know only a little of the wonders of this creature and it still has many secrets for us to discover. More research is needed on the herbal medicine and its related species.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Agaricoglycerides Protect against Hepatic Ischemia/Reperfusion Injury by Attenuating Inflammatory Response, Oxidative Stress, and Expression of NF-κB

Xiang-qian Zhao, Bin Liang, Yang Liu, and Xiao-qiang Huang

Hospital & Institute of Hepatobiliary Surgery, Chinese PLA General Hospital, Beijing 100853, China

Correspondence should be addressed to Xiang-qian Zhao; xiangqianzhao@126.com

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We have investigated the effects of agaricoglycerides (AG) in a mouse model of hepatic I/R injury. I/R triggered increases/changes in markers of liver injury, hepatic oxidative stress, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and nuclear factor κB (NF-κB). AG significantly reduced the extent of liver inflammation and oxidative stress and also attenuated the NF-κB activation as well as TNF-α and IL-1β production. Our results indicate that AG may represent a novel protective strategy against I/R-induced injury and inflammatory diseases.

1. Introduction

Inflammation has been recognized as a major risk factor for various human diseases. Hepatic ischemia reperfusion (IR) injury often arises from shock and various surgical procedures including liver transplantation. It has been demonstrated that hepatic IR injury can be characterized as an inflammatory response including activation of Kupffer cells, release of proinflammatory cytokines and chemokines, increased expression of adhesion molecules, and leukocyte infiltration [1]. This pathology leads to acute inflammatory responses including activation of Kupffer cells, release of proinflammatory cytokines, and leukocyte infiltration that ultimately causes hepatocellular damage and organ dysfunction [2]. So the pharmacological agents effective for the treatment of inflammatory diseases may also be employed in IR. New anti-inflammatory targets continue to be identified, which is an important area for translational medicine in IR.

Mushrooms have been considered as an edible and medicinal resource for thousands of years. *Grifola frondosa* (GF) is a Basidiomycete fungus belonging to the order Aphyllophorales and family Polyporeceae. It has recently attracted considerable attention for its various physiological activities [3–6]. The extracts from the basidiomas of GF exerted a highly significant hepatoprotective effect by reducing the paracetamol-induced acute elevation of the AST and ALT levels [7]. However, the ingredients accounting for its hepatoprotective effects were not yet understood in terms of modern pharmacological concepts.

The agaricoglycerides (AG) are a new class of fungal secondary metabolites that constitute esters of chlorinated 4-hydroxy benzoic acid and glycerol (Figure 1). Agaricoglycerides showed strong activities against neurolysin, a protease involved in the regulation of dynorphin and neurotensin metabolism, and even exhibited anti-inflammatory and antinociceptive properties [8, 9]. However, the effect of AG on liver I/R injury is poorly understood. In light of the previous protective effects of AG, we therefore investigate whether AG protects the liver from I/R in an experimental mouse model.

2. Material and Methods

2.1. Agaricoglycerides. The methodology for extraction of AG was described in detail by Han [10]. Briefly, mycelia were separated from fermented mushroom of GF by filtration and extracted twice with acetone in an ultrasonic bath. The extract was filtered, and the acetone was removed *in vacuo* to yield an aqueous residue. This residue was diluted with tap

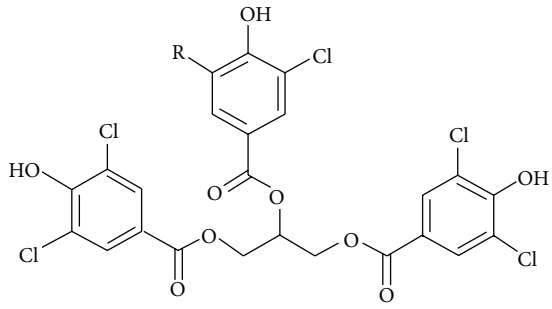


Figure 1: Chemical structures of agaricoglyceride.

water and subsequently extracted three times with EtOAc. The combined organic phases were dried over Na_2SO_4 and evaporated *in vacuo* to yield an oily residue.

2.2. Animals. Male C57BL/6J mice (25–30 g) were used in the study. All animal experiments followed the guidelines published by the Ministry of Science and Technology of China. Care was taken to minimize discomfort, distress, and pain to the animals.

2.3. Experimental Design. The animals were separated into five groups of ten mice each. The first group served as sham (SHAM). The second group was I/R group (I/R). Group I and group II were treated orally by distilled water for 30 days, respectively. Group III (AG-100), Group IV (AG-300), and Group V (AG-500) were treated orally by AG (100, 300, and 500 mg/kg/day, resp.) for 30 days followed by I/R.

Mice were anesthetized with pentobarbital (65 mg/kg intraperitoneally (ip)). A midline laparotomy incision was performed to expose the liver. The hepatic artery and the portal vein were clamped using microaneurysm clamps. This model results in a segmental (70%) hepatic ischemia as described [11, 12]. The duration of hepatic ischemia was 60 min, after which the vascular clips were removed and liver was reperfused for 2, 6, or 24 h, as indicated. Sham surgeries were identical except that hepatic blood vessels were not clamped with a microserrefine. The liver was kept moist at 37°C with gauze soaked in 0.9% saline. Body temperature was maintained at 37°C using a thermoregulatory heating blanket and by monitoring body temperature with a rectal temperature probe. After reperfusion, blood was collected and liver samples were removed and snap-frozen in liquid nitrogen for determining biochemical parameters or fixed in 4% buffered formalin for histopathological evaluation.

2.4. Estimation of Serum ALT and AST. Serum ALT and AST activity was measured colorimetrically using a diagnostic kit (procedure number 505, Sigma Chemical Co., St Louis, MO) according to the instructions provided.

2.5. Estimation 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).

2.6. Estimation of Liver TNF-α and IL-1β. Liver samples were disintegrated in 5 volumes of ice-cold RIPA buffer. After incubation on ice for 30 minutes, samples were centrifuged twice at 20,000 ×g for 15 minutes at 4°C. The resulting supernatants were used for assay. The hepatic concentration of TNF-α and IL-1β was measured by the way introduced by Moon et al. [12] using a commercial ELISA kit (Shanghai Jinma Biological Technology Inc., China) following the manufacture's instruction.

2.7. Quantification of NF-κB Activity. Liver tissue extracts were obtained by homogenization of snap-frozen liver tissue in Cell Lysis Buffer, subsequent sonication, and centrifugation. Activated NF-κB was quantified in liver 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.

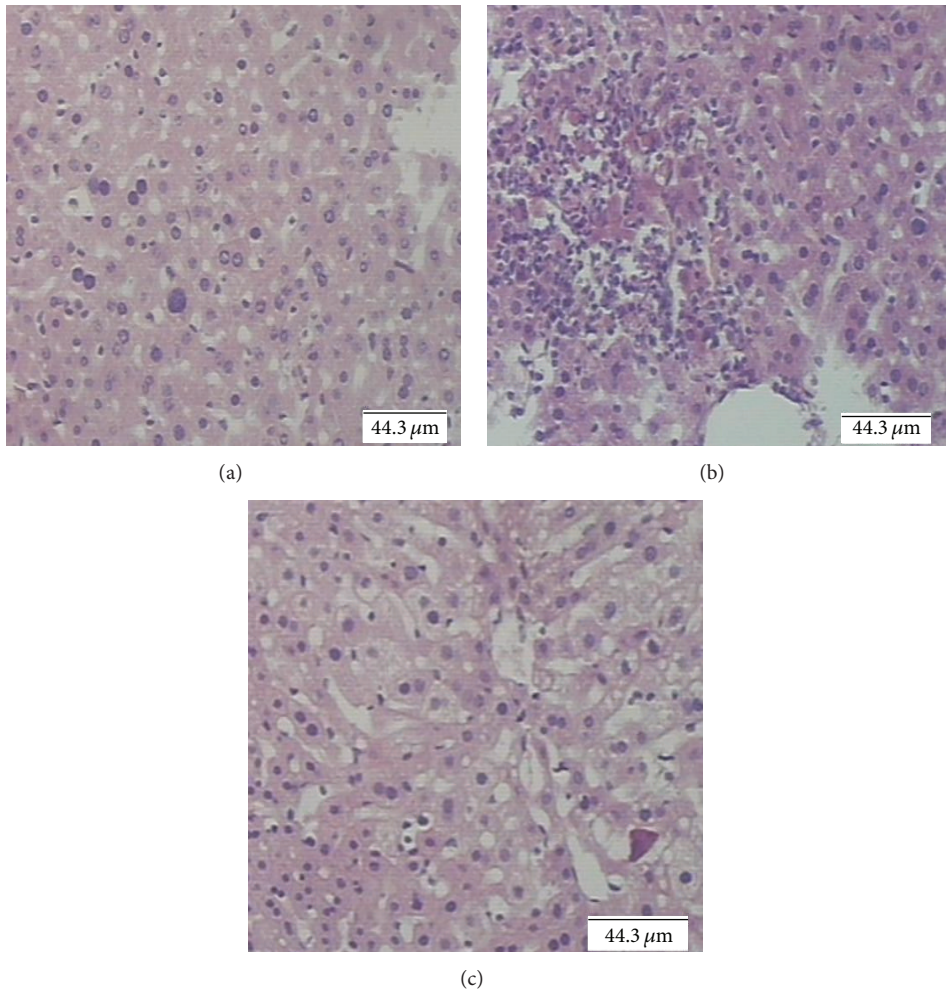
2.8. Measurement of Total Antioxidant Status. The total antioxidant status (TAOS) of liver was determined as previously described by Han [9]. The increase of absorbance at 405 nm was measured by a microplate reader (Shanghai Xunda Medical Technology Inc., China).

2.9. Histological Examination of Liver Sections. Liver 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 H&E for subsequent light microscope examination. Histological evaluation was performed in a blinded manner. A minimum of two slides per rat were read.

2.10. Statistical Analysis. All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data were shown as the mean ± SEM. The significant level of 5% ($P < 0.05$) was used as the minimum acceptable probability for the difference between the means.

3. Results and Discussion

In the setting of transplantation, the "injury hypothesis" states that I/R injury to the organ activates a cascade of innate-immunity-dominated proinflammatory responses [13]. The recently described cholinergic anti-inflammatory pathway is a mechanism through which the central nervous system regulates excessive inflammation and limits self-damage [14]. Agaricoglycerides (AG) are a new class of fungal secondary metabolite that has been shown to exert antioxidant and anti-inflammatory effects both in vitro and in various preclinical models of inflammatory disorders [8, 9]. In this study, we demonstrated that AG exerts protective effects against liver



(a) (b)

(c)

Figure 2: AG decreases I/R-induced neutrophil infiltration after I/R injury. I/R followed by 24 h of reperfusion dramatically increased neutrophil infiltration into the livers (b), which was attenuated by AG pretreatment (c). In livers of sham-operated mice there was no tissue inflammatory cell infiltration (a).

I/R reperfusion damage by attenuating major proinflammatory and NF-κB signaling pathways as well as oxidative stress.

3.1. Effect of AG on Liver Enzymes. The magnitude of hepatic damage is assessed by measuring the level of released cytosolic transaminases including ALT and AST in circulation, since AST and ALT are sensitive indicators of liver cell injury [15]. In the present study, the I/R-induced increase in serum ALT and AST activity was attenuated by AG. Table 1 illustrated that a significant increase of ALT and AST level was observed in the I/R group, as compared to the sham mice ($P < 0.05$). AG-500 suppressed this response ($P < 0.05$). On histologic analysis, I/R induced marked congested central veins and blood sinusoids as well as many hepatocytes with deeply stained acidophilic cytoplasm and dark stained nuclei (Figure 2(b)). It was dramatically reduced and became more focal in AG-500 treated mice (Figure 2(c)). The results of the current study provide noticeable evidence that oral administration of 500 mg AG/kg/day for 30 days prior to ischemia protected the liver from I/R injury.

Table 1: Effect of AG on ALT and AST level.

Different groups	ALT (U/L)	AST (U/L)
Sham group	31.0 ± 3.2*	36.0 ± 6.2*
I/R group	110.2 ± 21.0	111.5 ± 11.3
AG-100 group	97.1 ± 10.3	92.0 ± 8.0
AG-300 group	83.1 ± 8.8	63.6 ± 9.2
AG-500 group	45.1 ± 8.0*	42.6 ± 8.1*

Values are shown as means ± SEM, * $P < 0.05$ versus I/R group.

3.2. Effect of AG on MDA Level and TAOS. Hepatic MDA activity is commonly used as an indicator of liver tissue damage involving a series of chain reactions [16]. Accordingly, we sought to determine whether AG would provide antioxidation by measuring the MDA level. The hepatic tissues from sham animals contained low MDA level. MDA in I/R group was significantly higher than that of sham group ($P < 0.01$). MDA level in AG-300 and AG-500 group was significantly lower than that of I/R group ($P < 0.05$

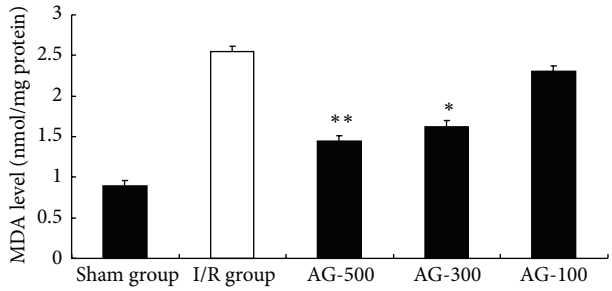


Figure 3: Effect of AG on MDA level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus I/R group. $^{**}P < 0.01$ versus I/R group.

Table 2: Effect of AG on TAOS activity (μM L-ascorbate).

Different groups	TAOS activity (μM L-ascorbate)
Sham group	$28.41 \pm 3.17^{**}$
I/R group	80.33 ± 9.32
AG-100 group	$72.22 \pm 2.78^{*}$
AG-300 group	$64.30 \pm 3.38^{*}$
AG-500 group	$56.35 \pm 4.33^{**}$

Values are shown as means ± SEM, $^{*}P < 0.05$ versus I/R group, $^{**}P < 0.01$ versus I/R group.

and $P < 0.01$, resp.) (Figure 3). Growing evidence showed that oxidative stress was involved in liver damage after I/R. MDA is an end-product of free radical formation and lipid peroxidation as well as an index of ROS-mediated injury that results from an imbalance between radical generating and scavenging systems leading to cell membrane impairment or DNA damage [17]. In the present study, administration of AG led to significant reductions in hepatic MDA content compared to levels in the I/R hosts, indicating a reduction in oxidative stress and an enhanced antioxidant state in these hosts' livers. These results indicated that the free radicals being released in the liver were effectively scavenged by AG, which may also account for its anti-inflammatory properties.

The TAOS is an indication of O_2^- and other oxidant species. We measured TAOS activity as an indirect indication of the formation of O_2^- and other oxidant species. The results of hepatic TAOS are shown in Table 2. TAOS in the I/R group was significantly ($P < 0.01$) higher than those in the sham group. Those in the AG-300- and AG-500-treated groups were significantly lower than those in the ethanol-treated group ($P < 0.05$ and $P < 0.01$, resp.). O_2^- is produced by polymorphonuclear leukocytes and macrophages from the enzyme activity of NADPH oxidase and xanthine oxidase at inflammatory sites. The AG groups had the lower level of TAOS activity in comparison to I/R group. We hypothesized that AG produces anti-inflammatory effect through decreasing the levels of TAOS activities.

3.3. Effects of AG on Liver TNF-α and IL-1β Level. TNF-α and IL-1β are proinflammatory cytokines which play a critical role in the initiation and progression of hepatic I/R. One of the hallmarks of I/R is the elevated TNF-α and IL-1β level in serum at the early phase of reperfusion [18]. Therefore,

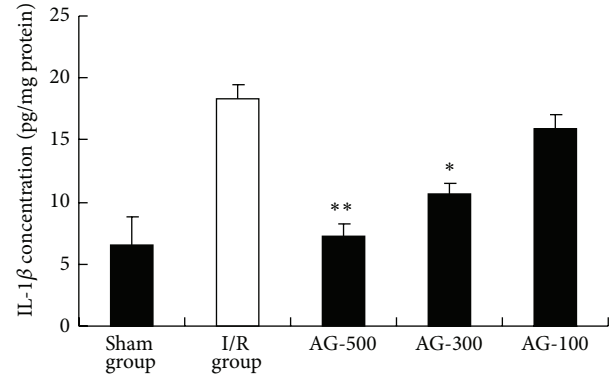


Figure 4: Effect of AG on IL-1β level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus I/R group. $^{**}P < 0.01$ versus I/R group.

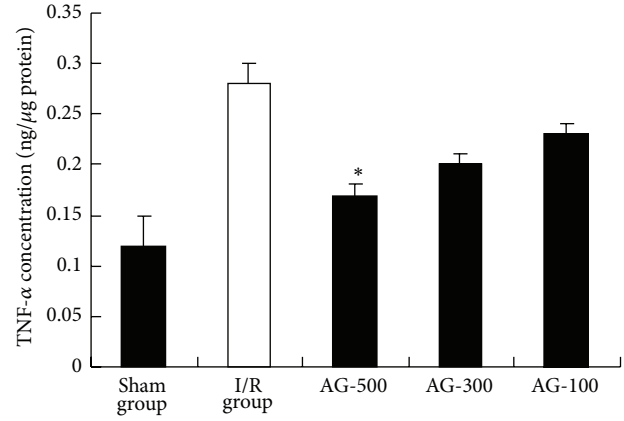


Figure 5: Effect of AG on TNF-α level. Values represent the mean ± SEM. $^{*}P < 0.05$ versus I/R group.

the effect of AG on TNF-α production was determined by ELISA. In comparison to I/R group (Figure 4), treatment with AG-300 and AG-500 resulted in a marked decrease in IL-1β levels compared with those in I/R group ($P < 0.05$ and $P < 0.01$, resp.). In addition, the levels of TNF-α were significantly increased in I/R group (Figure 5). AG-500 suppressed I/R-induced TNF-α production ($P < 0.05$). As a proinflammatory cytokine, TNF-α can result in liver microcirculation disturbance by interacting with neutrophils and endothelial cells [19]. Besides, IL-1β is considered a valid target for therapeutic intervention in diseases associated with tissue remodeling [20]. Thus, downregulating the level of TNF-α and/or IL-1β will be beneficial for the alleviation of I/R injury.

3.4. Effects of AG on Protein Expression of NF-κB. To further investigate the mechanisms of AG in hepatic I/R, the protein expression of NF-κB was detected after liver transplantation. Substantially, hepatic I/R induced a predominant increase in nuclear translocation of NF-κB (Figure 6(a)). Conversely, level of NF-κB protein decreased in the nucleus of liver cells of AG-500 group (Figure 6(b)). NF-κB is recognized to have pivotal roles in the inflammatory response to hepatic I/R [21]. Moreover, it is reported that the generation of NF-κB can

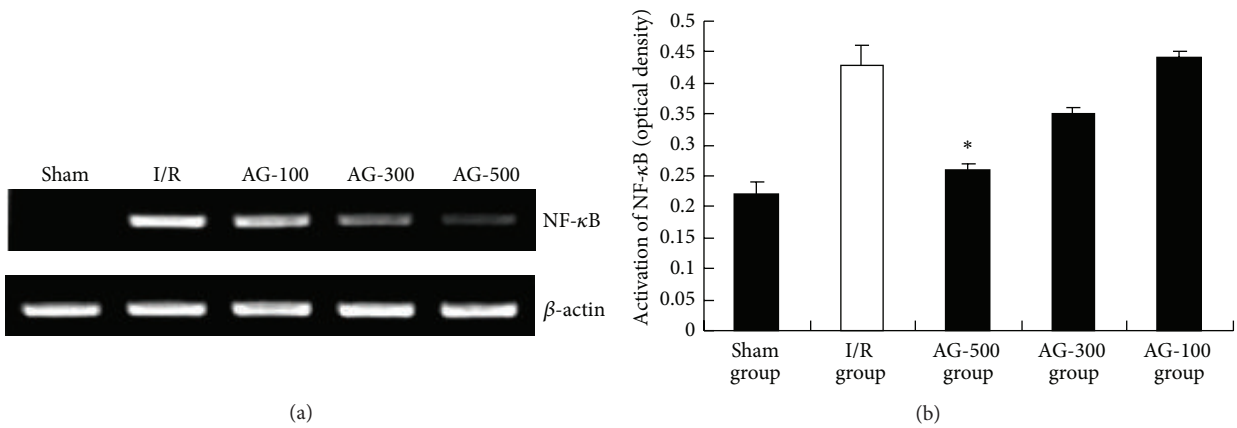


Figure 6: Effect of AG on protein expression of NF-κB. Values represent the mean ± SEM. $^{*}P < 0.05$ versus I/R group.

induce the increase of proinflammatory cytokines, such as TNF-α and IL-1β, which can further make I/R injury more severe [22].

As expected, nuclear protein levels of NF-κB increased after reperfusion in the present study. The increased NF-κB protein expression was attenuated by AG-500. So we can speculate that AG performs its liver protective effect partly due to the regulation on NF-κB, which can further influence the expression of TNF-α and IL-1β.

Collectively, our results indicate that AG may represent a novel protective strategy against I/R-induced injury and inflammatory diseases by attenuating the acute and chronic proinflammatory responses, oxidative stress, and expression of NF-κB depending on the dose employed.

Conflict of Interests

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

Acknowledgment

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Research Article

Protective Effect of Polysaccharides from *Inonotus obliquus* on Streptozotocin-Induced Diabetic Symptoms and Their Potential Mechanisms in Rats

Bao-zhong Diao,[1] Wei-rong Jin,[1] and Xue-jing Yu[2]

[1] *Department of Pharmaceutical Preparations, Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University, Liaocheng, Shandong 252000, China*
[2] *Department of Endocrinology, The First Affiliated Hospital of Jiamusi University, Jiamusi, Heilongjiang 154002, China*

Correspondence should be addressed to Xue-jing Yu; xuejingyujms@yeah.net

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The present study aimed to evaluate the therapeutic effects of polysaccharides from *Inonotus obliquus* (PIO) on streptozotocin- (STZ-) induced diabetic symptoms and their potential mechanisms. The effect of PIO on body weight, blood glucose, damaged pancreatic β-cells, oxidative stresses, proinflammatory cytokines, and glucose metabolizing enzymes in liver was studied. The results show that administration of PIO can restore abnormal oxidative indices near normal levels. The STZ-damaged pancreatic β-cells of the rats were partly recovered gradually after the mice were administered with PIO 6 weeks later. Therefore, we may assume that PIO is effective in the protection of STZ-induced diabetic rats and PIO may be of use as antihyperglycemic agent.

1. Introduction

Diabetes mellitus is a metabolic disorder affecting carbohydrate, fat, and protein metabolism. Long-standing diabetes is prone to various complications which include cardiac, kidney, and eye problems [1]. More effective and safer treatment modalities for diabetes mellitus need to be investigated. PIO is a mushroom habiting in the cold latitudes of Europe and Asia, which was used as traditional Chinese medicine for a long time. In the last decade, several studies have reported biological activities of PIO such as anticancer, antioxidation, anti-inflammatory, and antihyperglycemic activities and enhancement of immunity [2–5]. Up to now, however, no detailed investigation has been carried out on the effective constituents of PIO for antihyperglycemic activities. At the same time, the limited natural resources of *I. obliquus* limit its role as therapeutic agent for diabetes mellitus.

Many studies have shown that polysaccharides from PIO possessed clear antioxidant activities [6, 7]. There is growing evidence that free-radical-mediated oxidative processes are involved in the pathogenesis of diabetic complications and oxidative stress is implicated in cardiac dysfunction, leading to heart failure in diabetes [8]. In the present study, the purpose is to focus on the isolation and hypoglycemic properties of polysaccharide fractions from fermented mushroom of PIO for seeking new natural functional ingredients used in food and pharmaceutical industry to alleviate the diabetes mellitus.

2. Materials and Methods

2.1. Fermented Mushroom of Inonotus obliquus. A strain of PIO was used in this study. The seed was grown at 27°C for 7 days on PDA slants (1,000 mL 20% potato extract liquid +20.0 g dextrose +20.0 g agar). 10 pieces of the mycelia of *Inonotus obliquus* were transferred from a slant into each Erlenmeyer flask containing 50 mL seed medium with the sterilized self-designed cutter. The culture was incubated at 27°C-28°C on a rotary shaker at 180 rmp for 8 days.

2.2. Preparation of Polysaccharides from I. obliquus (PIO). Dried mycelium of *I. obliquus* was extracted with distilled water (600 mL) at 121°C for 2 h. After cooling and filtration,

the extract was concentrated to one-tenth of the volume and precipitated with 4 vol of 95% ethanol at 4°C for 24 h. Polysaccharides were precipitated from resuspended extracts using 75% ethanol followed by exhaustive dialysis with water for 48 h, giving thewater-soluble polysaccharide of PIO.

2.3. Animals. Healthy male adult Wistar rats (2 months old and weighing 200 ± 20 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 of the animals. Experimental diabetes was induced by intraperitoneal (i.p.) injection with freshly prepared solution of STZ (Sigma, USA) dissolved in citrate buffer (pH 4.5) at the dose of 35 mg/kg body weight. Only rats with blood glucose concentration more than 240 mg/dL were considered diabetic and used for the study. Glucose level was assessed by using enzymatic glucose oxidase peroxidase commercially available kit method, 72 h after STZ induction. The rats with blood glucose concentration more than 240 mg/dL were considered diabetic and used for the study.

2.4. Treatment Schedule and Experimental Protocol. Forty hyperglycemic rats were selected and allocated equally into 4 groups and administered orally saline, PIO (10 mg/kg/d), PIO (20 mg/kg/d), and PIO (30 mg/kg/d), respectively. The other 10 normal rats were administered orally with the saline and used as the control group.

Body weight of all animals was recorded on 0, 1st, 2nd, 3rd, 4th, 5th, and 6th week of treatment. Blood of all animals was collected through retroorbital route initially and on 6th week of treatment to measure the serum glucose levels. Then, the rats were sacrificed. The blood sample was allowed to clot for 20 minutes at refrigerator temperature. The blood samples were then shifted to clean centrifuge tubes. Lithium heparin was added to obtain plasma. The withdrawn blood was separated by centrifugation at 4000 rpm for 10 minutes to obtain serum. The serum was stored in freezer until analysis. The liver was dissected out for the measurement of IL-1β and TNF-α. The pancreas was reserved for pathological histology using hematoxylin and eosin (H&E) staining.

2.5. Measurement of IL-1β and TNF-α Level in Liver. The liver was dissected out for the measurement of hepatic glycogen. The liver TNF-α and IL-1β were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Jinma Biological Technology, Inc., China) following the manufacture's instruction.

2.6. Measurement of Lipid Profile. Total cholesterol (TC), triglycerides (TAG), low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol were determined in the serum samples using commerciallyavailable kits (Shanghai Jinma Biological Technology, Inc., China).

2.7. Measurement of Glucose Metabolizing Enzymes. The liver homogenate was used to assess metabolizing enzymes. Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and lactate dehydrogenase (LDH) were measured using commerciallyavailable kits (Shanghai Jinma Biological Technology, Inc., China).

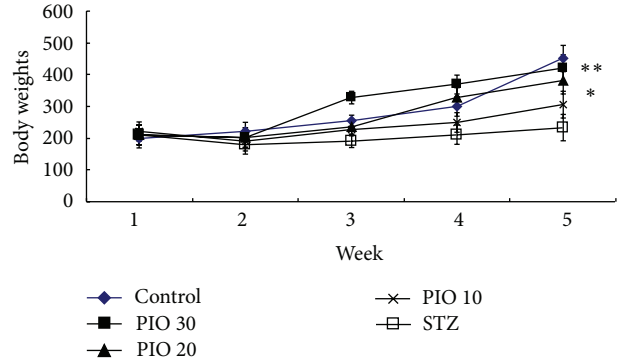


Figure 1: Effect of PIO administration on diabetic rats' body weight. Values represent the mean ± SEM. $^{*}P < 0.05$ versus STZ group.

2.8. Estimation of the Total Antioxidant Activity. The total antioxidant status (TAOS) of hepatic tissue was determined by the way introduced by Laight et al. [9]. The increase in absorbance at 405 nm was measured by using a microplate reader (Shanghai Xunda Medical Technology, Inc., China).

2.9. Statistical Analysis. All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data represents means and standard deviations. The significant level of 5% ($P < 0.05$) was used as the minimum acceptable probability for the difference between the means.

3. Results and Discussion

The objective of this study was to investigate whether the polysaccharides from *I. obliquus* (PIO) could produce hypoglycemic activity in STZ-induced diabetic rats. STZ is an antibiotic extracted from *Streptomyces achromogenes* and is diabetogenic due to a selective cytotoxic action upon pancreatic β-cell [10]. In the present investigation, STZ injected rats exhibit clinicopathological features including biochemical, oxidative, and metabolic changes. These changes were halted in PIO treated animals.

Many studies have shown an association between hyperglycemia and decreased body weight of diabetic animals [11]. As shown in Figure 1, the STZ-treated animals had significantly reduced body weight than the control rats ($P < 0.01$). When compared with STZ-treated animals, the body weight gains were significantly increased in groups of PIO-treated animals ($P < 0.05$; $P < 0.01$) in a dose-dependent manner.

STZ in the experimental diabetic model leads to defective glucose oxidation and causes hyperglycemia [12]. Our study is in agreement with this report. The blood glucose level in normal rats remained constant for six weeks and was

Table 1: Effect of PIO on blood glucose levels in STZ-hyperglycemic rats.

Different groups	Blood glucose (mmol/L)
STZ group	22.2 ± 2.2
PIO (30 mg) group	10.1 ± 3.2*
PIO (20 mg) group	15.5 ± 2.0
PIO (10 mg) group	18.6 ± 3.0
Control group	5.9 ± 1.2

Values are means ± SEM; $n = 10$. *$P < 0.05$ versus STZ group.

Table 2: Effect of PIO on TAOS activity (μM L-ascorbate).

Different groups	TAOS activity (μM L-ascorbate)
Control group	28.40 ± 3.10
STZ group	81.33 ± 5.32
PIO (10 mg) group	72.24 ± 2.78*
PIO (20 mg) group	65.30 ± 3.31*
PIO (30 mg) group	56.30 ± 4.34**

Values are shown as means ± SEM; *$P < 0.05$ versus STZ group; **$P < 0.01$ versus STZ group.

significantly ($P < 0.01$) lower than those of streptozotocin-induced diabetic rats (Table 1). Upon treatment with PIO for six weeks, the blood glucose levels of all diabetic rats were markedly diminished in a dose-dependent manner, suggesting that DPM is a potent therapeutic agent against diabetes.

The hypoglycemic mechanisms of many polysaccharides are closely related to their antioxidant activity [13]. Hence, it is plausible that the hypoglycemic effect of PIO may be due to the effect on alleviating oxidative stress. The TAOS is an indication of O_2^- and other oxidant species. We measured TAOS activity as an indirect indication of the formation of O_2^- and other oxidant species. The results of hepatic TAOS are shown in Table 2. The STZ treatment increased TAOS. TAOS in the PIO-20- and PIO-30-treated groups were significantly lower than those in the STZ-treated group ($P < 0.05$ and $P < 0.01$, resp.).

It has been observed that over 75% of early deaths in diabetes are related to coronary artery disease caused by abnormal lipid metabolism, which often leads to altered lipid profile of the victim [14]. Lipid peroxidation is one of the characteristic features of chronic diabetes. The increased free radicals produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation. It will, in turn, result in the elevated production of free radicals [15]. In the present experiment, significantly increased lipid peroxidation products were observed in STZ-induced diabetic rats. Treated with PIO-20 and PIO-30 for 6 weeks, LDL level was reduced ($P < 0.05$), whereas HDL cholesterol was increased ($P < 0.05$) (Table 3). These results further confirm that there is a strong correlation between oxidative stress and diabetes occurrence.

It was suggested that the STZ-induced weight loss in animal was the result of protein wasting in a situation of unavailability of carbohydrate for utilization as an energy source [11]. In diabetes, cytoplasmic enzymes such as GOT, GPT, and LDH pass into blood plasma and their activities in serum increase [16]. In the present study, oral treatment of PIO-20 and PIO-30 significantly ($P < 0.05$) restored the altered glycoprotein components of diabetic rats in a dose-dependent manner (Table 4).

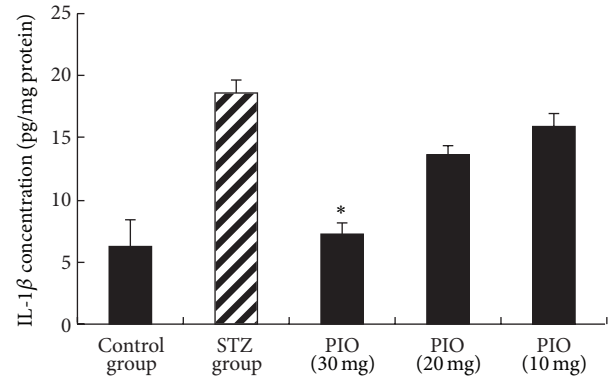


Figure 2: Effect of PIO on IL-1β level. Values represent the mean ± SEM. *$P < 0.05$ versus STZ group.

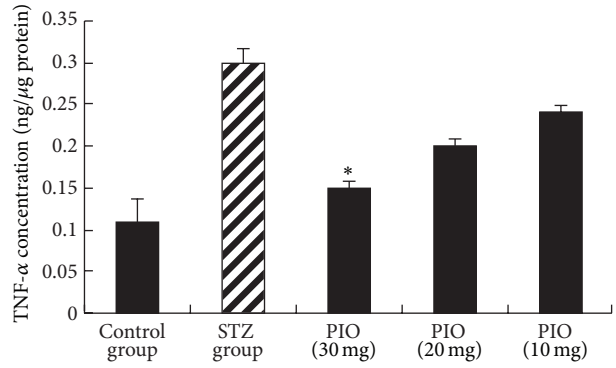


Figure 3: Effect of PIO on TNF-α level. Values represent the mean ± SEM. *$P < 0.05$ versus STZ group.

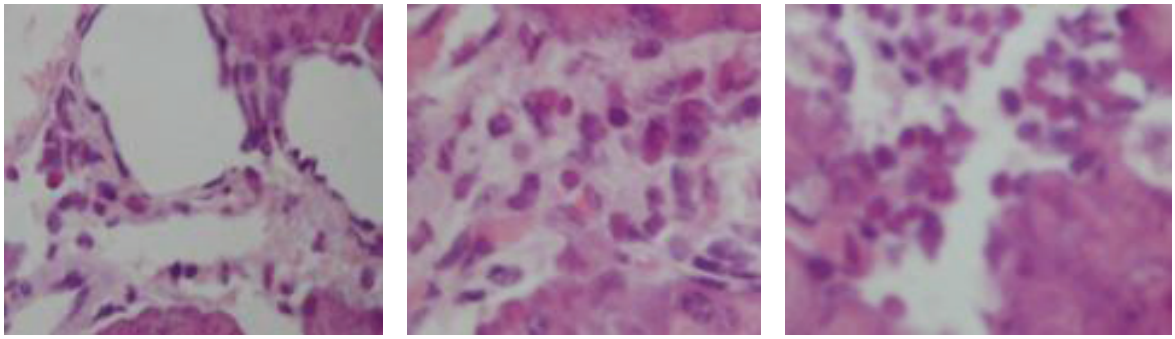
A chronic inflammation may have a role in the pathogenesis of metabolic disorders [17, 18]. Prospective studies have identified proinflammatory cytokines as predictors of diabetes [19]. TNF-α was the first proinflammatory cytokine implicated in pathogenesis of obesity-related insulin resistance and diabetes [20] and studies conducted with IL-1β antagonism beneficial effects on glycated hemoglobin and β-cell function [21]. Therefore, the effect of PIO on TNF-α and IL-1β production was determined by ELISA. In comparison to STZ group (Figure 2), treatment with PIO-30 resulted in a marked decrease in IL-1β levels ($P < 0.05$). In addition, PIO-30 suppressed STZ-induced TNF-α production ($P < 0.05$) (Figure 3).

STZ is a compound commonly used to induce diabetes in rodents. The mode of its action is mediated through the induction of severe damages to the β-cells [22]. The protective effect of PIO against the damages to β-cells induced by STZ toxicity was investigated. Selective destruction of pancreatic β-cells by STZ in the experimental diabetic model was

Table 3: Effect of PIO on changes in the levels of serum lipid profile.

Lipid profile mmol/L	Control group	STZ group	PIO (10 mg) group	PIO (20 mg) group	PIO (30 mg) group)
LPO	8.6 ± 0.51	13.3 ± 3.8	9.3 ± 0.31	8.9 ± 0.40	7.4 ± 0.48**
Cholesterol	4.30 ± 0.79	10.40 ± 0.85	8.30 ± 0.50	7.30 ± 0.80	4.34 ± 0.80
Triglycerides	0.80 ± 0.11	1.23 ± 0.10	0.98 ± 0.14	0.81 ± 0.09	0.78 ± 0.09
HDL	0.72 ± 0.09	0.85 ± 0.07	0.82 ± 0.25	0.86 ± 0.21*	0.74 ± 0.21**
LDL	0.28 ± 0.08	0.41 ± 0.09	0.37 ± 0.02	0.33. ± 0.05*	0.24 ± 0.05**

Values are shown as means ± SEM; * $P < 0.05$ versus STZ group; ** $P < 0.01$ versus STZ group.



(a) (b) (c)

Figure 4: Islet cell death and replication represented by hematoxylin-eosin. The islet cells of diabetic rat of STZ treatment (b) showed extensive cell lysis, representing loss of plasma membrane with condensed nuclei and dissolved cytoplasm in wide intercellular spaces. In contrast, the islet cell of PIO-fed rat (c) was partly recovered. (c) Islet from a normal rat.

Table 4: Effect of PIO on GOT, GPT, and LDH.

Groups	GOT (Unit L^{-1})	GPT (Unit L^{-1})	(Unit L^{-1})
Control group	79 ± 3.1	66 ± 3.3	48 ± 7.1
STZ group	288 ± 3.1	159 ± 3.7	316 ± 10.9
PIO (10 mg) group	185 ± 10.2	150 ± 11.3*	310 ± 11.1
PIO (20 mg) group	103 ± 4.0*	129 ± 30.0*	186 ± 10.2*
PIO (30 mg) group	88 ± 4.2*	81 ± 6.1*	77 ± 11.5*

Values are shown as means ± SEM; * $P < 0.05$ versus STZ group.

observed (Figure 4(b)). We observed focal necrosis, congestion in central vein, and infiltration of lymphocytes in the pancreas of STZ. Such lesions were considerably diminished by PIO-30 (Figure 4(c)). Further, β-cells structure of the PIO rats appeared normal. This indicated that PIO could significantly protect the β-cells from STZ-induced cell damage. This result strongly supported the therapeutic potential of PIO against diabetes.

In summary, we have shown that PIO has therapeutic effects against diabetes via multiple pathways. It displays antioxidant actions, hypolipidemic activity, and protects the pancreas from the diabetes induced injuries in STZ-treated rats. Therefore, PIO may provide a valuable therapeutic option against diabetes.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Research Article

Effects of Fermented Mushroom of *Cordyceps sinensis*, Rich in Selenium, on Uterine Cervix Cancer

Jing Ji,[1] Juan Liu,[2] Haijuan Liu,[1] and Yueling Wang[1]

[1] *Department of Gynecology and Obstetrics, First Affiliated Hospital of Medical School, Xi'an Jiaotong University, Xi'an 710061, China*
[2] *Department of Obstetrics, Maternal & Child Health Care Hospital of Shanxi Province, Xi'an 710003, China*

Correspondence should be addressed to Yueling Wang; yuelingwangdo@tom.com

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The purpose of this study was to investigate the effect of fermented mushroom of *Cordyceps sinensis* (CS), rich in selenium (Se-CS), on uterine cervical cancer in mice. The methylcholanthrene- (MCA-) induced tumor model was used in this paper. After the mice were administered Se-CS, the animals showed 40% tumor incidence ($P < 0.05$). Se-CS also enhanced the immune functions. Se-CS treatment showed significant ($P < 0.05$–0.01) restoration in the level of glutathione content, lipid peroxidation, glutathione peroxidase activity, glutathione reductase activity, catalase activity, Na^+/K^+-ATPase activity, and glutathione S transferase activity. This finding suggested that the concomitant use of Se and CS could be a potential therapeutic approach to improve the efficacy of therapy for uterine cervical cancer.

1. Introduction

Uterine cervical cancer is still the second most common cancer in women worldwide, despite the existence of effective screening methods [1, 2]. However, the treatment causes strong side effects such as digestive symptoms (vomiting and diarrhea) and bone marrow suppression. Thus, drugs with fewer side effects and a superior effect in combination are desired.

In humans, selenium (Se) is a trace element nutrient which functions as cofactor for reduction of antioxidant enzymes such as glutathione peroxidases. Several studies have suggested a possible link between cancer and selenium deficiency [3–5]. Some reports show that selenium administered to laboratory animals at levels above dietary requirements is capable of protecting against tumor formation in the mammary glands, liver, skin, colon, stomach, oral cavity, bladder, and pancreas [6–8]. However, it is toxic if taken in excess. Exceeding the tolerable upper intake level of 400 mg per day can lead to selenosis [9]. The present study evaluated the optimal concentration of Se, at which Se has equal efficacy but much lower toxicity. One novel selenium complex of Se-CS has been designed and evaluated. Using trace element at lower doses, in combination with edible mushrooms, has been ascribed as one potent way to reduce trace elements-associated toxicity and maintain their effect [10].

Cordyceps sinensis (CS) is a popular medicinal mushroom that has been used as a home remedy in traditional Chinese medicine for the prevention or treatment of a variety of diseases including cancer [11, 12]. Today, CS is recognized as a dietary supplement recommended in many countries as a cancer therapeutic. However, some of the trails were not well designed and lacked appropriate controls [2]. In the current study, we investigated the potential therapeutic efficacy of Se-CS for cervical cancer. The purpose of this study was to investigate the effect of fermented mushroom of Se-CS on methylcholanthrene-induced uterine cervical cancer in mice.

2. Materials and Methods

2.1. 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.

Female Kunming strain mice were maintained at room temperature under alternating natural light/dark photoperiod and had access to standard laboratory food and fresh water *ad libitum*.

2.2. Sodium Selenite Solution (SS). Sodium selenite was dissolved in saline solution (0.9% NaCl). An ampule was filled with 0.4 mL of SS and then was sterilized in a microwave oven for 3 minutes.

2.3. Se-Enriched Cordyceps sinensis (Se-CS). Se-CS was prepared according to study of Zhang et al. [9]. The only difference was that a series of 0 mg, 0.1 mg, 0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg, and 1 mg sodium selenite solutions was added to the substrate for obtaining Se-CS samples 1 (Se-0), 2 (Se-0.1), 3 (Se-0.2), 4 (Se-0.4), 5 (Se-0.6), 6 (Se-0.8), and 7 (Se-1), respectively [13, 14].

2.4. The Effect of Se on Growth of CS. The mycelia were harvested at the end of fermentation; then they were centrifuged at 12,000 g for 10 min and dried to constant weight at 60°C for sufficient time in laboratory vacuum ovens, and the dry weight of the mycelia was then measured.

2.5. Analysis of Organic Selenium in Se-CS. Se-CS was dialyzed against distilled water for 96 h by changing the water every 12 h until no Se was detected in the dialyzing water. Thus, Se compounds left in the dialyzed sample were considered to be organic selenium. The Se content was determined using graphite furnace atomic absorption spectrophotometry introduced by Zhang et al. [9]. Briefly, 100 grams of properly homogenized, dry mycelia was placed in a vitreosil crucible overnight at 410°C–440°C in an electric muffle furnace, maintaining the temperature 1200°C. The dry-ashing method destroys all of the organic materials present in the sample. The crucible containing pure ash was then taken out of the muffle furnace and kept in a desiccator [9]. Two grams of ash was digested with a mixture of hydrochloric acid and nitric acid in the ratio 1 : 3 [9]. The digested sample was dissolved in 50 mL of distilled water and used for analysis by means of an atomic absorption spectrometer (AAS-3200, Shanghai, China). The wavelength on selenium analyses was 196.0 nm.

Se concentrations in liver and kidney were determined using atomic absorption spectrometer (AAS-3200, Shanghai, China). The standard of selenium was purchased from Anhui Star New Material Technology Co., Ltd, China. Weighed aliquots of frozen tissue were digested in 3 stages: the first using 5 mL mixed acids (4 : 1 nitric acid : perchloric acid), the second using a combination of 2 mL HNO_3 and 30% H_2O_2, and finally using 2 mL HNO_3. All digestion stages were performed at 130°C until the acid was completely evaporated and the residue dried before the next acid stage was started. After the third acid treatment, 1% HNO_3 was added to the digests and heated at 80°C for 1 h. After cooling, the sample volume was measured and analyzed.

2.6. Tumor Induction by MCA. Murphy's string method [15] 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 (Group 1), MCA and Se-0.4 group (Group 2), MCA and SS group (Group 3), and MCA and Se-0 group (Group 4). The other 12 normal mice were used as the control group (Group 5). From then on, the 5 groups of mice were administered orally saline, Se-CS-0.4, SS-0.4, Se-CS-0, and saline, respectively. Body weight of the animals was recorded initially, at fortnightly intervals and at autopsy. All animals surviving after 90 days were killed. Tumor incidences in control and experimental groups were calculated [16]. Tissue samples of thymus, spleen, liver, and kidney were dissected from the visceral tissues. After washing with saline, the tissue samples were blotted dry and weighed. The uterus tissues of the mice were dissected for the estimation of various parameters related to oxidative stress. All samples were stored at −80°C for future analysis.

2.7. The Impact of Se-CS on Immune Organ. The impact of Se-CS on immune organ was evaluated based on the thymus index and spleen index [17]. The thymus or spleen index was calculated by the following formula: thymus (spleen) index = weight of thymus (spleen) (mg)/weight of mouse (g).

2.8. Biochemical Estimations. In uterus tissues, lactate dehydrogenase (LDH) was estimated using a method described by Lum and Gambino [18]. Serum was used for the assay of glutathione (GSH) content, lipid peroxidation (LPO), glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, catalase (CAT) activity, Na^+/K^+-ATPase activity, and glutathione S transferase (GST) activity by enzyme linked immunosorbent assay (ELISA) using DSL-10-1600 Active ELISA kit (Shanghai Jinma Biological Technology, Inc., China) [19–24].

2.9. Data Analysis. All data were analyzed by a one-way analysis of variance, and the differences between means were established by Duncan's multiple-range test. The data represents means and standard deviations. The significant level of 5% ($P < 0.05$) was used as the minimum acceptable probability for the difference between the means.

3. Results

3.1. The Effect of Se on Growth of CS. Se can be accumulated by CS even from low external concentrations (Figure 1). But it is also known that Se interpolates into disulfide bridges of protein, causing a structural weakness that leads to selenosis [25]. The biomass of mycelia was significantly influenced by the concentration of Se in the medium (Figure 1). A lower concentration of Se stimulated noticeably the growth of mycelium as compared to medium without selenium. But it was no significant variety as the concentration of Se between 0.1–0.4%. However, at 0.6% of Se the growth was clearly inhibited ($P < 0.05$).

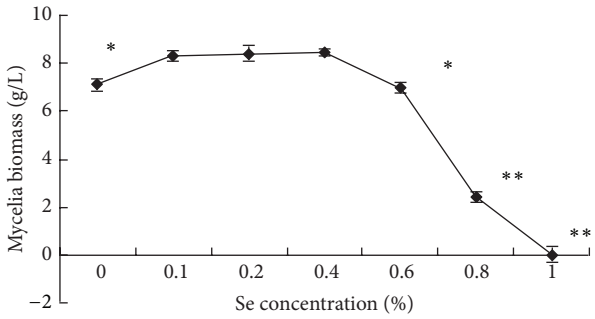

Figure 1: The effect of Se on growth of *Cordyceps sinensis*. The biomass of mycelia was the maximum when there was 0.4% Se in the medium. It declined rapidly when the concentration of Se exceeded 0.6%. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus 0 mg Se.

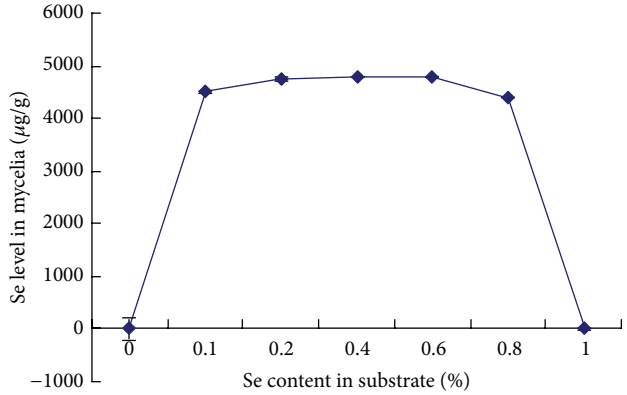

Figure 2: The content of Se in the mycelia. The content of Se accumulated in the mycelia was 4789.26 ± 13.0 μg/g, when the concentration of Se in the medium was 0.4%. $n = 6$. Values are means ± SEM.

3.2. Organic Selenium in Se-CS. The determination of selenium in mycelia obtained in the absence and in the presence of Se in medium demonstrated the incorporation of element to fungal cells (Figure 2). Apparently, there was no significant increase of selenium level in mycelia grown in 0.6% Se as compared to 0.4% Se ($P > 0.05$). Thus, we chose 0.4% Se as the optimal concentration of Se added in the liquid culture.

3.3. Effect of Se-CS on Survival of Mice Treated with MCA. The mice did not suffer from any apparent toxic effect of Se-0.4 during the observation period. Only a small number of mice died in certain groups (Table 1).

3.4. The Effect of Se-CS on Tumor Incidence. MCA-induced group administered orally saline showed 85.7% tumor incidence, whereas MCA and Se-0.4 group showed 40% tumor incidence ($P < 0.05$). SS-treated group and Se-0-treated group showed 77.7% and 70% tumor incidence, respectively. Although tumor incidence in SS-treated group and Se-0-treated group was lower than that of MCA-induced group, the difference was not significant ($P > 0.05$). Animals of control group showed no cervical tumor incidence (Table 2).

Table 1: Effect of SE-CS and other treatments on survival of MCA-treated mice.

Different groups	Number of mice	
	Initial	Final
MCA-treated	12	7
MCA and Se-0.4-treated	12	10
MCA and Se-0-treated	12	10
MCA and SS-treated	12	9
Control group	12	11

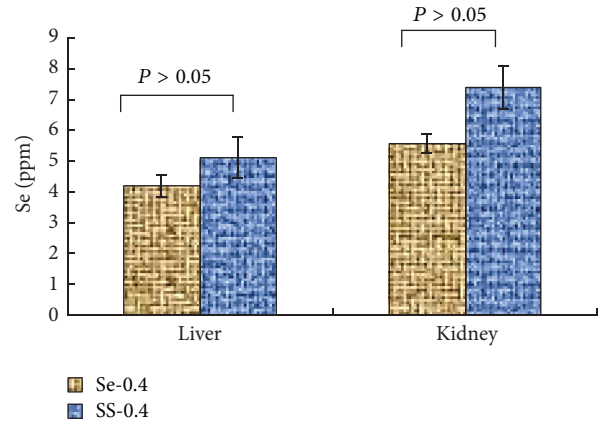

Figure 3: Se (ppm) in animal tissues ($n = 6$). The Se (ppm) was not significantly different between SS-0.4 and Se-0.4 group ($P > 0.05$).

3.5. The Effect of Se-CS on Immune Function. As shown in Table 3, compared with the Se-CS treated group, the thymus index and spleen index of mice in the MCA-treated group decreased significantly ($P < 0.05$). However, there was no significant difference between the MCA-treated group and the SS-treated group (Table 3). These results indicated that Se-CS could enhance the immune functions.

3.6. Se Accumulation in Liver and Kidney . The organ masses of liver and kidney were significantly different between SS and Se-CS group ($P < 0.05$). However, there was no significant difference of organ masses of liver and kidney between the Se-0.4 and Se-0 groups ($P > 0.05$). It is implied that SS-0.4 is toxic to animals, while Se-CS is essentially nontoxic (Table 4).

The Se contents in animal tissues were measured following the studies and the results are shown in Figure 3. The Se content in tissues of Se-0.4 group was 4.21 ppm and 5.56 ppm (liver and kidney) while SS-0.4 groups exhibited elevated levels of Se content but not to a statistically significant degree ($P > 0.05$). It is implied that CS could reduce Se accumulation in animal tissues to a certain extent.

3.7. The Effect of Se-CS on Various Parameters Related to Oxidative Stress. LDH was measured to evaluate the role of antioxidative stress in the protection of Se-CS. A significant increase in the activity of LDH in serum was observed in MCA group, as compared to the control

Table 2: Effect of SE-CS and other treatments on tumor incidence induction by MCA.

Different groups	Number of final mice (n)	Number of mice with cervical squamous cell carcinoma (n)	Tumor incidence (%)
MCA-treated	7	6	85.7
MCA and Se-0.4-treated	10	4	40*
MCA and Se-0-treated	10	7	70
MCA and SS-treated	9	7	77.7
Control group	11	0	0

* $P < 0.05$ versus MCA-treated group.

Table 3: The effect of SE-CS on immune function ($n = 7–11$).

Different groups	Number of mice (n)	Thymus index ($\times 10^3$)	Spleen index ($\times 10^3$)
MCA-treated	7	1.73 ± 0.980	5.17 ± 0.56
MCA and Se-0.4-treated	10	2.78 ± 0.06*	7.51 ± 0.10*
MCA and Se-0-treated	10	2.56 ± 0.03*	7.35 ± 0.23*
MCA and SS-treated	9	2.06 ± 0.09	5.94 ± 0.30
Control group	11	2.67 ± 0.23*	8.04 ± 0.29*

* $P < 0.05$ versus MCA-treated group.

Table 4: Effects of Se-CS on organ masses of mice ($n = 7–11$).

Mouse group	Number of mice (n)	Liver weight (g)	Kidney weight (g)
Control group	7	1.47 ± 0.05[a]	0.49 ± 0.04[a]
MCA-induced group	10	1.30 ± 0.07[b]	0.37 ± 0.03[b]
MCA and SS group	10	1.31 ± 0.06[b]	0.34 ± 0.03[b]
MCA and Se-0 group	9	1.48 ± 0.07[a]	0.48 ± 0.04[a]
MCA and Se-0.4 group	11	1.45 ± 0.06[a]	0.46 ± 0.02[a]

The different letters in the same column indicate a statistical difference ($P < 0.05$).

group ($P < 0.01$), whereas Se-CS treatment significantly ($P < 0.05$) resulted in decreased serum LDH levels when compared with MCA group mice (Table 5). Concentrations of GSH were lower in MCA group than those in control group (Table 5). Se-CS produced the increase in the level of GSH ($P < 0.05$). The level of LPO content adds to the proof of the increased peroxidative damage during MCA. A significant increase ($P < 0.001$) in the content of LPO was observed in the MCA group when compared with the control group. In the Se-CS group, a significant decrease ($P < 0.05$) was seen in the level of LPO when compared with the MCA group (Table 5).

Activities of various antioxidant enzymes and Na^+/K^+-ATPase of different groups have been listed in Table 6. The activity of endogenous antioxidant enzymes was decreased significantly ($P < 0.01$) in the MCA group, as compared to the control group, whereas, in the Se-CS group, Se-CS treatment showed significant ($P < 0.05–0.01$) restoration in the level of various enzymes as compared with MCA group.

4. Discussion

In the current study, we investigated the potential therapeutic efficacy of Se-CS for cervical cancer. Se is required in biosynthesis of important selenoenzymes. Some of them are active as catalysts for reduction of extracellular oxidants, thereby protecting cells from potential damage by these hazardous compounds. CS is recognized as a dietary supplement recommended in many countries as a cancer therapeutic. So the coeffect of CS and selenium on uterine cervix cancer was studied.

During the production of Se-CS, the biomass of mycelia was significantly influenced by the Se in the medium (Figure 1). This result is consistent with the findings of another study by Han et al. [13]. The possible mechanism could be that vanadium influences the stability of cell membranes, as well as the syntheses of nucleic acids and the stability of the double helix of DNA while forming hydrogen bonds.

Survival of animals receiving Se-CS was significantly longer than the groups receiving only CS or Se. The shortest

Table 5: Effect of SE-CS and other treatments on serum LDH, GSH, and LPO levels.

Different groups (n)	LDH (IU/L)	GSH (nmol/mg protein)	nmol/g protein
Control (7)	$85.222 \pm 2.561^{**}$	$1.39 \pm 0.003^{**}$	$13.21 \pm 0.26^{**}$
MCA-induced group (10)	181.111 ± 3.630	0.56 ± 0.021	22.20 ± 1.01
MCA and SS group (10)	170.121 ± 1.220	0.61 ± 0.011	22.11 ± 2.01
MCA and Se-0 group (9)	162.100 ± 2.130	0.79 ± 0.031	20.22 ± 3.22
MCA and Se-0.4 group (11)	$132.222 \pm 2.201^{*}$	$1.16 \pm 0.055^{*}$	$16.88 \pm 0.11^{*}$

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

Table 6: Effect of SE-CS and other treatments on the activity of various enzymes (nmol/g protein).

Different groups (n)	GPx	GR	GST	CAT	Na^+K^+-ATPase
Control (7)	$16.08 \pm 1.13^{***}$	$36.50 \pm 3.51^{***}$	$18.01 \pm 1.12^{**}$	$7.88 \pm 0.13^{*}$	$4.52 \pm 0.32^{*}$
MCA-induced group (10)	7.01 ± 0.32	20.89 ± 2.01	9.07 ± 1.11	3.20 ± 0.13	2.00 ± 0.13
MCA and SS group (10)	7.31 ± 0.30	21.86 ± 1.11	9.87 ± 2.21	3.88 ± 0.83	2.20 ± 0.11
MCA and Se-0 group (9)	7.98 ± 0.22	23.13 ± 2.31	10.07 ± 3.22	4.00 ± 0.83	3.00 ± 0.10
MCA and Se-0.4 group (11)	$10.10 \pm 0.32^{*}$	$25.01 \pm 2.12^{***}$	$11.60 \pm 0.88^{*}$	$4.78 \pm 0.23^{*}$	$3.23 \pm 0.11^{*}$

Values are shown as means ± SEM. $^{*}P < 0.05$ versus MCA group, $^{**}P < 0.01$ versus MCA group, and $^{***}P < 0.001$ versus MCA group.

survival times were observed in the no treatment group. The findings of the present study demonstrate that oral administration of Se-CS during the process of MCA-induced uterine cervix cancer results in significant reduction in the occurrence of cervical carcinomas ($P < 0.05$–0.01). CS could enhance the immune functions and reduce Se accumulation in animal tissues to a certain extent. The two agents achieve cytotoxicity through different means. It is possible that the bifunctional modulator of them inhibits tumor induction.

However, Se is also known to be toxic with a narrow range separating chronic conditions of deficiency and toxicity. The molecular toxicity of inorganic selenium was described in relation to its interaction with endogenous –SH groups. The animal studies have demonstrated that liver and kidney are the major target organs of Se toxicity [26]. The Se contents in animal tissues were measured following the studies. Ingestion of Se with CS reduced tissue metal accumulation, particularly for liver and kidney. At the same time, the organ masses of liver and kidney were significantly different between SS and Se-CS group ($P < 0.05$). The results indicate that Se-CS is less toxic to mice than SS.

Previous reports have shown that immunosuppression can be clearly detected in both cancer patients and tumor-bearing animals, demonstrating that the immune system plays an important role in immunosurveillance against malignant cells [27]. Many attempts have been made during the past years to develop immunostimulating approaches to cancer treatment. Thymus and spleen are important immunological organs that indirectly reflect humoral immunity. Some kinds of immune inhibitors could cause thymus and spleen atrophy. Our study demonstrated that spleen and thymus indexes were significantly decreased in MCA-treated group mice. This is consistent with the conclusion that immune system plays an important role in immunosurveillance against malignant cells [27]. However, the decrease of thymus and spleen indexes caused by MCA treatment in mice was alleviated by Se-CS. It demonstrated that improved immune function achieved by Se-CS may translate into an antitumor effect. Future clinical trials are required to confirm this finding.

It has been proposed that antioxidant changes reflect an altered redox balance in several pathological states [28]. LDH was measured to evaluate the role of antioxidative stress in the protection of Se-CS. It resulted in decreased serum LDH levels when compared with MCA group mice. 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 [29, 30]. Se-CS produced the increase in the level of GSH ($P < 0.05$). The large numbers of polyunsaturated fatty acids make cell membranes particularly vulnerable to lipid peroxidation. The oxidation of polyunsaturated fatty acids alters the structure of the membrane with resultant changes in fluidity and permeability. LPO can also inhibit the function of membrane bound receptors and enzymes [22]. In the Se-CS group, a significant decrease was seen in the level of LPO when compared with the MCA group. The antioxidants would be consumed in the reaction with free radicals. 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. Se-CS treatment showed a significant ($P < 0.05$–0.01) restoration in the level of various enzymes as compared with MCA group. These results suggested that Se-CS on uterine cervical cancer might be directly through a lower extent of oxidative stress in MCA mice.

5. Conclusions

Collectively, our results indicate that Se-CS may represent a novel protective strategy against uterine cervical cancer by attenuating oxidative stress and improving immune function in MCA mice. However, the antitumor effect of Se-CS-0 and

SS was not significant. It is implied that the antitumor effect was caused by the coeffect of CS and Se. Furthermore, our results also emphasize that Se-CS is less toxic to mice than SS. This finding suggested that the concomitant use of Se and CS could be a potential therapeutic approach to improve the efficacy of therapy for uterine cervical cancer.

Conflict of Interests

The authors declare that there is no conflict of interests.

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