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Effects of Mycelial Extract of *Phellinus linteus* on Ethanol-Induced Liver Injury in Rats

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We investigated the anti-inflammatory effects of mycelial culture extract from *Phellinus linteus* (MCPL) for suppression in the process of ethanol-induced inflammation in rat liver. Levels of aspartate amino-transferase (AST) and alanine aminotransferase (ALT) were significantly increased in the serum of ethanol-treated rats compared to normal. However, the level of ALT was arrested markedly in etha-nol-treated rats with MCPL compared to ethanol alone treated control ones. Severe histopathological changes of liver such as cloudy swelling, inflammatory cells infiltration, Kupffer cell reaction and focal necrosis were demonstrated in the rats challenged with ethanol compared with normal. Fewer scores of these changes were observed in MCPL-treated rat with recovered glycogen in centrolobular region of hepatic lobule. The Western analysis showed that the expression of inflammatory proteins such as cyclooxygenase (COX)-1, COX-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α were increased in the ethanol-treated rat. But decline of COX-2 and iNOS expression were observed in MCPL-treated rat. Immunohistochemical analysis showed that the expression of COX-2 and TNF-α tended to increase in ethanol-treated rat, but decrease of these reactions were induced by MCPL treatment. These results suggest that MCPL may act as a protective agent for alcohol-induced liver injury through a regulating inflammation-related proteins.

Key words — *Phellinus linteus*, mycelial culture, liver, alcohol

Introduction

Traditionally, *Phellinus linteus* commonly referred to as Sangwhang in Korea, has been used clinically as a medicinal mushroom for treatments of inflammatory diseases and cancer[8,13]. The uses of *P. linteus* are restricted by reasons of it being extremely rare to find in nature, but established mycelial culture enabled us to easily study the pharmacological activities[4].

Mycelial culture of *P. linteus* also draws great attention because of its medicinal value. The polysaccharide of mycelial culture of this mushroom stimulates humoral and cell-mediated immunity and exhibit a wider range of immunostimulation and anti-tumor activity as a fruiting body[6,7]. But mycelial culture of *P. linteus* has a wide application as a therapeutic agents and natural food for heath. Recently, we have discovered that mycelium of this fungus possesses some alcohol dehydrogenases and produces alcohol during mass-culture[1].

Despite extensive pharmacological studies, the investigations for the crude extract and chemical constituents of mycelial culture from *P. linteus* were mostly focused on their anti-tumor activities till now[8]. Especially, it also remains unclear whether the mycelial culture of *P. linteus* is effective on the treatment of alcohol-induced liver injury. In the present study, we investigated the effects of mycelial culture from *P. linteus* (MCPL) on alcohol-induced liver injury in rat focused on inflammation-related proteins.

Materials and Methods

Mycelial culture of *P. linteus*

Fresh fruiting bodies of *P. linteus* were obtained from a local farm. The medium components for the mycelial culture are as follows; 2.5% sucrose, 0.5% yeast extract and 0.1% MgCl$_2$ in distilled water. The culture medium was adjusted to pH 7.0. The aerated liquid culture was carried out in a 300 ml flask containing 100 ml of the medium and incubated at 25℃ for 6 days. The culture broth of *P. linteus* was filtered through a filter paper after cultivation. The 100 g of wet weight of mycelium was evaporated in vacua to give a solid extract (yield, 5 g) and a voucher specimens was deposed (DE-0401).

Antibodies and chemicals

Antibodies against nuclear factor (NF)-κBp65, inhibitory

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(I)-κBα, cyclooxygenase (COX)-1, COX-2, inducible nitric oxide synthase (iNOS)and tumor necrosis factor (TNF)-α and secondary horseradish peroxidase-conjugated rabbit immunoglobulin G antibodies were obtained from Santa Cruz Biotechnology (CA, USA). Avidin-biotin-peroxidase complex kit and substrate kit for peroxidase were purchased from Vector Lab (CA, USA). All other chemicals were obtained from Sigma (MO, USA).

Animals and ethanol and MCPL administration

Male Sprague-Dawley rats, weighing about 120 g on average, were obtained from Hyochang Science Co. in Korea. Rats were housed under conditions of 22℃ in 12 hr dark and light cycles, were fed a commercial diet, and allowed tap water *ad libitum* starting 2 weeks before and throughout the study. The experimental procedures were conducted under the ethical guidelines for investigations of experimental pain in conscious animals. Rats were administrated orally 3 ml of 40 % ethanol with or without MCPL (5 mg and 15 mg/Kg) a day for 10 days. Eight rats were used in each group.

Serum analysis

For the serum analysis, the blood was collected from the heart and immediately centrifuged at 3,000 rpm for 25 min and the plasma was stored at -20℃ for later analysis. Levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the Cobas Mira (Roche, Switzerland).

SDS-PAGE and Western blot analysis

Rat livers were washed in cold HEPES buffer, and homogenized in 9 volumes of potassium HEPES buffer containing 0.5% Triton X-100, 1 mM DTT, 5 mM sodium orthovanadate, 10 ug/ml aprotinin, 10 ug/ml leupeptin and 10 mM phenyl methylsulfonly fluoride. The homogenates were centrifuged at 14,000 rpm for 30 min at 4℃ and the supernatants served as liver protein extracts. Equal amounts of proteins were separated by 8-12% SDS-PAGE. The resulting gels were transferred to immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The membranes were incubated with the specific first antibodies for 2 hr at room temperature, and then the blots were incubated with horseradish peroxidase-conjugated secondary antibody. The antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham, Arlington Heights, IL, USA).

Histopathology and immunohistochemistry

The livers were fixed in 4% paraformaldehyde in PBS for 18 hr and dehydrated in a graded ethanol series. After embedment in paraffin, serial 5 μm thick sections were prepared. For histopathological examinations, hematoxylin-eosin stain and periodic acid Schiff's (PAS) reaction were used. For the immunohistochemistry, the sections were exposed to 0.3% methanolic hydrogen peroxide for 30 min, and washed with PBS. Tissues were then treated with goat normal serum at room temperature for 30 min followed by treatment with specific primary antibodies diluted for 1:500 in moisture chamber at 4℃ for 16 hr. After being washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit Ig G for 30 min and washed with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit at room temperature for 1 hr. Diaminobenzidine substrate kit for peroxidase was applied. For the controls, treatment with primary and secondary antibodies was omitted.

Data analyses

The data was analyzed for statistical significance using student's t-test. P-values less than 0.05 were considered to be significant.

Results

The levels of serum AST and ALT

Rats were administrated orally 3 ml of 40% ethanol with or without MCPL once a day for 10 days and serum AST and ALT, markers for liver function, were examined. As shown in Fig. 1, the levels of AST and ALT were significantly increased in the ethanol-treated rats compared with normal ones. But the level of ALT was markedly decreased in ehtanol-treated rat with MCPL administration compared to the ethanol alone-treated control one.

Histopathological analysis and glycogen distribution

Severe histopathological changes including cloudy swelling, inflammatory cells infilteration, Kupffer cell reaction and focal necrosis were demonstrated in ethanol-treated rat compared to normal. Fewer scores of the changes were observed in the ethanol-treated rat with MCPL administration compared to control one (Table 1). The distribution
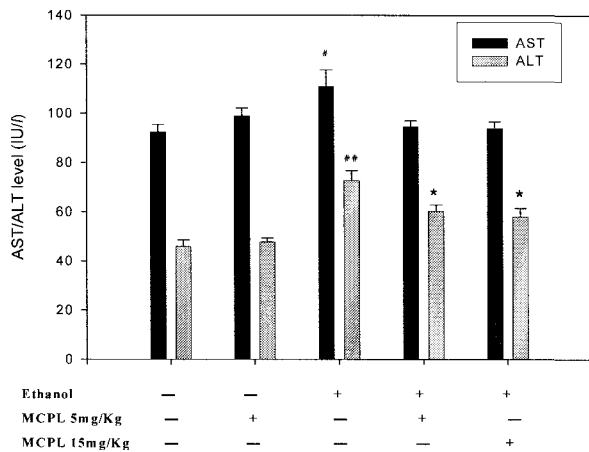
Fig. 1. Effects of MCPL on the serum AST and ALT levels in alcohol-treated rat. Rats were administrated 3 ml of 40% ethanol with or without MCPL for 10 days and examined by a blood serum analysis. Values are means±SEM of eight animals. [#], $P<0.01$ and [##], $P<0.001$ compared with normal rat; *, $P<0.05$ compared with ethanol alone treated rat.

of glycogen was decreased in the ethanol-treated rats, but these distribution were recovered by MCPL administration in centrolobular region of hepatic lobule (Table 2 and Fig. 2).

Western analysis

The inflammation-related proteins in ethanol-treated rat with or without MCPL administration were examined by Western analysis. As shown in Fig. 3, all proteins examined except I-κBα were increased in ethanol-treated rat compared to normal one, but these proteins, especially COX-2 and iNOS, were reduced by MCPL administration.

Immunohistochemical analysis

To confirm Western blot results, immunohistochemical analysis was performed as shown in Table 3 and Fig. 4. The COX-2 and TNF-α immunoreaction of hepatocytes,

Table 1. Histopathological findings of the liver in ethanol-treated rat with or without MCPL

Treatment and dose (mg/Kg)	CS	ICI	KC	FN
Normal	0	0	+	0
MCPL (5)	0	0	+	0
Ethanol	++	++	++	+
MCPL (5) + ethanol	++	+	+-++	0-+
MCPL (15) + ethanol	++	+	+-++	0-+

CS, cloudy swelling; ICI, inflammatory cells infilteration; KC, Kupffer cell reaction; FN, focal necrosis; MCPL, mycelial culture of *P. linteus*. 0-+++ indicate the relative changes of the histological finding: 0, faint and negligible; +, weak; ++, moderate; +++, severe.

Table 2. Histochemical findings of glycogen distribution in the liver of ethanol-treated rat with or without MCPL

Treatment and dose (mg/Kg)	Zone I	Zone II	Zone III
Normal	++	++	+++
MCPL (5)	++	++	+++
Ethanol	+-++	+-++	+-++
MCPL (5) + ethanol	+-++	+-++	++
MCPL (15) + ethanol	+-++	+-++	++

Zone I, peripheral region; Zone II, midlobular region; Zone III, centrolobular region. Other abbreviation listed in Table 1. 0-+++ indicate the relative intensity of PAS reaction: 0, faint and negligible; +, weak; ++, moderate; +++, intense.

especially in the inflammatory loci, were increased in ethanol-treated rat compared to normal one. But these immunoreactions were decreased in ethanol-treated rat with MCPL administration.

Discussion

The aqueous extract from the fruiting body or mycelia of *P. linteus* has been reported to produce anti-tumor activities and its polysaccharides are considered to be the active
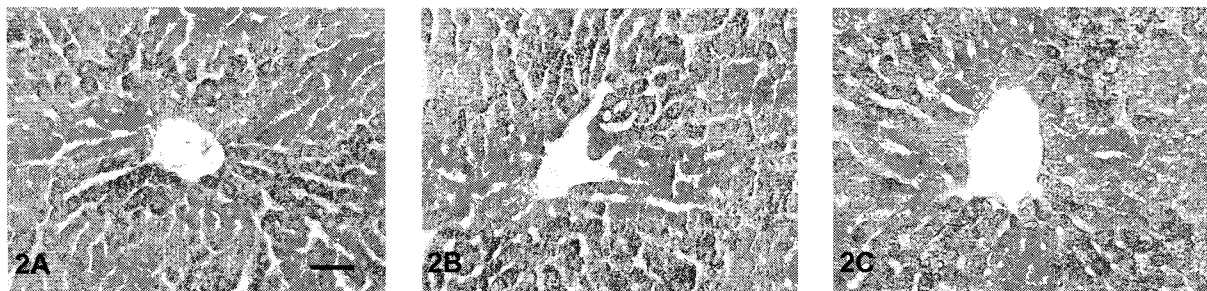

Fig. 2. Photomicrographs showing the liver of the rat in the normal (A), ethanol-treated (B) and ethanol-treated rat with MCPL administration (C). Weaker PAS reactions with stromatic changes including cloudy swelling were detected in the ethanol-treated rat compared with normal one. Scale bar = 50 μm.
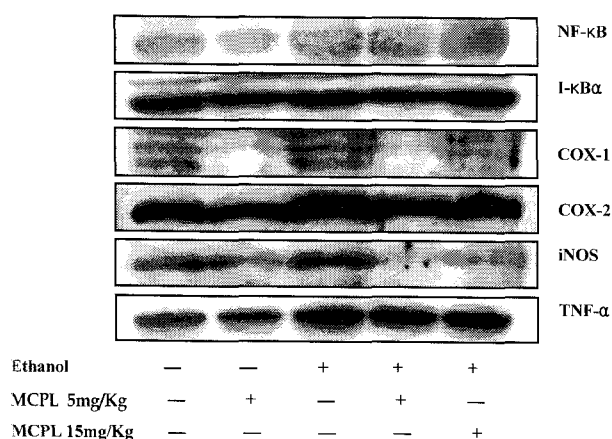
Fig. 3. Effects of MCPL on the expression of inflammation-related proteins in ethanol-treated rat liver. Rats were administrated 3 ml of 40% ethanol with or without MCPL for 10 days and examined by Western blot.

constituents[4,13]. *P. linteus* also strongly stimulates both cell-mediated and humoral immunity *in vivo* and *in vitro* conditions[7]. We discovered that mycelium of *P. linteus* possesses alcohol dehydrogenases and produces alcohol during mass-culture. Therefore, this study demonstrated

the hepatoprotective effect of MCPL in ethanol-administrated model.

The liver is one of the major organs to be damaged during the heavy intake of alcohol. Previous studies have demonstrated that liver disease results from the dose- and time-dependent consumption of alcohol[5,11]. In the present study, we have firstly observed that the effects of MCPL on the levels of serum AST and ALT. These levels are correlated with the degree of inflammation or cell death of the liver and served as a liver functional marker[9,16]. Lower levels of these enzymes, especially ALT, were demonstrated in the serum of ethanol-treated rats with MCPL administration compared with ethanol-treated control ones (Fig. 1).

The histopathological symptoms and severity of the ethanol-induced liver injury, cloudy swelling, inflammatory cells infilteration, Kupffer cell reaction in particular, were also significantly reduced by administering MCPL with the slight reduction of the local necrosis (Table 1). Ethanol alone treated rats showed a severe change on the distribution glycogen in centrolobular region of the liv-

Table 3. Immunoreactions of inflammation-related proteins in the liver of ethanol-treated rat with or without MCPL

Treatment and dose (mg/Kg)	Region	NF-κBp65	I-κBα*	COX-1	COX-2	iNOS	TNF-α
Normal	Zone I, II	0-+	++	0	0	0	+
	Zone III	+	++	0	0	+	+
MCPL (5)	Zone I, II	0-+	++	0	0	0	+
	Zone III	+	++	0	0	+	+
Ethanol	Zone I, II	0-+	++	0	0-++	0	+-++
	Zone III	+	++	0	0-+	+	+
MCPL (5) + ethanol	Zone I, II	0-+	++	0	0-+	0	+
	Zone III	+	++	0	0-+	+	+
MCPL (15) + ethanol	Zone I, II	0-+	++	0	0-+	0	+
	Zone III	+	++	0	0-+	+	+

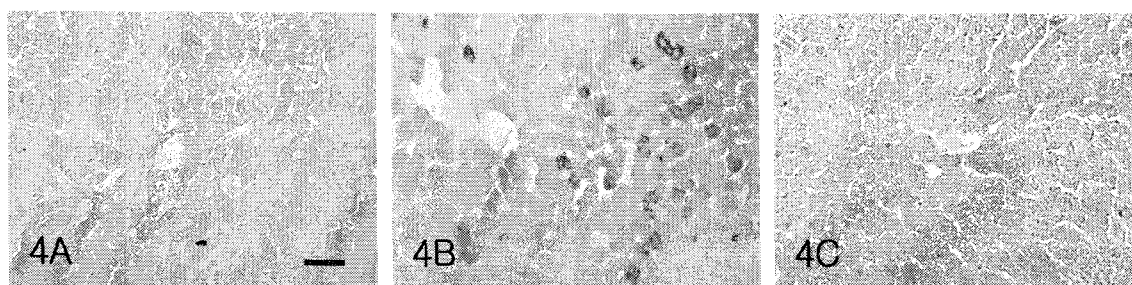* These reactions were mainly detected in nucleus. Abbreviations given in Tables 1 and 2.

Fig. 4. Immunoreaction of COX-2 in the liver of the rat in the normal (A), ethanol-treated (B) and ethanol-treated rat with MCPL administration (C). Note decline of COX-2 immunoreaction in the MCPL administrated rat compared with control one. Scale bar = 50 μm.

er, but the maintenance of glycogen showed in the MCPL aministrated rats (Table 2 and Fig. 2). These results suggest that MCPL may have some components to protect liver injury from alcohol.

The liver injury in response to alcohol is associated with an inflammatory process as an important precursor to the development of irreversible liver disease[12]. The NF-κB activation plays an important role in expression of inflammation-related proteins including multiple organ injury. NF-κB is dissociated from I-κB and is translocated into the nucleus where it induces transcriptional up-regulation of various proinflammatory mediators that contribute to the systemic inflammatory response such as iNOS, COX and TNF-α[10].

COX-2 is selectively expressed in response to various inflammatory stimuli and its induction is associated with the proliferation of hepatocarcinoma and liver injury[3]. Recent studies have shown increased levels of COX-2 in adjacent cirrhotic tissue of hepatocellular carcinoma[15]. The most iNOS is derived from inflammatory cells and Kupffer cell while hepatocyte is a weak inducer. During endotoxemia of the liver, expression of iNOS is seen in inflammatory cell, macrophages, hepatocytes and bile duct epithelium[17]. TNF-α is a principal regulator of inflammation and immunity eliciting a wide variety of biological effects[2]. The studies in ethanol-fed rats have shown that inhibition of TNF-α by curcumin leads to a decrease in the amount of fat storage in the liver[14].

In the present study, higher expression of inflammation-related proteins such as COX-2, iNOS and TNF-α were induced in the ethanol treated rats compared with normal ones. However, The declines of COX-2 and iNOS expression were observed in MCPL administrated rat compared to ethanol alone treated ones. Furthermore, immunohistochemical analysis confirmed that the administration of MCPL could induce a decreasion of COX-2 and TNF-α expression compared to control one. These results suggest that the expression of iNOS, COX-2 and TNF-α contributes to liver injury.

The inhibitory effect of MCPL on alcohol-induced expression of iNOS, COX-2 and TNF-α shown in Western and immunohistochemical analysis may be good evidence for hepatoprotective effects in alcohol-induced liver injury. Consequently, our results suggest that the co-administration of MCPL effectively suppresses and prevent ethanol-induced liver injury through regulating the expression

of inflammation-related proteins. Thus MCPL may be useful in the therapeutic agent or natural food for ethanol-induced liver injurie.

Acknowledgement

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초록 : 알코올성 간 손상에 대한 상황버섯 배양균사체 추출물의 효능

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상황버섯 배양균사체 추출물 (MCPL)이 알코올성 간 손상에 미치는 영향을 살펴보았다. Sprague-Dawley계 흰 쥐에 40% 알코올 3 ml을 MCPL (5 mg 및 15 mg/Kg)과 함께 1일 1회 10일간 투여하였다. 간 기능의 표지가 되는 혈청내 AST와 ALT값이 에탄올에 의해 현저히 증가하였으나 MCPL투여에 의해 저하되며 특히 ALT값이 유의성 있게 낮아졌다. 병리조직학적으로 살펴보면 알코올에 의해 염증세포의 침윤, 쿠퍼세포 반응 및 국소적 염증이 유발되나 MCPL투여에 의해 그 정도가 다소 완화되었다. 간소엽내 글리코겐 분포도 알코올에 의해 감소하나 MCPL투여에 의해 중심정맥주변 부위의 분포가 일부 회복되었다. 염증관련 단백질에 대한 Western blot 및 면역조직화학적 반응을 보면 에탄올 투여에 의해 COX-2, iNOS 및 TNF-α 면역반응이 증가하나 MCPL투여에 의해 발현의 감소를 볼 수 있었다. 이상의 결과로 보아 MCPL은 알코올에 의한 간 손상에 대해 보호 기능을 가짐을 알 수 있다.