Protection against D-Galactosamine-Induced Acute Liver Injury by Oral Administration of Extracts from *Lentinus edodes* Mycelia

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The development of oral medications to help prevent liver injury is desirable, and some mushrooms contain chemicals that show promise as such a treatment. Here, we tested whether a hot-water extract (L.E.M.) of the cultured mycelia of an edible mushroom, *Lentinus edodes*, could protect primary cultured hepatocytes from D-galactosamine (GalN)-induced injury. GalN induced cell death in the hepatocytes, and this effect was completely suppressed by the addition of 0.5 mg/ml L.E.M. Polyphenolic compounds contained in the L.E.M. seemed to be responsible for the protective effect. We next examined the protective effect of L.E.M. in a GalN-induced liver injury model in rats. In rats that had been treated with L.E.M. given orally or intraperitoneally, GalN caused less leakage of aspartate aminotransferase and alanine aminotransferase, markers for liver injury, and a lower decrease in serum protein content, than in non-L.E.M.-treated rats. Histological analysis of the liver also showed a protective effect of L.E.M. Our findings indicate that L.E.M. administration is a promising treatment for protecting the liver from acute injury.

Key words *Lentinus edodes*; hepatoprotective effect; D-galactosamine; polyphenol; oral administration

Lentinus edodes is a common mushroom that is indispensable for Japanese and Chinese cuisine. The mushroom has long been used as a folk medicine, and recently many substances that have immune-modulating, antitumor, antiviral, antibacterial, and antiparasitic effects have been isolated from various mushrooms.[1] *L. edodes* contains several compounds with physiological activities, such as an immunopotentiating effect,[2] anti-atherogenic action,[3] and anti-HIV effect.[4] The mycelia of *L. edodes* can be cultured in solid medium, and extracts obtained by hot-water treatment (L.E.M.) are commercially available as a nutritional supplement.

In our previous study, we found L.E.M. had a hepatoprotective effect on dimethylnitrosamine-induced liver fibrosis.[5] The intraperitoneal administration of L.E.M. seems to suppress the activation of hepatic stellate cells, which play a central role in liver fibrosis.[6,7] We believe that the polyphenols contained in the fraction extracted from L.E.M. using ethanol and methanol (ESMe) are responsible for this effect. The finding of antifibrotic activity in the L.E.M. is expected to be helpful in the development of hepatoprotective agents that have few side effects.

In this study, we investigated whether L.E.M. administered per os would protect hepatocytes from D-galactosamine (GalN)-induced acute liver injury. The induction of liver injury by GalN is a suitable experimental model of human liver failure.[8] The reduction in the intracellular level of uridylate is responsible for the GalN-induced apoptosis and necrosis in primary cultured rat hepatocytes.[9,10] Here, we show that L.E.M. protected cultured hepatocytes and the *in-vivo* liver from GalN-induced injury.

MATERIALS AND METHODS

Animals Wistar and Sprague-Dawley (SD) male rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 25±2 °C and 50±5% humidity under a 12 h light/dark cycle. L.E.M. was administered orally or intraperitoneally, at 600 or 100 mg/kg body weight, respectively, to 9-week-old Wistar rats, for 10 d. Hepatic injury was induced by intraperitoneal administration of GalN (Wako Pure Chemicals, Osaka, Japan) at 300 mg/kg body weight, after 9 d of L.E.M. treatment. The blood samples were collected at 6 and 24 h after GalN administration from the orbital sinus, and 48 h after this treatment from the vena cava; the samples were analyzed for the transaminase and protein content. The animal experiments were conducted according to the ethical guidelines of Kobayashi Pharmaceutical Co., Ltd.

Media The basal medium consisted of 50 U/ml penicillin G, 50 μg/ml streptomycin (ICN Biochemicals, Inc., Costa Mesa, CA, U.S.A.), 1 μM insulin, 1 μM dexamethasone (Wako), and 10% fetal bovine serum (FBS, Thermo Trace Ltd., Melbourne, Australia) in William's medium E (WE, Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

Culture Conditions Hepatocytes were isolated from male SD rats weighing 150—200 g by perfusing the liver with collagenase (from *Clostridium histolyticum* Type IV; Sigma), according to the method of Seglen.[11] Cells were seeded at a density of 1×10^5 cells/cm^2 into multi-well culture plates pre-coated with collagen type I (Asahi Technoglass Co., Chiba, Japan). Four hours after the seeding, the basal medium was replaced with medium containing L.E.M. and/or GalN and without insulin and dexamethasone, and the cells were cultured for 24 h.

Preparation of L.E.M. L.E.M. was prepared as previously reported.[5] Briefly, *Lentinus edodes* mycelia were cultivated in solid medium composed of sugar-cane bagasse and defatted rice bran. To make the culture extract, the medium including the mycelia was digested with mycelial enzymes by incubation in water at 30—55 °C. The incubation temperature was increased to 90 °C to inactivate the enzymes and

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sterilize the extract. The digest was filtered, lyophilized, and used as the L.E.M. preparation.

To make an alcoholic extract of the polyphenols, the L.E.M. was then incubated in 50% ethanol for 3 d in the dark, and the soluble fraction was dried under vacuum, yielding a brownish fraction (ES).[5] The ES was dissolved in a small amount of ethanol and further purified by Sephadex LH-20 column chromatography. After an elution with distilled water, the fraction containing polyphenols was obtained by elution with methanol. The eluate (ESMe) was concentrated under vacuum and lyophilized.

Assays Viable cells were counted using Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan). The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using the Quickauto-Neo assay kit (SHINO-TEST Co., Tokyo, Japan). The determination of total protein was performed using the Biuret method with a commercial kit, Clinimate TP (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Total polyphenols in L.E.M. and ESMe were determined by Folin-Denis method.[12]

Histological Analysis The excised liver was fixed in 20% formalin, embedded in paraffin, cut into 5 μm-thick sections, and stained with hematoxylin–eosin.

Statistics The data were analyzed for statistical significance using the Tukey test.

RESULTS

The *in vitro* protective effect of L.E.M. against GalN treatment was examined using primary cultured rat hepatocytes (Fig. 1). We examined various concentrations of GalN and chose 1 mM to give injury on hepatocytes *in vitro* because the 1 mM treatment significantly and stably decreased the viability in our preliminary experiments. The addition of 1 mM GalN decreased the number of viable cells by inducing apoptotic or necrotic cell death. The addition of L.E.M. increased the number of viable cells in a dose-dependent manner. The addition of 0.5 mg/ml L.E.M. completely suppressed the GalN-induced decrease in viability. These results show that L.E.M. protected hepatocytes from GalN-induced cell death *in vitro*.

The addition of 1.0 mg/ml L.E.M. significantly increased the viable cell number over that obtained under normal culture conditions. Since isolated hepatocytes do not proliferate *in vitro* in the absence of growth factor, L.E.M. probably blocked the death of some percentage of cells that normally occurs after plating. ESMe had an even stronger effect on the post-plating maintenance of hepatocyte viability than L.E.M. The addition of 0.1 mg/ml ESMe completely suppressed the GalN-induced decrease in viable cells. At higher levels of ESMe, the cell viability increased above that of cells cultured in the absence of GalN, in a dose-dependent manner to a concentration of 0.5 mg/ml. To the contrary, the enhancing effect on the viability disappeared at a concentration of 1.0 mg/ml. Considering the decreasing effect at high concentration of ESMe, we used L.E.M. for in the following *in vivo* experiments.

We next investigated whether the oral administration of L.E.M. could effectively protect the liver *in vivo* from GalN-induced injury. Intraperitoneal administration was also performed for comparison. After 9 d of L.E.M. administration, GalN was injected intraperitoneally, and the protective effect was evaluated by measuring the leakage of transaminases into the serum. Figure 2 shows the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum. Almost no activity was detected in the serum from normal rats. In the GalN-treated positive-control rats, very high levels of AST and ALT were detected in serum collected 24 h after the GalN-treatment. Thus, the liver was injured by the GalN treatment under our conditions. Both oral and intraperitoneal administrations of L.E.M. significantly lowered the leakage of AST and ALT into the serum, and there was no significant difference in the serum transaminase levels between the rats given oral and intraperitoneal L.E.M. Although the protective effects tended to decrease by 48 h following the GalN-treatment, a significant difference was still observed between the positive-control and L.E.M.-treated rats.

Since GalN inhibits RNA and protein synthesis through the depletion of UTP,[13] we next examined the effect of L.E.M. administration on the total protein content in the serum. As shown in Fig. 3, the GalN treatment significantly decreased the total protein content in the serum. Both oral and intraperitoneal administration of L.E.M. attenuated this

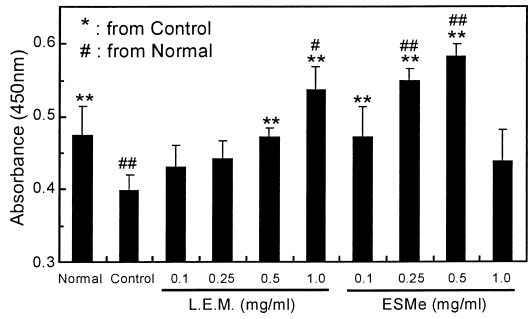

Fig. 1. L.E.M. and ESMe Protect Hepatocytes from GalN-Induced Death

Rat hepatocytes were cultured for 24 h in the presence or absence of additives, such as GalN, L.E.M., and ESMe. The viable cells were then counted. Hepatocytes cultured in the absence of additives and the presence of 1 mM GalN are indicated as Normal and Control, respectively. L.E.M. or ESMe was added to the culture medium at the concentrations indicated at the same time as GalN. Values are the means±S.D. of six experiments. * and #, significant difference from the values of Control and Normal, respectively. The data were analyzed by the Tukey test. (** $p<0.01$; ## $p<0.01$; # $p<0.05$).

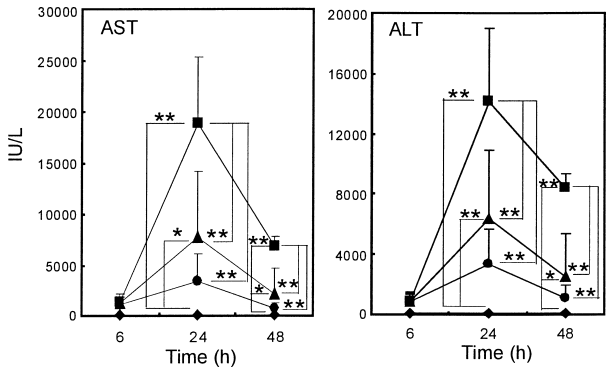

Fig. 2. Suppression of AST and ALT Levels by L.E.M. Treatment in GalN-Injured Rats

Blood samples were collected from normal (diamond), GalN-injured control (square), oral L.E.M.-treated (triangle), and intraperitoneal L.E.M.-treated (circle) rats, 6, 24, and 48 h after the GalN injection. Values are means±S.D. of five different animals. The data were analyzed by the Tukey test (** $p<0.01$; * $p<0.05$).

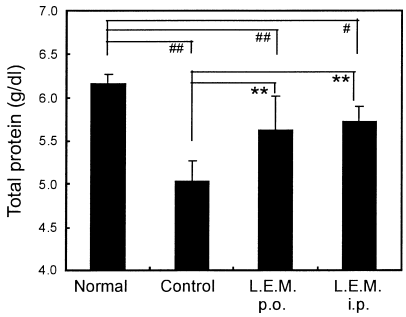

Fig. 3. Protective Effect of L.E.M. Treatment on the GalN-Induced Decrease in Serum Protein Content in Rats

Blood samples were collected from normal, GalN-injured control, oral L.E.M.-treated, and intraperitoneal L.E.M.-treated rats 48 h after the GalN injection. Values are means±S.D. of five different animals. The data were analyzed by the Tukey test. Symbols, * and #, indicate a significant difference from the values of Control and Normal rats, respectively (** $p<0.01$; ## $p<0.01$; # $p<0.05$).

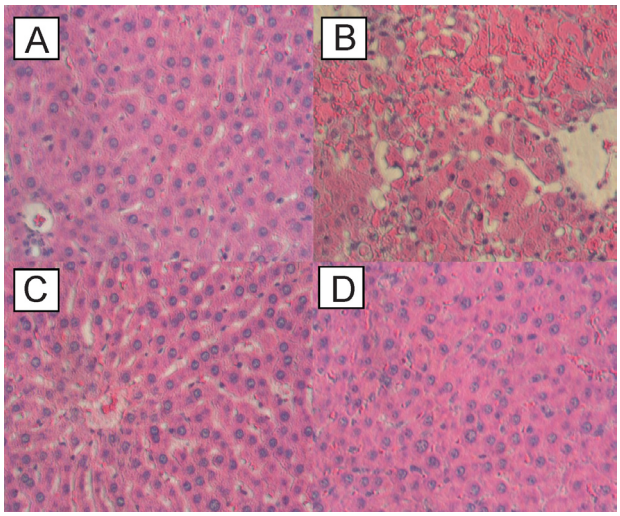

Fig. 4. Suppression of GalN-Induced Liver Injury in Rats Given L.E.M.

Hematoxylin–eosin staining was performed on paraffin-embedded sections of liver tissues. The liver was excised from normal (A), GalN-injured control (B), oral L.E.M.-treated (C), and intraperitoneal L.E.M.-treated (D) rats 48 h after GalN injection. Magnification for all photographs, ×400.

decrease in the protein content. Since the amount of proteins contained in L.E.M. was much less than that of rat diet, the effect of L.E.M. administration seems to be derived from the protection of liver against GalN treatment.

To look for histological evidence of protection from injury, liver sections were prepared and stained with hematoxylin and eosin; representative pictures are shown in Fig. 4. The normal architecture of the sinusoid was observed in sections from normal, untreated rats. In contrast, the sinusoid architecture was disorganized in liver sections from the GalN-treated control rats, showing that the liver was injured by the intraperitoneal injection of GalN. The disorganization caused by the GalN treatment was somewhat (Fig. 4C) to very obviously (Fig. 4D) reduced in the sections from the rats given oral and intraperitoneal L.E.M., respectively. These results indicate that the oral and intraperitoneal administration of L.E.M. protected the rats from GalN-induced liver injury.

DISCUSSION

The oral administration of medications has the advantage of higher patient compliance compared with invasive procedures such as injections. In this study, we showed that orally administered L.E.M. suppressed GalN-induced acute liver injury in rats. The protective effect of oral L.E.M. was comparable to that observed from giving L.E.M. intraperitoneally. An effective component of the L.E.M. appears to have been absorbed, to have reached the liver without degradation in the gastrointestinal tract, and to have had a cytoprotective effect. The mycelia of *L. edodes*, the source of the L.E.M., can be cultured on a large scale under completely controlled conditions inside a plant factory, and a constant supply of raw material can be obtained without regard to seasonal conditions or the external environment. Thus, L.E.M. has an advantage over other plant-derived medicines extracted from the leaves or stem. These advantages for production suggest that L.E.M. could be a promising agent for liver-supporting therapy.

In this study, we used GalN to damage rat primary cultured hepatocytes *in vitro* and rat liver *in vivo*. Besides the well-known effect of reducing the intracellular pool of uracil nucleotides in hepatocytes,[13] it has been reported that GalN induces apoptosis and necrosis in hepatocytes by increasing oxidative stress, *e.g.*, the intracellular production of hydrogen peroxide.[14] L.E.M. has strong antioxidative activities including superoxide dismutase-like activity, radical-scavenging ability, and the suppression of lipid peroxidation. L.E.M. contains 3.3 g of polyphenols in 100 g. It is well known that polyphenolic compounds scavenge oxygen free radicals.[15] The radical scavenging activity of the polyphenols in L.E.M. was approximately 30% of catechins contained in green tea powder (data not shown). Here, the polyphenols were further concentrated by extraction with ethanol and methanol, as described in Materials and Methods. The extracted fraction (ESMe) contained 15.8 g polyphenols in 100 g, and had a stronger radical-scavenging activity (data not shown) and protective effect on the viability of GalN-treated hepatocytes than L.E.M. Therefore, the ability of L.E.M. to protect the viability of GalN-treated hepatocytes seems to be derived from the antioxidative activity of polyphenols. However, the addition of high concentration of ESMe eliminated the enhancing effect on hepatocyte viability *in vitro*. When 1.0 mg/ml ESMe was added to the normal hepatocytes, no toxic effect was observed on the viable cell number (data not shown). It is possible that inhibitory substance(s) might counteract the enhancing effect of polyphenols at high concentration of ESMe in the GalN-injured cells. Further purification of ESMe is necessary to analyze the mechanism of *in vitro* maintenance effect on hepatocytes.

When administered to rats whose liver was subsequently injured, L.E.M. suppressed the leakage of AST and ALT into the serum, indicating that hepatocyte death was protected by L.E.M. treatment *in vivo*. One liver-specific function is to produce serum proteins, such as albumin and blood coagulation factors. The L.E.M. treatment suppressed the GalN-induced decrease in serum protein content, probably by protecting the hepatocytes from apoptosis or necrosis. In our previous study, we found that L.E.M. inhibits the proliferation and activation of hepatic stellate cells, which play a central role in liver fibrosis.[5] Moreover, we recently found that L.E.M. inhibits the proliferation of hepatoma cells (published elsewhere). Thus, L.E.M. has pleiotropic effects on various

cells and could be useful for preventing acute liver injury, suppressing liver fibrosis, and inducing the regression of liver cancer.

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