SHORT COMMUNICATION

FERMENTED ORIENTAL MEDICINAL INSECT EXTRACT HAS A SYNERGISTIC EFFECT ON INHIBITING TGF- β 1- OR H₂O₂-INDUCED HEPATIC FIBROGENESIS IN LX-2 AND HEPG2 CELLS

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Edible insects have received global attention as a potential alternative food source because they are nutritionally dense, containing high levels of dietary energy, proteins, fatty acids, fibers, vitamins (1). As such, in some regions, insect consumption is traditionally practiced for nutritional and medicinal purposes (2, 3). Recently, the Korea Ministry of Food and Drug Safety (MFDS) temporarily registered four edible insects, *Allomyrina dichotoma*, *Protaetia brevitarsis*, *Tenebrio molitor*, and *Gryllus bimaculatus*, in consideration of safety and management for use as food materials (4). In particular, *P. brevitarsis seulensis* belongs to the insect order Coleoptera (beetles) and its larvae have been used in traditional Korean medicine for treating liver-related diseases such as liver cirrhosis, hepatitis, and hepatic cancer (5).

In addition, fermented foods (such as soybean paste or natto) created using bacterial strains from the *Bacillus* genus have been distributed in Asian countries and consumed as healthy food for centuries (6). *Bacillus* strains produce high-efficiency proteases and digest high-molecular-mass proteins into smaller forms of nutrients (amino acids and peptides) (7), thus improving their nutritional value. However, little is known about the synergistic biomedical efficacies of *B. subtilis*-fermented *P. brevitarsis* larvae extract (F-Pbl-E) on liver fibrosis. In this study, we investigated the synergistic biological activity of F-Pbl-E with respect to suppression of TGF- β 1- or H₂O₂-induced hepatic fibrosis in LX-2 and HepG2 cells.

EXPERIMENTAL

Preparation of F-Pbl-E

Air-dried *P. brevitarsis* larvae were ground into a powder and autoclaved at 121°C for 15 min. A subcultured *B. subtilis* pellet (OD at 660 nm = 1.7–1.8) was resuspended in ddH₂O (deionized distilled water) after centrifugation. The pasteurized powder was inoculated with *B. subtilis* at a ratio of 1 : 1 (w/v) and incubated at 30°C for 0–3 days, followed by drying in a hot air oven at 50°C for 5 h. Samples were extracted using ddH₂O at a ratio of 1 : 10 (w/v) at 37°C for 24 h in a heating mantle (MS-E103, Misung Scientific, Seoul, Korea). After water extraction, F-Pbl-E was filtered using 110 nm filter paper (No. 2, Advantec, Toyo, Japan) and dried using a vacuum freeze dryer (FDU-8606, Operon, Gimpo-si, Korea). The yield of freeze-dried F-Pbl-E was measured, and the material was dissolved in ddH₂O.

MTS assay

The LX-2 cells were seeded in a 96-well (4 × 10³ cells/well) plate and treated with TGF- β 1 (10 ng/mL) alone or in combination with different doses of F-Pbl-E for 48 h. After incubation, 20 μ L of MTS working solution (2 mg/mL in phosphate buffered saline (PBS)) was added to each well; then cells were further incubated at 37°C for 4 h. Absorbance at 490 nm was measured using a microplate reader (Victor2 1420 Multilabel Counter, PerkinElmer, MA, USA) and cell viability was determined as the

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percentage of MTS reduction, by assuming the absorbance of control cells to be 100%.

qRT-PCR

Quantitative real time-PCR (qRT-PCR) analysis of *COLIA1* was performed in LX-2 cells after treatment with TGF- β 1 (10 ng/mL) alone or in combination with different doses of F-Pbl-E. The relative mRNA levels of *COLIA1* were assessed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The primer pairs (sense/antisense) were as follows: *COLIA1*, 5'-TGG ATT CCA GTT CGA GTA TG-3' and 5'-AGG TGA TGT TCT GGG AGG CC-3'; GAPDH, 5'-AGG GAT CTC GCT CCT GGA AG-3' and 5'-TGA AGG TCG GTG TCA ACG GA-3'. qRT-PCR was performed using a real-time PCR system (RG-6000, Corbett Research, CA, USA). Quantitative

assessment of cDNA was performed using the SYBR Green Master Mix Kit (QuantiMix SYBR Kit, PCR Biosystems, PA, USA). qRT-PCR data were recorded and analyzed with Rotor-Gene Q software. Results are expressed as fold change relative to the control.

Intracellular ROS measurement

Generation of intracellular ROS was measured with the fluoroprobe DCF-DA (2°C, 7°C- dichloro-fluorescein diacetate). HepG2 cells were stimulated with 100 μ M H₂O₂ (29-32% purity) for 1 h after treatment with F-Pbl-E at different concentrations. Fluorescence intensity was measured by fluorescence microscopy (NIKON; excitation 488 nm, emission 513 nm) and quantified using a fluorometer (PerkinElmer).

Figure 1. F-Pbl-E inhibits TGF- β 1-induced cell proliferation and gene expression in LX-2 cells. (A) Cell proliferation assays were carried out using the MTS colorimetric assay in LX-2 cells. LX-2 cells were treated with TGF- β 1 (10 ng/mL) alone or in combination with different concentrations of F-Pbl-E (50, 250, 500 μ g/mL for F-Pbl-E (3 days) and 500 μ g/mL for F-Pbl-E (0 days)). The concentration of DPI used was 10 ng/mL. Error bars represent standard deviations (SDs) of three biological replicates (* p < 0.01, Student's t -test). (B) HEK-293 cells were treated with F-Pbl-E, and cell viability at 24 h was determined using the MTS colorimetric assay. Error bars represent standard errors (SEs) of three biological replicates. (C) Quantitative real time-PCR (qRT-PCR) analysis of *COLIA1* in LX-2 cells treated with TGF- β 1 (10 ng/mL) alone or in combination with different concentrations of F-Pbl-E. The concentration of DPI used was 10 ng/mL. Error bars represent SDs of three biological replicates (* p < 0.01, # p < 0.05, Student's t -test)

Figure 2. F-Pbl-E reduces ROS, AST and ALT levels in HepG2 cells. (A) Endogenous levels of intracellular ROS were determined using the fluoroprobe DCF-DA in HepG2 cells. HepG2 cells were treated with H₂O₂ alone or in combination with different concentrations of F-Pbl-E (50, 250, 500 µg/mL for F-Pbl-E (3 days) and 500 µg/mL for F-Pbl-E (0 days)). The concentration of DPI used was 10 ng/mL. Error bars represent SDs from three biological replicates (**p* < 0.01, Student's *t*-test). (B) Enzyme activity was analyzed with cell culture supernatants from HepG2 cells treated with H₂O₂ alone or in combination with different concentrations of F-Pbl-E. The concentration of DPI used was 10 ng/mL. Supernatant AST and ALT levels were determined using the commercial kit following the manufacturer's instruction. Error bars represent SDs of three biological replicates (**p* < 0.01, #*p* < 0.05, Student's *t*-test). N.S.; not significant

AST and ALT activity assay

AST and ALT activity was measured using the commercial kit (Asan Pham, Seoul, Korea) following the manufacturer's instructions. HepG2 cells were stimulated with 3000 µM H₂O₂ (29-32% purity) for 1 h after treatment with F-Pbl-E at different concentrations. Luminescent emissions were recorded at 490 nm using a microplate reader and quantified using a fluorometer (PerkinElmer).

RESULTS AND DISCUSSION

Hepatic fibrosis is a "scarring" process due to acute or chronic liver injury, eventually leading to liver cirrhosis, liver cancer and hepatic failure (8). The main event underlying liver fibrosis is caused by the activation of hepatic stellate cells (HSCs), which excessively accumulate extracellular matrix (ECM) proteins. Their activation is considered an

the important feature in the development of liver fibrosis (9, 10). The human hepatic cell line LX-2 is a useful tool for studying hepatic fibrosis and liver disease because it preserves the phenotype of activated stellate cells (11). Hence, LX-2 cells were utilized in this study to investigate the cellular effects of F-Pbl-E in hepatic fibrosis.

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used to determine the effect of F-Pbl-E on the TGF-β1-induced proliferation of LX-2 cells. Transforming growth factor (TGF-β1), a profibrogenic cytokine secreted by activated Kupffer cells and sinusoidal endothelial cells activates HSC and triggers hepatic fibrogenesis (12). As shown in Figure 1A, treatment of LX-2 cells with TGF-β1 stimulated their growth compared to control cells. However, 3 days of F-Pbl-E treatment significantly inhibited TGF-β1-induced HSC growth in

a dose-dependent manner (Fig. 1A). Similar results were observed in diphenyliodonium (DPI) (13)-treated cells, with a NAD(P)H oxidase inhibitor serving as a positive control (Fig. 1A). Meanwhile, F-Pbl-E (0 day; Pbl extract just before fermentation) showed only moderate inhibition.

Therefore, these results suggest that treatment with F-Pbl-E for 3 days provides a synergistic effect on suppressing the proliferation of activated HSCs and may prevent liver fibrosis. On the other hand, F-Pbl-E did not induce cytotoxicity in human kidney epithelial cells (HEK-293), suggesting that F-Pbl-E does not have a negative effect on normal human cells (Fig. 1B).

Since HSCs are a major source of collagen type I alpha 1 chain (COLIA1), increased expression of COLIA1 is observed in injured livers, which have increased numbers of proliferating HSCs, eventually leading to liver fibrosis and cirrhosis (14). On the other hand, expression of COLIA1 in HSCs remains quiescent in the intact liver (12). As shown in Figure 1C, treatment of LX-2 cells with F-Pbl-E (3 days) significantly reduced TGF- β 1-induced COLIA1 expression in a dose-dependent manner. These results suggest that treatment with F-Pbl-E (3 days) decreases the proliferation of activated LX-2 cells by suppressing TGF- β 1-induced gene expression compared to F-Pbl-E (0 day).

In addition, hepatic oxidative stress is a known main factor in the progression of liver fibrosis. In particular, the production of reactive oxygen species (ROS) plays a critical role in hepatic fibrogenesis by activating HSCs (15). In this study, human liver cancer cells (HepG2) were employed to investigate the cellular effects of F-Pbl-E (3 days) on ROS in liver fibrogenesis. As shown in Figure 2A, F-Pbl-E (3 days) significantly reduced H₂O₂-induced ROS in HepG2 cells. These results suggest that F-Pbl-E (3 days) has a positive effect on H₂O₂-induced liver fibrogenesis by reducing ROS levels in HepG2 cells. Meanwhile, treatment with F-Pbl-E (0 day) showed a less pronounced positive effect.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are transaminase enzymes thought to be critical in the assessment of liver disease. When the liver is damaged, AST and ALT are released from damaged liver cells and move into the serum. Thus, serum AST and ALT levels are the most commonly used indicators to determine the degree of liver injury (16). As shown in Figure 2B, treatment with F-Pbl-E (3 days) reduced H₂O₂-induced AST and ALT activity in HepG2 cells. Therefore, this indicates that F-Pbl-E (3 days) ameliorates H₂O₂-induced liver damage in HepG2 cells

to a greater extent than treatment with F-Pbl-E (0 day).

CONCLUSION

In conclusion, treatment with F-Pbl-E (3 days) had a synergistic anti-fibrosis effect in LX-2 and HepG2 cells compared to F-Pbl-E (0 day). F-Pbl-E (3 days) more effectively inhibited TGF- β 1-induced cell proliferation and gene expression in LX-2 cells. Moreover, F-Pbl-E (3 days) more adequately reduced H₂O₂-induced reactive oxygen species (ROS), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels in HepG2 cells. Collectively, these data suggest that treatment with F-Pbl-E (3 days) can suppress TGF- β 1- or H₂O₂-induced liver fibrosis in LX-2 and HepG2 cells. Therefore, F-Pbl-E (3 days) plays a synergistic functional role in protecting hepatocytes and could be effective in minimizing liver fibrosis.

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