Protective Effects of *Puerariae flos* Against Ethanol-Induced Apoptosis on Human Neuroblastoma Cell Line SK-N-MC

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ABSTRACT—-*Puerariae flos* (PF) is an oriental medical herb for alcohol abuse. To investigate whether PF possesses protective effects against ethanol (EtOH)-induced cytotoxicity in the central nervous system, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometric analysis, DNA fragmentation assay, and reverse transcription-polymerase chain reaction were performed on SK-N-MC human neuroblastoma cells. Cells treated with EtOH exhibited several apoptotic features, while those pre-treated with PF prior to EtOH exposure showed a decreased occurrence of apoptotic features. In addition, PF pre-treatment inhibited the EtOH-induced increase in caspase-3 mRNA expression. These results suggest that PF may exert protective effects against EtOH-induced apoptosis in human neuroblastoma cells.

**Keywords**: *Puerariae flos*, Ethanol, Apoptosis

*Puerariae flos* (PF), an oriental medical herb, is the flowers of *Pueraria thunbergiana*. It has been used traditionally to treat alcohol abuse, and medications based on PF were found to be useful in the treatment of anti-intoxication and anti-drinking (1).

Ethanol (EtOH) consumption during developmental central nervous system (CNS) leads to substantial neuronal loss in multiple brain regions (2) and results in serious symptoms including CNS dysfunction (3). However, the exact mechanism behind the induction of brain damage by EtOH is still elusive. Recently, the role of EtOH as an inducer of apoptosis has been described in cultured rat astroglia (3) and neuroblastoma cell line (2). In addition, Ikonomidou et al. (4) have reported that EtOH induces apoptotic neurodegeneration in the developing rat brain. In the CNS, apoptosis (programmed cell death) plays an indispensable role in the development and maintenance of homeostasis within all organisms (5). However, excessive apoptosis has been suggested to underlie the neuronal loss associated with the exposure to various neurotoxicants (6). The caspases, a class of cysteine proteases, are considered central players of the apoptotic process and trigger a cascade of proteolytic cleavage event in mammals (7, 8). Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executors of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many proteins (7, 8). Because of the involvement of apoptosis in EtOH-induced neuronal cell death, it appears logical that a drug that inhibits apoptosis may be of use in reducing EtOH-induced CNS damage. However, it has not yet been reported whether PF possesses protective effects against EtOH-induced cell death in the CNS. In this study, such effects of PF on neuroblastoma cell line SK-N-MC were investigated.

PF was obtained from Kyung-Dong Market place (Seoul, Korea). After washing, the PF was immersed in cold water for 12 h, and aqueous extracts from the PF were made by using a rotary evaporator. The human neuroblastoma cell line SK-N-MC was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO₂, 95% O₂; in a humidified cell incubator. Cell viability was determined by an MTT assay kit as per the manu-
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For analysis of the protective effects of PF against cell death induced by EtOH, cells were pretreated with 0.1 and 1 mg/ml PF for 24 h, before 100 mM EtOH was applied for 3 h. The control group was left untreated. To observe morphological changes after treatment with PF and EtOH, cells were analyzed by phase-contrast microscopy (Olympus, Tokyo). Flow cytometric analysis was performed as previously described (9). After treatment with PF and EtOH, cells were collected and fixed with 75% ethanol in phosphate-buffered saline (PBS) at −20°C for 1 h. Then the cells were incubated with 100 μg/ml RNase and stained with 20 μg/ml propidium iodide (Sigma, St. Louis, MO, USA) in PBS. The stained cells were incubated for 30 min at 37°C and were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA). For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed with an ApopLadder EX™ DNA fragmentation assay kit (Takara, Shiga). Cells were treated with PF and EtOH and then lysed with 100 μl of lysis buffer. The lysate was incubated with 10 μl of 10% SDS solution containing 10 μl of Enzyme A at 56°C for 1 h followed by treatment with 10 μl of Enzyme B at 37°C for 1 h. After, adding 70 μl of precipitant and the resultant pellet resuspended in TE (Tris-EDTA) buffer, DNA fragmentation was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide. To identify expression of caspase-3 mRNA, the reverse transcription-polymerase chain reaction (RT-PCR) was performed. Total RNA was isolated from SK-N-MC cells using RNAzol™B (TEL-TEST, Friendswood, TX, USA) as per the manufacturer’s instructions. RNA was then reverse-transcribed using random hexamers (Promega, Madison, WI, USA), AMV reverse transcriptase, 10 mM dNTP, RNasin and 10 × AMV RT buffer. The generated complementary DNA was amplified using 10 pM primers, 10 × RT buffer, 2.5 mM dNTP and Taq DNA polymerase (Takara). Exact primer sequences used in this study were designed according to Wang et al. (10). For caspase-3, the primer sequences were 5'-CTCGGTCTGGTACAGATGATG-3' (a 24-mer sense oligonucleotide starting at position 412) and 5'-GGTAAACCGGGTAAGATGTGCA-3' (a 24-mer anti-sense oligonucleotide starting at position 922). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCTCAGTTCGATGATGCA-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTGGCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 533 bp (for caspase-3) and 299 bp (for cyclophilin). For caspase-3, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94°C, annealing at 58°C, and extension at 72°C for 30 s respectively, with an additional extension step at the end of the procedure at 72°C for 5 min. For cyclophilin, the PCR was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically with Molecular Analyst™ software version 1.4.1 (Bio-Rad, Hercules, CA, USA). Results were expressed as mean ± S.E.M. The data was analyzed by one-way ANOVA followed by the Scheffe’s post-hoc test using SPSS. Differences were considered statistically significant for P<0.05.

As shown in Fig. 1, the viabilities of cells incubated with EtOH at concentrations of 10 mM, 50 mM, 100 mM,
500 mM and 1 M were 71.72 ± 3.09%, 67.18 ± 1.91%, 32.17 ± 1.79%, 26.00 ± 1.20% and 22.20 ± 0.84% of the control value (P<0.05), respectively. A trend of decreasing viability with increasing EtOH concentration was observed. The viability of cells treated with PF at 0.1 and 1 mg/ml for 24 h were 100.72 ± 2.10% and 107.11 ± 4.70% of the control value. The viability of cells exposed with 100 mM EtOH was 29.89 ± 1.60% of the control value (P<0.05), while cells pre-treated with PF at 0.1 and 1 mg/ml before exposure to EtOH was increased in a statistically significant fashion to 52.33 ± 2.24% and 77.03 ± 0.73%, respectively (P<0.05). Results of the MTT assay showed a significant decrease in the viability of EtOH-treated cells, while PF was shown to exert a protective effects against EtOH-induced cytotoxicity. In Fig. 2, the protective effects of PF against EtOH-induced cytotoxic changes were examined by phase-contrast microscopy. Cells treated with EtOH were seen to have detached from the dish, with cell rounding, cytoplasmic blebbing and irregularity in shape, while cells pre-treated with PF prior to EtOH exposure appeared to be similar to the control. Through flow cytometric analysis, the protective effects of PF against EtOH-induced cell death were determined. The population of cells in the sub-G1 phase in the EtOH group increased from 32.21% to 60.93%, while the figure was decreased with PF pre-treatment to 45.76%, a value similar to that of the control (Fig. 2).

In order to ascertain the protective effects of PF against EtOH-induced cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As shown in Fig. 3, EtOH treatment resulted in the formation of definite fragments that could be seen via electrophoresis as a characteristic ladder pattern; PF pre-treatment resulted in a significantly decreased intensity of EtOH-induced DNA laddering. RT-PCR analysis of the mRNA level of caspase-3 was performed. The mRNA level of caspase-3 of control cells was set at 1.00, markedly increased to 14.54 ± 1.44 in cells treated with EtOH and

**Control**  
**EtOH**  
**PF + EtOH**

![Fig. 2. Protective effects of *Puerariae flos* (PF) on the changes in human neuroblastoma cell line SK-N-MC induced by ethanol (EtOH). Cells were treated with 0.1 and 1 mg/ml PF for 24 h, followed by exposure to 100 mM EtOH for 3 h. Above: Phase-contrast microscopic examination. It showed cells shrinkage, irregularity in shape and cellular detachment in the EtOH-treated cultures; these morphological changes were, although present, far lower intensity in the PF pre-treated cells. Scale bar represents 100 μm. Below: Results of flow cytometric analysis. The fraction of cells in the sub-G1 phase was increased in the EtOH-treated cells compared to the control, but was reduced again in the PF pre-treated cells. All experiments were independently performed in triplicate.](image-url)
decreased to 2.84 ± 0.18 and 1.29 ± 0.19 in cells pre-treated with PF at 0.1 and 1 mg/ml, respectively (Fig. 3).

EtOH intake is associated with various CNS impairments; particularly, EtOH consumption during pregnancy is associated with various teratogenic effects on the fetus (2). Recently, it has been demonstrated that EtOH induces apoptotic neurodegeneration in the developing rat brain (4). In this study, MTT assay and phase-contrast microscopic examination revealed that EtOH exerts cytotoxicity on human neuroblastoma cell line and EtOH cytotoxicity was significantly protected by PF pre-treatment. From flow cytometric analysis, an increase in the fraction in the sub-G1 phase, which could be seen as a peak positioned close to the sub-G1 phase, was observed in the EtOH-treated group, and this increase was reduced in the PF pre-treated group. DNA fragmentation assay revealed that EtOH-treated group exhibits the distinctive ladder pattern characteristic of apoptosis, while the PF pre-treated group showed a noticeable decreases in the intensity of EtOH-induced DNA laddering. These results demonstrated that EtOH induces apoptosis in human neuroblastoma cell line; this observation was similar to the previous reports (2, 3, 4, 11) and PF shows protective action against EtOH-induced apoptosis. Recent reports indicate that caspases may play a role in neuronal cell death (12, 13) as well as after neuronal injury (14). Deaciuc et al. (15) reported that caspase-3 activity is significantly increased in EtOH-treated rats in vivo. The present results also showed marked increase in caspase-3 mRNA level in EtOH-treated cells, while PF inhibits EtOH-induced overexpression of caspase-3 mRNA. Based on the results, it can be suggested that PF possesses protective effects against EtOH-induced apoptosis in neuroblastoma cells probably by inhibiting caspase-3 mRNA.

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