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五加皮煎湯液이 에탄올에 의해 유발되는 TM3 mouse Leydig 세포주의 apoptosis에 미치는 영향

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Protective effects Acanthopanax senticosus extracts on ethanol-induced apoptosis in TM3 mouse Leydig cells

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Abstract

목적: 오가피가 mouse testis에서 유래된 Leydig 세포주에서 에탄올에 의해 유발된 아폽토시스에 미치는 영향을 조사하였다.

방법: TM3 세포주에서의 아폽토시스 변화를 관찰하기 위해서 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI), DNA fragmentation assay, 및 reverse transcription-polymerase chain reaction (RT-PCR) 방법을 이용하였다.

결과: MTT assay를 이용하여 분석한 결과 농도에 따른 세포독성의 효과가 에탄올 투여부터 관찰되었다. 또한 오가피로 전처치하고 에탄올을 처치하였을 때 세포독성이 크게 감소되었다. DAPI staining에서 오가피 투여군은 에탄올 투여군에 비해서 fragmentation이 억제되었다. RT-PCR의 분석에 의하여 caspase-3 mRNA 발현이 오가피 투여군은 알코올 투여군보다 유의성있게 억제됨을 보여주었다.

결론: TM3 Leydig 세포주에서 에탄올에 의해 유발된 아폽토시스는 전형적인 세포사멸 형태를 나타내었다. 반면에 오가피 투여군은 에탄올에 의해서 유발된 아폽토시스에서 세포보호 효과가 있음이 확인되었다.

Key words : *Acanthopanax senticosus*, ethanol, apoptosis, Leydig cell.

Ⅰ. Introduction

Acanthopanax senticosus (AS), a member of the araliaceae family, is a herb which has been used traditionally for improving ischemic injury, rheumatism, weakened physical status, and its hypoglycemic action[1-3]. The protective effects of AS against rat gastric ulcer under stressful stimuli were reported by Fujikawa et al[4].

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Alcohol consumption induces apoptotic cell death in various types of organs including brain[5], liver[6], thymus[7], and testis[8]. Alcohol has received much attention worldwide as alcohol disturbs endocrine functions and in particular, causes testicular atrophy, gonadal dysfunction, erectile dysfunction, and male infertility by triggering apoptosis in testicular germ cells in alcoholic men[8,9].

Apoptosis, also known as programmed cell death, is a biological process that plays a crucial role in normal development and tissue homeostasis[10]. However, inappropriate cell death contributes to a variety of human disorders[11]. Apoptosis is characterized by morphological changes including progressive cell shrinkage with condensation, and fragmentation of nuclear chromatin and membrane blebbing[12].

In numerous studies, the process of apoptosis has been shown to be regulated by the expression of several proteins. Caspases, a class of cysteine proteases, are considered to be central players in the apoptotic process, and they trigger a cascade of proteolytic cleavage events in mammals. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis[13,14]. Caspase-3 in its activated form has many cellular targets which, when severed and/or activated, produce the morphologic features of apoptosis[15].

Leydig cells, situated in the interstitial component of the mammalian testis, are responsible for most of the testosterone produced by males[16], and it has been reported that alcohol suppresses secretion of testosterone in the adult male rat[17,18]. In the present study, the effect of aqueous extracts of AS on EtOH-induced cytotoxicity was investigated in the TM3 mouse Leydig cells *via* 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) and DNA fragmentation assay, and reverse transcription -polymerase chain reaction (RT-PCR).

Ⅱ. Materials and Methods

1. Drugs and Reagents

Acantopanax senticosus (AS) was obtained from Kyung-Dong Market place (Seoul, Korea). After washing, a total of 50 g of AS was added to 500 ml of water and boiled for 2 hrs, filtered and the concentrated to 200 ml. This decoction was spray-dried to give a powdered extract.

Ethanol (EtOH) was purchased from Merck (Darmstadt, Germany). DAPI were obtained from Sigma Chemical Co. (St. Louis, MO, USA). MTT assay kit was purchased from Boehringer Mannheim (Mannheim, Germany). DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Japan).

2. Cell culture

Mouse Leydig cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in Dulbeccos Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL) at

37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator, and the medium was changed every 2 days.

3. MTT cytotoxicity assay

Cell viability was determined using MTT assay kit as the manufacturer's protocol. For analysis of the protective effects of AS against cell death induced by EtOH, cells were pre-treated with AS at concentration 0.1 mg/ml and 1 mg/ml for 24 hr. After cells were treated with AS, EtOH was applied at a concentration of 100 mM for 3 hr. The control group was left untreated. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 hr. The cells were then incubated in 100 ㎕ of the solubilization solution for 12 hr, and the absorbance was measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

4. DAPI staining

In order to determine whether EtOH induces apoptosis, DAPI staining was performed according to previously described protocols[8]. Cells were cultured on 4-chamber slides. Cells were pre-treated with AS at a concentration 1 mg/ml for 24 hr. After cells were treated with AS, EtOH was applied at a concentration of 100 mM for 3 hr. Cells were washed twice with PBS and fixed by incubation in 4% PFA for 30 min. Following a second washing in PBS, cells were incubated in 1 ㎍/ml DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope (Zeiss, Oberköchen, Germany).

5. DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using ApopLadder EX™ DNA fragmentation assay kit. Cells were lysed with 100 ㎕ of lysis buffer. The lysate was incubated with 10 ㎕ of 10 % SDS solution containing 10 ㎕ of Enzyme A at 56 ℃ for 1 hr followed by treatment with 10 ㎕ of Enzyme B at 37 ℃ for 1 hr. This mixture was then centrifuged for 15 min after adding 70 ㎕ of precipitant and 500 ㎕ of ethanol. The DNA was extracted by washing the resultant pellet in ethanol and resuspending it in TE (Tris-EDTA) buffer. DNA fragmentation was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide.

6. RNA isolation and RT-PCR

Total RNA was isolated from TM3 cells using RNAzol™ B (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instructions. Two ㎕ of RNA and 2 ㎕ of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65 °C for 10 min. One ㎕ of AMV reverse transcriptase (Promega), 5 ㎕ of 10 mM dNTP (Promega), 1 ㎕ of RNasin (Promega), and 5 ㎕

of 10 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μℓ with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 hr. For *caspase-3*, the primer sequences were 5-CTTGGTAGATCGGCCATCTGAAAC-3 (a 24-mer sense oligonucleotide starting at position 1141) and 5-GGTCCCGTACAGGTGTG CTTCGAC-3 (a 24-mer anti-sense oligonucleotide starting at position 1521). For *cyclophilin*, the internal control used in the study, the primer sequences were 5'-ACCCC ACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 405 bp (for *caspase-3*) and 299 bp (for *cyclophilin*). PCR amplification was performed in a reaction volume of 40 μℓ containing 1 μℓ of the appropriate cDNA, 1 μℓ of each set of primers at a concentration of 10 pM, 4 μℓ of 10 x reaction buffer, 1 μℓ of 2.5 mM dNTP, and 2 units of *Taq* DNA polymerase (TaKaRa). 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 30 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec, with an additional extension step at the end of the procedure at 72 °C for 5 min. For *cyclophilin*, the PCR procedure 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 using Molecular Analyst™ software version 1.4.1 (Bio-Rad, Hercules, CA, USA).

7. Statistical analysis

Statistical analysis was performed using Student's *t*-test and results were expressed as mean ± standard error mean (S.E.M.). Differences were considered significant for $P < 0.05$.

Ⅲ. Results

1. Protective effects of AS on EtOH-induced cytotoxicity

The viability of TM3 cells pre-treated with AS for 24 hr before exposure to EtOH for 3 hr is shown in Fig. 1. The viability of cells treated with AS only at concentration of 0.1 mg/ml and 1 mg/ml for 24 hr were 95.81 ± 1.21 % and 103.30 ± 4.44 % of the control value. The viability of cells exposed to 100 mM EtOH without AS pre-treatment was 30.63 ± 0.73 % of the control value. The viability of cells pre-treated with AS at concentration of 0.1 mg/ml and 1 mg/ml before exposure to EtOH was increased to 81.35 ± 2.26 % and 83.91 ± 2.14 %, respectively.

2. Morphological changes

To observe the protective effects of AS against EtOH-induced cytotoxic changes in

morphology, cells were examined by phase-contrast microscope. Cells treated with EtOH only were seen to have detached from the dish, with cell rounding, cytoplasmic blebbing, and irregularity in shape, while cells pre-treated with AS prior to EtOH exposure appeared to be morphologically similar to the control. In DAPI staining, cells were observed *via* fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. The assay has revealed that the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon EtOH treatment. On the other hand, cells pre-treated with AS prior to EtOH exposure appeared to be morphologically similar to the control.

3. Characterization of apoptosis via examination of DNA fragmentation

In order to ascertain the protective effects

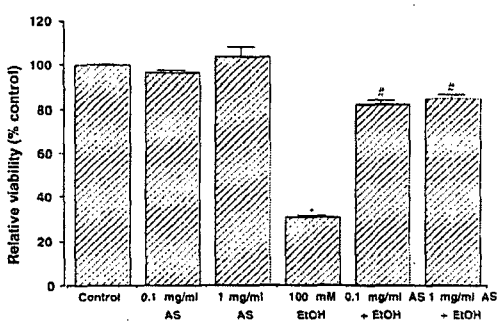

Fig. 1. Effect of *Acanthopanax senticosus* (AS) on cell viability. Viability was determined *via* MTT assay: AS was shown to exert a protective effect against ethanol (EtOH)-induced cytotoxicity. Results are presented as mean ± standard error (bars) for two independent experiments. *represents $P < 0.05$ compared to the control, #represents $P < 0.05$ compared to the EtOH-treated group.

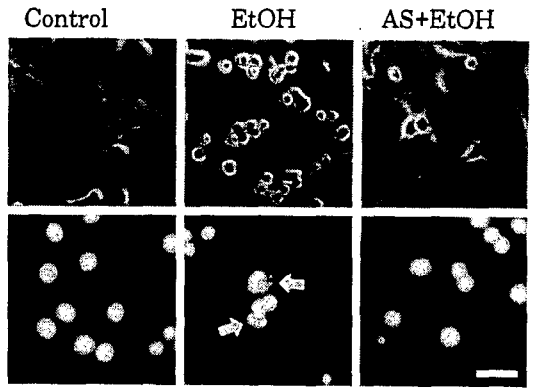

Fig. 2. Characterization of ethanol-induced cell death in TM3 cells. Top; Phase-contrast microscopy showed cell shrinkage, irregularity in shape, and cellular detachment in the EtOH-treated cultures; these morphological characteristics were not observed in the control (untreated), and although present, with a far lower intensity in the AS pre-treated group. Bottom: TM3 cells stained with DAPI. The white arrows indicate condensed nuclei. Scale bar represents 100 μm.

of AS against EtOH-induced cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As seen in Fig. 3, EtOH treatment resulted in the formation of definite fragments which could be seen *via* electrophoresis as a characteristic ladder pattern; AS pre-treatment resulted in a significantly decreased intensity of EtOH-induced DNA laddering.

4. Effects of EtOH and AS on mRNA expression of caspase-3

RT-PCR analysis of the mRNA level of *caspase-3* was performed in order to provide an estimation of the relative levels of expression of these genes. The mRNA level of *caspase-3* of control cells was set at 1.00, markedly

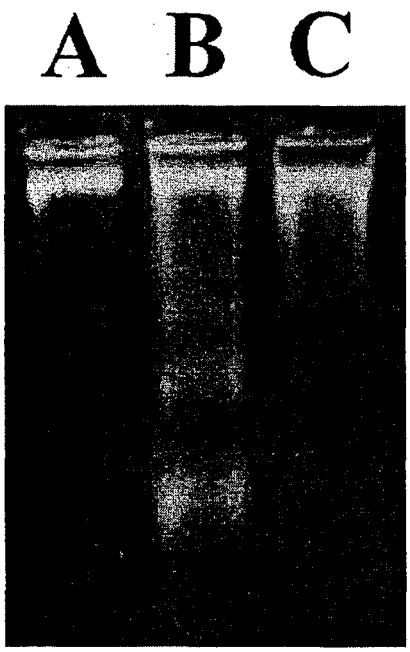

Fig. 3. Electrophoretic examination of genomic DNA of TM3 cells. Genomic DNA was extracted and analyzed *via* electrophoresis on a 2% agarose gel containing ethidium bromide. Cells of both groups exhibited the ladder pattern characteristic of apoptosis, although this pattern was of lower intensity in the case of the AS pre-treated group. A; Control, B; EtOH-treated group, C; AS pre-treated group.

increased to 6.88 ± 0.71 in cells treated with EtOH only and decreased to 4.84 ± 0.18 and 1.63 ± 0.22 in cells pre-treated with AS at concentrations of 0.1 mg/ml and 1 mg/ml, respectively.

Ⅳ. Discussion

The purpose of the present study was to find out whether AS exerts protective effects against EtOH-induced apoptosis in cells of the Leydig cell line TM3. It is well demonstrated

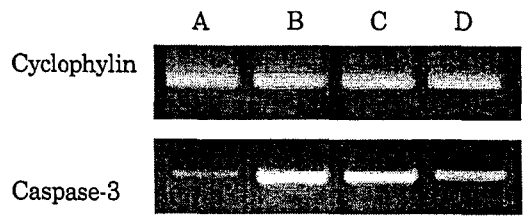

Fig. 4. Results of RT-PCR analysis of the mRNA levels of *caspase-3*. The level of expression of *caspase-3* in cells treated with AS was significantly lower than that in the cells exposed to EtOH. As the internal control, *cyclophilin* mRNA was also reversetranscribed and amplified. A; Control, B; EtOH-treated group, C; 0.1 mg/ml AS pre-treated group. D; 1 mg/ml AS pre-treated group.

that alcohol intake is associated with various tissue damage[11], particularly in reproductive organs[8]. In recent years, apoptotic cell death in testicular cells has been studied intensively, and apoptosis has been observed in testicular cells exposed to various pathologic factors[19]. It is known that EtOH may induce apoptosis in hepatocytes[6], thymocytes[7], and astroglia[5]. In the present study, apoptosis induced by alcohol on Leydig cells was observed. Assessment of cell viability in the present study *via* MTT assay confirmed that EtOH is dose-dependent in its cytotoxic effects and the administration of AS was shown to have a protective effect against the cytotoxic actions of EtOH. It is known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments, which appear as a characteristic ladder pattern upon agarose gel electrophoresis[20]. To provide evidence supporting the involvement of apoptosis in EtOH-induced cytotoxicity and the protective action of AS

against this effect, DNA fragmentation assay was performed. EtOH-treated group revealed the distinctive ladder pattern characteristic of apoptosis. On the other hand, the AS pre-treated group showed a noticeable decreases in the intensity of EtOH-induced DNA laddering.

In addition to the above effects, EtOH was also seen to cause characteristic changes in the morphology of TM3 cells and AS was seen to have a noticeable protective effect against these alterations. Under the phase-contrast microscope, cells of the AS pre-treated group appeared morphologically similar to those of the control group, but changes in appearance, including cell shrinkage, cytoplasmic condensation, and irregularity in shape, were seen in cells of the EtOH-treated group.

Understanding the molecular events triggering apoptosis is an important step toward the development of effective treatment strategies for such pathological diseases.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway[13]; caspase-3 in particular, when activated, has many cellular targets that, when activated, produce the morphologic features of apoptosis. The present data has also revealed increased expression of caspase-3 mRNA activity in cells exposed to EtOH, while cells pre-treated with AS showed a remarkable decrease. Thus, it was shown that AS inhibits EtOH-induced overexpression of the apoptotic pathway gene, caspase-3.

V. Conclusion

In the present study, it was investigated whether AS exerts anti-apoptotic effect on EtOH-induced cytotoxicity in Leydig cell line TM3. Based on the results, it can be suggested that AS possesses protective effects against EtOH-induced apoptosis in Leydig cells.

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