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원 저

The Effects of *Artemisia capillaries* Herbal Acupuncture on Ethanol-induced Apoptosis in Neuroblastoma Cell Line

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茵蔯 약침액이 신경아세포주에서 에탄올에 의해 유발된 아폽토시스에 미치는 영향

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목적 : 茵蔯 약침액이 SK-N-MC 신경아세포주에서 에탄올에 의해 유발된 아폽토시스에 미치는 영향을 조사하였다.
방법 : SK-N-MC cell line에서의 아폽토시스 변화를 관찰하기 위해서 MTT assay, DAPI staining 및 flow cytometric analysis 방법을 이용하였다.
결과 : MTT assay를 이용하여 분석한 결과 농도에 따른 세포 독성의 효과가 에탄올 투여로부터 관찰되었다. 또한 인진 약침액으로 전처치하고 에탄올을 처치하였을 때 세포 독성이 크게 감소되었다. DAPI staining에서 인진 약침액 투여군은 에탄올 투여군에 비해서 fragmentation이 억제되었다. Flow cytometry를 통하여 인진 약침액 투여군은 에탄올 투여군에 비하여 세포주기 중 sub G1 분획의 증가가 억제되었다.
결론 : SK-N-MC 신경아세포주에서 에탄올에 의해서 유발된 아폽토시스는 전형적인 세포사멸 형태를 나타내었다. 또한 인진 약침액은 에탄올에 의해서 유발된 아폽토시스에서 세포보호 효과가 있음이 확인되었다. *(J Korean Oriental Med 2001;22(1):90-95)*

Key Words: *Artemisia capillaries*, apoptosis, neuroblastoma, ethanol, herb acupuncture

INTRODUCTION

The developing central nervous system (CNS) is one of the major targets for the toxic action of EtOH[1], and EtOH consumption during neural development leads to substantial neuronal loss in several brain regions[2]. But these mechanism of EtOH-induced brain damage in the developing nervous system are still elusive. In recent years, however, the role of EtOH as an inducer of apoptosis has been described in astroglia[3], neuroblastoma[4], hepatocytes[5] and thymocytes[6]. In addition, Ikonomidou et al.[7] have reported that EtOH induced apoptotic neurodegeneration in the developing rat brain in vivo.

Programmed cell death, or apoptosis, is currently one

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of the hottest areas of modern biology. It describes the orchestrated collapse of a cell, staging membrane blebbing, cell shringkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by neighbouring cells. The excitement ensued when it became clear that apoptosis is and essential part of life for any multicellular organisms and that the way in which most cells die is conserved from worm to mammal[9].

Artemisia capillaries (AC) is a famous traditional Oriental medicine and used mainly as a choleretic, anti-inflammatory, and diuretic agent in the treatment of epidemic hepatitis[9]. It has been used for treatment of various liver disease, including alcoholism, in Korea. Recently, Hu et al.[10] AC induced apoptosis in human hepatoma cell line SMMC-7721.

Several reports suggested that ethanol-induced apoptosis may contribute to the pathophysiology of neurodegenerative disease. However, relatively few studies have been published concerning the prevention of apoptosis. In the present study, to determine the anti-apoptotic effects of AC on ethanol-induced apoptosis in the neuroblastoma cell line SK-N-MC, we have performed morphological analyses.

MATERIALS AND METHODS

1. Drugs & Reagents

AC was purchased from Kyung-Dong market (Seoul, Korea). After washing, AC was immersed in cold water for 12 hrs, and aqueous extract from AC was made by using rotatory evaporator. The total extract (AC, 50g) was dissolved in 500 ml water. Ethanol was obtained from Merck (Darmstadt, Germany). 4,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and paraformaldehyde (PFA) were obtained from Sigma chemical Co. (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Boehringer Mannheim (Mannheim, Germany).

2. Cell culture

The human neuroblastoma cell line, SK-N-MC cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured by previous reported method[11]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media (Gibco BRL, Grand Island, NY, USA). These media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA) at 37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator. Cells were plated at a density of 1×10^6 cells in culture dish (Corning Incorporated, NY, USA) and the media was changed once per 2 days.

3. MTT cytotoxicity assay

Cell viability was determined using a MTT assay kit as per the manufacturer's protocol. In order to determine cell viability of EtOH, cell were treated with EtOH at a concentration of 10 mM, 50 mM, 100 mM and 500 mM for 3 hrs. For analysis of the protective effects of AC against cell death induced by EtOH, cells pre-treated at concentration 10 mg/ml and 1 mg/ml with AC for 24 hrs. After cells treated with AC, EtOH exposed at concentration 100 mM for 3 hrs. And then, 10 μl of the MTT labeling reagent was then added to each well and the plates were incubated for 4 hrs. After cells were incubated in 100 μl of solubilization solution for 12 hrs, 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 result of the substraction of the absorbance at the reference wavelength from that of 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 using morphologic markers whether protective effects of AC against apoptosis induced by EtOH, DAPI staining procedure was performed according to previously described protocols[12,13]. In brief, SK-N-MC cells were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL) at a density of 2 x 10^4 cells/chamber. After treatment with AC (1 mg/ml) for 24 hrs and next with 1 M of EtOH for 3 hrs, the cells were washed twice with phosphate-buffered saline (PBS; Sigma) and fixed by incubation in 4% paraformaldehyde (PFA; Sigman, St. Louis, MO) for 30 min. Following a second washing in PBS, cells were incubated in 2 μg/ml DAPI solution for 30 min in the dark. The cells were observed with a fluorescence microscope (Zeiss, Oberkchen, Germany)

5. Flow cytometric analysis

Flow cytometric analysis was performed as previously described method (Yim et al., 2000). Briefly, after treatment with AC at concentration of 1 mg/ml for 24 hrs, and cells were treated with EtOH at a concentration of 1 M for 3 hrs. The collected cells were washed twice with PBS, and fixed with 75 % ethanol in PBS at 20 ℃ for 1 hr. After washing twice with PBS, the cells were incubated with 100 g/ml RNase (Sigma, St. Louis, MO) and stained with 20 g/ml of propidium iodide (PI; Sigma, St. Louis, MO) in PBS. The stained cells were incubated for 30 minutes at 37℃ and were analyzed with a FACScan (Becton Dickinson, San Jose, CA).

6. Statistical analysis

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

RESULTS

1. MTT assay for cells viability

1.1. The effects of EtOH on SK-N-MC cells viability

In order to find out the concentration at which the cytotoxicity effects of EtOH on the SK-N-MC cell line become evident, cells were cultured with EtOH at final concentrations of 10 mM, 50 mM, 100 mM and 500 mM for 3 hrs, and MTT assays were carried out, with cells cultured in EtOH-free media as the control. The viabilities of cells incubated with EtOH at concentrations of 10 mM, 50 mM, 100 mM and 500 mM were 71.72 ±3.09%, 49.31 ±2.35%, 42.76 ± 1.90% and 33.56 ±2.32% of the control value respectively. A trend of decreasing viability with increasing EtOH concentration was observed(Fig. 1).

1.2. The preventive effects of AC on EtOH-induced cytotoxicity

The viability of SK-N-MC cells pretreated with AC for 24 hrs before exposure to EtOH for 3 hrs is shown in

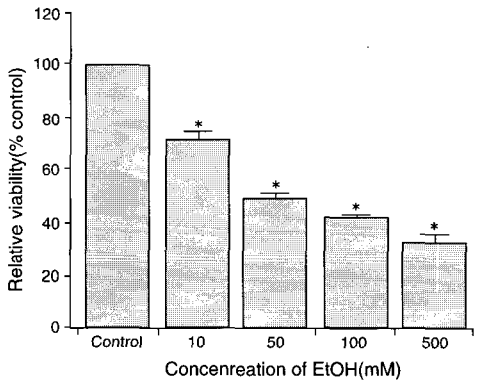

Fig. 1. Cytotoxic effects of ethanol (EtOH) on SK-N-MC cells.
Cells were incubated with EtOH at various concentrations (10 mM, 50 mM, 100 mM and 500 mM) for 3 hrs prior to the determination of cellular viability through MTT assay. Relative viability is shown as the percentage absorbance of the sample with respect to that of the control (% control), i.e. the untreated cells. Results are represented as mean standard error (bars). Values significantly different from the control at $P < 0.05$ are indicated with asterisks.

Fig. 2. Viability of cells pre-treated with AC at concentrations of 1 mg/ml and 10 mg/ml for 24 hrs were 122.56 ±1.4%, 64.96 ±3.68%. And viability of cells exposed to 100 mM of EtOH for 3 hrs with AC pre-treatment for 24 hrs at a concentrations of 1 mg/ml and 10 mg/ml were 61.99 ±2.00 %, 65.31 ±2.89% respectively, which viability of cells exposed to only EtOH was 42.76 ±1.90 % of the control value.

2. The preventive effects of AC on ethanol-induced apoptosis

To observe the protective effects of AC against EtOH-induced apoptosis by cell morphology, cells were examined by phase-contrast microscopy. As shown in Fig. 3, 100 mM EtOH-treated cell for 3 hrs were seen to have detached from the dish, with cell rounding and cytoplasmic blebbing and irregularity in shape, while cells pre-treated with AC at a concentration 1 mg/ml for 24 hrs prior to EtOH exposure showed similar shape to the control. To further confirm protective effects of AC against EtOH-induced apoptosis in SK-N-MC cells, biochemical analysis was performed via DAPI staining assay. Stained cells were observed via fluorescence microscopy 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-treated cells, while cell pre-treated with AC prior to EtOH exposure showed similar morphological shape to the control.

3. Cell cycle distribution change

Through flow cytometric analysis, protective effects of AC against EtOH-induced apoptosis was determined. As shown Fig. 3, 100 mM EtOH-treated cells for 3 hrs increased from 32.21 % to 60.93% of total cells number in the sub-G1 phase while number of cells in the sub-G1 phase was decreased to 34.13 % with pre-treated with 1 mg/ml of Ac for 24 hrs before exposed to EtOH

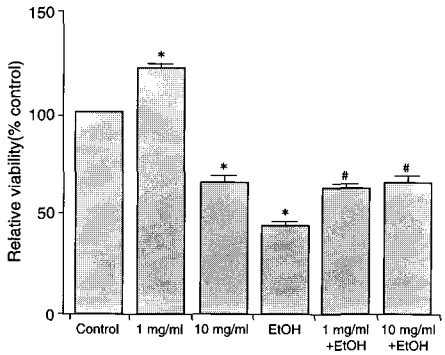

Fig. 2. The protective effect of *Artemisia capillaries* (AC) on ethanol-induced apoptosis in SK-N-MC cells by MTT assay.
Cells were treated with AC at two concentrations (1 mg/ml, 10 mg/ml) for 24 hrs, followed by exposure to EtOH at a concentration of 100 mM for 3 hrs in some of the cultures. In cultures which were exposed to alcohol after AC treatment, it was shown to exert a protective effect. Results are represented as mean standard error (bars). Values significantly different from the control at $P < 0.05$ are indicated with asterisks.

same the control value. In this study, AC may exerts protective effects against EtOH-induced apoptosis.

DISCUSSION

The purpose of the present study was to find out whether AC exert protective effects against EtOH-induced apoptosis in cells of the neuroblastoma cell line SK-N-MC. EtOH intake is associated with various CNS impairments. However, the mechanisms of EtOH-induced brain damage in the developing nervous system are poorly understood. Recently previous studies have demonstrated that EtOH induces apoptotic neurodegeneration in the developing rat brain[7]. It is known that apoptosis, a mode of refulated cell death, is an important mechanism in the developing CNS. Apoptosis is a genetically controlled programmed cell death mechanism serving physiologic and homeostatic functions. It is defined by a number of features,

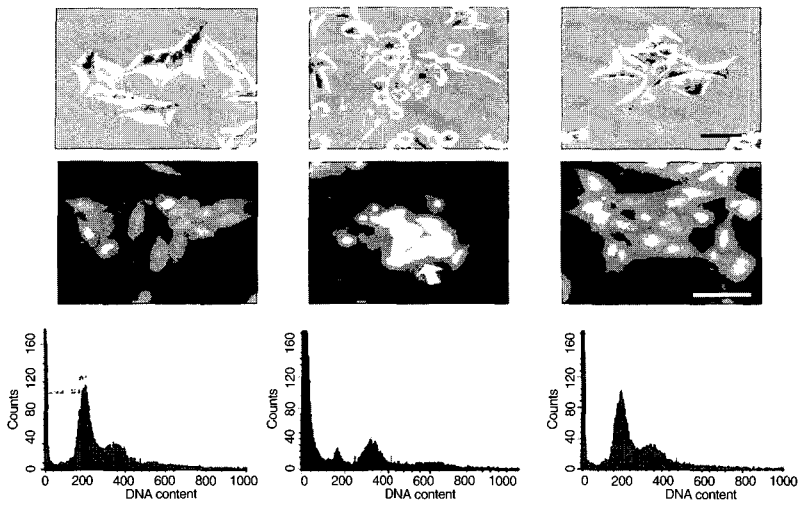

Fig. 3. Characterization of the effects of AC on the changes in SK-N-MC cells induced by EtOH.
Cells were treated with AC at a concentration of 1 mg/ml for 24 hrs, followed by exposure to 1 M EtOH for 3 hrs. Left: Control, Center: EtOH, Right: AC + EtOH, Top: Morphology. Phase-contrast microscopy showed cell shrinkage, irregularity in shape, and cellular detachment in the EtOH-treated group (B); these morphological characteristics were not observed in the control (A), and although present, with a far lower intensity in the AC-treated cells (C). Scale bars represent 100 μm. Center: DAPI staining. Nuclear condensation, while not present in the control (D) and AC-treated (F) groups, was observed in the EtOH-treated (E) group. White arrows indicate condensed nuclei. Bottom: Results of flow cytometric analysis. The fraction of cells in the sub-G1 phase was increased in the EtOH-treated cells (H) compared to the control (G) but was reduced again in the AC-treated cells (I), to a level comparable to that seen in the control. All experiments were triplicated independently.

including decrease in cell size, condensation of the cytoplasm, blebbing of the plasma membrane, collapse of the chromatin, fragmentation of DNA into oligonucleosome-length and apoptotic bodies. Ultimately, the apoptotic bodies are phagocytosed and degraded by neighboring cells. It is a normal process during development and a morphologically distinct form of cell death which is involved in the pathogenesis and pathophysiology of several known human disease, such an autoimmune dysfunction, cancer, stroke and neurodegenerative diseases. To date ethanol and apoptosis are known to perform very important roles in physiological phenomena and the pathophysiology of various diseases.

Assessment of cell viability via MTT assay confirmed that EtOH is dose-dependent in its cytotoxic effects and the administration of AC prior to exposure to EtOH was shown to have a protective effects against the cytotoxic actions of EtOH. From flow cytometric analysis of DNA content using the DNA-specific dye PI, 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 cells treated with 100 mM EtOH; this observation was similar to the results reported by Holownia et al.[3] from their study involving cultured rat astroglia. This increase in the sub-G1 phase fraction was reduced, to a level comparable to that seen in the control, in cells treated with AC prior to exposure to EtOH.

In the present study, we investigate whether Ac influence anti-apoptotic effects on EtOH-induced cell death in neuroblastoma cell line SK-N-MC. Based on the results, we suggested that AC possess protective effects against EtOH-induced apoptosis in neurob-

lastoma cells.

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