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원저

Phellodendri Cortex Herbal-acupuncture Solution Induced Apoptosis in Human Cervical Cancer Cells, SNU-17

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Abstract

Phellodendri Cortex (PC) has been used traditionally in Korea for damp heat leukorrhea with thick, yellow, discharge, foul-smelling diarrhea or dysentery. We investigated whether the Phellodendri Cortex Herbal-acupuncture Solution (PCHS) induced cell-death on SNU-17, human cervical cancer cell. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to find out the cytotoxicity of PCHS. The cell death was identified as apoptosis from the results of 4, 6-diamidineo-2-phenylindole (DAPI) staining, terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. The expression of proapoptotic gene, Bax, was increased and the expression of apoptotic gene, Caspase-3, was also increased. Considering the above results, PCHS could induce the apoptosis on SNU-17, human cervical cancer cell, via Bax-related Caspase-3 activation. And it might provide the experimental data for the clinical use of Phellodendri Cortex on cervical cancer.

Key Words : Apoptosis, Phellodendri Cortex, Herbal-acupuncture Solution, Cervical Cancer Cells

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Ⅰ. Introduction

Apoptosis is known as programmed cell death which occurs in several pathological situations. It has been recognized that induction of apoptosis is a highly desirable mode as a chemopreventive strategy for cancer control[1-2].

Carcinoma of the uterine cervix is one of the most common neoplasms in women[3]. The incidence rates of cervical cancer differ greatly between developed and developing countries[4].

Recently, the development of new anticancer drug is a key issue for cancer chemotherapy because of the reality that cancer cells which are resistant to chemotherapy will eventually cause the mortality. Many researchers are tried to find the therapeutic key step of cervical cancer and to develop new anticancer compounds. Herb medicines as substitute cancer remedies have attracted a great deal of interest because of their low toxicity and costs.

Phellodendri Cortex (PC, the peel of Phellodendron amurense Rupr.) has been traditionally used for damp heat leukorrhea with thick, yellow, discharge, foul-smelling diarrhea or dysentery, Damp Heat jaundice. And it is used for ascending kidney fire with such deficient Yin signs as steaming bone syndromes, night sweats, afternoon fevers and sweating, fire poison generated sores and damp lesions of the skin, sometimes accompanied by nocturnal emission and spermatorrhea[5]. In present, it has been examined the inhibitory effect of Lewis lung carcinoma[6] and its possibility for use in anticancer photodynamic therapy[7]. So we investigated whether the Phellodendri Cortex Herbal-acupuncture Solution (PCHS) induced cell-death on SNU-17, human cervical cancer cell.

The effects of PCHS on apoptosis was investigated through 3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay, 6-diamidineo-2-phenylindole (DAPI) staining, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. We also performed reverse transcription-polymerase chain reaction (RT-PCR) for apoptotic genes including Bax and Caspase-3.

Ⅱ. Materials and Methods

1. Materials

1) Preparation of Herbal- acupuncture solution

Phellodendri Cortex (Phellodendron amurense Rupr.) was purchased from Kyungdong market (Seoul, Korea). The sample was authenticated by college of Korean medicine, Semyung university, where the voucher specimen was preserved. 70% ethanol extracts of PC (yield: 16.52% of dry wt.) were obtained by 48h maceration at room temperature. The ethanol extract was filtered through a 0.45㎛ filter (Osmonics, Minnetonka, MN, USA), lyophilized, and kept at 4°C. The dried extract was re-solublized in saline before use PCHS.

2) Cell culture

The SNU-17 cells were purchased from Korean Cell Line Bank (KCLB, Seoul). SNU-17 cells were cultured in Rosewell Park Memorial Institue (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY). Cells were maintained in a humidified incubator at 37°C

in an atmosphere of 5% CO2 and 95% air, and the medium was changed every 2 days.

2. Methods

1) MTT cytotoxicity assay

Cytotoxicity was measured by MTT assay (Sigma, St. Louis, MO, USA) as previously described[2]. In order to detect the cytotoxicity of PCHS, SNU-17 cells were treated with PCHS at concentrations of 10, 50, 100 and 500 ㎍/㎖ at 24h. The control group was treated with the same amount of vehicle. Cells with or without PCHS extract were washed and treated with MTT labeling reagent. After the cells were incubated in the dark for 4h, absorbance at a test wavelength of 595㎚ with a reference wavelength of 690㎚ were measured using a microtiter plate reader (Bio-Tek, Winooski, VT, USA). The optical density (O.D.) was calculated as the difference between the absorbance from reference wavelength and from test wavelength. Percent viability was calculated as (O.D. of drug-treated sample / O.D. of none treated sample) ×100.

2) DAPI staining

Apoptotic cells induced by PCHS were determined by DAPI staining (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol[8]. SNU-17 cells were cultured on four-chamber slides (Nalge Nunc International, Naporvile, IL). After 300㎍/㎖ PCHS treatment, the cells were fixed by incubation in 4% paraformaldehyde for 30min, and were incubated for 30min in the dark, including 1㎍/㎖ DAPI solution. Then, treated cells were observed through a fluorescence microscopy (Zeiss, Oberköchen, Germany).

3) TUNEL assay

For in situ detection of apoptosis cells, TUNEL assay was performed using Roche in site cell death detection kit (Roche, Indianapolis, IN, USA). After treatment with PCHS (300㎍/㎖) for 12h, the cells were fixed in acetic acid for 5min at 20℃. The fixed cells were incubated with TUNEL-reaction mixture of enzyme solution (terminal deoxynucleotidyl transferase) and label solution (nucleotide mixture) for 1h at 37℃, and were then washed at room temperature. A converted-POD (antibody conjugated with peroxidase) was added and the cells were incubated for 30min. The DNA fragments were stained using 3, 3'-diaminobenzidine (Sigma, St. Louis, MO, USA) as the substrate for the peroxidase.

4) RT-PCR analysis

Total RNA was isolated from SNU-17 cells with RNAzolTMB (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instruction. RT-PCR was performed with following primers for Bax (5'-AAC ATG GAG CTG CAG AGG ATG ATT-3', 5'-CTG GTC TTG GAT CCA GCC AGC CCA ACA G-3') and for Caspase-3 (5'-CTT GGT AGA TCG GCC ATC TGA AAC-3', 5'-GGT CCC GTA CAG GTG TGC TTC GAC-3'). The Cyclophilin(5'-ACC CCA CCG TGT TCT TCG AC-3', 5'-CAT TTG CCA TGG ACA AGA TG-3') was used as an internal control. The RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

5) Statistical analysis

Results were expressed as mean±SEM. The data were analyzed by one-way ANOVA followed by Dunnett's post-hoc analysis using

SPSS. Differences were considered significant at $P < 0.05$.

Ⅲ. Results

1. Cytotoxicity of PCHS on SNU-17 cells

The effects of PCHS on the viability of SNU-17 cells are shown in Fig. 1. PCHS treatment displayed significantly dose-dependent decrease of cell viability. Viability of cell treated with PCHS at concentrations of 10, 50, 100 and 500㎍/㎖ for 24h was 104.1 ± 15.8%, 96.6 ± 5.6%, 89.4 ± 2.9% and 61.8 ± 6.9% of control group value, respectively (Fig. 1). This result indicates that PCHS induced cell death in SNU-17 cells dose-dependent manner.

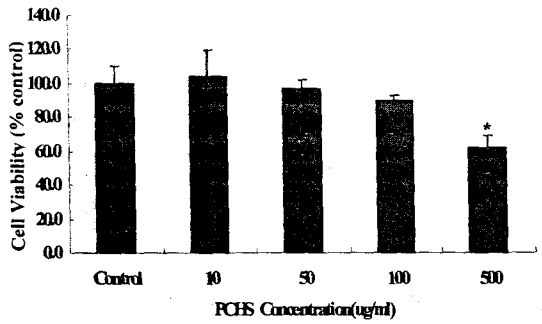

Fig. 1. Effect of PCHS on SNU-17 cells cytotoxicity

Cytotoxicity of Phellodendri Cortex Herbal-acupuncture Solution (PCHS) on the viability of human cervical cancer cells, SNU-17, for 24h by MTT assay. Results are presented as mean±SEM. The experiments were done triplicate. (* represents $P < 0.05$ compared to the control group).

2. Morphological changes induced by PCHS

DAPI staining and TUNEL reaction were performed to detect whether the death of SNU-17 cells was caused by apoptosis. SNU-17 cells treated with PCHS (300㎍/㎖) for 12 h revealed apoptotic cellular bodies through phase-contrast microscopy (Fig. 2B), whereas apoptotic cells were not observed in the untreated cells (Fig. 2A). DAPI staining showed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon PCHS treatment (Fig. 2D), while the untreated cells were not shown apoptotic features (Fig. 2C). As shown in Fig. 2F, analysis through TUNEL assay was ascertained that DNA strand breaks occur, and

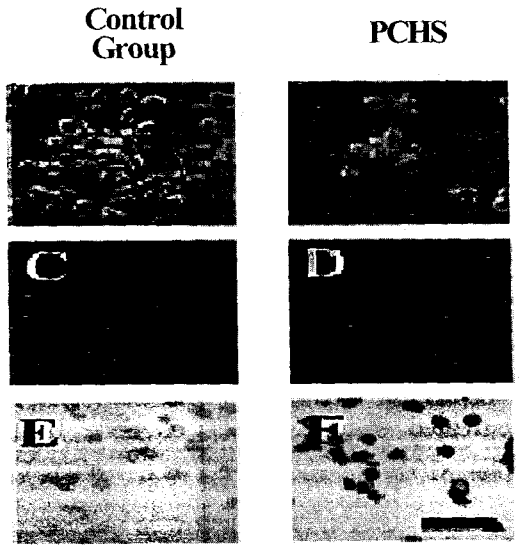

Fig. 2. Characterization of PCHS-induced cell death in SNU-17 cells

Characterization of Phellodendri Cortex Herbal-acupuncture Solution (PCHS) induced cell death in human cervical cancer cells SNU-17. Cells were cultured without PCHS (A, C and E) or with 300 ㎍/㎖ of PCHS (B, D and F). Morphology (top): phase-contrast microscopy showed cell shrinkage, irregularity in shape and cellular detachment in PCHS-treated cultures (B). DAPI staining (middle): SNU-17 cells stained with DAPI. Condensed nuclei were revealed by DAPI staining (D). TUNEL assay (bottom): SNU-17 cells stained using TUNEL method. Condensed and marginated chromatin showed to be stained dark brown (F). Scale bar, 100㎛.

it indicated the induction of apoptosis by PCHS in SNU-17 cells. TUNEL-positive cells were stained with dark brown. In Fig. 2E, the untreated control group was not revealed TUNEL-positive cells. This result showed that treatment of 300㎍/㎖ PCHS induced apoptosis in SNU-17 cells.

3. mRNA expression of apoptotic genes by PCHS

The effect of PCHS was examined on mRNA expressions of pro-apoptotic genes (Bax and Caspase-3). In cells treated with 300 ㎍/㎖ of PCHS, mRNA expressions of Bax and Caspase-3 were increased compared with none-treated cells (Fig. 3).

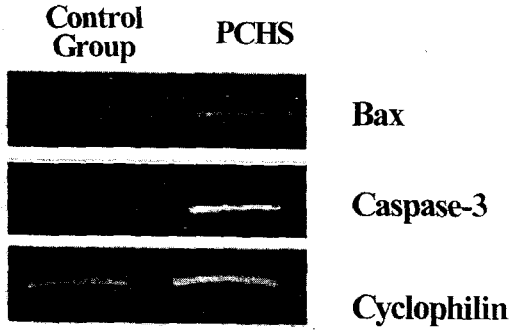

Fig. 3. Results of RT-PCR analysis of the mRNA of Bax and Caspase-3

RT-PCR analysis of apoptotic genes. Apoptotic genes (Bax and Caspase-3) were reverse-transcribed and amplified by RT-PCR. Cyclophilin mRNA was used as the internal control. Independent experiment was performed three times. Test group was treated with 300㎍/㎖ of PCHS (Phellodendri Cortex Herbal-acupuncture Solution).

Ⅳ. Discussions

Carcinoma of the uterine cervix is one of the most common neoplasms in women[3]. The incidence rates of cervical cancer differ greatly between developed and developing countries[4].

Phellodendri Cortex has been traditionally used for damp heat leukorrhea with thick, yellow, discharge, foul-smelling diarrhea or dysentery, damp heat pouring downward or swollen leg and painful knee, legs or foot, damp heat jaundice. And it is used for asending kidney fire with such deficient Yin signs as steaming bone syndromes, night sweats, afternoon fevers and sweating, fire poison generated sores and damp lesions of the skin, sometimes accompanied by nocturnal emission and spermatorrhea[5].

There have been several reports on the biological actives of compounds from Phelloderi Cortex such as responses of the blood pressure[9] and joint arthritis on the reduce of inflamation content induced by lipopolysaccharide[10].

Apoptosis, a form of cell death, is known as programmed cell death which occurs in several pathological situations in multicultural organism[11], as well as, is characterized as series of conserved steps. The early steps include chromatin condensation, nuclear membrane blebbing and cytoplasmic shrinkage. The late steps include loss of adherence, DNA degradation, compacted organelles, condensed cytoplasm and nuclear material into membrane-bound apoptotic bodies and phagocytosis by the neighboring cells[12-13]. It can be triggered by numerous stimuli, including antigens, carcinogens, extracellular calcium, UV irradiation, growth factor depletion and chemotherapeutic agents[12,14].

The cell viability through MTT assay in present study verified that PCHS reflects a cytotoxic effect on SNU-17 cells in dose-dependent manners. PCHS also showed change in morphology of SNU-17 cells. It has been reported that cells undergoing apoptosis exhibit cytoplasmaic blebbing, nuclear shrinkage, chromatin condensation, irregularity

in shape and retraction[8], and the characteristic changes of the apoptosis were caused in morphology of PCHS-treated SNU-17 cells. In this study, we observed the apoptotic morphology of cellular bodies and the chromatin condensation in DAPI staining. In addition, it is known that DNA strand breaks occur during the process of apoptosis, and the nicks in DNA molecules can be quantitatively and qualitively detected the apoptosis status of cells through TUNEL assay[15]. In present study, typical TUNEL distinction of apoptosis was observed in PCHS-treated cells.

In this study, our result showed that treatment of PCHS induced dose-dependent cell death in SNU-17 cell. Upregulated mRNA expressions of Bax and Caspase-3 indicated that the cell death was due to apoptosis.

Bax, a pro-apoptotic member, was upregulated, in the end, those were disrupted mitochondrial membrane potential[16-17]. Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway: Caspase-3, in particular, is believed to be one of the most commonly involving in the execution of apoptosis in various cell types[18]. Thus, based on the apoptotic method for cervical cancer, we investigated more detail antitumor activity on SNU-17, human cervical cancer cell line, with PCHS showing significant apoptotic activity in preliminary experiment.

In a number of studies, it has been documented that the progress of apoptosis is regulated by the expression of several transcriptional proteins. Bax, a pro-apoptotic protein of the family, is expressed abundantly and selectively during apoptosis, promoting cell death[19]. Moreover, the execution phase of apoptosis involves a series of morphological and biochemical changes that appear to be resulted from the action of cysteine-dependent asparatate-directed proteases, Caspases[20]. Particularly, Caspase-3 not only is a member of the CED-3 subfamily of caspases, but also cleaves most of caspases-related substrates, including key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase[21], the inhibitor of caspase-activated deoxynuclease[22], and gelsoline and fodrin, which induced in apoptosis regulation[23]. Our data showed the increased exhibition of Bax, as well as, Caspase-3 protein after treatment of PCHS on SNU-17.

Based on the results, PCHS appears to activate specific intracellular death-related pathways, leading to Bax-dependent Caspase-3 activation and induction of apoptosis in SNU-17 cells. These results suggested that the treatment of PCHS affected anticancer activity on SNU-17, human cervical cancer cell, through regulation of Bax and potent activation of Caspase-3, and PCHS might be used for therapeutic anticancer drug.

V. Conclusions

To detect the activity of Phellodendri Cortex Herbal-acupuncture Solution (PCHS) on apoptosis-inducing effects in human cervical cancer cell line SNU-17, we performed various experiments on cell viability, morphological changes, expression of Bax, Caspase-3 mRNA.

The results are as followings:

1. The viabilities of SNU-17 cells exposed to PCHS were significantly reduced dose-dependent manner in MTT assay.
2. In cells treated with PCHS, the occurrence of apoptotic features has increased morphological changes in SNU-17 cells.
3. In cells treated with PCHS, mRNA expression of Bax and Caspase-3 were

increased compared with none-treated cells.

Considering the above results, It was demonstrated that PCHS could induce the apoptosis on SNU-17, human cervical cancer cells.

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