대한침구학회지 제24권 제4호(2007년 8월)
The Journal of Korean Acupuncture & Moxibustion Society

원 저

Spatholobi Caulis Herbal-acupuncture Solution Induced Apoptosis in Human Cervical Cancer Cells, SNU-17

Seo Bo-myung*, Lee Yoon-kyung*, Lee Bong-hyo*, Lee Kyung-min*, Lim Seong-chul*, Jung Tae-young** and Seo Jung-chul*

*Department of Acupuncture & Moxibustion, College of Oriental Medicine, Daegu Haany University
**Je-Han Oriental Medical Academy

국문초록

계형등 약침액이 자궁경부암 세포주 SUN-17의 세포 사멸에 미치는 효과

서보명* · 이윤경* · 이봉효* · 이경민* · 임성철* · 정태영** · 서정철*

*대구한의대학교 한의과대학 침구경혈학교실
**제한동의학술원

목적 : 본 연구는 계혈등 약침액이 자궁경부암 세포주 SNU-17에서 세포 사멸 효과가 있는지 알아보고자 하였다.

방법 : 자궁경부암 세포주 SNU-17에서 세포 사멸의 변화를 관찰하기 위해서 MTT cytotoxicity assay, DAPI staining, TUNEL assay, RT-PCR analysis 방법을 이용하였다.

결과 : 세포독성 검사에서 계혈등 약침액은 자궁경부암 세포주 SNU-17에 농도 의존적으로 세포독성을 나타내었다. 이러한 계혈등 약침액의 세포독성이 세포사멸로 인한 것인지 다른 기전에 의한 것인지 알아본 결과 계혈등 약침액에 의한 세포독성은 DAPI staining과 TUNEL assay에서 세포사멸의 특징적인 소견들을 나타내었다. 계혈등 약침액이 Bax, Caspase-3의 발현에 미치는 영향을 RT-PCR로 관찰한 결과 계혈등 약침액은 Bax, Caspase-3의 발현을 증가시켰다.

결론 : 이상의 결과 계혈등 약침액이 자궁경부암 세포주 SNU-17에서 세포 사멸을 야기하여 자궁경부암의 치료에 유용할 것으로 사료된다.

핵심단어 : 계혈등, 약침액, 세포사멸, 자궁경부암 세포주, SNU-17

· 접수 : 2007년 7월 13일 · 수정 : 2007년 7월 25일 · 채택 : 2007월 7월 28일
· Corresponding author : Seo Jung-chul, Department of Acupuncture & Moxibustion, Daegu Haany University Oriental Hospital, #165, Sang-dong, Suseong-gu, Daegu, Republic of Korea
Tel. 82-53-770-2112 E-mail : acumox@hanmail.net

I. Introduction

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 tring to find the therapeutic key step of 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.

Carcinoma of the uterine cervix is one of the most common neoplasms in women[1]. The incidence rates of cervical cancer differ greatly between developed and developing countries[2].

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[3-4].

Spatholobi Caulis (SC, the stem of *Spatholobus suberectus Dunn*) has been traditionally used to harmonize and tonify blood and alleviate dysmenorrhea, irregular menstration or amenorrhea accompanied abdominal pain. It activates the meridian and relaxes the symptom such as numbness of the extremities, low back pain, knee pain or generalized joint soreness from wind damp painful obstruction with either deficient blood or congealed blood. And it is used to dry dampness and transform phlegm[5]. Besides, it has been used in Oriental medicine to treat cancer and blood stasis[6]. So we investigated whether the Spatholobi Caulis Herbal-acupuncture Solution (SCHS) induced cell-death on SNU-17, human cervical cancer cells.

In this study, The effects of SCHS 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

Spatholobi Caulis (*Spatholobus suberectus Dunn*) 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 SC (yield : 11.4% 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 SCHS.

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 described4). In order to detect the cytotoxicity of SCHS, SNU-17 cells were treated with SCHS 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 SCHS 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 SCHS were determined by DAPI staining (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol[7]. SNU-17 cells were cultured on four-chamber slides (Nalge Nunc International, Naporvile, IL, USA). After 300 ㎍/㎖ SCHS 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 SCHS (300㎍/㎖) for 12h, the cells were fixed in acetic acid for 5min at 20°C. The fixed cells were incubated with TUNEL-reaction mixture of enzyme solution (terminal deoxynucleotidyl transferase) and label solution (nucleotide mixture) for 1h at 37°C, 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 SCHS on SNU-17 cells

The effects of SCHS on the viability of SNU-17 cells are shown in Fig. 1. SCHS treatment displayed significantly dose-dependent decrease of cell viability. Viability of cells treated with SCHS at concentrations of 10, 50, 100 and 500㎍/㎖ for 24h was 100.6 ± 9.6%, 86.2 ± 4.2%, 78.6 ± 7.1%, and 62.3 ± 6.7% of control value, respectively (Fig. 1). This result indicates that SCHS induced cell death in SNU-17 cells with dose-dependent manner.

2. Morphological changes induced by SCHS

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 SCHS (300㎍/㎖) for 12h revealed apoptotic cellular bodies through phase-contrast microscopy (Fig. 2B), whereas apoptotic cells were not observed in the

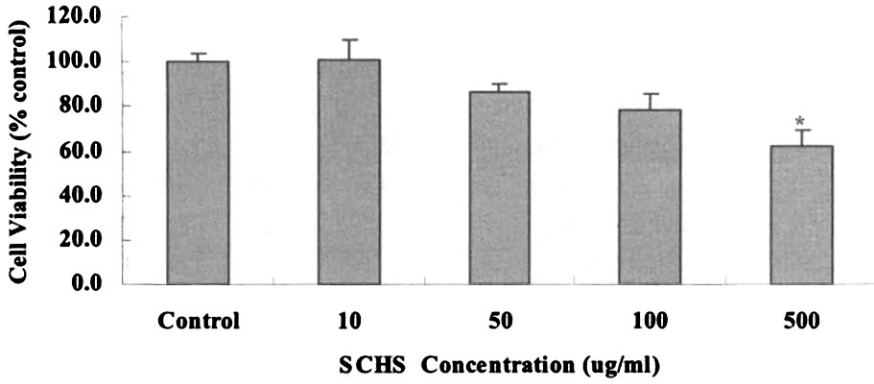

Fig. 1. Effect of SCHS on SNU-17 cells cytotoxicity

Cytotoxicity of Spatholobi Caulis Herbal-acupuncture Solution (SCHS) 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).

un-

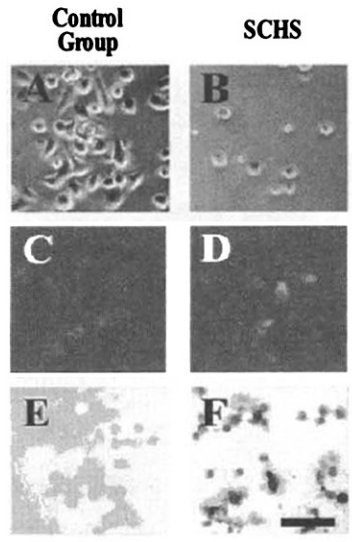

Fig. 2. Characterization of SCHS-induced cell death in SNU-17 cells

Characterization of Spatholobi Caulis Herbal-acupuncture Solution (SCHS) induced cell death in human cervical cancer cells SNU-17. Cells were cultured without SCHS (A, C and E) or with 300㎍/㎖ of SCHS (B, D and F). Morphology (top) : phase-contrast microscopy showed cell shrinkage, irregularity in shape and cellular detachment in SCHS-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㎛.

treated cells (Fig. 2A). DAPI staining showed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon SCHS 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 it indicated the induction of apoptosis by SCHS 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㎍/㎖ SCHS induced apoptosis in SNU-17 cells.

3. mRNA expression of apoptotic genes by SCHS

The effect of SCHS was examined on mRNA ex-

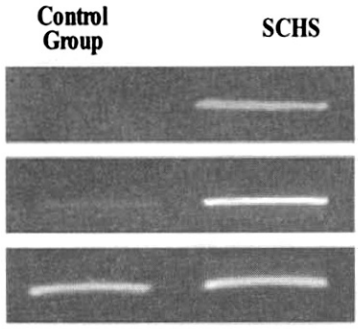

Fig. 3. Results of RT-PCR analysis of the mRNA of Bax and Caspase-3

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 SCHS (*Spatholobi Caulis* Herbal-acupuncture Solution).

pressions of pro-apoptotic genes (Bax and Caspase-3). In cells treated with 300㎍/㎖ of SCHS, mRNA expressions of Bax and Caspase-3 were increased compared with none-treated cells (Fig. 3).

Ⅳ. Discussions

Apoptosis, a form of cell death, is known as programmed cell death which occurs in several pathological situations in multicultural organism[8], 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[9-10]. It can be triggered by numerous stimuli, including antigens, carcinogens, extracellular calcium, UV irradiation, growth factor depletion and chemotherapeutic agents[9,11].

In Oriental medicine, the stem of *Spatholobi Caulis* has been used for the removal of blood stasis associated with menorrhalgia, arthralgia and muscular pain[5,12]. Blood stasis is associated with a cancer metastasis because a blood clot produced by platelets and tumor cells induces cancer metastasis[13]. Previously, it was reported that the ethyl acetate fraction of *Spatholobi Caulis* blocked tumor cell-induced platelet aggregation and tumor cell invasion[12].

There have been several reports on the biological activities of compounds from Spatholobi Caulis such as inhibition of prostaglandin biosynthesis and platelet aggregation[14], inhibitory activity on 3-hydroxysteroid dehydrogenase and anti-inflammatory effect[15], and proliferation inhibition of human uterine leiomyoma cell and expression of gene related cell apoptosis[16].

The cell viability through MTT assay in present study verified that SCHS reflects a cytotoxic effect on SNU-17 cells in dose-dependent manners. SCHS 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[7], and the characteristic changes of the apoptosis were caused in morphology of SCHS-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[17]. In present study, typical TUNEL distinction of apoptosis was observed in SCHS-treated cells.

In this study, our result showed that treatment of SCHS 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[18-19]. 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[20]. Thus, based

on the apoptotic method for cervical cancer, we investigated more detail antitumor activity on SNU-17, human cervical cancer cell line, with *Spatholobi Caulis* Herbal-acupuncture Solution (SCHS) 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[21]. 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[22]. 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[23], the inhibitor of Caspase-activated deoxynuclease[24], and gelsoline and fodrin, which induced in apoptosis regulation[25]. Our data showed the increased exhibition of Bax, as well as, Caspase-3 protein after treatment of SCHS on SNU-17.

Based on the results, *Spatholobi Caulis* Herbal-acupuncture Solution (SCHS) 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 might suggest the possible usage of SCHS on cervical cancer prevention.

V. Conclusions

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

The results are as followings :

1. The viabilities of SNU-17 cells exposed to SCHS were significantly reduced in dose-dependent manner by MTT assay.
2. In cells treated with SCHS, the occurrence of apoptotic features has increased morphological changes in SNU-17 cells.
3. In cells treated with SCHS, mRNA expression of Bax and Caspase-3 were increased compared with none-treated cells.

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

VI. References

1. Ku JL, Kim WH, Park HS, Kang SB and Park JG. Establishment and characterization of 12 uterine cervical-carcinoma cell lines : common sequence variation in the E7 gene of HPV-16-positive cell lines. Int J Cancer. 1997 ; 72(2) : 313-20.
2. Parkin DM, Pisani P and Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer. 1999 ; 80(6) : 827-41.
3. Kelloff GJ, Boone CW, Steele VE, Crowell JA, Lubet RA, Greenwald P, Hawk ET, Fay JP, Sigman CC. Mechanistic considerations in the evaluation of chemopreventive data. IARC Sci Publ. 1996 ; 139 : 203-19.
4. Jang MH, Shin MC, Shin HS, Kim KH, Park HJ, Kim EH, Kim CJ. Alcohol induces apoptosis in TM3 mouse Leydig cells via bax-dependent caspase-3 activation. Eur J Pharmacol. 2002 ; 449 : 39-45.
5. Dan B, Andrew G. Chinese Herbal Medicine. 8th edn. Eastland Press : Seattle. 1986 : 386-7.
6. Ha ES, Lee EO, Yoon TJ, Kim JH, Park JO, Lim NC, Jung SK, Yoon BS, Kim SH. Methylene chloride fraction of Spatholobi Caulis induces apoptosis via caspase dependent path-

way in U937 cells. Biol Pharm Bull. 2004 ; 27(9) : 1348-52.
7. Yim SV, Kim KH, Kim CJ and Chung JH. Serotonin induces apoptosis in PGT-beta pineal gland tumor cells. Jpn J Pharmacol. 2000 ; 84 : 71-4.
8. DeLong MJ. Apoptosis : a modulator of cellular homeostasis and disease states. Ann N Y Acad Sci. 1998 ; 842 : 82-90.
9. Gooch JL, Yee D. Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. Cancer Lett. 1999 ; 144 : 31-7.
10. Kerr JF, Wyllie AH, Currie AR. Apoptosis : a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer. 1972 ; 26 : 239-57.
11. Borther CD, Cidlowski JA. Cellular mechanisms for the repression of apoptosis. Annu Rev Pharmacol Toxocol. 2002 ; 42 : 259-81.
12. Kang IC, Kim SA, Song GY, Baek NI, Park YD, Ryu SY, Saiki I, Kim SH. Effects of the ethyl acetate fraction of Spatholobi caulis on tumour cell aggregation and migration. Phytother. Res. 2003 ; 17 : 163-7.
13. Giancotti FG, Mainiero F. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim Biophys Acta 1994 ; 1198 : 47-64.
14. Goda Y, Shibuya M and Sankawa U. Inhibitor of prostaglandin biosynthesis from Mucuna Birdwoodiana. Chen Pharm Bull. 1987 ; 35 : 2675-7.
15. Kwon YS, Lee JH and Kim CM. Inhibitory activities of three compounds from Mucuna Birdwoodiana on 3-hydroxysteroid dehydrogenase. Saengyak Hakhoechi. 1999 ; 30 : 216-21.
16. Lee HW, Kwon YG, Lee TG, Jo CH, Kim SC, Back SH. The effect of Millettia Reticulatas on the proliferation inhibition of human uterine leiomyoma cell and expression of gene related cell apoptosis. Deptartment of Oriental Medicine Graduate School Daeguhanny University. 2004.
17. Qiao L, Hanif R, Sphicas E, Shiff SJ, Rigas B. Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. Biochem pharmacol. 1998 ; 55 : 53-64.
18. Gilmore AP, Metcalfe AD, Romer LH and Streuli CH. Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. J Cell Biol. 2000 ; 149 : 431-46.
19. Rytomaa M, Lehmann K and Downward J. Matrix detachment induces caspase-dependent cytochrome c release from mitochondria : inhibition by PKB/Akt but not Raf signaling. Oncogene. 2000 ; 19 : 4461-8.
20. Izban KF, Wrone-Smith T, Hsi ED, Schnitzer B, Quevedo ME, Alkan S. Characterization of the interleukin-1 beta-converting enzyme/ced-3-family protease, caspase-3/CPP32, in Hodgkin's disease : lack of caspase-3 expression in nodular lymphocyte predominance Hodgkin's disease. Am J Pathol. 1999 ; 154 : 1439-47.
21. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993 ; 74 : 609-19.
22. Thornberry NA, Lazebnik Y. Caspases : enemies within. Science. 1998 ; 281 : 1312-6.
23. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature. 1995 ; 376 : 37-43.
24. Sakahira H, Enari M, Nagatg S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature. 1998 ; 391 : 96-9.
25. Kamada S, Kusano H, Fujita H, Ohtsu M, Koya RC, Kuzumaki N, Tsujimoto Y. A cloning method for caspase substrates that uses the yeast two-hybrid system : cloning of the antiapoptotic gene gelsolin. Proc Natl Acad Sci. 1998 ; 95 : 8532-7.