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活絡效靈丹 추출물의 인간 유방암세포 MCF-7에 대한 성장억제 효과

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ABSTRACT

Anti-proliferative effects of Whalakhyoryoung-Dan extract on MCF-7 cells

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Purpose : 이 연구는 MCF-7 인간 유방암 세포주에 대한 活絡效靈丹 추출물의 증식억제효과, 세포독성효과, 세포사 유발효과를 확인하기 위하여 이루어졌다.

Methods : MCF-7 인간 유방암 세포주는 Dulbecco's modified Eagle's medium/F12 (DMEM/F12)에 10% fetal bovine serum (FBS)와 항생제를 가하여 만든 배지를 이용하여 배양하였고 MCF-7 세포를 96-well plate에 접종한 후 다양한 농도 (0 ~ 2000 g/㎖)의 活絡效靈丹이 든 배지로 처리한 후 72시간 동안 배양하였고 또한 1000g/㎖의 活絡效靈丹이 든 배지로 처리한 후 48, 96, 192 시간 동안 배양하여 각각 MTS assay kit로 세포생존율을 측정하였다. 세포독성은 Sulforhodamine B assay 방법을 이용해 측정하였고 세포사 과정에서 MCF-7 세포에서의 caspase 활성화를 측정하기 위해 Western blotting을 수행하여 poly ADP ribose polymerase (PARP)의 절단을 확인하였다.

Resuluts : 실험결과 活絡效靈丹 추출물에 의한 세포성장 및 독성효과는 시간 및 농도에 비례하는 것으로 나타났고 세포고사과정에서 작용하는 caspase의 전기질인 PARP 절단량이 活絡效靈丹 처리 농도에 비례에 증가하였다.

Conclusions : 活絡效靈丹은 다양한 기전에 의해서 유방암 세포에 대한 억제효과를 가질 수 있는 것으로 인식할 수 있다.

Key words : breast cancer, MCF-7, Whalakhyoryoung-dan, anti-proliferation, cytotoxicity, apoptosis

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I. Introduction

In the western country, female breast cancer is the disease with high death rate and high incidence. But nowadays the incidence of breast cancer has been increased even in korea due to the change of dietary life and westernization and become conspicuous as the disease threatening female health[1].

Currently, the medical treatments of breast cancer are classified into local therapy and general therapy[2]. Despite modern advancements in diagnosis, prevention, and therapy, cancer treatment have not been fully effective against the high incidence or low survival rate of most cancer[3]. Thus, these days the development of anticancer drugs using natural products and the study about the effects of herb medicine on the cancer cell are being actively progressed all over the world[4-12].

Whalakhyoryoung-Dan(WHD) being used as herbs for invigorating blood and eliminating the stagnant was mentioned in "Uihakchungjungchamseorok (醫學衷中參西錄)" authored by Jang Seok-Sun (張錫純) for the first time. It has been being used for stagnation of Gi and blood, stringlike mass in hypochondriac region, abdominal mass, pain in the abdomen and below the xiphoid process, thigh pain and arm pain, internal and external sores, abdominal mass of the internal organs and blood stagnation in the meridian system[13].

In "Uihakchungjungchamseorok", it was recorded the case of curing abdominal mass occurred to a 30-year-old female and "Uihakjeongjeon (醫學正傳)" asserted that the method of clearing the normal flow Gi and activating blood should be used at the early stage of breast cancer. Therefore it is thought that WHD invigorating blood and eliminating the stagnant have clinically good effects on the breast cancer.

Lim's study[14] on the effects of WHD on blood stasis model reported that it has significant effects on Endotoxin-induced blood stasis model and Hydrocortisone acetate-induced blood stasis model. Shon's study[13] on the effects of WHD on anti-metasis and Hwang's study[15] on angiogenic inhibition effects of WHD plus Sagunja-tang reported that it has anti-tumor, anti-matasis and angiogenic inhibition effects. Though it is found that WHD has anti-tumor and anti-metasis effects through these studies, on what kind of tumor cell it has effect and in which mechanism it works have not been sufficiently studied yet.

Thus we reports that WHD shows significant effects on MCF-7 cell in the experiment to find out what kind of breast cancer cell WHD has effects on.

Ⅱ. Materials and methods

1. Materials

1) Chemicals and laboratory wares

Unspecified, chemicals and laboratory wares used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plastic Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA).

CellTiter 96 Aqueous One Solution Cell Proliferation Assay was from Promega (CA, USA). Media were purchased from GIBCO BRL (Life Technologies, CA, USA). Poly (ADP-ribose) polymerase (PARP) antibody was purchased from Cell Signaling Technology, and beta-actin antibody was purchased from Sigma.

2) Extract of WHD

WHD is composed of the four herbs shown in Table 1. Each herb of WHD was obtained from an oriental drug store, Songsan Oriental Pharmacy (Gwangju, Republic of Korea) and authenticated by Professor S.J. Yang, College of Oriental Medicine, Dongshin University.

An extract of WHD was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 2 h. The decoction was filtered, vacuum evaporated, freeze dried and kept at 4 ℃. The yield of extraction was about 54.1 % (w/w). The ingredients of 86.56 g WHD was comprised of 40g of Angelica giigas radix (Umbelliferae), 40g of Salviae miltiorrhizae radix respectively, 40g of Olibanum, and 40g of Myrrha, respectively.

Table 1. The Composition of WHD.

Herb	Scientific Name	Weight(g)
當歸	*Angelica giigas radix (Umbelliferae)*	40
丹蔘	*Salviae miltiorrhizae radix*	40
乳香	*Olibanum*	40
沒藥	*Myrrha*	40
Total amount		160

2. Methods

1) Cell culture and treatment

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10 % fetal bovine serum (FBS: Gibco) and antibiotics. 1×10^{6} cells were seeded in 60-mm

culture and 5×10^3 cells/well were seeded in 96-well flat-bottomed plates, respectively. At varying times after extract treatment, cells were harvested with scraper and processed for analysis of protein expression, proliferation, cytotoxicity and apoptosis.

2) The assessment of proliferation

MCF-7 cells were seeded in 96-well flat-bottomed plates. After 24 hr incubation cells were treated with varying concentration of extract. After appropriate time, 20 ㎕/well of CellTiter 96 Aqueous One Solution Reagent (Promega Corp.) was added. After 3 hrs at 37℃ in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader. A reference wavelength at 65 nm was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance.

3) The assessment of cytotoxicity

The cellular cytotoxic effect of extract was measured using Invitro Toxicology Assay Kit (Sigma). MCF-7 cells were seeded in 96-well flat-bottomed plates. After 24 hr incubation cells were treated with varying concentration of extract a. After appropriate time, fixed the cells by gently layering 1/4 volume of cold 50% TCA on top of the growth medium. After 1 hr incubation at 4℃, rinsed the cells with water several times to remove TCA and air dried. Sulforhodamin B solution (0.4%) was added onto dried cells in an amount sufficient to cover the culture surface area and then allowed to stain for 20 minutes. After being rinsed with 1% acetic acid several times, the cells were dried completely. The incorporated dye was then solubilized in a volume of Sulforhodamine B Assay Solubilization Solution (10 mM Tris) equal to the original volume of culture medium. Allowed cultures to stand for 5 minutes at room temperature with gentle stirring in a gyratory shaker and then measured absorbance at a wavelength of 565 nm.

4) The assessment of apoptosis

Aliquots of MCF-7 cells in 60-mm culture dishes were treated with extract for varying times and varying concentrations and then attached and floated cells were harvested altogether. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis so the PARP cleavage was checked by western blot analysis using PARP antibody.

5) Western blot analysis

Added 50 ㎕ NP-40 lysis buffer (50 mM Tris-Cl (pH7.4), 150 M NaCl, 50 mM NaF, 0.5% NP-40, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF) to 60 mm plate scale and scraped cells with cell scraper. Transfered the supernatant to eppendorf tube and vortexed moderately. Centrifuged the tube for 30 min. at 14,000 rpm at 4℃ and then transferred the supernatant to new eppendorf tube. Cell lysates that

contained 35 ㎍/㎖ of total protein were subjected to 7.5-12% SDS-PAGE and transferred by electroblotting to nitrocellulose membranes. The membranes were blocked with 50 mM Tris (pH 7.5) containing 500 mM NaCl, 5 % non-fat dried milk. The blots were probed with specific primary antibodies and visualized using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturer's instructions. Polyclonal antibody specific for PARP was purchased from Cell Signaling Technology and monoclonal antibodies for beta-actin were purchased from Sigma.

Ⅲ. Results

1. The effect of WHD on the proliferation of MCF-7 cells

The effect of extract on the proliferation of MCF-7 human breast cancer cells was examined. As shown in Fig. 1, extract of WHD induced cell growth inhibition and morphological change of MCF-7 cells and inhibited proliferation of MCF-7 cells in a dose dependent manner (Fig. 2).

In MCF-7 cells, the inhibition of cell growth by WHD was so obvious that one could observe the difference easily under a microscope (Fig. 1). This inhibitory effect was dose-dependent (Fig. 2). Treatment with 500 ㎍/㎖ for 72 hr could result in a nearly 50 % inhibition (Fig. 2).

Similarly, WHD inhibited proliferation of MCF-7 cells in a time-dependent manner (Fig. 3). When the cells were treated with 500 ㎍/㎖ of WHD for 96 hr, almost 50 % inhibition of MCF-7 cell growth was observed. These findings indicate that WHD strongly inhibits the proliferation of MCF-7 cells in a dose and time-dependent manner.

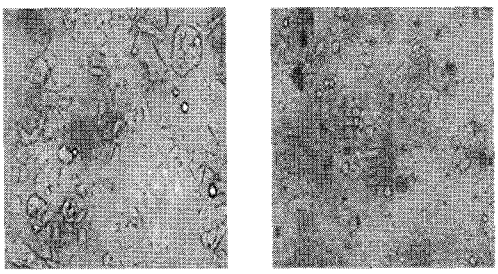

Fig. 1. Microphotographs showing the inhibitory effect of WHD on cell growth. MCF-7 cell lines were plated onto 6-well plates and treated with drug-free media (control) or media containing 500 ㎍/㎖ of WHD for 72 hr. The photographs were taken directly from culture plates using a phase microscope (×100 magnification).

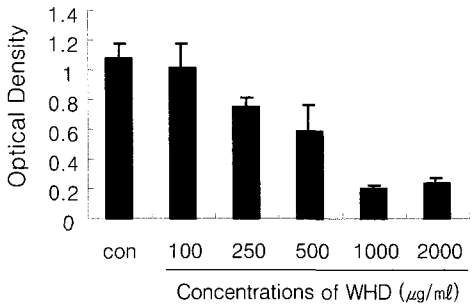

Fig. 2. Dose-dependent effects of WHD on cell growth. MCF-7 cells were plated onto 96-well plates and treated with or without (control, con) varying concentrations (100, 250, 500, 1000 and 2000 ㎍/㎖) of WHD extract for 72 hr. The number of viable cells in each well was quantified by using MTS assays. Data are representative of at least three independent experiments. Error bars represent mean±SD.

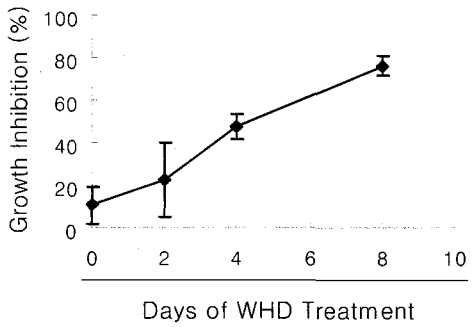

Fig. 3. Time-dependent effects of WHD on cell growth. MCF-7 cells were plated onto 96-well plates and treated with 500 ㎍/㎖ concentration or without of WHD extract for 48, 96 and 192 hr. The number of viable cells in each well was quantified by using MTS assays. Results (optical densities) were calculated as the percentage of unexposed control cultures. Data are representative of at least three independent experiments. Error bars represent mean±SD.

2. The cytotoxic effect of WHD on MCF-7 cells

To characterize the mechanism by which WHD inhibits the proliferation of MCF-7 cells, we examined whether WHD exerted a cytotoxic effect on breast cancer cells by using both an Sulforhodamine B assay, a means of measuring total biomass by staining cellular proteins with the Sulforhodamine B. As shown in figure 4, Sulforhodamine B showed that the addition of WHD extract reduced the viability of MCF-7 cells in a dose-dependent manner.

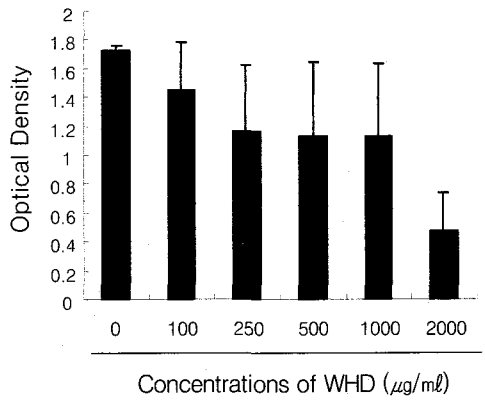

Fig. 4. Cytotoxic effects of WHD in MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of WHD for 48 hr. The cells were then processed for Sulforhodamine B assay. Each bar represents the mean±SD values of three separate experiments.

3. The effects of WHD on apoptosis

To assess the potential role of caspases in the WHD, whole cell lysates were prepared after the herb extract

treatment. Caspase-3 activity in the whole-cell lysates was examined by Western blot assay on one of its major substrate, poly[ADP (ribose)] polymerase (PARP) and the metabolite.

Figure 5. shows the cleavage of the full-length PARP (116 kDa) to generate the 89-kDa cleaved PARP fragment, indicating the activation of caspase-3. Figure 6. represents the concentration- dependency of WHD extract- treatment.

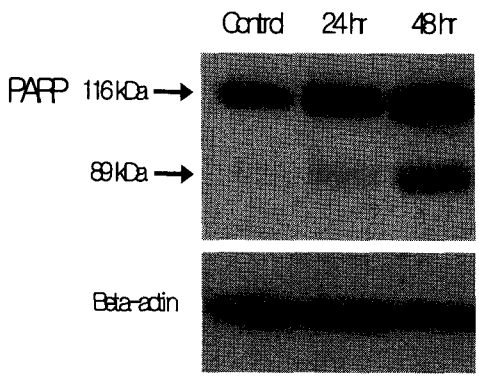

Fig. 5. Caspase activation in WHD extract treated MCF-7 cells. The whole cell lysate from WHD extract-treated cells was assayed by Western blotting after a 24 and 48-hr exposure period. Poly[ADP(ribose)] polymerase (PARP), a major substrate for caspase-3 was extensively cleaved in the WHD-treated cells, while control cells failed to show PARP cleavage.

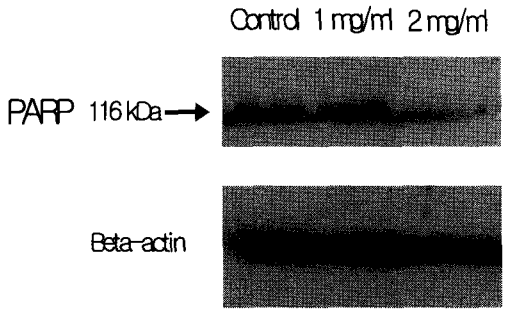

Fig. 6. Caspase activation in WHD extract treated MCF-7 cells. The whole cell lysate from WHD extract-treated cells with 1, 2 mg/ml concentration or without extract was assayed by Western blotting after a 24 hr exposure period. Poly[ADP(ribose)] polymerase (PARP), a major substrate for caspase-3 was extensively cleaved in the WHD-treated cells, while control cells failed to show PARP cleavage.

Ⅳ. Discussion

Breast cancer is one of the leading causes of premature death in women. In North America approximately one in nine women will develop this disease during their life time[16]. Breast cancer is thought to be promoted by prolonged estrogen stimulation in women with the requisite genetic makeup. Physiologically, prolonged estrogen stimulation occurs with early menarche, late menopause and in the absence of pregnancy. In Western countries, a trend toward younger age at menarche, late menopause, decreased parity, and later age at first pregnancy is being occurred[17,18].

But nowadays the incidence of breast cancer has been increased even in korea due to the change of dietary life and westernization and become conspicuous as the disease threatening female health[1].

Currently, the medical treatments of breast cancer are classified into local therapy and general therapy. There are surgical operation and radiological treatment in the local therapy, anticancer chemotherapy and anticancer endocrinotherapy in the general therapy[2]. But cancer treatment by chemotherapeutic agent, surgery and radiation have not been fully effective against the high incidence or low survival rate of most cancer. The development of new therapeutic approach to breast cancer remains one of the most challenging area in cancer research[19].

Thus, these days the development of anticancer drugs using natural products and the study about the effects of herb medicine on the cancer cell is being actively progressed all over the world[4-12].

So far, Kamisodokeum[20], Chungkan-Haewul- Tang[21], and Euonymus alatus (Thunb.)[19] have been studied as useful herb medicines to breast cancer. But much more studies will be required.

WHD being used as herbs for invigorating blood and eliminating the stagnant was mentioned in "Uihakchungjungchamseorok (醫學衷中參西錄)" authored by Jang Seok-Sun (張錫純) for the first time. It has been being used for stagnation of Gi and blood, stringlike mass in hypochondriac region, abdominal mass, pain in the abdomen and below the xiphoid process, thigh pain and arm pain, internal and external sores, abdominal mass of the internal organs and blood stagnation in the meridian system etc., and consists of Angelica giigas radix (enriching and invigorating the blood), Salviae miltiorrhizae radix (eliminating the stagnant and activating the blood), and Olibanum · Myrrha (invigorating blood and alleviating pain)[13].

In "Uihakchungjungchamseorok", it was recorded the case of curing abdominal mass occurred to a 30-year-old female and "Uihakjeongjeon (醫學正傳)" asserted that the method of clearing the normal flow Gi and activating blood should be used at the early stage of breast cancer. Therefore it is thought that WHD invigorating blood and eliminating the stagnant have clinically good effects on the breast cancer.

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Sagunja-tang reported that it has anti-tumor, anti-matasis and angiogenic inhibition effects. Though it is found that WHD has anti-tumor and anti-metasis effects through these studies, on what kind of tumor cell it has effect and in which mechanism it works have not been sufficiently studied yet. This study was made to identify anti-proliferation, cytotoxic and apoptosis inducing effect of WHD extract to MCF-7 human breast cancer cell lines.

To examine the effect of extracts proliferation of MCF-7 cells , MCF-7 cell lines were plated onto 6-well plates and treated with drug-free media (control) or media containing 500 ㎍/㎖ of WHD for 72 hr. The photographs were taken directly from culture plates using a phase microscope (×100 magnification). In MCF-7 cells, the inhibition of cell growth by WHD was so obvious that one could observe the difference easily under a microscope

To assess Dose-dependent effects of WHD on cell growth, MCF-7 cells were plated onto 96-well plates and treated with or without (control) varying concentrations (100, 250, 500, 1000 and 2000 ㎍/㎖) of WHD extract for 72 hr. The number of viable cells in each well was counted by using MTS assays. This inhibitory effect was dose-dependent. Treatment with 500 ㎍/㎖ for 72 hr could result in a nearly 50 % inhibition

Similarly, WHD inhibited proliferation of MCF-7 cells in a time-dependent manner. MCF-7 cells were plated onto 96-well plates and treated with 500 ㎍/㎖ concentration or without of WHD extract for 48, 96 and 192 hr. The number of viable cells in each well was counted by using MTS assays. Thus, when the cells were treated with 500 ㎍/㎖ of WHD for 96 hr, more than 60 % inhibition of MCF-7 cell growth was observed. These findings indicate that WHD strongly inhibits the proliferation of MCF-7 cells in a dose and time-dependent manner.

To characterize the mechanism by which WHD inhibits the proliferation of MCF-7 cells, we examined whether WHD exerted a cytotoxic effect on breast cancer cells by using an Sulforhodamine B assay, a means of measuring total biomass by staining cellular proteins with the Sulforhodamine B. As shown in figure 4, Sulforhodamine B showed that the addition of WHD extract reduced the viability of MCF-7 cells in a dose-dependent manner.

To assess the potential role of caspases in the WHD extract inducing apoptosis, The whole cell lysate from WHD extract-treated cells was assayed by Western blotting after a 24- and 48-hour exposure period. PARP, a major substrate for caspase-3 was extensively cleaved in the WHD treated cells, while control cells failed to show

PARP cleavage.

To confirm Caspase activation in WHD extract treated MCF-7 cells ,the whole cell lysate from WHD extract-treated cells with 1, 2 mg/ml concentration or without (control) extract was assayed by Western blotting after a 24 hour exposure period. PARP was cleaved in the WHD-treated cells.

Therefore we have demonstrated that extract of WHD has anti-proliferation with inhibiting cell growth and morphological change of MCF-7 cell. This inhibitory effect was dose-response and time-relation manner. Moreover, it was found that extract of WHD induces a cytotoxic effect and apoptosis on breast cancer. Thus this study suggests that WHD may play a role in anti-breast cancer agent. However more research needs to be done prior to incorporating these findings into clinical recommendation.

V. Conclusion

To investigate the effect of Whalakhyoryoung-Dan against breast cancer, MCF-7cells were treated by Whalakhyoryoung- Dan at varying time-concentration.

The changes of MCF-7 cells, antiproliferation, cytotoxicity, apoptosis were reserved and the number viable cell, total biomass, 89kd cleaved PARP fragment indicating the activation of caspase-3, were measured. The result were as follows.

1. In MCF-7 cells, the inhibition of cell growth by Whalakhyoryoung-Dan was so obvious that one could observe the difference easily under a microscope. The inhibition of cell growth was dose-response and time-relation. Treatment with 500μg/ml for 72 h could result in a nearly 50 % inhibition.
2. It is founded that the addition of Whalakhyoryoung-Dan extract reduced the viability of MCF-7 cells. It is measured total biomass by staining cellular proteins with the Sulforhodamine B. The reduction of the viability of MCF-7 cells in a dose-response manner.
3. It is observed that caspases in the Whalakhyoryoung-Dan extract induced apoptosis. The PARP, a major substrate for caspase-3, was extensively cleaved in the treated cells with Whalakhyoryoung-Dan, while control cells failed to show PARP cleavage.
4. The 89-kd cleaved PARP fragment increased according to the concentration-dependency of Whalakhyoryoung-Dan treatment.

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