대한한방부인과학회지 pISSN 1229-4292 / eISSN 2508-3619
J Korean Obstet Gynecol. VOL.37 NO.1 : 001-022 (2024)
https://doi.org/10.15204/jkobgy.2024.37.1.001

Antitumor Effects of *Cistanchis Herba* Aqueous Extracts on MCF-7 Human Breast Cancer Cell-Xenograft Athymic Nude Mice, through Potent Immunomodulatory Activities

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국문초록

유방암 세포(MCF-7) 이식 누드 마우스에서 육종용 열수 추출물의 항암 효과 평가

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목 적: 본 연구의 목적은 유방암 세포(MCF-7) 이식 누드 마우스 모델을 이용하여 육종용 열수 추출물의 면역활성 효과를 통한 항암 활성을 체계적으로 평가하는 것이다. 육종용 열수 추출물은 유방암 치료제로 자주 사용되는 대표적인 경구용 항암제인 tamoxifen 경구 투여군과 비교하여 분석 연구하였다.

방 법: 총 110마리의 6주령 암컷 누드 마우스를 준비하여, 7일간 적응 후 체중이 일정한 마우스를 선정하여 우측 둔부 피하부위에 MCF-7 세포를 이식하였다. 종양세포 이식을 한 지 20일 후, 종양 크기 및 체중을 기준으로 그룹 당 8마리씩 본 실험에 사용하였다. MCF-7 이종 이식 21일 후부터 매일 1회씩 35일간 육종용 열수 추출물을 10 ml/kg의 용량(400, 200 및 100 mg/kg)으로 경구 투여하였으며, tamoxifen 역시 10 ml/kg의 용량(20 mg/kg)으로 경구 투여하였고, 정상 및 종양 이식 매체 대조군에서는 멸균증류수만 종양 이식 21일 후부터 동일한 방법으로 35일간 경구 투여 하였다.

결 과: 본 실험의 결과, MCF-7 세포 이식을 함으로써 현저한 비장 및 하악하 림프절 무게, 혈중 IFN-γ의 함량, IL-1β 및 IL-10의 함량, 비장내 TNF-α, 비장세포 및 복강 대식구의 활성의 감소가 관찰되었고, 비장 및 하악하 림프절의 림프구 감소에 의한 조직병리학적 위축 또한 관찰되었다. 그리고 체중 및 증체량의 감소 역시 관찰되었으며, 혈중 IL-6 함량의 증가, 난소 주위의 지방 무게의 감소 및 조직병리학적으로 난소 주위의 축적 지방 조직 위축 현상이 인정되어, 종양 이식 후에 전형적인 종양과 관련된 면역억제와 악액질 현상이 유발된 것으로 판단되었다. 한편 육종용 열수 추출물 400, 200 및 100 mg/kg 경구 투여군에서는 종양 이식 대조군에 비해 유의성 있는 현저한 항암활성이 투여 용량 의존적으로 관찰되었다. 또한 tamoxifen 20 mg/kg 경구 투여군에서는 종양 관련 악액질 소견이 오히려 악화되는 반면, 육종용 열수 추출물 경구 투여군에서는 면역활성 및 악액질 억제 효과가 관찰되었다.

결 론: 본 연구 결과, 육종용 열수 추출물의 적절한 경구 투여는 심각한 부작용 없이, 종양 관련 악액질 소견을 포함하여, 효과적인 유방암 치료 수단을 제공할 수 있을 것으로 기대된다.

중심단어: 유방암, MCF-7 세포, 육종용, 한약, 항신생물제

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Ⅰ. Introduction

Breast cancer is cancer that develops from breast tissue. And it is one of the highest morbidity form of neoplasia in women accounting for almost a third of all new cases of women's cancer[1]. In 2008, there were about 420,000 cases of breast cancer and about 129,000 women died because of this disease in Europe[1]. And eventually about 20~50% of early breast cancer patients develop to metastatic disease, depending on patient and tumor characteristics[2]. Therefore palliation and survival extension are the main treatment goals in breast cancer.

MCF-7 cells are representative adenocarcinoma cell originated from human breast cancer, and one of the most frequently used breast cancer cell lines in anti-tumor research fields[3]. Also they showed estrogen positive (ER+) growth and sensitive react to tamoxifen[4].

Although tamoxifen (Nolvadex^{TM}) has been used for breast cancer as a nonsteroidal estrogen agonist-antagonist antineoplastic agent, there is also risk of endometrial cancer[5], reproductive organ damages mediated by hormone imbalances[6] in some individuals. And hot flushes, vaginal discharge, weight loss and menstrual irregularities are common side effects related to tamoxifen treatment[7]. Therefore it needs to develop medicine which is more effective and has less side effects.

Cistanchis Herba (CH) is Yuk-jong-yonga in Korean Medicine, Yang-invigorating Chinese tonic herb that is primarily used to treat kidney deficiency with symptoms such as impotence, infertility, premature ejaculation[8]. Besides, CH treatments induced significant immunomodulatory effects on 4T1, tumor-bearing by increasing cytokines interleukin (IL)-2 and interferon (IFN)-γ and modulation of regulatory T-cells[8]. Furthermore, CH treatments did not stimulate but suppressed human triple-negative MDA-MB-231 breast xenografts growth in immunodeficiency mice, through their potent immunomodulatory activities[8].

Therefore it intended to systemically observe the anti-tumor, anti-cachexia and immunomodulatory effects of CH as compared to tamoxifen in MCF-7 cell xenograft athymic nude mice in this experiment. We observed the changes on body weights, tumor weights and volume, spleen weights, submandibular lymph node weights, periovarian fat pad weights, serum IL-6 and IFN-γ levels, natural killer cell (NK cell) activities, splenic cytokine contents, tumor mass histopathology and immunohistochemistry, spleen histopathology, submandibular lymph node histopathology and periovarian fat pad histopathology. So we report the experiment through this paper.

Ⅱ. Materials and methods

1. Animals and husbandry

110 virgin, SPF/VAF CAnN.Cg-Foxn1

nu/CrljOri mice - Nude mice (6 week old - body weight ranges 16~20 g) were used after 7 days acclimatization. Animals were allocated 4 to 5 in each filter which cap polycarbonate cage in 45~55% humidity and 20~25℃ temperature. Light and dark cycle was 12 hours and 12 hours and sterilized feed and water were supplied freely. In this experiment, 100 mice were used as tumor-bearing/xenograft mice and 10 mice were used as intact control as remainder. 20 days after tumor inoculation, 8 mice in each group showing regular body weights (intact mice: 20.31±1.40 g, ranged in 18.20~22.20 g; tumor bearing mice: 20.34±0.95 g, ranged in 18.70~22.30 g) and tumor volumes (114.93±18.49 mm^3, ranged in 100.26~168.36 mm^3) were selected and used further experiments (Table 1). Experimental groups were 6 groups, 8 mice in each group. All animals were treated according to the animal control international regulations guidelines about the usage and welfare of laboratory animals. And it is approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea). (Approval No DHU2022-032, March 24, 2022).

Table 1. Experimental Designs Used in This Study

Groups	Xenograft	Dose	Animal No.
Effects on MCF-7 cell xenograft nude mice			
Control	Saline	Vehicle 10 ml/kg	M01~M08
Control	MCF-7 cells	Vehicle 10 ml/kg	M09~M16
Reference	MCF-7 cells	Tamoxifen (20 mg/kg)	M17~M24
Active	MCF-7 cells	CH* (400 mg/kg)	M25~M32
Active	MCF-7 cells	CH* (200 mg/kg)	M33~M40
Active	MCF-7 cells	CH* (100 mg/kg)	M41~M48

*CH : *Cistanchis Herba*

2. Preparations test materials

CH, deep brown powders, were prepared by Ockchundang (Daegu, Korea). Tamoxifen, white crystalline powders, were used as control drug by Kunshan SanYou Pharmaceutical Material Co., Ltd., (Suzhou, China).

3. Cell culture

MCF-7 (Human breast adenocarcinoma cells; Korea Cell Line Bank - KCLB, Seoul, Korea) cells were treated in RPMI 1640 media (Gibco BRL, Grand Island, NY, USA). And it contains fetal bovine serum 10% (FBS; Gibco BRL, Grand Island, NY, USA), 100 μg/ml streptomycin (Sigma-Aldrich, St. Louise, MO, USA) and 100 U/ml penicillin (Sigma-Aldrich, St. Louise, MO, USA) at 5% CO_2 and 37℃ conditions by using commercial CO_2 incubator (Model 311, Thermo Forma, Marietta, OH, USA)[9].

4. Tumor cells xenograft and drug administration

The dosage of tamoxifen was selected as 20 mg/kg based on the previous efficacy test in xenograft mice[10]. In addition, the dosages of CH was selected as 400, 200 and 100 mg/kg based on the previous in vivo and in vitro efficacy tests[8]. Maintained MCF-7 cells were suspended in 1×10^8 cell/ml concentration saline, and 0.2 ml cell suspensions (2×10^7 cell/mouse) were inoculated on the each mouse's right dorsal hip skins. And equal volume of saline was also injected subcutaneously in intact control mice, instead of tumor cell suspensions[9]. CH were dissolved as 40, 20 and 10 mg/ml concentrations in distilled water, and administered orally in 10 ml/kg (of body weight) in repeat oral administration in mouse the recommended volumes[11], once a day for 35 days from 21 days after tumor cell implantation, as equivalence 400, 200 and 100 mg/kg[9]. Tamoxifen was also dissolved in distilled water as 2 mg/ml concentrations, and administered orally in a volume of 10 ml/kg, once a day for 35 days from 21 days after tumor cell implantation, as equivalence 20 mg/kg[10]. In intact and tumor-bearing (TB) controls, only distilled water 10 ml/kg was orally administered once a day for 35 days, also from 21 days after tumor cell inoculation, to provide same restrained stresses from gastric gavage[9].

5. Body weight measurements

Body weight changes were taken a measurement at 1 day before administration (20 days after tumor cell inoculation), initiation of administration, and also 1, 3, 7, 14, 21, 28, 34 and 35 days after initiation by sacrifice using an automatic electronic balance[9]. At initial administration and termination, all animals were overnight fasted (water was not; about 18 hours) for reducing the differences from feeding[9]. In addition, the body weight changes based on that at sacrifice and first administration were calculated.

6. Tumor volume measurements

Each tumor-bearing mouse's tumor width (short axis) and length (long axis) were measured at first day before administration. 1, 3, 7, 14, 21, 28, 34 and 35 days using electronic digital caliper[9].

7. Tumor weight measurements

At Sacrifice, each mouse's tumor masses were collected after eliminations of the surrounding skins, muscles, any debris and connective tissues[9]. Tumor's weight was measured at g levels regarding absolute wet-weights[9]. The relative tumor's weight (%) was calculated by using absolute tumor weights and body weight at sacrifice for reducing the individual body weight differences.

8. Lymphatic and periovarian fat pad weight measurements

Left periovarian fat pads and left

submandibular lymph node and spleen in each mouse at sacrifice were collected after eliminations of the surrounding connective muscles, tissues and any debris[9]. The organ's weight was measured at g levels regarding absolute wet-weights[9].

9. Serum IL-6 and IFN-γ level measurements

About whole blood 1 ml was collected from vena cava under inhalation anesthesia with 3% isoflurane in the mixture of 70% nitrous oxide (N_2O) and 28.5% oxygen (O_2), and separated the serum[9] at sacrifice. All samples of serum were frozen at -150℃ till they were assayed, using ultradeepfreezer. Serum IL-6 levels were investigated by enzyme-linked immunosorbent assay (ELISA) kit as pg/mld, and serum IFN-γ levels were calculated also using mouse IFN-γ ELISA Kit according to manufacturer's recommended protocols, using a microplate reader, as pg/ml levels[9].

10. NK cell activity measurements

Peritoneal and splenic NK cell activities were measured by using of a standard 51Cr release assay. Shortly, all mice were killed at sacrifice, and peritoneal macrophages and splenocytes were collected. And spleen 10~20 mg were separated and washed twice by RPMI-1640 medium at 4℃. And homogenates were prepared by using ultrasonic cell disruptor and bead beater.

11. Splenic cytokine content measurements

Tumor necrosis factor (TNF)-α, IL-1β, and IL-10's splenic concentrations were measured by ELISA commercially available kits - TNF-α Mouse ELISA Kit, IL-1β Mouse ELISA Kit and IL-10 Mouse ELISA Kit[9]. Approximately 10~15 mg of tissue samples were homogenized by bead beater and ultrasonic cell disruptor containing lysis buffer 1 ml as described by previous report[12]. Analysis was carried with 100 ml of standard or 10, 50, or 100 ml of tissue homogenate[9]. Data are expressed as pg/mg of protein[9].

12. Histopathology

After weight measurement at sacrifice day, some parts of tumor mass, left periovarian fat pads and spleen and submandibular lymph node were separated and fixed in neutral buffered formalin 10% for 24 hours at least. Then paraffin embedding was conducted using embedding center and automated tissue processor. And it was sectioned at thickness of 3-4 μm using microtome for routine histological methods. For general histopathology each slide was stained with Hematoxylin and eosin (HE).

13. Immunohistochemistry

Other prepared serial sectioned tumor mass tissues were immunostained by an ABC methods and peroxidase substrate kit[9] after citrate buffer antigen retrieval pretreatment. Shortly, endogenous peroxidase activity was blocked by incubated in

0.3% H_2O_2 and methanol for 30 minutes. Also non-specific binding of immunoglobulin was blocked with normal horse serum.

14. Statistical analyses

All numerical values are represented as mean±standard deviation (SD) of 8 athymic nude mice[9]. Variance homogeneity was examined by using the Levene test[16]. If there is no significant deviations by the Levene test, the data were analyzed by 1 way analysis of variance (ANOVA) test followed by Tukey's honest significant difference (THSD) test for determining which pairs of group comparison were significantly different. In case of significant deviations were observed at Levene test from variance homogeneity, Dunnett's T3 (DT3) test was executed for determining which pairs of group comparison were significantly different. Differences were considered significant at $p<0.05$. Statistical analyses were conducted by using SPSS.

Ⅲ. Results

1. Effects on body weights

Animals were selected at 20 days after MCF-7 cell implantation base on tumor volumes and body weights, and significant decreases of body weights were demonstrated in TB control mice as compared with intact control. Accordingly, the body weight gains during 35 days of oral administration periods were also significantly decreased in TB control as compared with intact control. Although no significant changes on the body weights were demonstrated in tamoxifen 20 mg/kg treated mice as compared to those of TB control, marked decreases of body weight gains during 35 days of oral administration periods were observed in tamoxifen 20 mg/kg treated mice as compared with TB (Table 2). On the contrary, CH 400 and 200 mg/kg administrated mice showed significant increases of body weights from 14 or 21 days as compared to those of TB control and the body weight gains in CH 400 and 200 mg/kg treated mice also showed significantly increases of body weight gains during 35 days as compared to those of TB control dose-dependently. In addition, CH 100 mg/kg treated mice showed non-significantly but dramatically increases of body weight gains during 35 days of oral administration periods as compared to those of TB control ($p<0.01$ or $p<0.05$) (Table 2).

The body weight gains during 35 days of administration period in TB control were changed as -75.71% as compared with intact control, but they were changed as -75.29, 223.53, 163.53 and 62.35% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 2).

Table 2. Body Weight Gains

Groups	Periods body weights (g)			Body weight gains [B-A]
	Before	At first administration [A]	At sacrifice [B]	
Controls				
Intact	20.31±1.40	18.35±1.39	22.73±0.83	4.38±0.80
TB*	20.33±1.04	18.24±1.04	19.30±1.05	1.06±0.44
Tamoxifen				
20 mg/kg	20.33±0.67	18.33±1.65	18.59±0.84	0.26±0.48
CH†				
400 mg/kg	20.38±1.27	18.31±1.22	21.75±1.12	3.44±0.61
200 mg/kg	20.36±1.07	18.36±1.08	21.16±0.67	2.80±0.68
100 mg/kg	20.31±0.83	18.36±1.98	20.09±0.94	1.73±0.41

*Tumor-bearing, †CH : *Cistanchis Herba*

2. Effects on tumor volumes

Significant decreases of tumor volumes were detected in tamoxifen 20 mg/kg treated mice as compared with those of TB control from 7 days after initial administration ($p<0.01$ or $p<0.05$). Accordingly, the tumor volume changes after end of 35 days continuous oral administration in tamoxifen 20 mg/kg treated mice were also significantly decreased as compared with TB control ($p<0.01$) (Table 3). And all three different dosages of CH 400, 200 and 100 mg/kg administered mice showed significant decreases in tumor volumes from 14 or 21 days after initial administration as compared to those of TB control, dose-dependently. Accordingly, the tumor volume changes after end of 35 days continuous oral administration in CH 400, 200 and 100 mg/kg treated mice were also significantly decreased as compared with TB control ($p<0.01$) (Table 3). Especially, CH 400 mg/kg showed effective inhibitory activities on MCF-7 cell xenograft tumor volume increases as comparable to those of tamoxifen 20 mg/kg (Table 3).

The tumor volume changes in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -82.26, -86.11, -75.04 and -60.37% as compared with TB control, respectively (Table 3).

Table 3. Tumor Volume Changes

Groups	Periods tumor volume (mm^3) First administration [A]	Sacrifice [B]	Changes (mm^3) [B-A]
Controls			
TB*	114.14±22.44	512.71±79.40	397.44±81.02
Tamoxifen			
20 mg/kg	114.57±20.67	186.14±7.71	70.50±15.62
CH†			
400 mg/kg	115.03±18.25	170.93±12.45	55.19±10.36
200 mg/kg	115.62±19.02	214.48±17.94	99.21±11.45
100 mg/kg	115.30±16.69	272.56±43.04	157.49±42.90

*Tumor-bearing, †CH : *Cistanchis Herba*

3. Effects on tumor weights

Significant decreases of tumorweights were made observation in all test and reference material administered mice including CH 400 mg/kg treated mice as compared with TB control. Especially, CH 400, 200 and 100 mg/kg inhibited the MCF-7 cell xenograft tumor weights, dose-dependently (Table 4 and 5).

The absolute tumor weights in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -49.81, -50.58, -36.46 and -29.91% as compared with TB control, respectively (Table 4 and 5).

The relative tumor weights in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -48.32, -56.32, -42.29 and -32.87% as compared with TB control, respectively (Table 4 and 5).

Table 4. Absolute Tumor Mass and Organ Weights

Organs / Groups	Tumor mass	Spleen	Submandibular lymph node	Periovarian fat pad
Controls				
Intact		0.173±0.010	0.020±0.003	0.178±0.027
TB*	0.097±0.012	0.079±0.009	0.006±0.002	0.064±0.008
Tamoxifen				
20 mg/kg	0.049±0.012	0.057±0.005	0.002±0.001	0.031±0.011
CH†				
400 mg/kg	0.048±0.014	0.144±0.012	0.014±0.003	0.147±0.013
200 mg/kg	0.062±0.008	0.122±0.012	0.012±0.002	0.116±0.017
100 mg/kg	0.068±0.008	0.109±0.010	0.010±0.001	0.094±0.010

*Tumor-bearing, †CH : *Cistanchis Herba*

Table 5. Relative Tumor Mass and Organ Weights

Groups \ Organs	Tumor mass	Spleen	Submandibular lymph node	Periovarian fat pad
Controls				
Intact		0.761±0.043	0.088±0.015	0.784±0.113
TB*	0.507±0.075	0.408±0.045	0.031±0.007	0.331±0.052
Tamoxifen				
20 mg/kg	0.262±0.058	0.305±0.032	0.012±0.005	0.184±0.062
CH†				
400 mg/kg	0.221±0.061	0.663±0.073	0.066±0.015	0.674±0.059
200 mg/kg	0.292±0.034	0.578±0.047	0.057±0.009	0.545±0.069
100 mg/kg	0.340±0.041	0.544±0.051	0.051±0.005	0.471±0.059

*Tumor-bearing, †CH : *Cistanchis Herba*

4. Effects on spleen weights

Significant decreases of absolute and relative weights of spleen were observed in TB control as compared with intact control. However, CH 400, 200 and 100 mg/kg administered mice showed significant and dose-dependent increases of spleen weights as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant decreased absolute and relative splenic weights as compared with those of TB control($p<0.01$)(Table 4 and 5).

The absolute spleen weights in TB control were changed as -54.49% as compared with intact control mice, but they were changed as -27.98, 82.83, 55.64 and 38.79% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

The relative spleen weights in TB control were changed as -48.40% as compared with intact control mice, but they were changed as -25.08, 62.68, 41.75 and 33.45% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

5. Effects on submandibular lymph node weights

Significant decreases of relative and absolute weights of submandibular lymph node were observed in TB control mice as compared with intact control mice. However, CH 400, 200 and 100 mg/kg administered mice showed significant and dose-dependent increases of submandibular lymph node weights as compared to those of TB control mice, respectively ($p<0.01$). On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant decreased relative and absolute weights of submandibular lymph node as compared with those of TB control ($p<0.01$)(Table 4 and 5).

The absolute submandibular lymph node weights in TB control were changed as -69.81% as compared with intact

control mice, but they were changed as -62.50, 135.58, 100.00 and 70.83% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

The relative submandibular lymph node weights in TB control were changed as -64.76% as compared with intact control mice, but they were changed as -60.67, 115.13, 83.41 and 65.12% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

6. Effects on periovarian fat pad weights

Significant decreases of relative and absolute weights of periovarian fat pad were observed in TB control mice as compared with intact control. However, CH 400, 200 and 100 mg/kg administered mice showed great and dose-dependent increases of periovarian fat pad weights as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant decreased periovarian fat pad weights of relative and absolute as compared with those of TB control ($p<0.01$) (Table 4 and 5).

The absolute periovarian fat pad weights in TB control were changed as -64.31% as compared with intact control mice, but they were changed as -60.67, 115.13, 83.41 and 65.12% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

The relative periovarian fat pad weights in TB control were changed as -64.76% as compared with intact control mice, but they were changed as -60.67, 115.13, 83.41 and 65.12% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 4 and 5).

7. Effects on serum IL-6 and IFN-γ levels

Significant decreases of IFN-γ levels and increases of serum IL-6 levels were observed in TB control mice as compared with intact control. However, CH 400, 200 and 100 mg/kg administered mice showed great and dose-dependent increases of IFN-γ levels and decreases of serum IL-6 levels as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant increases of serum IL-6 levels with significant decreases of IFN-γ levels as compared with those of TB control ($p<0.01$).

The serum IL-6 levels in TB control were changed as 445.37% as compared with intact control mice, but they were changed as 42.42, -60.58, -45.50 and -32.78% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively.

The serum IFN-γ levels in TB control were changed as -56.28% as compared

with intact control mice, but they were changed as -44.70, 80.54, 53.28 and 36.17% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively.

8. Effects on NK cell activities

Significant decreases of peritoneal and splenic NK cell activities were observed in TB control mice as compared with intact control mice. However, CH 400, 200 and 100 mg/kg administered mice showed great and dose-dependent increases of peritoneal and splenic NK cell activities as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant decreases of splenic and peritoneal NK cell activities as compared with those of TB control ($p<0.01$).

The splenic NK cell activities in TB control were changed as -64.65% as compared with intact control mice, but they were changed as -36.54, 88.13, 67.45 and 49.36% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively.

The peritoneal NK cell activities in TB control were changed as -69.38% as compared with intact control mice, but they were changed as -39.98, 113.21, 74.69 and 57.47% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively.

9. Effects on splenic cytokine contents

Significant decreases of splenic TNF-α, IL-1β and IL-10 contents were observed in TB control as compared with intact control mice. However, CH 400, 200 and 100 mg/kg administered mice showed significant and dose-dependent increases of splenic cytokine contents as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg oral administrated mice showed significant decreases on the splenic cytokine contents TNF-α, IL-1β and IL-10 as compared with those of TB control ($p<0.01$) (Table 6).

The splenic TNF-α contents in TB control were changed as -70.66% as compared with intact control mice, but they were changed as -46.35, 146.14, 115.39 and 77.06% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 6).

The splenic IL-1β contents in TB control were changed as -75.87% as compared with intact control mice, but they were changed as -46.40, 155.09, 117.33 and 81.73% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 6).

The splenic IL-10 contents in TB control were changed as -77.62% as compared with intact control mice, but they were changed as -40.23, 145.46, 112.89 and 77.88% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control,

respectively (Table 6).

Table 6. Splenic Cytokine Contents

Items / Groups	Tumor necrosis factor-α	Interleukin-1β	Interleukin-10
Controls			
Intact	93.89±17.35	45.92±10.57	91.23±12.60
TB*	27.55±7.70	11.08±1.85	20.42±2.80
Tamoxifen			
20 mg/kg	14.78±4.10	5.94±2.02	12.20±2.67
CH†			
400 mg/kg	67.80±12.85	28.27±10.05	50.12±15.56
200 mg/kg	59.33±10.97	24.08±10.47	43.47±11.92
100 mg/kg	48.77±6.79	20.14±5.55	36.32±6.03

*Tumor-bearing, †CH : *Cistanchis Herba*

10. Effects on tumor mass histopathology and immunohistochemistry

CH 400, 200 and 100 mg/kg effectively increased apoptotic cells, and related decreased the tumor cell volumes in MCF-7 cell xenograft tumor masses, dose-dependently, more favorable or as comparable to those of tamoxifen 20 mg/kg in CH 400 mg/kg. In addition, all three different dosages of CH 400, 200 and 100 mg/kg treated mice showed significant increases of cleaved caspase-3, and poly adenosine diphosphate-ribose polymerase (PARP), and inducible nitric oxide synthases (iNOS) and TNF-α immunoreactivities, along with decreases of cyclooxygenase-2 (COX-2) immunolabeled cells in tumor mass as compared with TB control mice. Marked and significant increases of cleaved caspase-3 and PARP immunoreactivities, and decreases of COX-2 immunolabeled cells were also demonstrated in tamoxifen 20 mg/kg administrated mice as compared to those of TB control, but treatment of tamoxifen 20 mg/kg did not showed any significantly changes on the tumor mass iNOS and TNF-α immunoreactivities as compared with TB control mice ($p<0.01$).

The tumor cell volumes in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -61.52, -62.47, -43.13 and -29.93% as compared with TB control, respectively.

The apoptotic cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as 540.86, 572.46, 423.89 and 200.04% as compared with TB control, respectively.

The caspase-3 immunolabeled cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg

administered mice were changed as 1330.75, 1416.53, 1233.26 and 641.47% as compared with TB control, respectively.

The PARP immunostained cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as 866.03, 944.50, 639.44 and 349.91% as compared with TB control, respectively.

The COX-2 positive cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -53.21, -55.66, -45.80 and -36.65% as compared with TB control, respectively.

The iNOS immunopositive cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as 2.20, 991.45, 697.94 and 346.11% as compared with TB control, respectively.

The TNF-α immunoreactive cell percentages in tumor mass of tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice were changed as -5.12, 990.52, 558.38 and 346.91% as compared with TB control, respectively.

11. Effects on spleen histopathology

Atrophic changes about the splenic white pulp lymphoid cells decrease were detected in TB control as compared with intact control. Consequently the total splenic thicknesses, white pulp numbers and diameters were significantly decreased in TB control as compared with those of intact control. However, these splenic atrophic changes were dramatically inhibited by treatment of CH 400, 200 and 100 mg/kg as compared to those of TB control, dose-dependently. On the contrary, significant decreases of total splenic thicknesses, white pulp numbers and diameters were detected in tamoxifen 20 mg/kg mice as compared with those of TB control ($p<0.01$) (Table 7).

The total splenic thicknesses in TB control were changed as -28.31% as compared with intact control mice, but they were changed as -19.31, 26.38, 20.38 and 14.68% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 7).

The splenic white pulp numbers in TB control were changed as -59.44% as compared with intact control mice, but they were changed as -42.47, 72.60, 45.21 and 35.62% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 7).

The splenic white pulp diameters in TB control were changed as -38.88% as compared with intact control mice, but they were changed as -17.69, 42.92, 25.06 and 18.50% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 7).

Table 7. Spleen Histomorphometry

Groups \ Items	Total thickness (μm/central regions)	White pulp numbers (/mm^2)	White pulp diameters (μm/white pulp)
Controls			
Intact	1710.85±191.38	22.50±4.34	609.44±112.16
TB*	1226.49±66.02	9.13±1.13	372.51±26.29
Tamoxifen			
20 mg/kg	989.69±115.98	5.25±1.16	306.60±28.80
CH†			
400 mg/kg	1550.00±130.25	15.75±2.76	532.40±42.18
200 mg/kg	1476.42±105.69	13.25±1.58	465.84±37.18
100 mg/kg	1406.54±42.96	12.38±0.74	441.40±34.74

*Tumor-bearing, †CH : *Cistanchis Herba*

12. Effects on submandibular lymph node histopathology

Marked atrophic changes about the lymphoid cells decrease were detected in the submandibular lymph nodes of TB control as compared with intact control. Consequently the total and cortex thicknesses and follicle numbers were significantly decreased in TB control as compared with intact control. However, these submandibular lymph node atrophic changes were markedly and significantly inhibited by treatment of CH 400, 200 and 100 mg/kg as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg treated mice showed marked and significant decreases of the total and cortex thicknesses, follicle numbers in the submandibular lymph nodes as compared with those of TB control mice ($p<0.01$) (Table 8).

The total submandibular lymph node thicknesses in TB control were changed as -52.34% as compared with intact control mice, but they were changed as -31.90, 62.10, 52.53 and 28.75% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 8).

The submandibular lymph node cortex follicle numbers in TB control were changed as -58.16% as compared with intact control mice, but they were changed as -50.85, 76.27, 64.41 and 44.07% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 8).

The submandibular lymph node cortex thicknesses in TB control were changed as -53.74% as compared with intact control mice, but they were changed as -30.78, 50.66, 31.99 and 15.62% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 8).

Table 8. Submandibular Lymph Node Histomorphometry

Groups \ Items	Total thickness (μm/central regions)	Cortex lymphoid cell follicle numbers (/mm^2)	Cortex thickness (μm/lymph node)
Controls			
Intact	1349.36±228.85	17.63±1.85	849.34±63.59
TB*	643.11±46.65	7.38±1.06	392.94±22.85
Tamoxifen			
20 mg/kg	437.99±67.69	3.63±1.06	272.00±39.55
CH†			
400 mg/kg	1042.50±146.21	13.00±1.07	592.01±71.19
200 mg/kg	980.93±84.53	12.13±0.99	518.66±84.78
100 mg/kg	828.01±72.21	10.63±0.74	454.33±33.20

*Tumor-bearing, †CH : *Cistanchis Herba*

13. Effects on periovarian fat pad histopathology

Significant atrophic changes about the sizes of white adipose cells decreases were detected in periovarian fat tissues of TB control as compared with those of intact control. Consequently the total deposited fat thicknesses and mean diameters of white adipocyte were significantly decreased in TB control as compared with intact control. However, these atrophic changes on the white periovarian adipose tissues were markedly and significantly inhibited by treatment of CH 400, 200 and 100 mg/kg as compared to those of TB control. On the contrary, tamoxifen 20 mg/kg treated mice showed significant decreases of the total periovarian deposited fat thicknesses and mean diameters of white adipocyte as compared with TB control ($p<0.01$) (Table 9).

The total periovarian fat pad thicknesses in TB control were changed as -57.80% as compared with intact control mice, but they were changed as -25.58, 86.98, 51.10 and 38.38% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 9).

The mean diameters of periovarian white adipocyte in TB control were changed as -47.36% as compared with intact control mice, but they were changed as -36.55, 52.30, 40.95 and 29.20% in tamoxifen 20 mg/kg, CH 400, 200 and 100 mg/kg administered mice as compared to those of TB control, respectively (Table 9).

Table 9. Periovarian Fat Pad Histomorphometry

Items / Groups	Total thickness (μm/central regions)	White adipocyte diameters (μm)
Controls		
Intact	2362.18±356.92	66.45±10.62
TB*	996.80±95.19	34.98±4.20
Tamoxifen		
20 mg/kg	741.85±99.17	22.19±5.09
CH†		
400 mg/kg	1863.83±281.22	53.27±6.88
200 mg/kg	1506.15±289.19	49.30±4.65
100 mg/kg	1579.32±176.28	45.19±3.39

*Tumor-bearing, †CH : *Cistanchis Herba*

Ⅳ. Discussion

Breast cancer is cancer that develops from breast tissue and one of the highest morbidity form of neoplasia in women accounting for almost a third of all new cases of women's cancer[1]. MCF-7 cells are representative adenocarcinoma cell originated from human breast cancer, and one of the most frequently used breast cancer cell lines in anti-tumor research fields[3] and they showed estrogen positive (ER+) growth and sensitive react to tamoxifen[4].

Although tamoxifen has been used for breast cancer as a nonsteroidal estrogen agonist-antagonist antineoplastic agent[18], it has been also reported that diverse side effects as bone loss, endometrial cancer, reduced cognition and hemolytic anemia[19,20]. And also hot flushes, vaginal discharge, weight loss and menstrual irregularities are common side effects related to tamoxifen treatment[7].

Medicinal herbs gain importance and space in the pharmaceutical industry and inspire the search for new possible materials of bioactive molecules[21]. CH is a Yang-invigorating Chinese tonic herb that is primarily used to treat kidney deficiency with symptoms such as impotence, infertility, premature ejaculation[8]. Modern pharmacological studies have shown that CH can stimulate immunity and anti-osteoporotic activities[8]. In fact, the estrogenic effect of CH was reported in a previous study, in which CH extracts-fed mice increased the viability of MCF-7 cells[22]. Besides, CH treatments induced significant immunomodulatory effects on 4T1, murine mammary tumor-bearing mice by increasing cytokines interleukin IL-2 and interferon IFN-γ and modulation of regulatory T-cells[8]. CH in immunodeficiency mice didn't stimulate but suppressed human triple-negative MDA-MB-231 breast xenografts growth through their potent immunomodulatory activities[8].

In this experiment, the anti-tumor,

anti-cachexia and immunomodulatory effects of CH were systemically observed in MCF-7 cell xenograft athymic nude mice. The dosages of CH was selected as 400, 200 and 100 mg/kg based on the previous in vivo and in vitro efficacy tests[8]. The results were compared with tamoxifen, an antineoplastic agent and a nonsteroidal estrogen agonist-antagonist used for breast cancer[11].

In the present study, significant and dose-dependent decreases of tumor weights and volumes were detected in CH 400, 200 and 100 mg/kg administrated mice as compared to those of TB control. Especially, CH 400 mg/kg showed more favorable or as comparable inhibitory activities against MCF-7 cell xenograft tumor volume and weight increases. These findings are considered as direct evidences that CH 400, 200 and 100 mg/kg oral administration showed effective and dose-dependent inhibitory activities on MCF-7 xenograft tumor mass growths, more favorably or as comparable to those of tamoxifen 20 mg/kg oral administration.

In this experiment, CH 400, 200 and 100 mg/kg administration showed marked increase of body immune systems. These findings are considered as definitive evidences that dose-dependent and favorable antitumor activities of CH 400, 200 and 100 mg/kg quite differed to those of tamoxifen. Once again, apoptotic tumor cells increases and tumor volumes and weights decreases were observed in CH 400, 200 and 100 mg/kg administered mice.

The cytokine TNF-α was associated with critical events leading to T-lineage differentiation and commitment. TNF-α can enhance the in vivo immune-response at doses much lower than that cause weight loss or tissue toxicity, also proliferation of T and B cells. In addition, it enhances augments IL-2 stimulated natural killer cell activity and monocytes proliferation and IL-2-induced immunoglobulin production[24]. They are both glycoproteins of 17 kDa. IL-1 is necessary for the immune response successful initiation[24]. IL-10 is an immune-suppressive glycoprotein of 19～21 kDa, secreted by Th2 cells and activated macrophages. IL-10 primarily acts on activated macrophages to suppress TNF-α, IL-1, IL-12, and reactive oxygen radicals secretion. IFN-γ is a glycoprotein of 20 to 25 kDa. It effect on T and B cell functions and enhance the macrophages activities and NK cell[24]. We observed that decreases of splenic TNF-α, stimulatory cytokines and IL-1β contents and blood IFN-γ levels, and the inhibitory cytokine levels[24]. However, these splenic and blood cytokine decreases were inhibited by treatment effectively of all three different dosages of CH 400, 200 and 100 mg/kg, dose-dependently, well corresponded to the lymphatic organ weights and histopathological inspections. On the contrary, significant decreases on the splenic cytokine contents - TNF-α, IL-1β and IL-10 were observed in tamoxifen 20 mg/kg treated mice as compared with those of TB control.

As a cancer related immunosuppress, these immune cells's activation were highlight as a new treatment regime for cancer. Marked decreases of NK cell activities were demonstrated after tumor cell inoculations, and more decreases of NK cell activities were induced by tamoxifen 20 mg/kg oral treatment as compared with those of TB control. But all three different dosages of CH 400, 200 and 100 mg/kg oral treatment dose-dependently increased the NK cell activities suggesting immunomodulatory effects of CH, quite differed to those of tamoxifen.

Apoptosis occurs through two pathways. One is an extrinsic pathway that is involving the interaction of death ligands. And the another is an intrinsic pathway, initiated by insults that damage the DNA. Eventually both pathways result in mitochondrial damage with release caspase-3. And other downstream caspases's activation results in cleavage of cellular proteins[13]. PARP cleavage contributes to the progression of apoptosis and results in a decreased enzymatic repair function[25]. Caspase-3 is responsible for cleavage of critical nuclear targets in the apoptotic cascade. These mean the inhibitor of caspase-activated deoxynuclease and PARP results in a defective DNA repair function[13]. The activated PARP and caspase-3 in the tumor mass are indicated apoptosis of tumor cells[9]. Increases of cleaved caspase-3 and PARP immunoreactivities were demonstrated in the tumor masses as tamoxifen and CH administration related tumor cell apoptosis.

Especially, CH 400, 200 and 100 mg/kg administered MCF-7 xenograft athymic nude mice showed significant and clear dose-dependent increases in caspase-3 and PARP as direct evidences that CH showed effective dose-dependent anti-tumor activity on MCF-7 human breast cancer, more favorably or as comparable to those of tamoxifen 20 mg/kg. Furthermore, decreases of COX-2 is also involved in angiogenesis and progression[15], immunoreactivities were also demonstrated in all tamoxifen 20 mg/kg, and CH 400, 200 and 100 mg/kg administrated mice. Especially CH 400, 200 and 100 mg/kg administrated mice also showed clear dose-dependent decreases of tumor mass COX-2-immunopositive cells, more favorably or as comparable to those of tamoxifen.

Generally, the increases of iNOS activities regarding to the proinflammatory agents can be induced shock and over inflammatory responses in the body, and over expressions of iNOS also induced tumor neovascularization. However, iNOS can be induced tumor cell apoptosis and related tumor regressions[26]. In the present study, marked iNOS increases were detected in the tumor mass of all three different dosages of CH 400, 200 and 100 mg/kg, dose-dependently. But treatment of tamoxifen 20 mg/kg did not influence on the tumor mass iNOS immunoreactivities as compared with TB control. These increases of iNOS immunoreactivities and

tumor mass are regarded as secondary changes from immunostimulatory effects of CH related to NK cell activity. Furthermore, significant increases of tumor mass TNF-α were also demonstrated in CH 400, 200 and 100 mg/kg administrated mice as compared to those of TB control, also dose-dependently. But the treatment of tamoxifen 20 mg/kg did not influence on the tumor mass TNF-α immunoreactivities as compared with those of TB control[27].

Cancer cachexia is the syndrome that worsens patients's the quality of life[9]. Numerous studies have suggested that IL-6 plays an important role in cancer-induced cachexia[9]. According to the present study, marked increases of serum IL-6 levels can decrease body weight, reduce and atrophic changes of deposited fat pads were observed after MCF-7 cell transplantation, but these changes related to cachexia were dramatically inhibited by oral treatment of CH, dose-dependently. These findings are direct evidences that CH 400, 200 and 100 mg/kg oral administration can be effectively control the cancer related cachexia. On the contrary, tamoxifen 20 mg/kg treated mice showed more profound cancer-related cachexia in the present study[28].

Therefore it is expected that appropriate oral administration of CH will provide effective and novel anti-tumor alternative therapeutic regimes including cancer cachexia control without serious side effects. Additional oral administration of all three different dosages of CH 400, 200 and 100 mg/kg effectively inhibited MCF-7 cell xenograft tumor mass growth dose-dependently. In addition, CH 400, 200 and 100 mg/kg administered mice also showed favorable and dose-dependent inhibitory activities on cancer-related immunosuppress and cachexia in MCF-7 xenograft mice, as compared with those of TB control. Marked decreases of tumor volumes and weights were also demonstrated in tamoxifen 20 mg/kg treated mice and also marked increases of tumor mass cleaved caspase-3 and PARP immunoreactive tumor cells and decreases of COX-2 immunoreactivities were observed in tamoxifen 20 mg/kg treated mice as compared to those of TB control. However, tamoxifen 20 mg/kg treatment deteriorated the cancer cachexia and immunosuppress as compared with TB control, without meaningful changes on tumor mass iNOS and TNF-α immunoreactivities.

CH has favorable activities on breast cancer in the MCF-7 cell xenograft athymic nude mice as compared with tamoxifen 20 mg/kg. Considering the side effects of tamoxifen like weight loss and hot flushes, CH will be as a new potent alternative refinement agents to treat breast cancer. However CH has various active ingredients, the screening of the biological active compounds should be conducted in future with more detail mechanism studies.

V. Conclusion

Through this study, I found the following conclusion.

1. Marked decreases of tumor volumes and weights were also demonstrated in tamoxifen 20 mg/kg treated mice with decreases of tumor cell volumes in tumor masses.
2. Marked increases of tumor mass cleaved caspase-3 and PARP immunoreactive tumor cells and decreases of COX-2 immunoreactivities were observed in tamoxifen 20 mg/kg treated mice as compared to those of TB control mice.
3. Tamoxifen 20 mg/kg treatment deteriorated the cancer cachexia (more decreased in the body weight gains, periovarian fat depositions and elevation of serum IL-6 levels) and immunosuppress (more decrease of lymphatic organ weights, serum IFN-γ levels, NK cell activities, splenic TNF-α, IL-1β and IL-10 contents, histopathological atrophic changes of lymphatic organs) as compared with TB control mice.
4. There are no meaningful changes on tumor mass iNOS and TNF-α immunoreactivities, at least in a condition of this experiment.

Therefore, it is considered that CH can be a effective and novel anti-tumor alternative therapeutic regimes on breast cancer from MCF-7 cell.

☐ Received : Jan 08, 2024
☐ Revised : Jan 15, 2024
☐ Accepted : Feb 23, 2024

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