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Effects of Astragalus injection on cervical immortalized epithelial cell growth and its cell cycle regulation mechanism

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ABSTRACT

Objective: To study the regulating effect of Astragalus injection on cervical immortalized epithelial cell growth and cell cycle regulation mechanism. **Methods:** Cervical immortalized epithelial cells (H8 cells) were selected for experiment and processed with different concentrations of Astragalus injection, MTT method was used to detect cell viability, flow cytometry was used to detect the percentages of different cell cycles and the contents of different immune cell subsets, and PCR method was used to detect mRNA contents of cell cycle-related proteins. **Results:** Astragalus injection could reduce cell viability in a dose-dependent manner, and the inhibiting effect of 200 μg/mL concentration was the most significant; NK cell and $CD3^+CD4^+CD8^-$T cell contents as well as $CD3^+CD4^+CD8^-$/$CD3^+CD4^-CD8^+$T cell percentage of 200 μg/mL Astragalus–processing group was higher than those of 0 μg/mL Astragalus–processing group, and $CD3^+CD4^-CD8^+$T cell content was lower than that of 0 μg/mL Astragalus–processing group; percentages of G_0/G_1 phase and S phase cells as well as mRNA contents of cyclinA, cyclinB and cyclinD1 of 200 μg/mL Astragalus–processing group were lower than those of 0 μg/mL Astragalus–processing group, and percentage of G_2/M phase cell was higher than that of 0 μg/mL Astragalus–processing group. **Conclusion:** Astragalus injection has inhibiting effect on the growth of cervical immortalized epithelial cells, and its mechanisms include regulating immune cell contents, arresting cell cycle and down-regulating cell cycle-related protein expression.

1. Introduction

Cervical cancer is the most common type of malignant tumor of the female reproductive system. Epidemiological studies have shown that human papillomavirus (HPV) infection is detected in 99.8% of cervical cancer tissue and high-risk HPV infection is a risk factor for cervical cancer[1]. The majority of patients are self-limited after HPV infection, and only a minority of patients with HPV infection will develop into cervical intraepithelial neoplasia (CIN)[2]. In the case of high-risk HPV persistent infection, DNA fragments of viruses will be integrated into the host cells' genome and induce CIN and the progressive development into cervical cancer in the presence of factors such as smoking and sexually transmitted diseases. Surgery, radiotherapy and chemotherapy are important methods to treat cervical cancer, but radiotherapy and chemotherapy have low long-term remission and high toxic reaction, and the overall effect is not ideal[3]. In recent years, more and more clinical scholars have realized that detection and treatment of precancerous lesion of cervical cancer can clear the lesion tissue and prevent disease development into cervical cancer, thereby improving the prognosis of the disease[4]. Astragalus is a Chinese herbal medicine with antitumor effect, and in the following research, the regulating effect of Astragalus injection on cervical immortalized epithelial cell growth and cell cycle regulation mechanism were analyzed.

2. Materials and method

2.1. Materials

Human immortalized cervical squamous epithelial cell lines H8

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(purchased from Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences); Astragalus injection was from Shijiazhuang Shineway Pharmaceutical Co., Ltd., Approval No.: Z13020999 and specification: 10 mL each; serum for cell culture was from Hangzhou Evergreen Bioengineering Materials Co., Ltd, RPMI-1640 media were from Gibco Company, flow cytometry kits were from Roche Company, RNA extraction and PCR amplification kits were from TAKARA Company.

2.2. Cell culturing methods

Cells were recovered and then cultured with cell culture media containing 10% fetal bovine serum, culturing conditions were 37 ℃ and CO_2 saturation 5%, and an appropriate amount of penicillin and streptomycin were added to culture system.

2.3. MTT detecting methods

Cells were digested, then seeded in 96-hole cell plate with 1×10^4 cells in each hole and processed with different dosages of Astragalus injection, dosages were 0 μg/mL, 20 μg/mL, 100 μg/mL and 200 μg/mL respectively, parallel holes were set for each dosage, after 24 h of drug processing, 20 μL MTT was added to each hole, and 4 h later, absorbance (OD values) at 490 nm was detected in ELIASA.

2.4. Flow cytometry detecting methods

Cells were digested, seeded in Petri dishes with diameter of 10 cm and processed with different dosages of Astragalus injection, dosages were 0 μg/mL and 200 μg/mL respectively, and 24 h later, cells were digested, divided into two and collected in 15ml centrifuge tubes. One was added to 70% absolute alcohol, incubated for 24 h at -20 ℃, then centrifuged and washed with PBS twice, added to PI/RNase and incubated for 20 min, and percentages of different cell cycles were detected in flow cytometer; as for the other, CD3, CD4, CD8, CD16 and CD56 monoclonal antibodies were added, and after 20 min of incubation, contents of different immune cell subsets were detected in flow cytometer.

2.5. RNA extracting and PCR detecting methods

Cells were digested, seeded in 12-hole cell culture plate and processed with different dosages of Astragalus injection, dosages were 0 μg/mL and 200 μg/mL respectively, 24 h later, RNA extraction kits were used to extract RNA from cells for reverse-transcription, then PCR kits were used for amplification, amplified genes included cyclinA, cyclinB, cyclinD1 and β-actin, β-actin was used to standardize data among groups, and mRNA contents of cyclinA, cyclinB and cyclinD1 were calculated.

2.6. Statistical methods

SPSS 19.0 software was used for analysis of experimental data, comparison among groups was by variance analysis, comparison between two groups was by t test, and $P<0.05$ indicated statistical significance in differences.

3. Results

3.1. Cell viability

Variance analysis results of the detection of cell viability by MTT were as follows: OD values at 490 nm among four groups had differences. Results of LSD-t pair wise comparison showed that OD values of 20 μg/mL, 100 μg/mL and 200 μg/mL Astragalus–processing groups were lower than that of 0 μg/mL Astragalus–processing group, and OD value of 200 μg/mL Astragalus–processing group was lower than those of 20 μg/mL and 100 μg/mL Astragalus–processing groups.

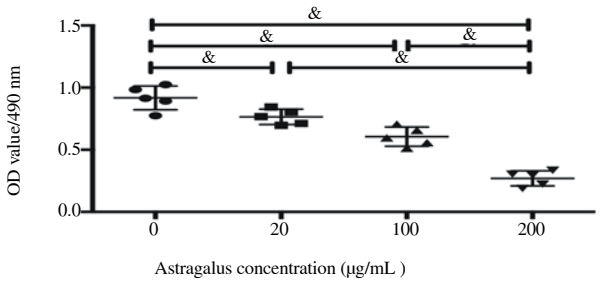

Figure 1. MTT detecting results after concentration gradient Astragalus processing.

3.2. Immune cell contents

T test analysis results of the contents of NK cell, $CD3^+CD4^+CD8^-$T cell and $CD3^+CD4^-CD8^+$T cell by flow cytometry were as follows: contents of NK cell and $CD3^+CD4^+CD8^-$T cell as well as $CD3^+CD4^+CD8^-$/ $CD3^+CD4^-CD8^+$T cell percentage of 200 μg/mL Astragalus–processing group was higher than those of 0 μg/mL Astragalus–processing group, and $CD3^+CD4^-CD8^+$T cell content was lower than that of 0 μg/mL Astragalus–processing group.

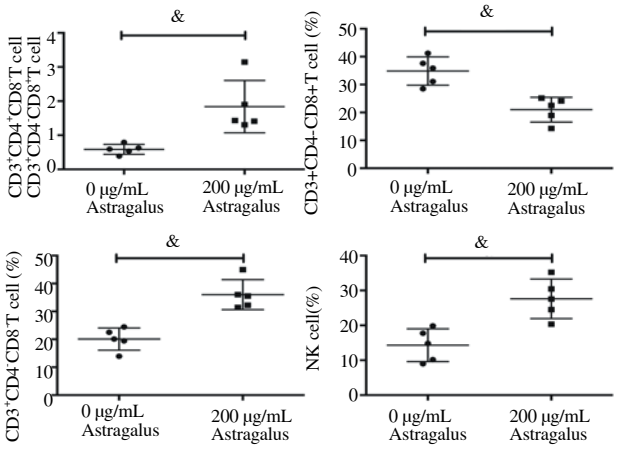

Figure 2. Detecting results of the contents of NK cell, $CD3^+CD4^+CD8^-$T cell and $CD3^+CD4^-CD8^+$T cell after 0 μg/mL and 200 μg/mL Astragalus processing.

Table 1
Cell cycle percentages after 0 μg/mL and 200 μg/mL Astragalus processing.

	12 h after processing			24 h after processing		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0 μg/mL Astragalus	40.49±5.96	37.91±4.16	21.60±2.52	37.58±4.15	33.48±3.59	28.94±3.51
200 μg/mL Astragalus	26.82±2.96	23.15±2.89	50.03±6.24	17.47±1.95	13.32±1.57	69.21±8.34
T	8.918	7.878	11.486	13.585	17.685	23.128
P	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05

3.3. Cell cycle percentages

12 h and 24 h after processing, cell cycle percentages of cervical immortalized epithelial cells were detected and analyzed, and results showed that the percentages of G_0/G_1 phase and S phase cells of 200 μg/mL Astragalus–processing group were lower than those of 0 μg/mL Astragalus–processing group, and the percentage of G2/M phase cell was higher than that of 0 μg/mL Astragalus–processing group.

Table 2
mRNA contents of cell cycle molecules after 0 μg/mL and 200 μg/mL Astragalus processing.

	12 h after processing			24 h after processing		
	CyclinA	CyclinB	CyclinD1	CyclinA	CyclinB	CyclinD1
0 μg/mL Astragalus	100.00±14.52	100.00±12.95	100.00±15.59	100.00±14.57	100.00±13.15	100.00±15.67
200 μg/mL Astragalus	34.22±3.74	22.45±3.10	37.56±5.91	28.79±3.16	41.46±4.66	35.36±3.94
T	18.383	23.282	15.613	20.984	13.182	17.855
P	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05

3.4. Expression levels of cell cycle molecules

CyclinA, cyclinB and cyclinD1 are involved in the regulation of cervical immortalized epithelial cell cycle; 12 h and 24 h after Astragalus processing, mRNA contents of cyclinA, cyclinB and cyclinD1 in cells were detected and analyzed, and results showed that mRNA contents of cyclinA, cyclinB and cyclinD1 of 200 μg/mL Astragalus–processing group were lower than those of 0 μg/mL Astragalus–processing group.

4. Discussion

Cervical intraepithelial neoplasia (CIN) is the precancerous lesion of cervical cancer, and diagnosis and treatment of the disease at this stage can improve the prognosis of disease. Loop electrosurgical excision procedure is the main means of clinical treatment of CIN, and although the surgery can remove lesion tissue, it fails to completely correct the pathological factors causing the occurrence of precancerous lesions at the molecular level, which will lead to greater risk of postoperative relapse. How to use drug therapy to correct the pathological factors of CIN and improve the effect of loop electrosurgical excision procedure is a hotspot of clinical research. Astragalus is a Chinese herbal medicine with effects such as supplementing Qi and up-bearing Yang, securing the exterior and checking sweating, closing sores and expelling pus as well as dispersing swelling and engendering flesh[5]. Experimental and clinical studies in recent years have approved that separate application of Astragalus has certain antitumor effect; combined application with chemotherapeutic drugs can enhance the antitumor effect of chemotherapeutic drugs and alleviate drug resistance as well as toxic and side effect of chemotherapy and it is a good synergistic antidote of chemotherapeutic drugs[6]. The molecular mechanisms of antitumor effect of Astragalus are complex and not completely clear, and links such as enhancing immune function, inhibiting cell proliferation, inducing cell apoptosis and reducing the number of new blood vessels are all related to antitumor effect of Astragalus[7,8].

Astragalus is currently used as a clinical complementary medicine to regulate body's immune state after chemotherapy, and studies of directly killing tumor cells are still stuck in experiments *in vitro*. In the research, cervical immortalized epithelial cells (H8 cells) were selected as research subjects, and the method of in vitro experiment was used to explore the regulating effect of Astragalus on cells as well as the specific mechanisms. As has been noted, HPV is a main carcinogenic factor of cervical cancer, and studies show that HPV16 alone is not enough to cause fully malignant transformation of human normal squamous epithelial cells, only reaching the "immortalized" state of cellular transformation. The cervical immortalized epithelial cells used in the research are equivalent to the clinical precancerous lesion stage of cervical cancer, and exploring the regulating effect of Astragalus on the growth of the cells can provide basis for clinical development of CIN treatment options. After Astragalus was used to process H8 cells, MTT method was used to detect cell viability, and results showed that Astragalus could reduce H8 cell viability in a dose-dependent manner, and the inhibiting effect of 200 μg/mL Astragalus on cell viability was the most significant.

Astragalus was first recognized and clinically used as a biological immunity preparation, and mainly exerted the regulating effect on body's immune function. In the occurrence and development process from precancerous lesion of cervical cancer to cervical cancer, there are pathological states such as immune imbalance and immune escape of cancer cells in the body[9]. T lymphocytes are important cell subsets involved in body's antitumor immune

response, T lymphocytes develop into mature $CD3^+CD4^+CD8^-$ cells and $CD3^+CD4^-CD8^+$T cells after positive and negative selection, the former are helper immune cells that can enhance the immune function of cytotoxic cells, and the latter are suppressor immune cells that can inhibit the immune function of cytotoxic cells[10,11]. NK cells are independent lymphocyte subsets involved in innate immune response and adaptive immune response, and have direct killing effect on tumor cells[12]. The specific immunomodulatory effects of Astragalus include enhancing the function of cytotoxic cells such as NK cells and regulating the balance of T cell subsets. In the research, after Astragalus processing, flow cytometry was used to detect the contents of NK cell and T cell subsets, and results showed that 200 μg/mL Astragalus processing could increase NK cell and $CD3^+CD4^+CD8^-$T cell contents as well as $CD3^+CD4^+CD8^-$/ $CD3^+CD4^-CD8^+$T cell percentage, and decrease $CD3^+CD4^-CD8^+$T cell contents.

Current studies about the molecular mechanisms of precancerous lesion of cervical cancer and cervical cancer believe that HPV-DNA integrity into cervical epithelial cells will affect the expression of a variety of cell cycle proteins, thereby causing uncontrollable growth cycle regulation and enhanced cell proliferative capacity[13]. According to above research results, Astragalus had inhibiting effect on cell proliferation, and therefore it was considered that Astragalus might affect cell cycle to inhibit cell proliferation. In the research, analysis of cell cycle showed that the percentages of G_0/G_1 phase and S phase cells of 200 μg/mL Astragalus–processing group were lower than those of 0 μg/mL Astragalus–processing group, and percentage of G_2/M phase cell was higher than that of 0 μg/mL Astragalus–processing group, which indicated that Astragalus processing could make cell cycle of cervical immortalized epithelial cells arrest in G_2/M phase. Overexpression of cell cycle-related proteins cyclinA, cyclinB and cyclinD1 is an important link causing enhanced proliferative capacity of cervical epithelial cells and they can be combined with cyclin dependent kinase 4 (CDK4) to cause the development of cell cycle[14,15]. In the research, analysis of the expression levels of above three cell cycle-related proteins showed that mRNA contents of cyclinA, cyclinB and cyclinD1 of 200 μg/mL Astragalus–processing group were lower than those of 0 μg/mL Astragalus–processing group.

Based on above discussion, it is believed that Astragalus injection has inhibiting effect on the growth of cervical immortalized epithelial cells, and its mechanisms include regulating immune cell contents, arresting cell cycle and down-regulating cell cycle-related protein expression.

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