Clinical study on black raspberry extract on the negative rate and effectiveness of patients with persistent human papilloma virus infection

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【Abstract】To investigate the negative rate and effectiveness of black raspberry extract in clearing persistent infection with human papilloma virus (HPV), three hundred patients are included in the randomized, double-blind, placebo-controlled human trial for multicenter indication. The distribution ratio of the tested group versus the control group is 2: 1 and the subject patients were given a preparation in the vagina before bed every other day, each dose was 3 g containing black raspberry extract (tested group) or placebo (control group). SPSS 20.0 was used for statistical analysis. Differences between groups were analyzed by the Rank Sum method or Chi-square method. The results showed that 191 subjects in the tested group completed the trial, of which 139 subjects (72.8%) were identified to have an effective clearance of HPV; 95 subjects in the control group completed the trial, of which only 15 subjects (15.8%) were identified to have effective clearance of HPV. The difference between the two groups was statistically significant ($\chi^2=82.9, P<0.001$). The HPV viral load of the tested group was decreased from 224.7 RLU/CO to 88.6 RLU/CO whereas HPV viral load of the control group was ascended from 218.4 RLU/CO to 266.1 RLU/CO. Thus, the present clinical observation suggested that black raspberry extract may be a potential candidate in targeting persistent infection with HPV.

【Key words】Human papillomavirus virus; cervical cancer; black raspberry extract
Human papillomavirus (HPV) infects epithelial cells of human skin and mucosa tissues, especially in the genitalia of men and women. HPV infection can lead to condyloma acuminatum, cervical erosion, chronic cervicitis, and even tumors \[1\]. As one of the most common malignant tumors in women, the incidence rate of cervical cancer is only inferior to that of breast cancer and colorectal cancer \[2\]. In China, an estimated 135,000 new cases and 80,000 deaths from cervical cancer per year. Epidemiological and molecular biological studies have confirmed that persistent infection of high-risk human papillomavirus (HPV) is a necessary condition for the occurrence of cervical cancer \[3\]. According to statistics, 99.7% of cervical cancer is caused by persistent HPV infection \[4\]. Therefore, control of HPV infection is the key to the prevention and treatment of cervical cancer.

At present, few drugs can effectively prevent and control HPV infection in the world except for the preventive vaccine \[5\]. It has been clinically confirmed that the HPV vaccine can almost 100% prevent the infection of some HPV strains, but it cannot prevent the infection of all types of virus strains. In the treatment, laser microwave therapy and drug therapy are mainly used. The course of drug treatment is long and the negative rate remains low. Laser microwave therapy is invasive and causes wound infection. Therefore, it is urgent to develop products and biological agents effectively against HPV infection.

A number of basic researches and animal experiments have confirmed that black raspberry and its active ingredients have a chemopreventive effect on the tumor. Hecht et al. \[6\] analyzed the active parts of the black raspberry extract by high-performance liquid chromatography and found that cyanidin-3-O-glucoside, cyanidin 3-O-(2\(^{\prime}\)-xylosylrutinoside) and cyanidin 3-O-rutinoside. These chemical components were good inhibitors from raspberry to inhibit BPDE induced NF\(\kappa\)B activity, which accounted for 3.4% of dry weight. Wang et al. \[7\] confirmed that black raspberry anthocyanins inhibit the occurrence of chemically induced esophageal cancer in rats. In recent years, it has been reported that black raspberry extract can effectively target a panel of cancer cells such as human oral cancer cells, colorectal cancer cells, and cervical cancer cells \[8,9\] \[10\]. Meanwhile, some studies have found that chemically modified lactoglobulin has highly effective antiviral activity against human papillomavirus (HPV) infection \[11\]. It mainly has a high clearance effect on HPV6, HPV16, and HPV18 type, and can be used as an effective and safe antiviral candidate for the treatment and prevention of HPV infection. In this study, we tried to use black raspberry extract combined with lactoglobulin to remove the HPV virus and prevent cervical cancer.

**Materials and methods**

**Subjects**

The 300 patients with HPV infection were recruited in this clinical observation confirmed by HPV E6/E7 mRNA or HPV DNA test and were randomly divided into an experimental group (n=200) and control group (n=100). These patients were detected in Tianjin Cancer Hospital, Tianjin Heping District Obstetrics and Gynecology Hospital, Tianjin Central maternity hospital, the fourth hospital of Hebei Medical University, and Harbin cancer hospital. The specific data were as follows: experimental group: age 20-50 years old, average (37.73 ± 3.67) years old. Control group: the age was 22-49 years old, with an average of (36.36 ± 4.89) years old. Two
groups of patients were matched with the distributions of age, weight, and HPV types. The trial is approved by the medical ethic committees of these hospitals.

Inclusion criteria

1. The detection of HPV E6/E7 mRNA or HPV DNA lasted for more than half a year and the number of examinations >2 times.
2. The results of cervical cytology were normal or inflammatory.
3. The patients with ASC-US or LSL were examined by histopathology, and the results were inflammation.
4. Women aged 50 or less who have sexual activity.
5. The patients should have proper nutritional status, BMI≥18 kg/m², body weight>40kg, serum albumin≥3g/dL.
6. Patients should be easy to accept the study and participate in follow-up. Patients who participated in the trial must be followed up in the research center where they participated. This means that patients participating in the trial must have reasonable geographical restrictions. The investigator should ensure that the patients randomly enrolled in the study can complete all the studies, HPV virus evaluation, adverse event evaluation and follow-up.
7. Patients are not allowed to participate in other clinical trials at the same time and agree not to participate in other interventional clinical trials during the protocol study. Except for the questionnaire survey or observational study.
8. Informed consent must be signed by the patient.

Drug administration and virus assessments

Cervical cells of all subjects were taken for HPV DNA test, HPV E6 / E7 mRNA detection, TCT test, secretion test, epidemiological examination, and quality of life assessment form 1 day before the study. After the start of the study, the control group and the experimental group were treated with 3 g once every other day for 3 months, except for the menstrual period. After 20 days of continuous use, the drug was stopped for 10 days, and then the next cycle was started. On the 3rd-7th day after 3 months, all the preparations in the experimental group and the control group were used TCT, HPV DNA, HPV E6 / E7 mRNA and secretion were detected. After an interval of 3 months, cervical cells of subjects in the experimental group and the control group were retaken for TCT, HPV DNA, HPV E6 / E7 mRNA detection and secretion examination.

Evaluation criteria of study endpoint

1. HPV clearance negative rate and effective rate evaluation standard
   - Turn negative: HPV E6 / E7 mRNA and HPV DNA test turned negative (3 points).
   - Remarkable effect: the viral load of HPV DNA detection was reduced by two or more orders of magnitude (2 points).
   - Improvement: the viral load of HPV DNA detection decreased by an order of magnitude (1 point).
   - Ineffective: HPV E6 / E7 mRNA and HPV DNA detection did not turn negative, and HPV viral load did not decrease or aggravate (0 point).
2. Secondary evaluation criteria
   - No inflammation was detected by TCT (2 points).
TCT was used to detect inflammation by one level or more (1 point).

TCT test showed that the inflammatory phenomenon aggravated or decreased by less than one level (0 point).

- Secretion: normal (1 point), abnormal (- 1 point)
- Recovery: 3 points
- Remarkable effect: the score is 2 points
- Invalid: score ≤ 1 point

Statistics

The subjects in each group were described by means and frequency counting methods. SPSS (version 20.0) software was used for all statistical analysis. The difference between groups was analyzed by the rank-sum test or chi-square test. It was considered that a P value less than 0.05 was statistically significant.

Results

1. Black raspberry extract can effectively eliminate persistent HPV infection

In this study, no serious side effects, including burning, tingling or other serious symptoms, were observed. Black raspberry extract is generally considered to be more comfortable, soft and moist in the body. The results of HPV clearance efficiency in the test group and control group are shown in Table 1.

|                         | Experimental group | Control group | χ²  | P       |
|-------------------------|--------------------|---------------|-----|---------|
| Number of participants  | 200                | 100           |     |         |
| Number of clinical trials completed | 191 | 95 |     |         |
| Number of HPV positive patients before the test (%) | 191 (100%) | 95 (100%) |     |         |
| Effective number of HPV clearance after test (%) | 139 (72.8%) | 15 (15.8%) | 82.9 | <0.001 |

As shown in Table 1, 191 patients in the experimental group completed the test, of which 139 patients had HPV effectively eliminated after using the black raspberry extract preparation. In the placebo control group, 5 patients were dropped off and 95 patients completed the test with 15 patients effectively cleared HPV virus. The chi-square test showed that the test group and the control group had a significant difference (P<0.001). Therefore, black raspberry extract can effectively eliminate persistent HPV infection.

2. The viral load of HPV decreased significantly after using black raspberry extract

As shown in Table 2, the HPV DNA viral load of each subject was detected by HPV DNA quantitative method. Before the experiment, the average viral load of 191 subjects in the experimental group was 224.7 RLU / Co, while the average viral load of each person in the control group was 218.4 RLU / Co. After the test, the average viral load of each person in the experimental group decreased to 88.6 RLU / CO. The control group had an increase in the average viral load of HPV to 266.1 RLU / CO. These data prove that the black raspberry extract can effectively reduce the viral load in patients and even eliminate them directly.
Table 2. The average viral load of HPV was compared before and after the two sets of tests

|                         | Experimental group (RLU/CO) | Control group (RLU/CO) | Z     | P       |
|-------------------------|----------------------------|------------------------|-------|---------|
| Before the test         | 224.7                      | 218.4                  | 0.762 | 0.446   |
| After the test          | 88.6                       | 266.1                  | 7.551 | <0.001  |

3. Effect of black raspberry extract on vaginal Microecology

As shown in Table 3, we studied the effect of the black raspberry extract on vaginal microecology. Before the experiment, the vaginal microecology of the experimental group and the control group had different proportions of abnormalities, including bacterial vaginitis, abnormal vaginal pH, increased vaginal cleanliness index, abnormal white blood cell count of vaginal secretions, and esterase activity of leukocyte. After the three-month treatment of black raspberry extract, although there was no significant difference in vaginal pH, white blood cell count, and esterase activity of leukocyte (P > 0.05), the percentage of bacterial vaginitis decreased greatly from 20.9% to 7.9%, which was significantly lower than the control group with a value of 18.9% (P < 0.05). The significant difference of vaginal cleanliness was also observed between experimental and control groups, with the percentage of 29.3% and 94.7%, respectively (P<0.05). These results confirm that black raspberry extract has the effect of improving vaginal micro ecological environment.

Table 3. The distribution and proportion of vaginal micro-ecological indicators

| Index                        | Number of abnormal vaginal samples (%) | Before the test | After the test |
|------------------------------|----------------------------------------|----------------|----------------|
|                              | Experimental group                     | Control group   | P              | Experimental group | Control group | P       |
| Vaginal pH                   | 126 (65.9%)                            | 61 (64.2%)      | 0.768          | 96 (50.3%)         | 54 (56.8%)    | 0.316   |
| Bacterial vaginitis          | 40 (20.9%)                             | 24 (25.3%)      | 0.409          | 15 (7.9%)          | 18 (18.9%)    | <0.05   |
| Vaginal cleanliness           | 163 (85.3%)                            | 80 (84.2%)      | 0.861          | 56 (29.3%)         | 90 (94.7%)    | <0.05   |
| White blood cell count of vaginal secretion | 171 (89.5%)                            | 87 (91.6%)      | 0.583          | 164 (85.9%)        | 89 (93.7%)    | 0.075   |
| Esterase activity of leukocyte| 168 (87.9%)                            | 89 (93.7%)      | 0.131          | 148 (77.5%)        | 83 (87.4%)    | 0.056   |

Discussion

Human papillomavirus infection is the main cause of cervical squamous intraepithelial neoplasia and cervical cancer, and persistent high-risk HPV infection is a necessary condition for the occurrence of cervical cancer [3]. The risk of HPV infection in a woman's life is 80%. Most HPV infections are transient and can be cleared by the immune system. However, 10%-15% of patients can not clear HPV virus in time, resulting in persistent infection of HPV virus, cervical cell lesions, and even developing into cervical cancer [12]. Therefore, active prevention and treatment of HPV infection are necessary to prevent and reduce the occurrence of cervical cancer.

In previous studies, it was found that bovine β-lactoglobulin modified by 3-hydroxyphthalic
anhydride has the potential to inhibit HIV\textsuperscript{[13]}. It was also found that bovine β-lactoglobulin modified by 3-hydroxyphthalic anhydride showed inhibitory activity on HIV, HSV-1, HSV-2 and some chlamydia.\textsuperscript{[14]} Lu\textsuperscript{[11]} found that chemically modified lactoglobulin showed highly effective antiviral activity against human HPV infection, including HPV6, HPV16 and HPV18. This anti HPV activity was related to the percentage of lysine and arginine residues in lactoglobulin. The chemically modified lactoglobulin was not cytotoxic at a concentration of 1 mg/ml and was highly stable at room temperature and 37 °C for at least 12 weeks. These results indicate that chemically modified lactoglobulin has potential as an effective and safe antiviral drug for the treatment and prevention of HPV infection.

Previous studies have shown that black raspberry contains a variety of bioactive substances, including a large number of compounds belonging to anthocyanins, which are efficient free radical scavengers and related to tumor chemoprevention \textsuperscript{[15]}. Through the antioxidant system, black raspberry powder and extract can reduce the growth rate of precancerous cells, promote cell apoptosis and cell differentiation, reduce inflammation and angiogenesis, eliminate the damage of oxygen free radical accumulation on DNA, reduce the risk of carcinogenesis, and restore the metabolic function of cells. At the same time, lactoglobulin is acidic under the action of raspberry powder. The acidified lactoglobulin carries a large number of negative charges on its surface, binding to human papillomavirus HPV L1 which has a large number of positively charged regions and forming a complex. Therefore, after adsorption, the virus cannot bind to the transport glycoprotein on the cell surface or enter the cell to form a new infection, leading to the blocking of the infection pathway of human papillomavirus.

\textbf{Conflict of interest} All authors declare that there is no conflict of interest and there is no conflict of interest with the Shu Jing Yue® bacteriostasis gel manufacturers.
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