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Original Article

The correlation between vaginal microecological changes and HPV outcome in patients with cervical lesions in the Inner Mongolia area of China

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Abstract: Objective: Through microecological evaluation and HPV detection in vaginal secretions obtained from women with cervical lesions before and after treatment, we explored the correlation between vaginal microecological changes and human papillomavirus (HPV) outcome. Methods: 136 patients with low-grade squamous intraepithelial lesions (LSILs), 262 patients with high-grade squamous intraepithelial lesions (HSILs), and 33 patients with cervical cancer were assigned into the research group. 100 healthy women were enrolled as controls. The indexes of vaginal secretions and HPV seroconversion rate were detected in order to explore the relationship between these two through data analysis. Results: (1) Differences in HPV infection rate and seroconversion rate among groups in all lesion levels were statistically significant ($P<0.05$). (2) Differences in vagina dominant bacteria, flora diversity and flora density between the high-lesion level groups and cervical cancer group were statistically significant ($P<0.05$). (3) The difference in H_2O_2 positive rate between the low- and high-lesion level groups was statistically significant ($P<0.05$). (4) Differences in SNa negative rate, LE negative rate and GADP negative rate among all lesion level groups were statistically significant ($P<0.05$). (5) Differences in GUS negative rate among the low-lesion level groups were statistically significant ($P<0.05$). Conclusion: Vaginal microecological imbalance is positively correlated with HPV infection, and vaginal microecological restoration is positively correlated with HPV outcome. Vaginal microecological changes can predict the development and prognosis of cervical lesions, and HPV outcome. The vagina microecological evaluation system has a guiding significance for the treatment of HPV.

Keywords: Cervical intraepithelial neoplasias, cervical cancer, vaginal microecological, HPV

Introduction

Cervical lesions include benign cervical lesions, cervical intraepithelial neoplasia (CIN) and cervical cancer. A study [1] confirmed that cervical lesion is an infectious disease, which is caused by human papillomavirus (HPV) infection. According to the classification in the 4th edition of the "Female Genital Tumor Classification" published by the World Health Organization (WHO) in 2014, CINI is a low-grade squamous intraepithelial lesion (LSIL), while CINII and CINIII belong to high-grade squamous intraepithelial lesion (HSIL). Since the cervix is exposed in the vagina, vaginal microecological changes would affect the cervical microenvironment to different extents [2]. The vaginal microecological system is one of the complex microecological systems in the human body, which consists of the vaginal flora, local immune function and endocrine regulation of the body [3]. Disease and lower immunity may cause vaginal flora imbalance, limited dominant bacterium function, or fewer numbers of dominant bacterium [4]. When this status persists for a long time, the invasion of exogenous harmful microbes and the mass propagation of endogenous pathogenic bacteria can easily occur; causing HPV infection [5, 6]. When the microenvironment is not recovered for a long time, cervical disease may occur and progress. In this study, from November 2012 to September 2015, vaginal secretions were obtained from patients before treatment in all lesion levels at three, six and twelve months after treatment. The test results and HPV serocon-

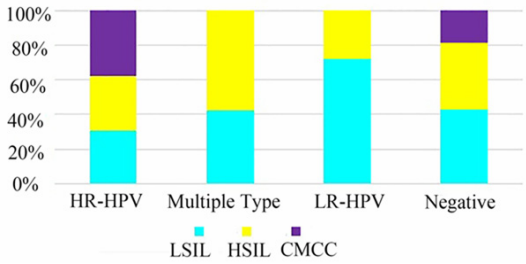

Figure 1. HPV Infection Rate of different Cervical Lesion Grades (n, %).

version rate were statistically analyzed. The results presented as follows.

Materials and methods

Subjects of the study

The present study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University, China. All subjects provided an informed consent prior to enrollment into this study. The research group was composed of 136 LSIL patients, 262 HSIL patients and 33 cervical cancer patients. All patients were diagnosed by the Affiliated Hospital of Inner Mongolia Medical University from November 2012 to September 2015. In addition, 100 healthy women were enrolled as controls. The average age of all subjects was 42.22 ± 8.76 years old, and differences in age among all groups were not statistically significant. The proportion of patients that were lost to follow-up was 18.3% in the LSIL group, 18.6% in the HSIL group, and 9.07% in the cervical cancer group.

Inclusion criteria for the study subjects: Subjects that met the following criteria were enrolled into this study: patients who have a history of sexual life and not in the gestation period; patients who have not taken systemic antibiotics for the last two weeks; patients who did not take sex hormones for the past three months; patients who did not undergo vaginal douche and medication for the past one week; patients with no sexual life, no tub bath, no vaginal douche and surgery in the previous 24 hours; patients who do not have a history of radiation and chemotherapy before sampling.

Exclusion criteria for the study subjects: Women in pregnancy or lactation; patients with various autoimmune diseases, malignant tumors, or women who used immune inhibitors and women with low immune function caused by other factors; women with mental illness, serious heart, liver, kidney and hematopoietic system diseases.

Sampling method

Vaginal secretion acquisition: The vagina was exposed using a vaginal dilator without lubricant. Three long sterile cotton swabs were rotated at the upper 1/3 segment of the vagina near the fornix for 10-15 seconds. When the swabs were found to have obvious secretion, they were immediately sent for testing. Two of the three cotton swabs were parallel rolled on a slide to prepare two smears, and the last one was used to detect the five preformed enzymes in the microecology.

HPV detection: A HPV-specific cervix brush was inserted into the opening of the cervix, and was spun clockwise for 3-5 laps. The brush head was placed into a sample tube with cell preservation solution for inspection.

Experiment

Vaginal microecological experiment method: Detection of the first smear with a microscope after gram stain: ① Bacteria concentration: Under a 10 × 100 times microscope with oil immersion, observation results were recorded as I-IV (denoted as + to ++++), according to the average number of bacteria in each visual field. When the average bacteria number was between 1-9 in the visual fields, the result was regarded as grade I (denoted as +); and when the average bacteria number was between 10-99 in the visual fields, the result was regarded as grade II (denoted as ++). Furthermore, when the average bacteria number was more than 100 or the bacteria occupied the whole visual field, this was regarded as grade III (denoted as +++); and when bacteria aggregated into a mass or densely covered the epithelium, it was regarded as grade IV (denoted as ++++). ② Flora diversity: According to the species number of viable bacteria, the results were classified into grade I-IV (denotDominanted as + to ++++). When 1-3 species of bacteria could be identified, the result was regarded as grade I (denoted as +); when 4-6 types of bacteria could be identified, it was regarded as

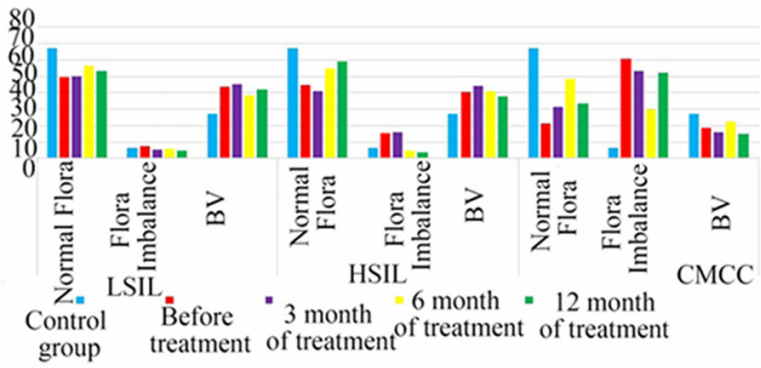

Figure 2. The proportion of Dominant Bacteria in different treatment time (n, %).

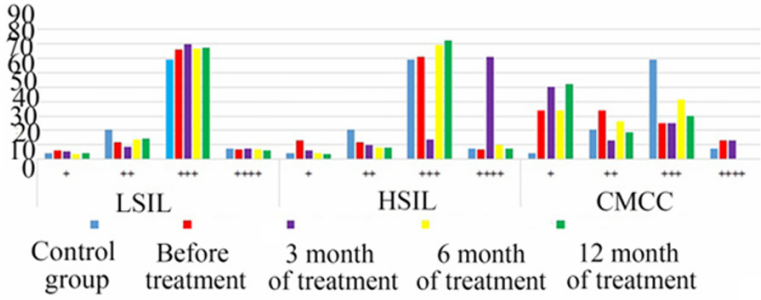

Figure 3. Diversity of Flora in different Cervical Lesion Grades of different Treatment Time (n, %).

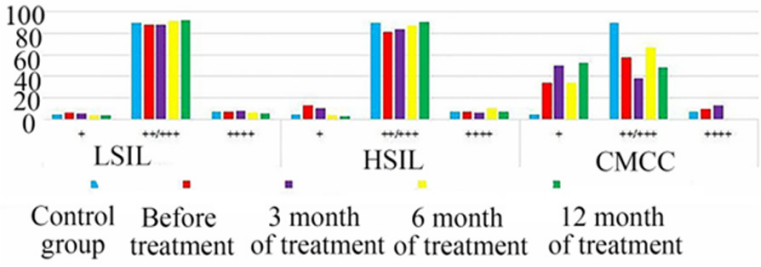

Figure 4. Density of Flora in different Cervical Lesion Grades of different Treatment Time (n, %).

grade II (denoted as ++); when 7-10 types of bacteria could be identified, it was regarded as grade III (denoted as +++); and when 11 or more types of bacteria could be identified, it was regarded as grade IV (denoted as ++++). ③ Dominant bacteria: Under the microscope, the microorganism species with the largest number was defined as the dominant bacteria.

The second smear was stained with hematoxylin and eosin (H&E). Then, the clue cells, trichomonad, candida and other pathogens were examined under a 10 × 40 times lens.

The remaining sample was used for the detection of the catalytic activities of catalase (H_2O_2), neuraminidase (SNa), leukocyte esterase (LE), beta glucuronidase (Gus) and coagulase (GADP) using a TORCH assay kit [9].

Experimental methods for HPV: After sampling, the samples were immediately sent to the Laboratory Department for testing. HPV infection was detected using the HPV gene detection method.

Diagnostic criteria for vaginal microecology

Flora density: ++ and +++ were defined as normal, while + and ++++ were defined as abnormal; Flora diversity: ++ and +++ were defined as normal, while + and ++++ were defined as abnormal; When the dominant bacteria were lactobacilli, the flora was determined as a normal flora. When clue cells appeared positive, the condition was determined as bacterial vaginosis (BV). When other pathogen infections appeared, the condition was assessed as flora imbalance, including aerobic vaginitis (AV); When H_2O_2 was ≥ 2 μmol/L, two was defined as positive, SNa ≥ 7 U/L was defined as positive, LE ≥ 9 U/L was defined as positive, GUS ≥ 15 U/L was defined as positive, and GADP ≥ 20 U/L was defined as positive. When H_2O_2 was positive, lactobacillus function was evaluated as normal; while when SNa, LE, GUS and GADP were all negative, it was defined as normal.

Statistical analysis

Data were analyzed in the Public Health College of Medical University of Tianjin, China using sta-

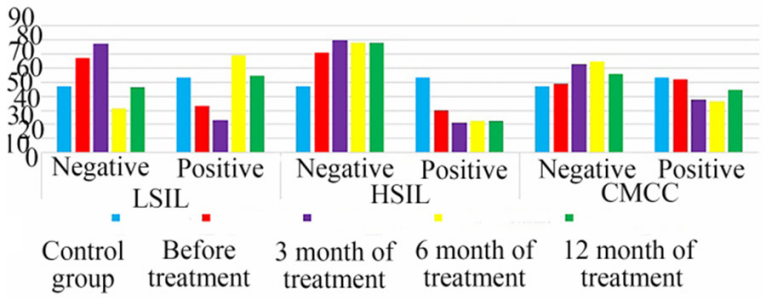

Figure 5. The Proportion of H_2O_2 in different Cervical Lesion Grades of different Treatment Time (n, %).

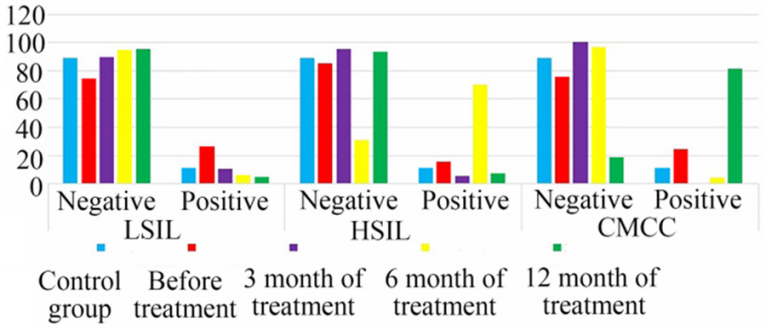

Figure 6. The Proportion of SNa in different Cervical Lesion Grades of different Treatment Time (n, %).

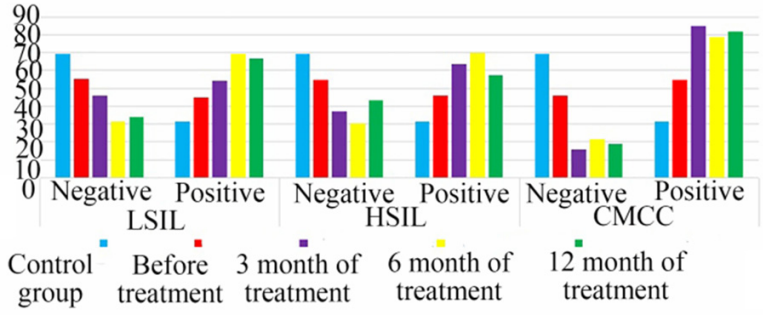

Figure 7. The Proportion of LE in different Cervical Lesion Grades of different Treatment Time (n, %).

tistical analysis software SAS 9.2. Quantitative data were expressed as mean ± standard deviation. Qualitative data were described by absolute and relative indices, such as the number of cases and the proportion. Statistical tests were conducted using the two-sided test. A P-value ≤ 0.05 was considered statistically significant.

Results

HPV infection rate

HPV infections in different lesion level groups were compared using Chi-square test, and the difference was statistically significant (P<0.0001) (**Figure 1**).

Composition of dominant bacteria

Dominant bacterium composition in patients in the LSIL groups at different times was analyzed using Chi-square test, and the difference was not statistically significant (X^2=10.2534, P=0.2477). The dominant bacterium composition in patients in the HSIL groups at different time points was analyzed using the Chi-square test, and the difference was statistically significant (X^2=50.109, P<0.05). Dominant bacterium composition in patients in the cervical cancer groups at different time points was analyzed using Chi-square test, and the difference was statistically significant (X^2=57.9128, P< 0.0001) (**Figure 2**).

Composition of bacterial diversity

Flora diversity in patients in the LSIL group at different times was analyzed using Chi-square test, and the difference was not statistically significant (X^2=7.4307, P= 0.8279). Flora diversity in patients in the HSIL group at different time points was analyzed using Chi-square test, and the difference was statistically significant (X^2=40.278, P<0.0001). Flora diversity in patients in the cervical cancer group at different time points was analyzed using Chi-square test, and the difference was statistically significant (X^2=64.1268, P<0.0001) (**Figure 3**).

Composition of bacterial density

Flora density in patients in the LSIL group at different time points was analyzed using the Chi-square test, and the difference was not statistically significant (X^2=2.0521, P=0.9794). Flora density in patients in the HSIL group at

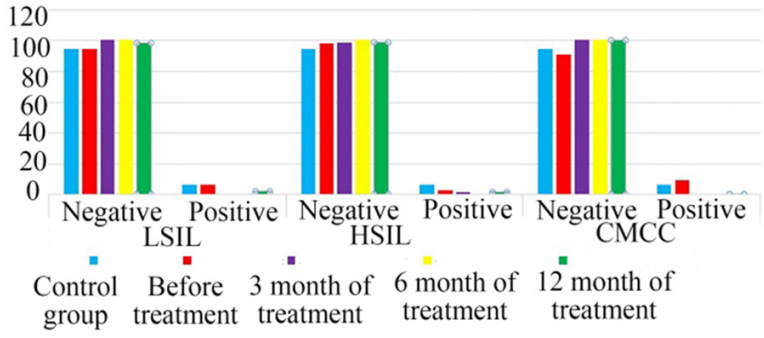

Figure 8. The Proportion of GUS in different Cervical Lesion Grades of different Treatment Time (n, %).

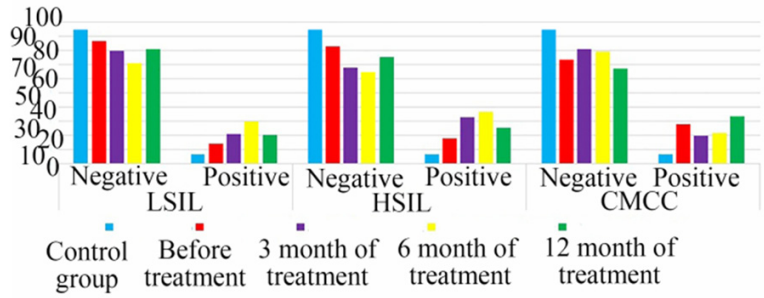

Figure 9. The Proportion of GADP in different Cervical Lesion Grades of different Treatment Time (n, %).

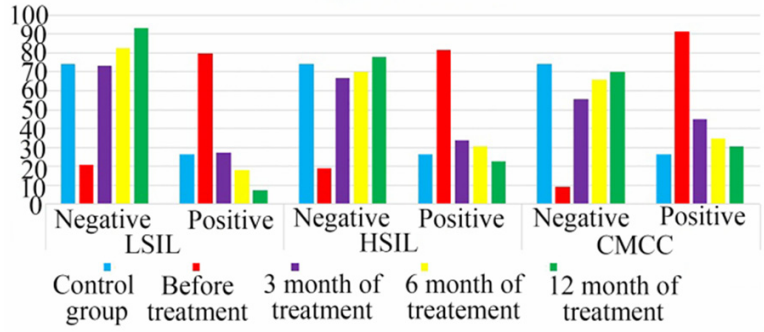

Figure 10. The HPV-Negative Rate in different Cervical Lesion Grades of the different Treatment Time (n, %).

different time points was analyzed using Chi-square test, and the difference was statistically significant (X^2=27.833, P<0.05). Flora density in patients in the cervical cancer group at different time points was analyzed using Chi-square test, and the difference was statistically significant (X^2=54.0783; P<0.0001) (**Figure 4**).

H_2O_2 composition

H_2O_2 composition in patients in the LSIL and HSIL groups at different time points was analyzed using Chi-square test, and the difference was statistically significant (P<0.05). H_2O_2 composition in patients in the cervical cancer group at different time points was analyzed using the chi-square test, and the difference was not statistically significant (P>0.05) (**Figure 5**).

SNa composition

SNa composition in patients in the LSIL, HSIL and cervical cancer groups at different times was analyzed using Chi-square test, the difference were statistically significant (P<0.05) (**Figure 6**).

LE composition

LE composition in patients in the LSIL, HSIL and cervical cancer groups at different time points was analyzed using Chi-square test, and the difference was statistically significant (P<0.05) (**Figure 7**).

GUS composition

GUS composition in patients in the LSIL group at different time points was analyzed using Chi-square test, and the difference was statistically significant (P<0.05). GUS composition in patients in the HSIL and cervical cancer groups at different time points was analyzed using Chi-square test, and the difference was not statistically significant (P>0.05) (**Figure 8**).

GADP composition

GADP composition in patients in the LSIL, HSIL and cervical cancer groups at different time points were analyzed using Chi-square test; and the differences were statistically significant (P<0.05) (**Figure 9**).

HPV seroconversion rate

HPV seroconversion rate in patients in all lesion level groups at different times were analyzed after treatment using Chi-square test, and the differences were statistically significant (X^2= 160.7805, P<0.0001; X^2=207.4137, P<0.0001; X^2=45.1629, P<0.05) (**Figure 10**).

Discussion

At least one in approximately 80% of women who have an active sexual life have HPV infection, but not all infected women would develop CIN or cervical cancer [7]. When the host defense mechanism defects or genes mutations occur, the HPV virus can induce the integration of the gene fragment of the HPV virus, and the unstable and transcriptional active regions of the genome of cervical epithelial cells; which progresses into persistent infection, CIN, and finally into cervical cancer [8]. Hence, HPV infection is not the only condition that may cause cancer. Other synergic factors may increase the risk of cervical cancer at the same time [9].

HPV infection and vaginal microecology: The cervix is exposed to the vagina. Vaginal microecological changes not only affect the microenvironment of the cervix, but also have certain correlations with HPV infection [10, 11]. The normal vaginal flora is dominated by lactobacillus, which accounts for more than 90%. It can convert glycogen into lactic acid in the epithelial cells of the vagina, and maintain an acidic vaginal environment, flora balance and inhibit the growth of other endogenous pathogenical bacteria [12]; blocking the invasion of exogenous pathogenic bacteria by competitive adhesion and nutritional competition [13]. Lactobacillus Crispatus can produce H_2O_2 and an inhibitor to suppress the growth of other microorganisms [14]. Preformed enzymes are a kind of enzymes produced by bacteria in the process of growth and reproduction, which is related to growth and the metabolism of bacteria. For different species of bacteria, the kinds of preformed enzymes differ [15]. Therefore, vaginal microecologically preformed enzymes are a good indicator for the common bacteria of vaginal infection. H_2O_2 is a product of some species of lactobacilli. When the number of lactobacilli producing H_2O_2 reduces or their functions decline, the important status of the lactobacillus in maintaining dynamic balance in the microecology would be affected [16]. Weakening of vaginal defense function increase the chances of infection of BV and AV. SNa is an enzyme produced by Gardner and some anaerobic bacteria, which is a good indicator of part of BV. GADP and GUS are produced by some aerobic bacteria, and LE is an indicator of the indirect reaction to the local vaginal inflammation [17]. Weakening of the vaginal defense function would increase the chances of infection on BV and AV. SNa is an enzyme produced by Gardner and some anaerobic bacteria, which is a good indicator of part of BV. GADP and GUS are produced by some aerobic bacteria, and LE is an indirect reaction to local vaginal inflammation [17].

In this study, through determining the HPV infection rate in groups of all lesion levels, it was found that with the increase of the cervical lesion levels, HPV infection rate exhibited a gradual upward trend; especially, high-risk HPV infection rate in the cervical cancer group was the highest, which was consistent with the results reported in related literature [18]. The detection of related microecological indicators before treatment revealed that lesion levels became elevated, normal flora, flora diversity and flora density decreased, abnormal flora including BV increased, and the amplitude of flora imbalance increased. These suggests that HPV infection is related to the imbalance of bacterium composition; namely, with the increase of the proportion of the abnormal component of bacteria, HPV infection rate increased [19]. The detection of preformed enzyme factors revealed that H_2O_2 positive rate, LE positive rate, GUS positive rate and GADP positive rate were all the highest in the cervical cancer group. Furthermore, SNa positive rate was the lowest in the HSIL group, but was higher in all the research groups, compared with the control group. These suggests that vaginal microecological imbalance is the most serious in the cervical cancer group, and HPV infection rate was the highest in this group. We consider that HPV infection and the occurrence of cervical diseases are related to the infection of vaginal pathogenic bacteria, which was consistent with the results reported in related studies [20]. The H_2O_2 positive rate in the cervical cancer group was the highest, namely, the production capac-

ity of H_2O_2 was the best. The causes maybe that patients with high lesion levels receive more attention, as well as timely diagnosis and treatment. This enabled lactobacilli producing H_2O_2 not to be completely inhibited for a short time.

Vaginal lactobacilli reduce or their function is inhibited; and the protective mechanism of the vaginal microenvironment is destroyed [21, 22]. This limits the growth of normal dominant bacteria, H_2O_2 positive rate declines, flora diversity and density changes, increase in the chance of infection of BV, AV and other pathogens [23], and rising SNa, GUS and GADP positive rates. Long term imbalance of the vaginal bacteria aggravates local inflammation, which result in an increase of LE positive rate. If patients cannot obtain timely diagnosis and treatment, this would provide an opportunity for HPV to invade and persistently infect, causing the occurrence and development of CIN, and even into cervical cancer [24-26]. It reveals that vaginal microecological imbalance was positively correlated with HPV infection, and long-term HPV infection can also reversely act on the microenvironment of the cervix; promoting the occurrence and development of cervical diseases.

HPV outcome and vaginal microecology: Vaginal microecology is determined by multiple factors. Any of these factors are affected or interactions between them lose balance cause microecological imbalance [27]. This study revealed that after treatment, HPV negative rate increased with treatment time, and decreased with increasing lesion level. The detection of bacterial flora composition revealed that with the increase of lesion level, normal dominant bacteria, diversity and density were decreased gradually. Furthermore, the abnormal flora proportion increased gradually. There was no significant difference in the indexes of flora in the LSIL group. Since the degree of vaginal microecological imbalance is lower than in the lower lesion level group, and HPV seroconversion rate is the highest. We consider that the normal vaginal flora can promote clearance of persistent viral infection. Composition of the bacteria in the HSIL group gradually improved with treatment. However, before and after treatment remains different from that in the control group. This revealed that microenvironment unbalance persisted for a longer period of time in the higher lesion level groups, and the difficulty for recovery would also increase. This would result in a lower seroconversion rate of HPV, compared with the low lesion level groups. Normal flora in the vagina revealed a recovery trend in the cervical cancer group 3-6 months after. However, the treatment 12 months after treatment caused the recovery trend to be deviated. This suggests that the imbalance degree of the vaginal flora in the cancer group is severe, which provides the condition for the continuous infection of HPV. In addition, long-term infection exhausted the anti-infection ability of the vagina, and inhibited the function of lactobacillus [28, 29]; resulting in a lowest seroconversion rate of HPV in this group.

In this study, it was found that during the course of treatment, H_2O_2 positive rates gradually recovered as the treatment time extended in the LSIL and HSIL groups. Furthermore, this was the most significant in the LSIL group; and the seroconversion rate of HPV was the highest in this group. This suggests that H_2O_2 recovery is conducive to the seroconversion of HPV in the low lesion level groups. Although the reason why the cervical cancer group revealed the highest H_2O_2 positive rate was maybe that these patients received more attention, and were given timely and effective treatments. The differences in SNa negative rate between all research groups and the control group were statistically significant. This suggests that the infection rate of BV in cervical lesion group was higher than in the control group. The negative rates of SNa increased with treatment time in the LSIL and HSIL groups, and the infection rate revealed a decreasing trend. Furthermore, seroconversion rate of HPV showed an increasing trend. These results revealed that the inhibition of vaginal infection can promote the seroconversion of HPV in the treatment course. Reduction of BV infection relieved the inhibition effect on the function of the lactobacillus that produces H_2O_2 [30-33]. Hence, this can play a certain role to recover the microecological environment. SNa negative rate was decreased 12 months after treatment in the cervical cancer group. The reason may be that many cervical cancer patients are in the peri-menopausal period or have undergone the resection of double uterine appendix [34]. Their decreased estrogen level causes the blocking of the pathway where lactobacillus maintains the flora bal-

ance through the decomposition of glycogen, resulting in an increased infection rate of some anaerobic bacteria. LE negative rate fluctuates during treatment in each group without an obvious improvement trend, but HPV negative rate gradually improved. This suggests that in addition to HPV infection, other infections affected the recovery of local inflammation. Hence, chronic inflammatory conditions increased the risk of the persistent HPV infection [35]. GADP negative rate gradually decreased with treatment time in the HSIL and cervical cancer groups. This suggests that there were infections of aerobic bacteria such as Staphylococcus Aureus in the two groups, leading to a lower seroconversion rate of HPV, compared with the low-lesion level groups. GADP negative rate was decreased at first, and increased in the LSIL group, which was the highest in 12 months after treatment; and the seroconversion rate of HPV was the highest in this stage. From this, we can determine that this type of infection is gradually controlled, which has a certain impact on the outcome of HPV in the treatment. GUS is produced by aerobic bacteria such as Escherichia coliand group B streptococcus. Since the number of such infections is fewer, GUS negative rate was statistically significant only in the LSIL group; and its proportion in the treatment process gradually approached the level of the control group. Furthermore, the seroconversion rate of HPV also increased accordingly. In this study, GUS and GADP negative rates were always very close to or even over the control group in each treatment stage or in each research group. The reason may be that the number of AV cases was few, and AV was more difficult to be diagnosed, compared with other vaginitis; which often combines with other infections, increasing diagnostic difficulty [36-38].

Conclusion

In this experiment, through detecting the vaginal microecology and HPV negative rate in 432 research subjects and 100 controls, it was found that vaginal flora composition and function of lactobacillus and vaginal infections were correlated with the occurrence and development of cervical disease and HPV seroconversion [39]. Vaginal microecological disturbance provided an environment for HPV growth, and long term imbalance would make vaginal bacteria functions decline. Furthermore, the difficultly of recuperation increases, and further provides conditions for the long-term infection of HPV and the development of cervical diseases; while the persistence of HPV also increased the chances of other vaginal infections. In the process of treatment, the gradual recovery of the microecology has a promoting effect on HPV outcome and disease prognosis [40]; that is, with the restoration of vaginal microecology, HPV negative rate increases. This suggests that vaginal microecological restoration is positively correlated with HPV outcome.

With the continuous strengthening of people's awareness, screening for cervical lesions is more and more becoming popular. Through more convenient and simple means, vaginal microecological detection method can be used to predict the development of cervical lesion level, evaluate the prognosis of lesions, and provide clinical guidance to HPV treatment. This would be a very important reference that will be significant for further research in the future.

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Disclosure of conflict of interest

None.

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