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Effect of radiofrequency therapy on HPV16-E7 lentivirus infection in the reproductive tract of mice and its effect on immune function of splenic lymphocytes

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Abstract
Objective: To investigate the effect of radiofrequency therapy (RFT) on HPV16-E7 lentivirus infection in the reproductive tract of mice and reveal its effect on immune function of splenic lymphocytes. *Materials and Methods*: The mouse reproductive tract model was established by infection with HPV16-E7 lentivirus. Fluorescence microscope was used to evaluate successful injection. The expression of HPV16-E7 protein was detected by Western blotting test. The levels of CD4+ and CD8+ were determined by flow cytometry, and the ratio was calculated. The proliferation of splenic lymphocytes was detected by MTT assay. Expression of Interleukin (IL)-2 and interferon-γ (IFN-γ) messenger RNA (mRNA) in lymphocyte was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *Results*: Fluorescence microscope determined the successful injection of HPV16-E7 lentivirus. Compared with model group, RFT treatment decreased HPV16-E7 protein, and increased CD4+/CD8+ ratio and the proliferation activity of splenic lymphocytes. Besides, RFT treatment increased the mRNA expression levels of IL-2 and IFN-γ compared to the model group. In particular, the proliferation activity of spleen lymphocytes and the expression levels of IL-2 mRNA and IFN-γ mRNA in RFT were higher at 12 days than at 6 days after treatment. *Conclusion*: RFT could eliminate HPV16-E7 lentivirus infection in the reproductive tract of mice, and the mechanism was related to the immune system.

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Introduction

Cervical cancer (CC) is one of the most common malignant tumors of female reproductive system, ranking second in the list of malignant tumors. The initial onset of cervical cancer is found in the cervix, and the initial symptoms are not significant. Late onset of common symptoms primarily includes vaginal bleeding and drainage. Coupled with current changes in women's lifestyles and eating habits, the morbidity and mortality of cervical cancer are increasing at younger age. Among the causes, human papillomavirus (HPV) accounts for a larger share. It causes cervical cancer in about half a million women each year. Currently, HPV vaccination is an effective method to prevent cervical cancer, but clinically, effective treatment methods to eliminate genital tract HPV infection are still inadequate.[1,2]

Radiofrequency therapy (RFT) is a new hyperthermal technology developed in recent decades. This method of treatment can effectively treat malignant tumors. The primary principle of RFT is high-temperature treatment, in which the needle is punctured at the tumor site, and the physical phenomenon of heat of the tissue appears on the tip of the needle. Heat changes the temperature of tumor cells, causing their protein to coagulate and wither necrosis, thereby destroying tumor cells and attaining the effect of antitumor treatment.[3] However, RFT is probably to induce a heat shock response to surrounding cells by local-generated heat. At present, RFT has been widely used in the treatment of functional uterine bleeding, uterine myoma, cervical erosion, condyloma acuminatum, and other gynecological diseases.[4] Tan and Repici *et al*.[6] demonstrated that RFT had a good clinical effect to clear HPV infection. However, specific mechanism of RFT on the therapeutic effect of HPV16-E7 lentivirus infection and splenic lymphocyte immune function has not been clarified and requires additional research and analysis.

The present study successfully established the reproductive tract HPV16-E7 lentivirus infection model and treated it with RFT. After 4 and 8 days of treatment, preliminary experiments were performed to explore the effect of RFT on HPV16-E7 lentivirus infection in mouse reproductive tract and reveal its effect on the immune function of splenic lymphocytes.

Materials and Methods

Chemicals and reagents

The expression plasmid PLV-UBC-GFP-3FLAG, 293T human renal epithelial cells and Lipofectamine 2000 were purchased from OBiO Technology (Shanghai) Co. Ltd (Shanghai, China). Fetal bovine serum and RPMI1640 medium were purchased from Gibco (Grand Island, NY, USA). Nonoxynol ether, β-estradiol, and hydroxymethyl cellulose were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Medical medroxyprogesterone acetate injection was purchased from Zhongshan Hospital (Xiamen, Guangdong, China). Restrictive endonuclease EcoR I, Nhe I, T4 ligase, and one-step reverse transcription fluorescence quantitative kit were purchased from Sangon Biotech (Shanghai) Co. Ltd (Shanghai, China). FITC anti-mouse CD4+ and PE anti-mouse CD8+ antibodies were purchased from BD Biosciences Inc. (San Jose, CA, USA). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase; ab9485) antibody and HRP-labeled secondary antibody (ab6721) were purchased from Abcam (Cambridge, MA, USA). The lentivirus packaging kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). HPV16-E7 (RS-0992R) antibody was purchased from Shanghai Yanjing Biotechnology Co. Ltd. (Shanghai, China).

Establishment of mouse reproductive tract model infected with HPV16-E7 lentivirus

Posted 4–6-week-old female BALB/c (concatenation of Bagg and Albino) mice, with a body weight of 20 g, were purchased from Nanjing University Model Animal Research Institute (Jiangsu, China). HPV16-E7 lentiviral vector was constructed by referring to the method described by Zhou *et al*.[7] A total of 293 T cells were co-transfected with the lentiviral vector of *HPV16 E7* gene, lentiviral structural protein plasmid pCD/NL-BH, and lentiviral membrane protein plasmid pLTR-G to package HPV16-E7 lentivirus. The titer was diluted to 9×10^5 TU/mL. The protocol was performed based on the *Guide for the Care and Use of Laboratory Animals*,[8] and the ethical approval was obtained from the Ethical Committee of the Affiliated Hospital of Chengde Medical University.

The method described by D. Wang *et al*. was used to establish the mice reproductive tract HPV16-E7 lentivirus infection model.[9] The specific steps were as follows: each mouse was subcutaneously injected with 0.1-μg β-estradiol. After 24 h, the mice were subcutaneously injected with 3-mg medroxyprogesterone acetate to enter the estrous phase. After 12 h, the mice were anesthetized with gas anesthesia system (XGI-8), and their vaginas were chemically damaged by infusing 50-μL 4% nonoxenol ether. After 6 h, 25-μL HPV16-E7 lentivirus and 15-μL 4% hydroxymethyl cellulose were infused into the vagina of mice for infection. Mice in normal control group were treated with 40-μL 4% hydroxymethyl cellulose. After 48 h of infection, approximately 5 mm of tip of a 10-μL pipette was inserted into the vagina of mice. After injection and suction for two to three times, 10 μL was aspirated and placed on glass slides. Fluorescence microscope was used to observe the expression of Green Fluorescent Protein (GFP) in the vaginal smear of mice, indicating the successful establishment of mice model.

Eight normal female BALB/c mice were selected as normal control. Sixteen female BALB/c mice with HPV16-E7 lentivirus infection in the reproductive tract were randomly divided into two groups: the RFT group and the model group (n = 8 in each group). In the RFT group, mice infected with HPV16-E7 lentivirus were treated with RFT. The treatment process was as follows: after mice were anesthetized, approximately 5 mm of radiofrequency ablation knife was placed in the vagina of mice after prostrate fixation. The initial power of treatment was set at 20 W (at a temperature of 65°C, for 5 min), as reported previously.[10] Mice in the model group received HPV16-E7 lentivirus infection without RFT treatment. After 6 and 12 days

of RFT treatment, four mice in each group were sacrificed at the same moment; the spleen and vaginal tissues were removed by aseptic operation.

Western blotting test

Western blotting test was performed to detect the expression of HPV16-E7 protein in the vaginal tissues of mice. RIPA lysate, dissolved on ice for 20 min, was added to tissues, and centrifuged at 13,000 rpm at 4°C for 20 min. The supernatant was collected and the protein content was determined using BCA kit; 35-μg protein solution was taken for SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred, and sealed with blocking solution (2% BSA). Primary antibodies were added at 4°C overnight (with GAPDH antibody as reference). After washing, the secondary antibody was added and incubated at room temperature for 1 h. Exposure control limit (ECL) exposure was performed, and the results were analyzed using the Alpha Imager HP gel imaging system.

Preparation of splenic lymphatic single-cell suspension

Spleen tissues of mice were cut into pieces, placed on a sterile nylon net, set into the complete culture medium of RPMI1640, and grounded gently. After filtration using a 200-mesh steel sieve and centrifuged at 1500 rpm for 5 min, the supernatant was discarded. Precipitation was resuspended with erythrocyte lysate at room temperature for 5 min. Equal volume of phosphate buffered saline (PBS) was added to neutralize and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, washed with PBS, and resuspend in RPMI1640 medium (containing 1-mM/L glutamate, 100-IU/mL penicillin, 100-μg/mL streptomycin, and 10% fetal bovine serum). The survival rate of cells detected by trypan-blue staining was more than 95%.

Percentage of CD4+ and CD8+ T cells in spleen cells was determined by flow cytometry

Splenic lymphocyte fluid, 100 μL, was taken from mice and added into a flow cytometry tube. In a blank group, no antibodies were added; FITC anti-mouse CD4+ and PE anti-mouse CD8+ were added and protected from light for 30 min. Finally, 500 μL of PBS was added and washed for three times. The percentage of CD4+ and CD8+ T cells was analyzed by the CELLQuest software, and the ratio of CD4+/CD8+ cells was calculated.

Proliferation of splenic lymphocytes was detected by MTT assay

Splenic lymphocytes were inoculated into 96 well plates at 2×10^3 per well for 24, 48, and 72 h. MTT solution (10 μL) was added to each well for 4 h. The supernatant was discarded and dimethyl sulfoxide (DMSO) was added to dissolve the crystals. The absorbance of each well was detected by automatic microplate analyzer at 490-nm wavelength. The cell proliferative rate of each group was calculated as follows:

Proliferation rate (%) = optical density (OD) value of experimental group - OD value of background group/OD value of normal control - OD value of background group × 100

The messenger RNA (mRNA) expression levels of interleukin (IL)-2 and interferon-γ (IFN-γ) in spleen lymphocyte cells were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Splenic lymphocyte fluid (2 mL) was taken to detect the mRNA expressions of IL-2 and IFN-γ according to the instructions of one-step reverse transcription fluorescence quantitative kit, with *GAPDH* as an internal reference gene. ΔCt (i.e., ΔCt(n)=Ct(gene)−Ct(internal control)) was used for relative quantitative analysis: IL-2, F: *ATGAACTTGGACCTCTGCGG*, IL-2, R: *GTCC ACCACAGTTGCTGACT*; IFN-γ, F: *GAGGTCAACCAACACACAGGT*, IFN-γ, R: *GGGACAATCTCTTCCCCACC*; GAPDH, F: *TTCTTTTGC GTCGCCAGCC*, GAPDH, R: *CTTCCCGTTCTCAGCCTTGAC*.

Statistical analysis

All data were analyzed using the SPSS 21.0 software. The measurement data were expressed as (mean [X] ± SD). With metric data conforming to normal distribution, the two groups were compared using independent sample *t*-test and the comparison between multiple groups was performed by one-way analysis of variance (ANOVA). Enumeration data were expressed as percentage (%), and Chi-squared (χ^2) test was performed to compare intergroup rates. Pearson correlation analysis was used, and the test level was set as $\alpha = 0.05$. Difference was considered statistically significant at $P < 0.05$.

Results

GFP expression in the vaginal smear of mice infected with HPV16-E7 lentivirus

After 48 h of HPV16-E7 lentivirus infection, GFP expression was observed in the vaginal smear of mice infected with HPV16-E7 lentivirus using fluorescence microscope. As shown in Figure 1, the results demonstrated that no GFP expression was observed in the vaginal smear of mice in the normal control, but the GFP expression was highly expressed in the RFT group, indicating that the model of HPV16-E7 lentivirus infection in the reproductive tract of mice was established successfully.

Expression of HPV16-E7 protein in the vaginal tissues of mice after RFT

The expression of HPV16-E7 protein in the vaginal tissue of mice was significantly decreased after 6 and 12 days of RFT treatment as shown in Figure 2, and there was significant

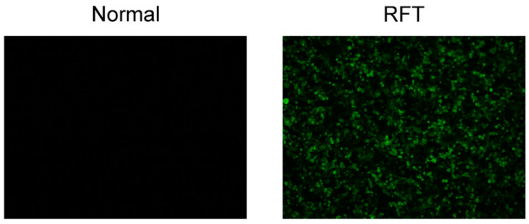

Figure 1 Green fluorescent protein (GFP) expression in the vaginal tissues of mice. The mouse lentivirus infection model of HPV16-E7 reproductive tract was established by the method described by D. Wang *et al.*[9] after −48 h of infection; fluorescence microscopic image observed GFP expression in the vaginal smear of mice. (A) Expression of GFP in the vaginal smear of mice infected with HPV16-E7 lentivirus by fluorescence microscope. (B) Fluorescence microscopic image shows no GFP expression in the vaginal smears of normal mice.

difference between the two groups (all P-values < 0.05). In particular, no expression of HPV16-E7 protein was detected in the normal control at any time point.

Levels of CD4+ and CD8+ and ratio of CD4+/CD8+ in the spleen of mice after RFT

As shown in Table 1 and Figure 3, compared with the normal control, the CD4+/CD8+ ratio in the spleen of mice at after 6 and 12 days of RFT was significantly increased in the RFT group (all P-values < 0.05). However, the ratio of CD4+/CD8+ in the spleen of model group was significantly decreased at each moment (all P-values < 0.05).

Proliferation of splenic lymphocytes in mice after RFT

As shown in Figure 4, the proliferative activity of splenic lymphocytes in the RFT group was 62.75 ± 1.11 and 76.00 ± 1.08 after 6 and 12 days of RFT, respectively, which was significantly higher than that in the model group and normal control (P < 0.05). As the number of treatment days increases, the proliferative activity of splenic lymphocytes in mice of the RFT group also increased significantly (P < 0.05).

Expression levels of IL-2 and IFN-γ mRNA in splenic lymphocytes after RFT

As shown in Figure 5, compared with the normal group, the mRNA expression levels of IL-2 and IFN-γ in splenic lymphocytes of mice in the RFT group were significantly increased after 6 and 12 days of treatment (all P-values < 0.05). However, the mRNA expression levels of IL-2 and IFN-γ in splenic lymphocytes of the model group was significantly decreased at each time point (all P-values < 0.05). As the number of treatment days increases, the mRNA expression levels of IL-2 and IFN-γ in splenic lymphocytes of mice in the RFT group also increased significantly (P < 0.05).

Discussion

Radiofrequency therapy is a commonly used minimally invasive hyperthermia technology. Its primary principle is that the radiofrequency current is emitted within a short time period by radiofrequency electrode, and the local

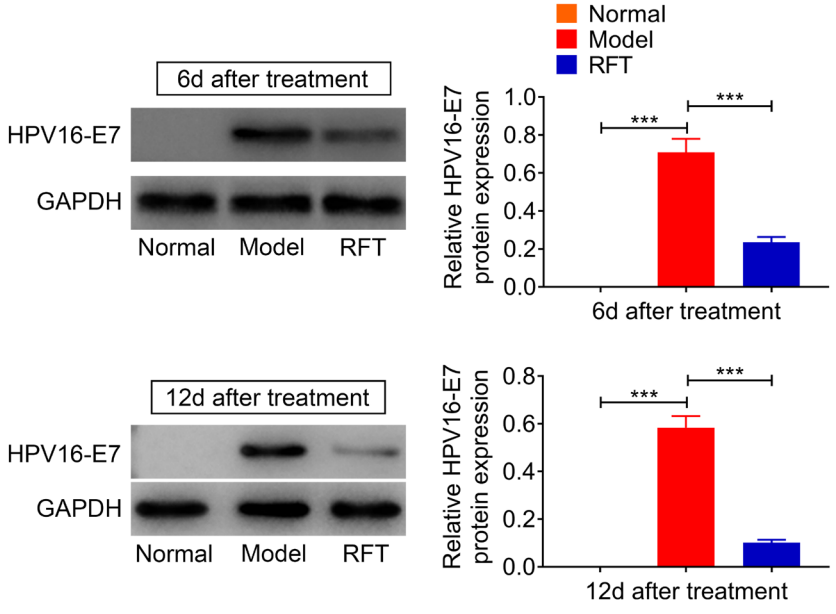

Figure 2 Expression of HPV16-E7 protein in the vaginal tissues of mice after radiofrequency therapy (RFT). (A) Alpha Imager HP gel imaging system was used to analyze the expression level of HPV16-E7 protein in the vaginal tissues of mice. (B) Western blot test was performed to detect the expression of HPV16-E7 protein in the vaginal tissues of mice. Data were expressed as mean ± SD. ***P < 0.001 represents statistically difference.

Table 1 Levels of CD4+ and CD8+, and ratio of CD4+/CD8+ in the spleen of mice in each group (x ± s#, n = 4).

Group		Radiofrequency therapy group	Model group	Normal control	*F#*	P
After 6 days of treatment	CD4+ (%)	18.92 ± 0.31	9.24 ± 0.25	7.67 ± 0.22	2152.00	0.00
	CD8+ (%)	10.43 ± 0.14	9.19 ± 0.48	10.07 ± 0.30	16.36	0.00
	CD4+/CD8+ (%)	1.82 ± 0.03***	1.01 ± 0.05***	1.31 ± 0.01	575.00	0.00
After 12 days of treatment	CD4+ (%)	25.68 ± 0.50	9.82 ± 0.53	7.66 ± 0.19	2049.00	0.00
	CD8+ (%)	12.34 ± 0.15	10.05 ± 0.20	10.09 ± 0.22	185.90	0.00
	CD4+/CD8+ (%)	2.08 ± 0.03***	0.98 ± 0.05***	1.32 ± 0.01	1088.00	0.00

* x ± s defined as mean ± standard deviation. F =MSR/MSE.

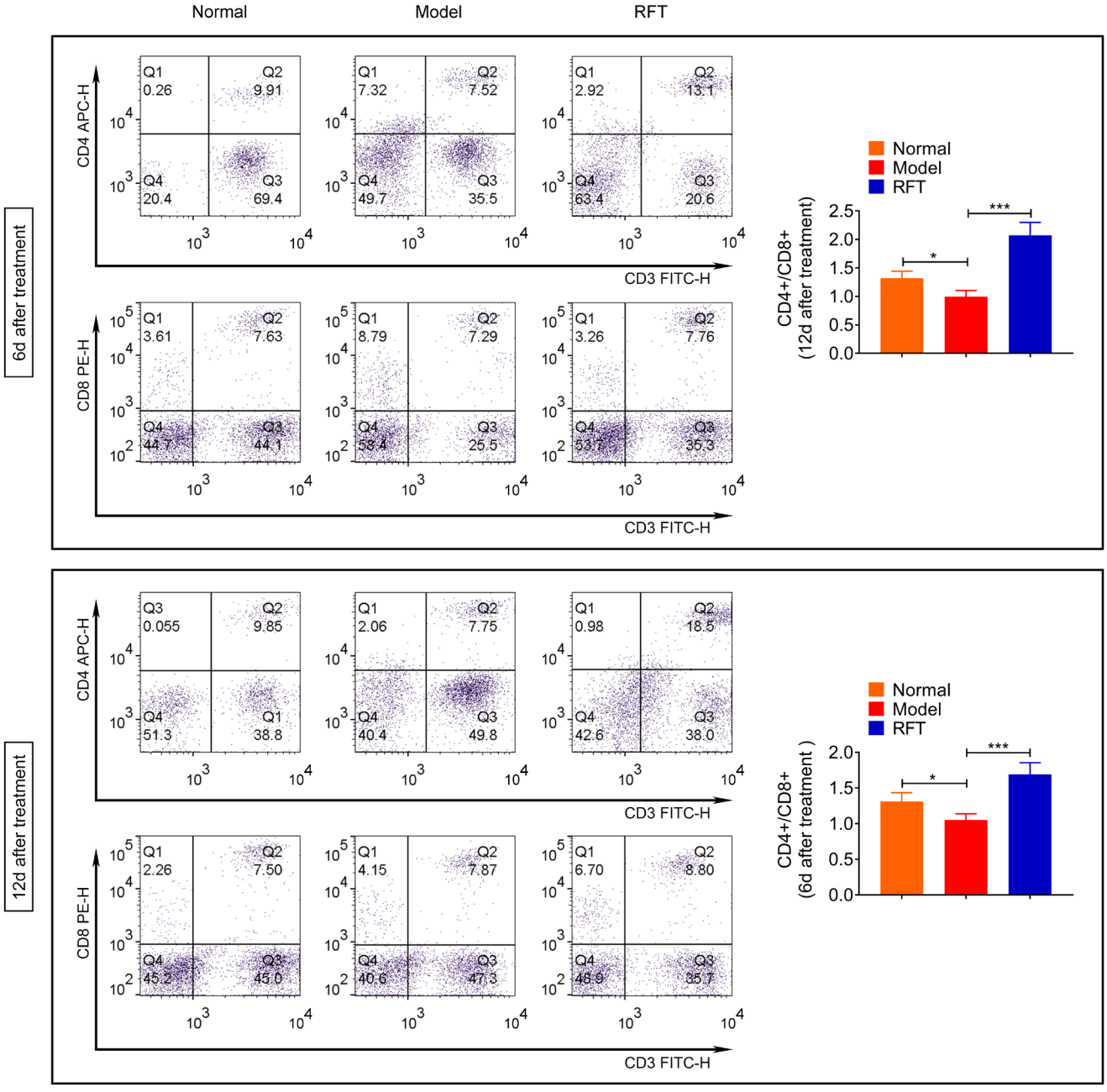

Figure 3 Levels of CD4+ and CD8+ and ratio of CD4+/CD8+ in the spleen of mice after radiofrequency therapy (RFT). Flow cytometry was used to determine, and the Cell Quest software was used to analyze the percentage of CD4+ and CD8+ T cells and calculate CD4+/CD8+ ratio in the spleen of mice in RFT group, model group, and normal group. Data were expressed as mean ± SD. $P < 0.05$ represents statistically difference.

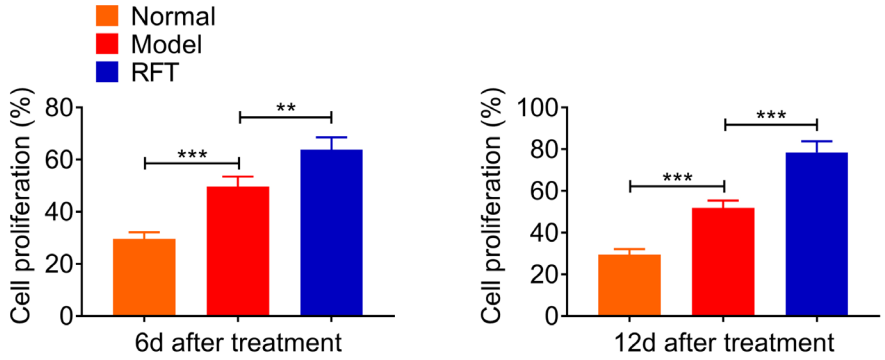

Figure 4 Proliferation of splenic lymphocytes in mice after radiofrequency therapy (RFT). After RFT, the proliferation of splenic lymphocytes in mice of RFT group, model group, and normal group was detected by MTT ASSAY. Data were expressed as mean ± SD. ***P < 0.001 represents statistically difference.

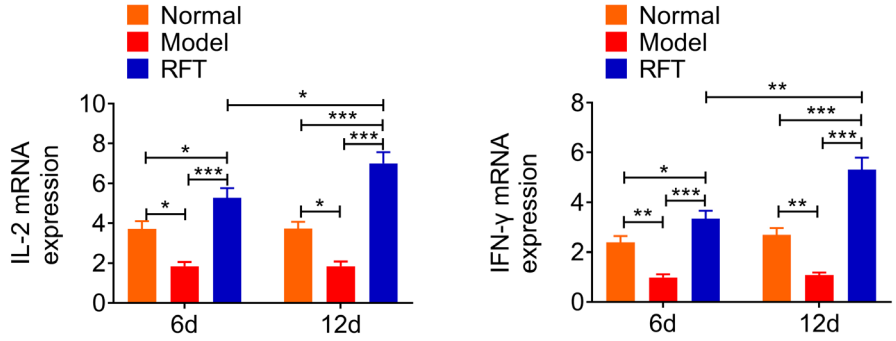

Figure 5 Expression levels of IL-2 and IFN-γ mRNA in splenic lymphocytes after radiofrequency therapy (RFT). (A) The expression level of IL-2 in the spleen lymphocytes of mice was measured by RT-qPCR after 6 and 12 days of RFT. (B) RT-qPCR determined the expression level of IFN-γ mRNA in spleen lymphocytes after 6 and 12 days of RFT. Data were expressed as mean ± SD. P < 0.05; ***P < 0.001 represents statistically difference.

temperature can reach 80–100°C, resulting in denaturation and necrosis of the surrounding cells. At present, domestic studies have established that RFT has a good effect on the radical treatment of arrhythmia, lumbar disc herniation, small liver cancer, and unresectable malignant tumors, with the advantages of less trauma and fewer complications.[11,12] In addition, RFT is also widely used in the treatment of functional uterine bleeding (FUB), uterine myoma, cervical erosion, and other gynecological diseases.[4,5]

The continuous infection of high-risk HPV is an important factor leading to cervical cancer, which causes different degrees of precancerous lesions in the cervical epithelial cells. Studies conducted by Chen g[13] and Hancock *et al.*[14] demonstrated that after HPV infection therapeutic drugs activated the immune system of patients to prevent or even clear the lesions and tumors caused by HPV infection. In this study, after RFT treatment in mice infected with HPV16-E7 lentivirus, it was found that the protein expression of HPV16-E7 in the vaginal tissues of mice was significantly decreased, indicating that RFT could reduce the infection of HPV16-E7 lentivirus in the reproductive tract of mice. This result was consistent with the conclusions of the study conducted by Hao Yuntao[5] and Repici *et al.*[6] In addition, Duan *et al.*[15] indicated that after RFT treatment, a large amount of heat shock proteins are produced in tumor tissues around the ablation area. These proteins play a role in antigen presentation and activate T-cell immunity. It is speculated that RFT locally kills HPV-infected cells and activates the immune system of mice to eliminate HPV virus.

The immune system is composed of immune cells, immune organs, and immune molecules. It plays an important role in the immunity of infectious diseases and tumors. The spleen is the largest peripheral immune organ present in human body, and spleen lymphocytes are the primary immune cells which are divided into T lymphocytes and B lymphocytes. These T and B lymphocytes of the spleen are activated after being stimulated by antigens, resulting in specific immune responses. Antibodies or cytokines are produced to recognize and remove foreign antigens so that the body returns to its normal physiological state. In order to verify the above speculation and explore the effect of RFT on the immune activity of splenic lymphocytes, this study evaluated the functional role of RFT on both spleen lymphocyte proliferation and proportion of lymphocyte subsets. The results found that RFT significantly promotes the proliferative rate of mice spleen lymphocyte, and the CD4+/CD8+ ratio was significantly higher than that in both model and control groups. This is consistent with the results established by Shaobin *et al.*[16] and Shi *et al.*,[17] suggesting that RFT could significantly promote the proliferation of splenic lymphocytes and improve the activity of immune cells. IL-2 and IFN-γ are cytokines that regulate cellular immunity. H. Wang *et al.* demonstrated that IFN-γ

can interfere with viral infection and replication.[18] Gaffen *et al*.[19] expressed that IL-2 could promote the proliferation of B lymphocytes (B cells), T lymphocytes (T cells), and natural killer (NK) cells as well as the synthesis of immunoglobulin, and could also induce the production of tumor necrosis factors (TNF) and IFN. Results of RT-qPCR assay were consistent with the reported investigations.[19,20] Zhang *et al*.[10] established that RFT could promote the production of IL-2 and IFN-γ secreted by lymphocytes and exerted active immune effects dominated by T helper type 1 (Th1) cells.

Conclusion

This study established that RFT could reduce HPV16-E7 lentivirus infection in the reproductive tract of mice, and the preliminary experiments revealed that the mechanism relates to activating the immune system in mice. It must be noted that the human immune system is extremely complex, and the indicators observed in this study cannot fully explain it. Therefore, additional studies are required to confirm these results in relation to human body.

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Competing interests

The authors state that there was no conflict of interest to disclose.

Contribution of authors

Penghua Cui and Lijing Li designed the study and supervised data collection. Zhiyan Li analyzed and interpreted the data. Yujuan Zhang prepared the manuscript for publication and reviewed the draft manuscript. All the authors read and approved the final manuscript.

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