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Original Article

Cervicovaginal secretions protect from human papillomavirus infection: Effects of vaginal douching

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Abstract

Objective: Cervicovaginal secretions (CVSs) are reported to protect against human papillomavirus (HPV) infection. Although vaginal douching is known to clear both viral inoculants and CVSs, its effect on CVSs in women with HPV infection is unknown.
Materials and Methods: The *in vitro* HPV pseudovirus infection system was used to test the protective activity of CVSs against HPV infection in samples collected before and after vaginal douching. To simulate different time points of vaginal douching in relation to viral exposure, the cell CVS reconstitute was washed after different viral exposure durations.
Results: In the CVSs of premenopausal and postmenopausal women who did not perform douching, the CVSs inhibited HPV infection by 56.7 ± 1.8% and 53.6 ± 2.5%, respectively; in women who had performed douching, the CVSs inhibited HPV infection by only 31.2 ± 7.1%, which was significantly lower ($p < 0.01$). Cell washing effectively cleared 60–90% of the infectious load with the greatest activity occurring within 30 minutes after inoculation. In the presence of CVSs, a sustained inhibition of HPV infection existed for up to 8 hours after HPV exposure, and cell washing increased the clearance to up to 82–93% of the infectious load.
Conclusion: This study confirms the protective activity of CVSs against HPV infection regardless of age. In this *in vitro* study, the net effect of douching was found to be beneficial.

Keywords: cervicovaginal secretion; human papillomavirus infection; vaginal douche

Introduction

Infection by human papillomavirus (HPV), one of the most common sexually transmitted infections, is necessary for cervical carcinogenesis. A global survey of HPV prevalence revealed an overall prevalence rate (95% confidence interval) of 10.5% (range: 9.9–11.0) [1]. With ample epidemiological evidences suggesting sexual behavior as the primary risk factor for HPV infection [2], the role of cervicovaginal secretions (CVSs) and the effect of vaginal douching around sexual intercourse on HPV infection are important issues that deserve detailed investigation. Notably, CVSs have selective antimicrobial activity against nonresident bacterial species in the vagina [3]. Various antimicrobial substances in CVSs play important roles in innate host defense of the lower genital tract [4]. For example, cationic polypeptide components of CVSs, such as alpha defensins, reportedly block 55% of *in vitro* HPV infections [5].

Vaginal douching is a common practice worldwide [6]. Frequent vaginal douching is associated with susceptibility to infectious pathogens, such as *Chlamydia*, *Gonococcus*, and human immunodeficiency virus [7–10], and increased risk for pelvic inflammatory disease [6]. Studies that investigated the effect of vaginal douching on HPV infection have produced inconsistent results [11–13]. In addition, the relationship between vaginal douching and the prevalence of cervical cancer

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is also controversial [14,15]. Although vaginal douching should simultaneously clear both infectious agents and protective CVSs from a theoretical perspective, the net effect of this practice is unknown and warrants thorough investigation.

Because the life cycle of HPV infection entirely relies on differentiation of the squamous epithelial cells, HPVs are difficult to propagate in culture. Detailed studies on the infectious process and inhibitors of infection were not been possible until studies on infectious HPV pseudovirus (PsV) expressing fluorescence marker were performed to monitor infectious events [16,17]. Like authentic HPV, the PsV has an icosahedral capsid resembling that of the native HPV [18]. Apparently, PsVs precisely replicate the initial phases of HPV infection, which makes them useful for elucidating HPV infection mechanisms [19]. In a mouse cervicovaginal infection model, Roberts et al [20] reported that mechanical disruption of the vagina enhances HPV infectivity and that the infectivity can be blocked by peptide antibiotics such as alpha defensins [5].

In this *in vitro* study, the effects of CVSs, which along with infectious agents, are readily cleared by douching, were analyzed in terms of their effects on infectivity of HPV PsV by simulating the effects of douching performed at different times after viral exposure.

Materials and methods

Clinical data and specimen collection

The CVS samples used in this study were collected before and after vaginal douching in eight premenopausal and eight postmenopausal women who had undergone regular Pap smear tests. After giving written informed consent, each woman underwent a cervical scraping for a Pap smear test. The CVS samples were collected by gently and systemically swabbing the cervicovaginal mucosa with a cytobrush. The same procedure was repeated with a second cytobrush. The vagina and cervix were then douched two times with a 3-cm sponge fully rinsed with normal saline. The CVSs were then collected again using the same procedure. The two cytobrushes containing CVSs were transferred into a centrifuge tube containing 3 mL phosphate-buffered saline (PBS) and stored at −80°C before use. This study was approved by the Institutional Review Board of the Tzu Chi General Hospital, Hualien, Taiwan.

Production of HPV 16 PsVs

The 293TT cell transfection method was used for efficient production of HPV 16 PsVs [21]. Plasmids (p16Shell) encoding codon-modified HPV 16 L1/L2 structure genes were co-transfected with a 5.9-kb target plasmid (pfwB) that expresses enhanced green fluorescent protein (GFP) under the control of human elongation factor 1 alpha promoter [17]. The HPV 16 L1/L2 vector (p16Shell) and GFP plasmid were provided by Dr Schiller [14]. Maps of L1/L2 vector (p16Shell) and GFP plasmid can be found at http://home.ccr.cancer.gov/lco/target.htm. Transfection, production, and harvest of PsVs from the 293TT cells were performed according to protocols posted online. The harvested PsVs were purified by iodixanol (OptiPrep, Axis-Shield) gradient centrifugation and allowed to mature *in vitro* [17]. The virus titer was determined using 293TT cells as targets and was calculated using the following formula: $(1 \times 10^5$ cells) × (1000 μL/mL) × (dilution factor) × (fraction of cells expressing GFP) = transducing units per milliliter (TU).

PsV infection assay

A 1:1 dilution of CVSs in PBS was filtered through a 0.45-μm filter before the experiments. For the PsV infection assay, 1.2×10^5 TU of PsVs in 500 μL PBS or filtered CVSs were inoculated into 1×10^5 HeLa cells grown overnight in a six-well plate. After 48 hours, the cells were trypsinized, and the proportion of GFP-positive cells (i.e., the infection ratio) was determined by flow cytometry. A potent HPV inhibitor, 1% carrageenan [20], was used as the control. The inhibition percentage was calculated using the following formula: 100 × [1 − (net percentage of GFP cells in test/net percentage of GFP cells in mock)]. To determine the period of inoculant removal, HeLa cells were washed once with PBS and fed with a fresh medium at different time points after inoculation. The infection ratio was determined at 48 hours. Experiments were repeated in triplicate. The typical infection ratio in the resulting PsVs was 20–30%.

Statistical analysis

Proportions of inhibition activity by CVSs and the effects of vaginal douching on infection ratios were compared by z test in a time-course study. All statistical analyses were performed using SAS software (version 8.2; SAS Institute, Inc., Cary, NC, USA). A p value less than 0.05 was considered statistically significant.

Results

CVSs protected cells from HPV16 PsVs infection

The inhibiting effects of the CVSs on HPV infection were tested in cultured cells through *in vitro* assays of PsV. The CVSs from premenopausal and postmenopausal women inhibited HPV infection by 56.7 ± 1.8% and 53.6 ± 2.5%, respectively (Fig. 1). The CVSs collected after vaginal douching had a significantly decreased inhibition ratio of 31.2 ± 7.1% ($p < 0.01$). In comparison, carrageenan, a known HPV infection inhibitor [20], inhibited infection by 98.2 ± 1.0%. The experimental results showed that the protective activities of CVSs against HPV infection are modest compared with those of carrageenan.

Protective effects of CVS at different viral exposure durations

Vaginal douching may be beneficial by removing HPV from the place of infection and by shortening the duration of

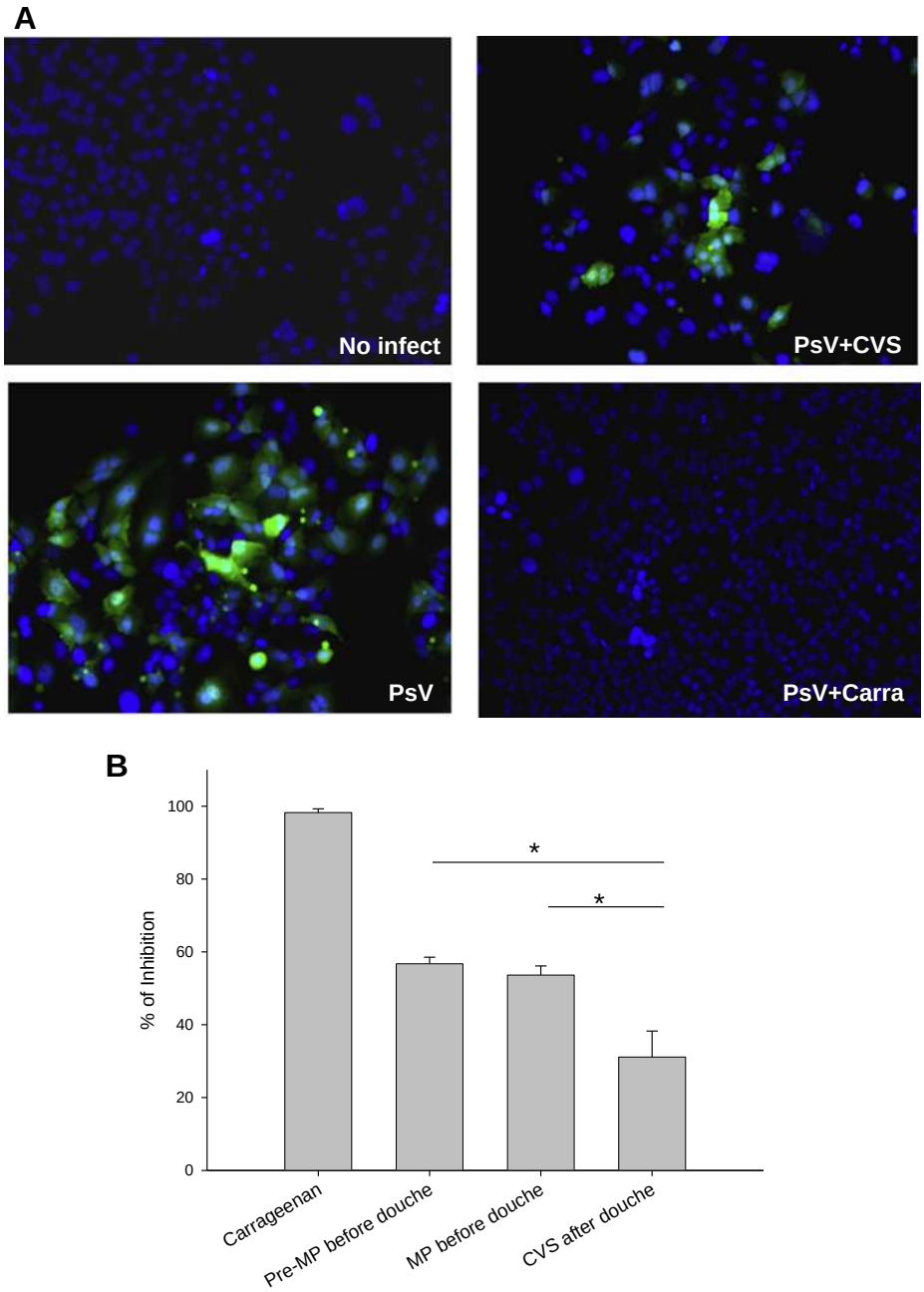

Fig. 1. Inhibiting activity of cervicovaginal secretions (CVSs) against human papillomavirus pseudoviruses (HPV PsVs) infection. (A) The HeLa cells were infected with green fluorescent protein-expressing HPV16 PsVs (PsV) in the presence or absence of CVS or 1% carrageenan (Carra) and then observed under a fluorescence microscope. (B) Inhibiting effects of CVSs on PsV infection in premenopausal (pre-MP) and menopausal (MP) women before and after vaginal douching. Carrageenan (1%) served as the positive control. $*p < 0.01$.

viral exposure. This study first tested the scenario of vaginal douching before intercourse when CVSs are cleared, and after intercourse when inoculated viruses are cleared. To mimic this scenario *in vitro*, PsVs were inoculated into cervical cells in the absence of CVSs and were washed off at different time points to shorten the duration of viral exposure. Early removal of inoculants markedly decreased infectivity to as low as 10% (Fig. 2), that is, 90% of the infectious load was cleared. This clearance effect was greatest within the first 30 minutes after inoculation. When cell washing was performed as late as 8 hours after inoculation, the clearance effect reduced to 60% (Fig. 2).

In the other mimicked scenario in which CVSs were present, the PsV-inoculated cervical cells showed a steady suppression of infectivity in general for up to 8 hours after inoculation. The effect of cell washing on inhibiting PsV infection was over 90% within 4 hours and over 80% at the 8th hour (Fig. 2). These experimental results indicate that CVSs have a general protective effect, and early douching after exposure helps to reduce the infectious load.

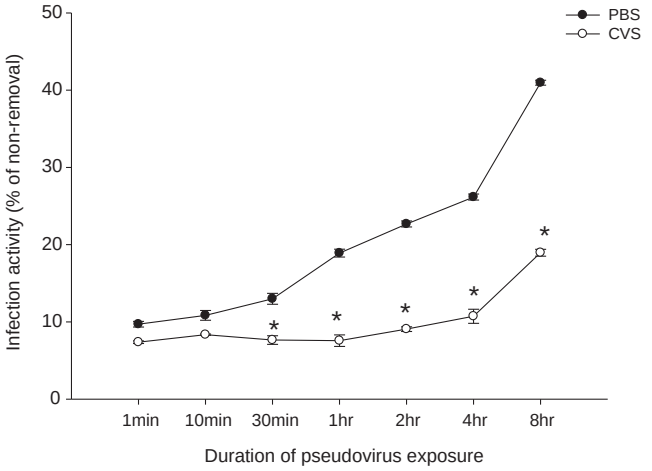

Fig. 2. Effect of time-course removal of inoculants in the presence or absence of cervicovaginal secretions (CVSs). The human papillomavirus pseudoviruses in the presence of CVSs or phosphate-buffered saline (PBS) were removed from cell cultures by washing with PBS at 1 minute, 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 8 hours after inoculation; infection ratios in HeLa cells were measured at 48 hours. Infection ratios with inoculants not removed in the presence of PBS and CVSs were 18.0 ± 0.4% and 6.1 ± 0.2%, respectively. *$p < 0.05$.

Discussion

This study explored the net effects of vaginal douching on HPV infection. A nationwide investigation of adult women in Taiwan demonstrated that 65% of Taiwan women douche [22]. Surveys show that most women use tap water, and <8% use feminine hygiene products [11]. Relatively higher rates of vaginal douching have been reported in developing countries such as Cambodia [23] and countries in the sub-Saharan African regions [10,24], where most adult women routinely practice douching.

By clearing the mucus barrier of CVSs and by disturbing innate immunity, vaginal douching may facilitate acquisition of HPV. However, this practice also reduces the infectious load. Epidemiological studies on the relationship between vaginal douching and HPV infection have produced conflicting results [11−13]. The *in vitro* HPV PsVs infection assay performed in this study revealed the net effect and the importance of timing of vaginal douching on HPV infection. Comparisons of CVSs data collected from women before and after vaginal douching showed that douching reduced infection rates by one-half. This finding is supported by a mouse model showing a high rate of HPV PsVs infection after vaginal brushing and a still higher rate of infection after using a vaginal microbicide [20].

Previous epidemiological studies have identified a second peak prevalence of HPV infection in older women, but the reason for the prevalence is unknown [2]. Among the different causes speculated, atrophy of the genital mucosa with a compromise of innate immunity is a likely cause. The CVSs collected from premenopausal and postmenopausal women in this study showed similar inhibiting effects on HPV infection, which excludes the possibility of a differing antiviral activity between pre menopause and postmenopause.

This study evaluated postcoital douching performed at different times after viral exposure by simulating the washing of host cells exposed to HPV for different periods. Experiments were performed in the absence of CVSs to simulate those areas that are not exposed to CVSs, for example, the vulva, and were also performed in the presence of CVSs to simulate the exposed areas such as the cervix and vagina. In the absence of CVSs, early washing reduced virus infectivity. Notably, 90% of viral infections were prevented when washing was done within the first 30 minutes after HPV exposure. This protection time window is consistent with the kinetics of early HPV infection; a critical period of approximately 30 minutes is needed for viruses to attach to cells [25,26]. This study shows that cleansing of HPV inoculants from the skin is most effective if washing is performed within 30 minutes. This study also showed that CVSs have a sustained inhibiting effect on HPV infection. In the presence of CVSs, HPV infection became very inefficient. Depending on the duration of viral exposure, CVSs protected against >90% of HPV infection in 4 hours and >80% of infection in 8 hours.

Clearly, this *in vitro* study is missing a number of important elements in the real life, such as *de novo* production of CVSs and continuous inoculation during sexual exposure. In addition, in the real life, vaginal douching may cause microtrauma to the vaginal mucosa, which is essential for HPV entry [19]. Microtrauma may facilitate self-inoculation of HPV that already exists in mildly dysplastic lesions, which typically hosts a highly productive virus life cycle. This explains the risky effect of vaginal douching on the persistence of low-grade squamous intraepithelial lesions in a large-cohort study [22].

In summary, this study provides *in vitro* evidence of a sustained protective role of CVSs, which is not adversely affected by vaginal douching. In areas lacking CVSs, a golden cleansing period of 30 minutes effectively prevents HPV entry.

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