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***In vivo* effects of nonoxynol-9 on endometrial immune cell populations**

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Introduction

Vaginally applied microbicides are being evaluated for efficacy in the prevention of sexual HIV transmission. These studies are based on a model in which the vagina and ectocervix are the anatomic sites of HIV transmission;¹ whether transmission can also occur in the upper genital tract (endocervix and endometrium; UGT) is not known. Vaginally-administered gels and creams rapidly gain entrance into the UGT.² Nonoxynol-9 (N-9) is a non-ionic detergent that failed to protect against HIV transmission in clinical trials and was associated with increased acquisition of HIV in high prevalence settings.³ This latter finding

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was attributed to N-9-associated lower genital tract (LGT) irritation and genital ulceration. Various subsequent microbicides were associated with no protection or potential increased risk of HIV acquisition, despite the apparent absence of inflammatory effects on the lower genital tract¹; microbicide-induced effects on the UGT may explain these findings. We undertook a pilot study of inflammatory effects of N-9 effects on the UGT using paired endometrial biopsy (EMB) specimens from women in the luteal phase of the menstrual cycle before and after vaginal exposure N-9.

Methods

The study protocol was approved by the UCSF Committee on Human Research. Inclusion criteria were: age 18-40 years; HIV-negative; 21-35 day menstrual cycles; agreement to avoid use of extraneous intravaginal products for the study duration. Exclusion criteria included: pregnancy or attempting to conceive; abnormal Pap test within the past 12 months; recent use of intravaginal or intrauterine contraceptives; use of exogenous sex hormones; immunocompromised status. Participants were tested for HIV, pregnancy and sexually transmitted infections, and instructed in the use of luteinizing hormone (LH) surge predictor kits (ClearBlue Easy Ovulation Test, Inverness Medical Innovations, Delaware USA). Subjects returned 8-10 days after the LH surge for the first (pre-N-9) EMB. Participants were then provided with intravaginal N-9 (4% N-9 contraceptive gel [Conceptral, Ortho, Raritan, NJ] and instructed to apply it intra-vaginally nightly, beginning after their next menses, and continuing until their next study visit, resulting in usage of approximately 2-3 weeks depending on cycle length. Participants returned 8-10 days after the next LH surge for post-N-9 EMB. Standard hematoxylin and eosin stained slides were prepared from formalin-fixed paraffin-embedded EMB samples for endometrial dating by two independent board-certified pathologists; in cases where the results were discordant, a third pathologist was consulted. Immunohistochemistry was used to identify T cells (CD4, CD8), macrophages (CD68), B cells (CD20), natural killer cells (CD56) and regulatory T cells (FoxP3+) using published techniques.^{4, 5} Image analysis software was used to measure the area of stromal regions of interest (excluding epithelium, glands and vessels). The number of stained cells (counted visually) was divided by the area of the region of interest to determine stromal density of each cell type.

Results

Forty-one women completed telephone screening. Twenty-seven women were invited for an initial screening and consent visit. Of those women, 19 presented for initial screening and consented to participate in the study. Two women were excluded secondary to pregnancy, and 14 presented for the first EMB visit. The cervix of one subject could not be dilated sufficiently for EMB and three subjects did not tolerate EMB, leaving 10 subjects who underwent at least one biopsy. Six subjects completed all procedures. One EMB sample obtained at the second biopsy visit was classified histologically as inadequate, and one sample was in the proliferative rather than secretory phase at the second visit, resulting in 4 paired secretory EMB sample sets for analysis. Of these, 2 sample sets had matched mid-secretory endometrium in both pre- and post-N-9 samples (N9011 and N9034), one set had matched early secretory endometrium (N906), and one was mid-secretory in the pre-N-9 and

early secretory in the post-N-9 sample (N9032). Results of immunohistochemical assessment (Figure 1) demonstrated increased densities of CD8+ cells and FOXP3+ cells following N-9 exposure in all 4 paired samples. The density of CD8+ cells increased 1.7-4.8 fold (mean 2.7). The density of FOXP3+ cells increased 1.2-3.8 fold (mean 2.3). The densities of other cell types did not demonstrate consistent patterns of change following N-9 exposure.

Discussion

Paired endometrial sampling before and after exposure to intravaginal products is a powerful research tool for assessing microbicide effects on the UGT, yet our results demonstrate the challenges of this experimental approach, due to difficulties in matching the phase of the cycle for sampling as well as heterogeneity of cell densities within EMB samples. Despite these shortcomings, our results indicate that vaginal N9 may have inflammatory effects on the endometrium as measured by increased densities of endometrial CD8+ cells and FOXP3+ cells. Further studies into the female UGT as a portal for HIV acquisition are needed to accurately assess the safety of vaginal microbicides. To this end, improved methods for the collection of cycle-matched, paired endometrial samples will be helpful.

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Figure 1.
N9 Effects on Endometrium