

Development of a 3D *in vitro* epithelial model to investigate host-pathogen interactions during *Candida albicans* and Herpes Simplex Virus 2 mono- and co-infections in a simulated vaginal environment

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Introduction. *Candida albicans* (Ca) and Herpes Simplex Virus 2 (HSV-2) are among the most prevalent pathogens affecting the human genital tract, frequently coexisting and potentially interacting in ways that influence infection dynamics and host responses. Increasing literature documents that the presence of a primary infectious agent can predispose the host to a secondary infection, suggesting a possible synergistic interplay between pathogens. We recently published the results of a study investigating Ca and HSV-2 mono- and co-infections of the A-431 epithelial cell line. These cells, derived from a human epidermoid skin carcinoma, form a monolayer after 24 hours of incubation or, after 5-day growth, a differentiated multilayer which closely mimics a reconstituted vaginal epithelium. Building on this foundation, the present study aims to develop a three-dimensional (3D) *in vitro* model of vaginal epithelium using A-431 cells cultured on inert scaffolds, enabling a detailed analysis of host-pathogen interactions during both single and dual infections, in the presence or absence of a synthetic vaginal fluid (SVF).

Materials and Methods. The A-431 epithelial cells were cultured on 3D inert scaffolds, incubated for 5 days in DMEM with 10% FBS, exposed or not to SVF, and infected with Ca and/or HSV-2. Epithelial viability was assessed by LDH release; fungal burden and viral load were also evaluated, at different time points and conditions, by CFU assay and PCR-based DNA quantification, respectively. Histological and immunohistochemical (IHC) staining with hematoxylin and eosin (H&E) and specific anti-cytokeratin 5/6 (CK 5/6) antibodies were performed on slides from the scaffolds.

Results. The A-431 line exhibited the ability to adhere to inert scaffolds and to grow as a multilayered epithelium, after 5 days of incubation, as shown by H&E staining. Specific IHC analysis revealed the highest levels of CK 5/6 expression in the presence of SVF, indicative of a high degree of epithelial cell differentiation. The addition of SVF enhanced the Ca survival and growth ability. Epithelial cell viability was significantly impaired by Ca infection, whereas HSV-2 had minimal or no effect; the dual infection resulted in slightly additive effects in terms of cell damage.

Discussion and Conclusions. The A-431 epithelial cells demonstrated the capacity to colonize and grow onto an inert 3D matrix, mimicking the structural and functional characteristics of human vaginal mucosa. Thus, the 3D inert scaffolds represent a useful tool to develop *in vitro* studies on polymicrobial infections, with the addition of SVF providing a prototype as similar as possible to the *in vivo* vaginal environment.