Novel 3D model to study feminine care ingredients

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ABSTRACT

In line with a growing demand for non-animal *in vitro* alternatives for the safety testing of feminine and cosmetic care products, the present study describes the in-house reconstruction of a vaginal organoid tissue model and demonstrates its use to evaluate topical ingredients commonly found in feminine care formulations. The 3D human vaginal epithelium (HVE) *in vitro* model reconstructed from isolated vulvar epithelial cells displayed *in vivo*-like structure and functionality from batch to batch, supporting its use as a reliable and reproducible *in vitro* tool to evaluate the safety and efficacy of intimate care ingredients and final products

The human vaginal epithelium is a non-keratinized stratified squamous epithelium composed of basal, parabasal, intermediate, and superficial layers that undergo dynamic remodeling across the menstrual cycle but also with woman's age and hormonal status.[1]

The basal layer is the deepest layer, which is mitotically active, continuously producing new cells. The parabasal cells are small, round cells that emerge from the basal layer and form several layers of tissue. The intermediate cells are characterized by foamy nuclei and larger cytoplasm rich in glycogen.

The most superficial layer consists of flattened, non-cornified cells with pyknotic nuclei.[2] This multilayered structure forms a robust barrier that protects underlying tissues from mechanical stress and chemical insults.

In a woman's life, main external stressors are hygiene soaps, moisturizing creams, local contraceptives or even menstrual pads. Yet, because those intimate care products are in direct and often prolonged contact with the vaginal tissue, ingredients with even sub-irritant effects can disrupt the epithelial barrier function and induce inflammation.

This is why feminine care products are subject to regulations aiming at protecting consumers from hazardous chemicals, including the assessment of the level of risk by in vitro safety assays ensuring the compatibility of topically applied women's health products and devices with the vaginal tissues.

The traditional method for safety testing has long been based on *ex vivo* animal vaginal tissues.[3] The shift in recent years toward non-animal and more ethical alternatives allowed the emergence of innovative *in vitro* models.

Conventional vaginal epithelial cell lines in monolayer culture are now available from providers. They are highly adapted to preliminary researches and early identifications of potential safety issues but like every 2D cell systems, fail to replicate the morphology and function of multilayered structure such as the vaginal epithelium.

In contrast, cells can be cultured in specific conditions to generate organ-like 3D systems that replicate the architecture and behaviour of native tissues. Such organotypic structures are more representative of the *in vivo* environment and considered nowadays as the gold standard in safety and efficacy assessment in preclinical studies.[4,5] Notably, organoid-based assays have demonstrated higher predictive accuracy for irritation and inflammation compared to monolayer cell cultures, providing a more reliable cell system for assessing product safety.[6]

The present study reports the newly reconstruction of a vaginal organoid tissue model and demonstrates its use to evaluate the safety of topical ingredients commonly found in feminine care formulations.

The *in vitro* 3D HVE model described here was reconstructed from isolated A431 vulval epidermoid carcinoma cells cultured on a microporous support at the air/liquid interface. Reconstructed HVE were maintained in a humid atmosphere at 37°C with 5% CO_2.

Hemalun/Eosin histological sections generated from paraffin-embedded tissues revealed an early stratification after three days of culture that could be increased until day 14 with no visible sign of mortality (Figure 1). Cells in the different layers were non-keratinized squamous cells similar to the basal layer of native vaginal tissues. After 18 days in culture, apical cells displayed a morphology close to the native suprabasal layer could be detected (Figure 1).

However, a loss of tissue architecture with increased intercellular spaces was also observed, predicting a reduction in cell survival and a visible failure of the barrier function at

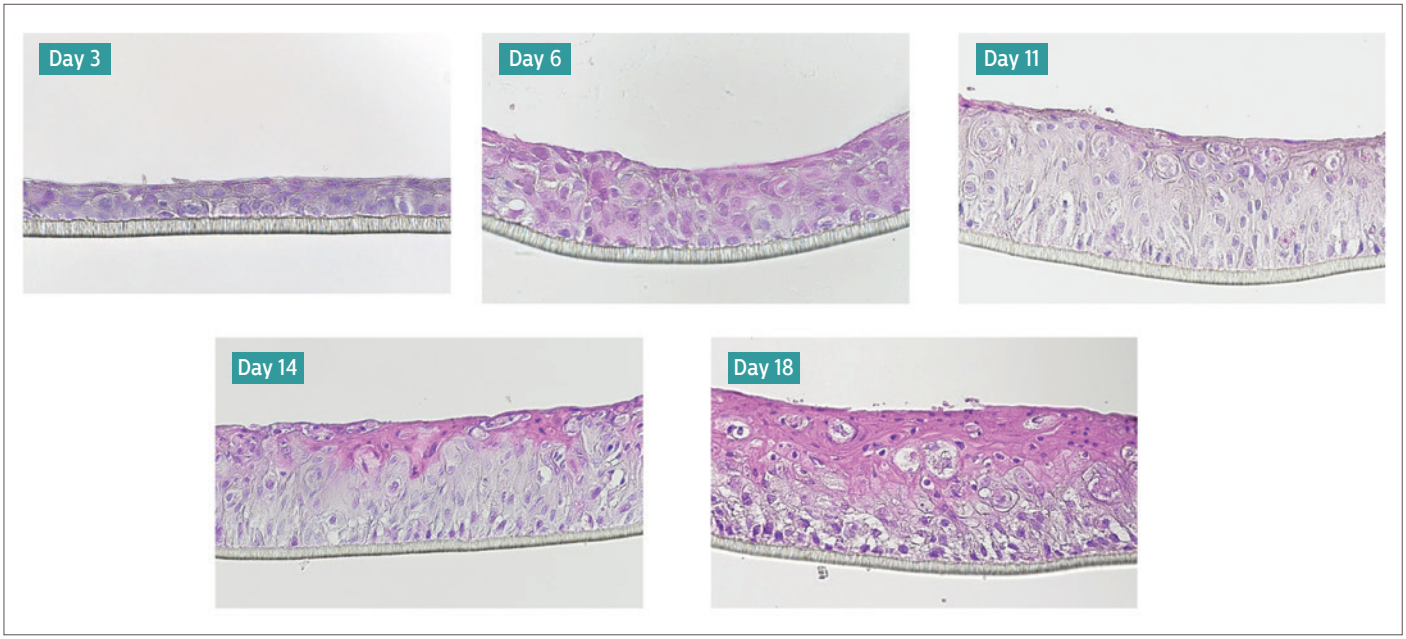

Figure 1: Light microscopy images of paraffin-embedded HVE stained with hemalum/eosin. Day 3 to 18: representative images after three, six, 11, 14 or 18 days at the air/liquid interface, respectively. Magnification 20x

this later stage of reconstruction. Consistent histological features were observed from one batch of reconstructed tissues to another, confirming the observed evolution of the 3D model with time of culture.

Characterization of the 3D HVE model

To further characterize the structure and organization of this new multilayered tissue, the expression and localization of vaginal biomarkers were analysed all along the 3D reconstruction of HVE. Functional and structural vaginal biomarkers were monitored by immunofluorescence in paraffin-embedded HVE after six, 11, 14 and 18 days at the air/liquid interface.

Monoclonal antibodies targeting the water-channel proteins Aquaporin-3 proteins (AQP3) that plays a key role in hydration, were used to evaluate the functionality of the reconstructed tissue. A double staining of the cytokeratins 13 (CK13) and 14 (CK14) was also performed to follow the expression of those structural proteins.

A third immunofluorescence staining was also conducted to track the expression of involucrin (IVL) since IVL, CK13 and CK14 proteins are typical epithelial differentiation markers of the basal layer. In parallel to the specific AQP3, CK13/CK14 and IVL immunostaining, cellular nuclei were labelled with DAPI.

Fluorescence microscopy images from AQP3 staining revealed the widespread expression of this hydration protein from the sixth to the 14th day, with a sudden reduction of fluorescence in HVE cultured for 18 days.

Likewise, the monitoring of the IVL biomarker revealed its increasing expression in the cytoplasm of the epithelial cells with the length of the air/liquid tissue culture (Figure 2), up to 14 days. IVL immunostaining in HVE tissues after 18 days in culture displayed less specificity, confirming the loss of functionality consequent to the loss of structure observed by hemalum/eosin morphological staining.

Finally, the pattern of CK13/CK14 cytokeratin expression was typical of basal stratified squamous epithelium with a limited expression of CK13 compared to a widespread expression of CK14 from day six to day 18, attesting on cell differentiation and functionality up to 14 days in this reconstructed tissue.

Altogether, histological analyses allowed to conclude that the herein reconstructed HVE replicates the stratified architecture and barrier properties as early as after 11 days at the air/liquid interface. The disorganized morphology observed at day 18 confirmed that the robustness of the 3D model declines over time of culture, suggesting its highest and optimal functionality between the 11th and the 14th day of culture.

Based on those conclusions, 11 days of culture was considered as the ideal time of air/liquid maintenance to generate a reproducible 3D model replicating the human vaginal

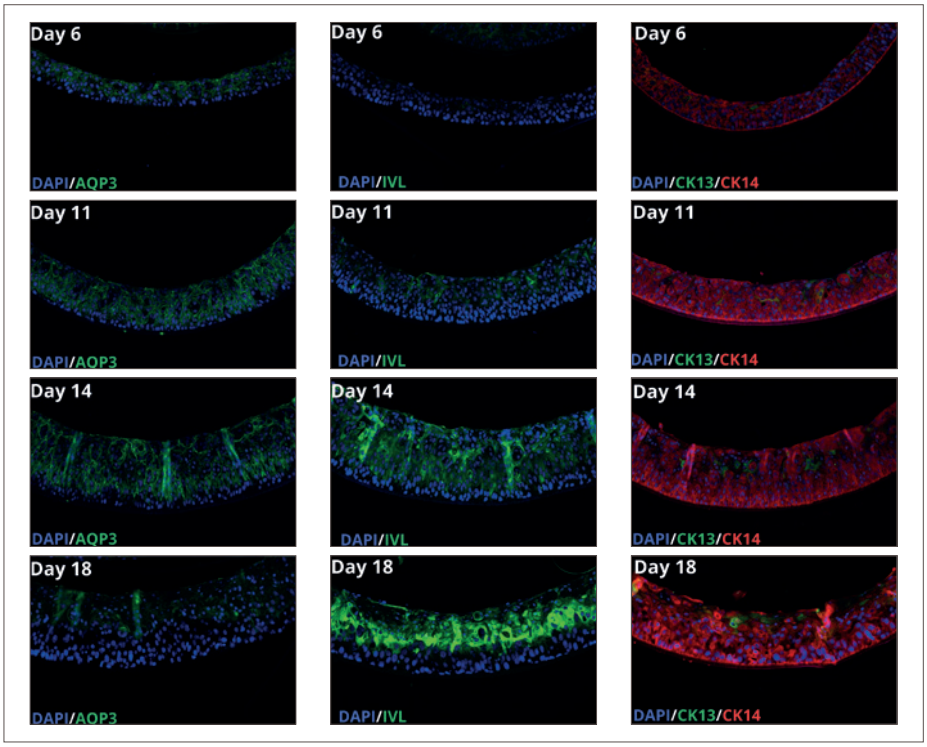

Figure 2: Fluorescent microscopy images of Aquaporin-3 (AQP3) and Involucrin (IVL) immunostaining, and of Cytokeratin 13 and 14 (CK13/CK14) co-immunostaining of HVE. Cell nuclei are stained with DAPI. Day 6 to 18: representative image after six, 11, 14 and 18 days at the air/liquid interface, respectively. Magnification 40x

environment. With this recommendation in mind, HVE tissues maintained in culture for 11 days were used to further evaluate their relevance as an *in vitro* tool to assess the risk of topically applied hazardous chemicals.

Viability assay

Irritant and corrosive substances cause harmful cell damages at the site of contact, reducing cell viability and initiating a protective inflammatory reaction. *In vitro*, cellular activity and viability is evaluated by a capacity to convert MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into a blue formazan salt, while inflammatory responses are detected by the quantification of inflammatory cytokines released by injured cells.

In order to validate the suitability of the 3D HVE model as an *in vitro* tool to assess the risk of hazardous chemicals, its responsiveness to Benzalkonium chloride (BZK) and Triton X100 (Tx100) has been defined first by a viability MTS assay. BZK and Triton X100 are toxic agents causing deleterious impact on the environment and human health.

BZK is commonly used at 0.02% as a preservative in various personal care solutions, with described adverse effects on human tissues.[7] Tx100, also known as octylphenol ethoxylate, is commonly used around 0.1% as an emulsifier in personal care products, with proven cytotoxic effect on mammalian cell lines.[8]

The impact of BZK 0.02% and Tx100 0.1% on the viability of the epithelial cells was evaluated by an MTS colorimetric assay after a 24 hours treatment on top of the HVE. Topical applications were performed by the use of a nylon mesh that ensures the complete spreading and equal repartition of chemical solutions on the surface of 3D models.

Besides the untreated tissue control, a placebo solution applied by a similar route was used as a negative control condition to evaluate the impact of the mesh application route on the viability of the model. Spectrophotometric quantifications revealed a statistical reduction of the metabolic activity under BZK and Tx100 treatments, compared to untreated and placebo tissues (Figure 3).

The absence of a statistical difference between the untreated HVE and the placebo confirmed that a nylon mesh can be used to apply compound on top of the 3D model without impacting its viability. Taken together, those data comforted on the responsiveness of the 3D HVE model to hazardous chemicals and on its use to evaluate the viability of vaginal tissues after topical application of irritant and corrosive products.

Inflammatory response to chemical threats

Irritation often triggers inflammation as a natural response to physical injuries or chemical exposure. Therefore, to correlate the observed loss of viability to an inflammatory status, a complementary quantification of cytokine biomarkers was conducted from HVE treated with BZK or Tx100.

Given the central role of Interleukin 1 beta (IL-1β) in recruiting immune cells to mediate inflammation at infectious site,[9] this biomarker was selected to evaluate the response of HVE to the two cytotoxic agents.

The concentration of IL-1β released into the culture media of HVE treated with BZK 0.02% and Tx100 0.1% from day 11 to day 12 of air/liquid culture was quantified by a specific ELISA assay. IL-1β was also quantified from the culture media of HVE treated with a placebo solution to evaluate the impact of the topical application on the inflammatory status.

As expected, the release of IL-1β was statistically increased in HVE treated for 24 hours with BZK 0.02% or with Tx100 0.1%, compared to untreated and placebo tissues (Figure 4). Combined to the previous observation of a loss of viability following BZK and Tx100 stimuli, the IL-1β quantification confirmed the responsiveness of the HVE model to chemical threats and therefore its use as a reliable *in vitro* tool to reflect physiological changes and biochemical reactions induced in natural human vaginal epithelium submitted to external stressors.

Conclusion

The present study reports a new 3D vaginal model that recapitulates key anatomical and biochemical features of the natural basal vaginal epithelium. This *in vivo*-like model displayed the expected cellular responses to

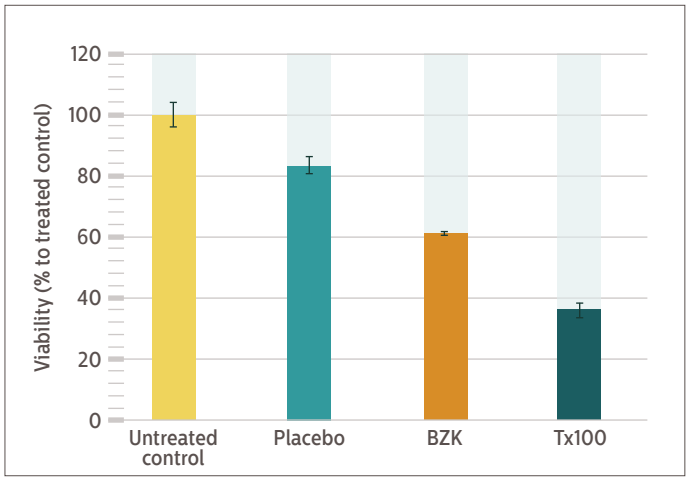

Figure 3: Percentage of total cell viability of HVE untreated (Untreated control), or treated with a solution without (Placebo) or with BZK 0.02% (BZK) or Triton X100 0.1% (Tx100). Treatments were performed from the 11th to the 12th day at air/liquid interface, using a nylon mesh. The experiment was performed in three biological replicates. Results are expressed as percentage of viability relative to untreated control, arbitrary set to 100%. Histogram represents mean ± standard deviation

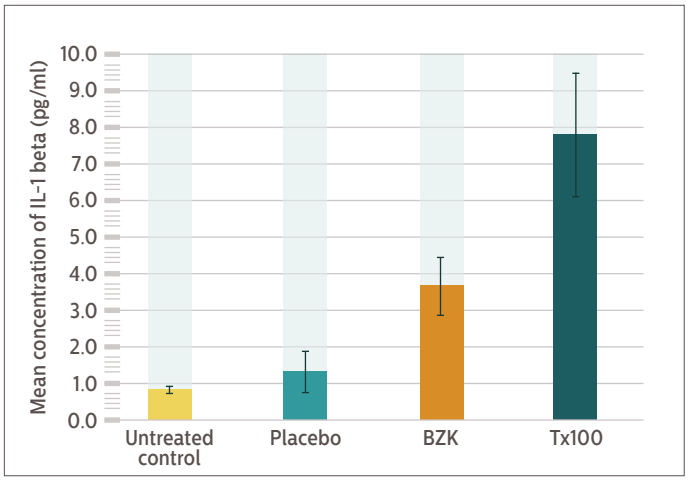

Figure 4: Mean concentration of interleukin 1 beta (IL-1β) quantified by ELISA assay from the cell culture medium of HVE untreated (Untreated control), or treated with a solution without (Placebo) or with BZK 0.02% (BZK) or Triton X100 0.1% (Tx100). Treatments were performed from the 11th to the 12th day at air/liquid interface, using a nylon mesh. The experiment was performed in three biological replicates. Histogram represents mean ± standard deviation

chemical threats, comforting on its use as a new tool to define the safety profile of feminine care products.

The herein described 3D HVE model was reconstructed at the air/liquid interface for up to 18 days. At that late stage of culture, histological and functional analysis revealed a disorganized morphology and loss of biological relevance, constraining to limit the use of the model between its 11th and 14th day of culture.

In the present study, HVE at day 11 challenged topically for 24 hours with hazardous agents displayed the expected tissue response as demonstrated by an MTS viability assay and the quantification of the inflammatory cytokines IL-1β. At that stage of reconstruction, HVE also exhibited robustness during transport at room temperature.

Assays performed on HVE at day 11 demonstrated that the integrity and functionality of the tissues were preserved in an agarose-based culture media, during a delivery lasting up to 48 hours (data not shown). This result comforts on the possible distribution of this new ready-to-use, non-animal alternative model to support and accelerate discoveries in the feminine care sector.

As most feminine care products are in direct contact with the surface of the vaginal epithelium, a topical application through a nylon mesh has been tested in the present study. This approach allowed a successful treatment of the HVE model without compromising its viability.

Other routes of application may however be tested in the future, including the dilution of ingredients in the culture medium of the 3D model to replicate the systemic application of orally prescribed medications, for example.

The robust release of IL-1β cytokine in response to BZK and Tx100 stimuli strongly indicates the biochemical response of HVE. It also confirms the suitability of the 3D HVE model to screen anti-inflammatory compounds.

The vaginal epithelium can also release Interleukin-1 alpha (IL-1α) in response to various stimuli, including irritating agents, infections from pathogens and in certain dysbiosis vaginal microbiota conditions. Secretion of Interleukin-1 alpha (IL-1a), IL-6 and IL-8 have been reported.[10]

The release of inflammatory cytokines is an active process, often preceding cell death, and contributes to the host's immune response, though the context of the release, such as the presence of tissue damage, influences its overall effect on the host's immune system.[11,12]

Monitoring inflammatory cytokines in cell culture supernatants can provide valuable insights into the anti-inflammatory potential of bioactive compounds. Quantification by ELISA assays represents a reliable and widely used method to evaluate cellular responses under stress conditions.

By applying this approach to the *in vitro* HVE model subjected to various stressors, it becomes possible to compare and characterize the anti-inflammatory efficacy of innovative active ingredients. Such analyses may provide a valuable assay to identify novel potent anti-inflammatory molecules aiming at reducing vaginal irritation and itching issues.

In conclusion, by using its strong tissue engineering skills, Straticell releases here a new reliable, animal-free 3D vaginal model not only to meet current safety regulatory expectations but also to actively participate to the comprehension and the objectivation of the efficacy of future women's health care products. **PC**

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