Validation of Vaginal Tissue Model for Toxicity and Microbiome Studies.

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Abstract

Background: Personal lubricants delivered to the vaginal canal can cause damage to the vaginal epithelium, induce inflammation, and alter the microbiome of the vaginal ecosystem. Such epithelial barrier damage by chemicals/therapeutics and microbial dysbiosis can make women vulnerable to sexually transmitted and other infections.Therefore, manufacturers of personal lubricants and medical devices are required to show biocompatibility and safety assessment data to support regulatory decision-making within a specified context of use. The goal of the study is to validate the use of the 3D human in vitro vaginal tissue model as an alternate to the rabbit vaginal irritation (RVI) assay requested for the regulatory evaluation of medical devices in contact with the vaginal mucosa. Additionally, we examined the utility of the model for microbiome study in the vaginal microenvironment.

Methods: A double-blinded study was conducted in vitro and in vivo with N=14 coded test articles (TAs) including materials intended to be in contact with vaginal tissues in the form of preservatives, contraceptives, solvents, viscosity enhancers, antiseptics, and cleansing agents (surfactants). The TAs were topically applied in vitro and in vivo at 2% dose with 5 repeat exposures over 6 days. Dose volumes were proportionally adjusted based on an estimated vaginal surface area. N=5 rabbits and N=3 EpiVaginal tissues were used per TA. RVI score was used to monitor in vivo irritation; for in vitro vaginal irritation assay, MTT, TEER, and histological analysis were used as endpoints. The variability of the reconstructed 3D vaginal tissues was further evaluated using tissues reconstructed from cells obtained from 4 donors. A total of 55 test articles (including those tested in RVI assay) commonly used in feminine care products were assessed using tissues from the four donors. For the in vitro assay triplicate vaginal tissues/per donor were topically exposed to TAs at a single concentration (2%) and incubated for 24 hr. After the 24 hr exposure, tissues were rinsed with PBS and examined for tissue viability (MTT assay) and for barrier integrity (TEER measurements). To study the vaginal microbiome, the effect of lactobacillus iners (normal flora in the vagina) alone or in the presence of Gardnerella vaginalis (associated with the disease bacterial vaginosis) on tissue viability (MTT), barrier integrity (TEER), and structural features (histology) were assessed.

Results: In the RVI assay, benzalkonium chloride (BZK) was identified as a mild/severe irritant and the effect of nonoxynol-9 (N9) in rabbits was highly variable. In vitro results showed that four TAs including the two known irritants, BZK and N9 were correctly predicted as irritants by MTT viability and TEER (<50% reduction at time 24 hrs). These results were reproducible in vaginal tissues reconstructed using cells from 4 donors. The results showed that 5 of the 55 test articles were found to be irritant in both the MTT and TEER assays in all donors. These TAs were Gynol containing 2% N9, BZK, sodium dodecyl sulfate, acetic acid, and Cremophor. These TAs resulted in >50% reduction in tissue viability and TEER values versus the saline controls. Only 1 test article, copper sulfate, showed low MTT viability (9.5%) but the TEER values for 3 of the 4 donors were non-irritating (>50%). The remaining test articles were found to be non-irritants in both MTT and TEER assays in all donors. In the microbiome study, exposure of the 3D vaginal tissue for 24 hrs to Lactobacillus iners followed by Gardnerella for 44 h results in no significant difference in MTT viability and TEER measurements. However, tissues exposed to Gardnerella showed perturbation of glycogen filled layer which was thinner in thickness compared to the no-bacteria tissues and tissues exposed only to lactobacillus.

Conclusion: Tissues from the four donors were highly reproducible and a decrease in MTT and TEER appears to be useful endpoints for preclinical toxicity screening of chemicals and feminine care products. The reconstructed 3D vaginal tissue model is also a useful tool to study microbial dysbiosis and symbiosis. The use of this in vitro system to assess the safety of topically applied vaginal care products, drugs, vaginally inserted medical devices is cost effective and could replace the use of animals for experimentation.

Methods

Tissue preparation: Vaginal-ectocervical (VEC) cells and fibroblasts were isolated from VEC tissue as obtained from healthy women undergoing hysterectomies for benign indications. VEC cells were seeded onto polycarbonate cell culture inserts (NalgeNunc Corporation; partial thickness), raised to the air liquid interface and cultured for approximately 2 weeks. Tissues that passed our standard quality control test were used in the various experiments.

Histology: EpiVaginal cultures were fixed in 10% formalin (overnight, room temperature), paraffin embedded, sectioned using a microtome, and stained with hematoxylin and eosin (H & E) according to standard procedures **(Figure 1)**.

MTT viability assay: Following treatment with the various chemicals, tissue viability was determined using the MTT assay. % viability was determined using the equation: % viability = OD (treated tissue)/OD (control tissue)*100. MTT results from EpiVaginal tissues exposed to test articles are shown in **Figure 2**. No reduction in MTT viability was noted for all tested lubricants. The positive control Gynol II (with 3% N9) showed a significant reduction in MTT viability of the tissue.

Transepithelial electrical resistance (TEER): To examine barrier function, TEER measurements were made using the EVOM volt-ohmmeter equipped with an Endohm electrode chamber (World Precision Instruments, Sarasota, FL). %TEER was calculated as TEER (Ohms*cm^2) of treated tissues (TTT) divided by the TEER of untreated tissues (TUT) times 100 (% TEER = (TTT/TUT*100).

Bacterial exposure: Designated tissues were exposed as follow. 1) N=4 tissued were used as negative control. Tissues were cultured for 48 hr. 2) N=4 tissues were exposed to 50 µL of Lactobacillus iners (10^7 c.f.u) and cultured for 4 hr in antibiotic free medium. Tissues were washed 3X with DPBS and cultured in antibiotic free medium for additional 44 hr. 3) N=4 tissues were exposed to 50 µL of Lactobacillus iners (10^7 c.f.u) and cultured for 4 hr in antibiotic free medium. Tissues were then washed 3X with DPBS and cultured in antibiotic free medium for additional 44 hr. 50 µL of Gardnerella (10^7 c.f.u) was applied topically to Lactobacillus iners exposed tissues for 4 hr in antibiotic free medium. Tissues were washed 3X with DPBS and cultured in antibiotic free medium for additional 44 hr.

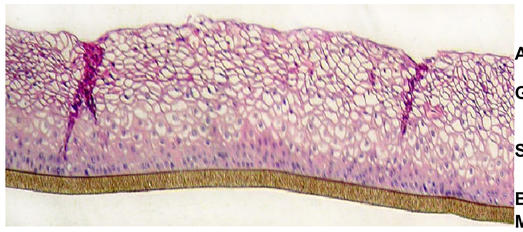


Figure 1: H & E stained cross-sections of partial thickness, epithelial VEC-100 tissue, **B.** The epithelium contains nucleated basal and suprabasal, and glycogen-filled cell layers.

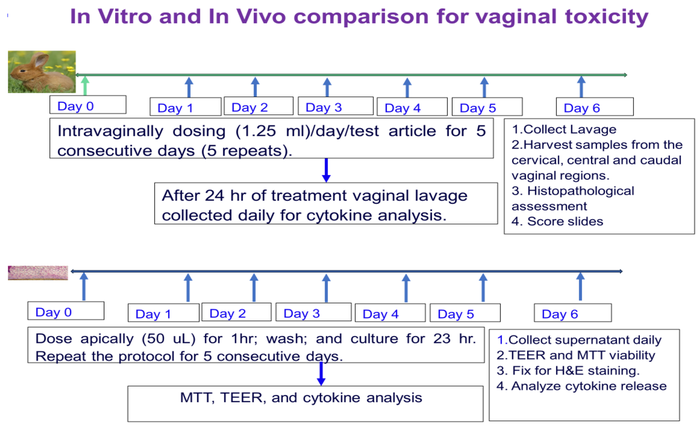


Table 1: Comparison of in vitro vs in vivo cytotoxicity and cytokine /chemokine release

				Cytokine/Growth Factor Analysis							
				In Vitro (Fold Increase)				In Vivo (#positives/total # rabbits			
				Il-1α		IL-1β		IL-1β	GROα	MIP-1α	VEGF
TEST ARTICLE	Coded Test Article*	RVI Score	MTT (%)	1X	5x	1x	5x	1x	1x	1x	1x
SA0823-001	Calcium acetate (2%)	2	95.0	1.2	0.8	1.0	1.1	ND	ND	ND	ND
SA0823-002	Cellulose (2%)	2	89.4	1.9	0.8	1.5	0.9	ND	ND	ND	ND
SA0823-003	Gynol II (2% N9)	2	12.0	49.6	4.4	2.1	1.2	ND	2/5	2/5	1/5
SA0823-004	SDS (2%)	3	10.1	61.1	0.1	5.3	0.6	ND	ND	ND	ND
SA0823-005	Bentonite (2%)	2	93.5	1.7	1.3	1.4	1.1	ND	ND	ND	ND
SA0823-006	Urea (2%)	2	94.7	1.3	1.1	1.2	1.1	ND	ND	ND	ND
SA0823-007	Calcium lactate (2%)	2	101.3	1.3	1.0	1.4	1.0	ND	ND	ND	ND
SA0823-008	Corn oil (2%)	2	95.1	1.3	1.1	1.3	1.1	ND	ND	ND	ND
SA0823-009	Benzalkonium chloride (2%)	4	20.7	30.3	0.0	1.3	6.9	4/5	2/5	2/5	2/5
SA0823-010	Saline	3	103.1	1.5	1.2	1.3	0.9	ND	ND	ND	ND
SA0823-011	Citric acid (2%)	3	93.3	1.6	0.9	1.2	1.0	ND	ND	ND	ND
Control	No treatment	3	100.0	1.0	1.0	1.0	1.0	ND	ND	ND	ND
Irritation		Minimal	<50%	>2.0 = +							

Table 2: Reproducibility of in vitro irritation assay using MTT % viability measurement as an end point. 4 donors, 3 tissues/test article/donor, 2% concentration (50 uL/tissue), and 24 hr incubation.

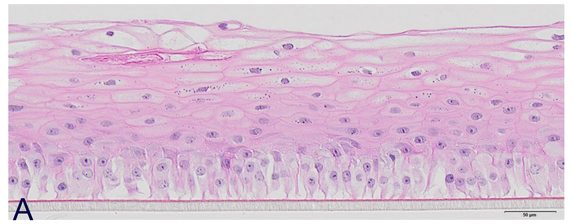
		% Viability by MTT					
		Donors					
#	Chemical Name	V19	V1201	V1004	V1105	Mean	StDev
1	Untreated	103.0	119.2	109.4	113.4	111.3	6.8
2	Saline	100.0	100.0	100.0	100.0	100.0	0.0
3	Gynol II (Nonoxynol-9)	6.0	5.6	5.9	8.5	6.5	1.4
4	Benzalkonium chloride	17.7	9.4	7.1	12.7	11.7	4.6
5	Sodium dodecyl sulfate	5.9	4.0	4.1	4.8	4.7	0.9
6	Calcium acetate	91.9	89.3	89.3	89.9	90.1	1.2
7	Citric acid	81.7	78.3	89.6	87.4	84.2	5.2
8	Corn oil	97.4	99.9	101.2	96.6	98.8	2.1
9	Cellulose	94.8	98.0	98.2	92.1	95.8	2.9
10	Aluminum sulfate	96.4	100.3	104.9	108.1	102.4	5.1
11	Beta cyclodextrin	92.4	81.8	119.7	114.3	102.0	17.9
12	Copper Sulfate	6.7	8.5	16.2	6.4	9.4	4.6
13	Diacetin	94.8	89.7	90.8	114.4	97.4	11.5

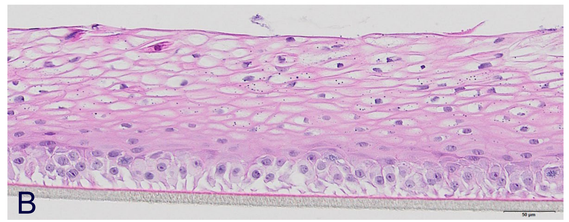
Table 3: Percent viability of treated tissues compared to untreated negative control tissues.

	OD (570 nm)					
Treatment	Tissue# 1	Tissue# 2	Mean OD	SDV	%Viability	%CV
Untreated - Control	2.053	2.211	2.132	0.1	100.0	0.1
Lactobacillus	1.942	2.086	2.014	0.1	94.5	0.1
Lactobacillus for 24 hours then apply Gardnerella for 44 hours	2.762	3.057	2.9095	0.2	136.5	0.2

Table 4. Percent TEER of treated tissues compared to untreated negative Control tissues.

Treatment	%Viability
Untreated - control	100.0
Lactobacillus	100.2
Lactobacillus for 24 hours then apply Gardnerella for 44 hours	104.6





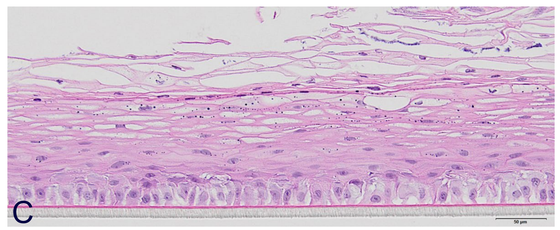


Figure 2: H&E staining showing a) control tissue, B) tissues exposed to Lactobacillus iners, and C) tissues exposed to Lactobacillus iners followed by Gardnerella vaginalis.

Summary

- Histological features of EpiVaginal tissue model mimics the invitro counterpart **(Fig 1)**
- The known human irritant, Nonoxynol 9 (N-9) showed variable results in RVI assay but detected as irritant in the EpiVaginal tissue model in a reproducible manner (**Table 1**).
- Test articles that were determined as vaginal irritant in vitro (copper sulfate and sodium dodecyl sulfate (SDS)) were not identified as irritant in the RVI test (**Table 2**).
- Exposure of EpiVaginal tissues to Lactobacillus iners followed by Gardnerella for 44 h results in no significant difference in MTT viability and TEER measurements (Tables 3 and 4) but showed perturbance of the glycogen filled layer (Fig 2).
- The in vitro assay method is sensitive, predictive, reproduceable, and reliable tool to assess vaginal toxicity of lubricants, drugs, and medical devices.

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