CURRENT STATUS OF AVAILABLE IN VITRO TESTS FOR VAGINAL IRRITATION ASSESSMENT

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ABSTRACT

The vaginal mucosa provides an effective barrier against numerous pathogens as one of the body's host defense and immune surveillance components. However, some feminine-care and cosmetic products may induce irritation of the vaginal epithelium, consequently making the tissues more susceptible to infections. Therefore, it is important that the compatibility of newly developed cosmetic or personal care products with the human mucosal surface be assessed before the product is marketed. The most frequently used test to screen for vaginal mucosal irritation is the *in vivo* rabbit vaginal irritation model. However, the current emphasis and preference in toxicology is to use alternative, *in vitro* methods that Reduce, Refine, or Replace the use of animals in testing programs. To that end, a clear understanding of the current status, applicability, and limitations of available *alternative* tests for vaginal irritation assessment is critical when companies are building their safety testing strategies. We present an overview of the available alternative and *in vitro* techniques for vaginal irritation assessment, from simple cell cultures to more complex explants and reconstructed tissues. We further assess their advantages and disadvantages compared to whole animal test systems and their role in the safety assessment strategy used for a wide array of active ingredients or final formulations.

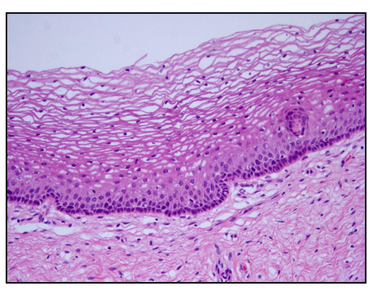
INTRODUCTION

The safety testing of personal care, cosmetic and pharmaceutical products has traditionally been performed in animals. Due to ethical and scientific concerns, non-animal human cell-based *in vitro* methods for eye and skin irritation have been proposed and validated. However, the current preclinical test for the assessment of vaginal irritation required by the U.S. Food and Drug Administration (FDA) for the regulation of spermicides and microbicides (regulated as drugs), and menstrual tampons and pads (regulated as devices) is the *in vivo* rabbit vaginal irritation (RVI) model. There are, however, other product types for intimate use (baby diapers, incontinence products, feminine deodorants and moisturizers, moist toilet tissues, personal lubricants, bath and body washes) for which the RVI is not specifically required, but is often used. The use of alternative, *in vitro* methods, which reduce, refine, and replace the use of animals, and model and predict the human responses is of particular interest to personal care and cosmetic industries that are becoming legally and ethically restrained in their use of animals for safety testing. Currently there is no alternative *in vitro* method validated and/or accepted by U.S. or European regulatory agencies for vaginal irritation assessment although several promising methods are being investigated. Here we provide an overview of the existing alternative *in vitro* pre-clinical methods with the goal of introducing the need for validation of pre-clinical *in vitro* methods that can accurately predict the effects of personal care, cosmetic and pharmaceutical products for vaginal use on humans to the representatives of the regulatory community, industry and organizations supporting alternative methods.

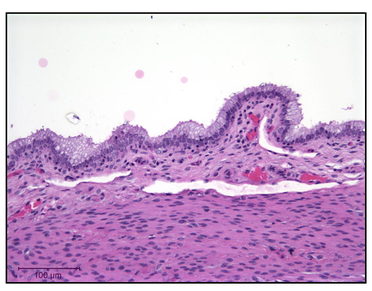
	Whole animal test systems		***In vitro* alternative models**		
	Rabbit	Slug (*Arion lusitanicus*)	Cell culture systems	Reconstructed tissues	Explants
Use of the model	• Preclinical safety testing of microbicides • Testing of vaginal tolerance to spermicidal preparations • Testing of long-lasting vaginal delivery systems for contraceptives • Studies on contraceptives efficacy	• Preclinical safety screening of new vaginal formulations	• Studies on cervico-vaginal physiology • Testing of pharmacological agents for intravaginal application • Preclinical evaluation of topical vaginal microbicides • Studies on the mechanisms of bacterial adherence to vaginal epithelial cells	• Studies of Human Immunodeficiency Virus (HIV)-1 and other sexually transmitted infections • Potential use for screening and assessment of the irritation, penetration, metabolism, or efficacy of active ingredients or final formulations for vaginal application • Studies on the expression and role of the *C. albicans* proteinases during infection and tissue damage of vaginal epithelium • Studies on the estrous cycle • Toxicity studies of feminine hygiene, vaginal care, and microbicide products	• Safety evaluation and risk assessment of ingredients and finished products • Human explants: research into mechanisms of early events in HIV infections and as bridge between the preclinical and clinical phase of microbicide candidates evaluation
Experimental setup	• 3-4 mature rabbits are treated with the test material (1 mL) daily, for 10 days. • Alternatively, 3 mature rabbits are exposed to the test material (1 mL) daily, for 5 days (as per ISO protocol 10993-10). • The external genitalia are observed daily for signs of erythema, edema, and discharge as a reaction to the exposure to the test material. • At the end of the experiment, parts of the cervico-vagina, mid-vagina and uro-vagina are fixed, paraffin-embedded and stained with Hematoxylin & Eosin (H&E) and are scored for epithelial ulceration, leukocyte infiltration, edema and vascular congestion.	• The irritation potential of a test material is evaluated by placing 5 slugs on the undiluted test material for contact periods of 30 minutes for five successive days and then measuring the amount of mucus produced. • After each 30 minutes contact period, the amount of mucus produced, the reduction of body weight, and release of enzymes (lactate dehydrogenase [LDH] and alkaline phosphatase [ALP]) from the body tissue are quantified.	• Cells are grown until confluence and then are incubated with the test materials for various times (10 minutes – 24 hours). • The 3-[4,5 – dimethylthiazol-2-yl] – 2,5 – diphenyltetrazolium bromide (MTT) reduction assay is performed; the medium may be collected for subsequent cytokine determination.	• Time-to-toxicity approach. • Several exposure times are tested relevant to product class (15 minutes – 24 hours). • Tissues are exposed to 83 µL of test material (volume may vary depending on the protocol suggested by the manufacturer of each tissues model). • Tissues are rinsed and viability is assessed with MTT. • ET_{50} values are interpolated from exposure time-response curves. • ET_{50} values are evaluated (rank order of test formulations, comparison to reference materials, evaluation by prediction model, etc.).	• Assessment of tissue morphology, cellular distribution and permeability. • Viability of tissues treated with the test materials is assessed by MTT.
Endpoints/scoring	Endpoints: epithelial ulceration, leukocyte infiltration, edema, and vascular congestion *Individual* irritation scoring 0 = no irritation 1 = minimal irritation 2 = mild irritation 3 = moderate irritation 4 = intense irritation The *total* scoring system correlates to human irritation potential as follows: - scores of 0 - 8 are acceptable - scores of 9 - 10 indicate borderline irritation potential - scores of 11 and above are indicative of significant irritation potential	Endpoints: the amount of mucus produced, the reduction of body weight, and release of proteins and specific enzymes (LDH, ALP) Irritation scoring - low total mucus production (<15% BW), a low protein release, and no enzyme release = non-irritating - no additional effect on the protein and enzyme release, induced mucus production of 15 - 20% BW = mildly irritating - induced mucus production of ≥20% BW = moderately irritating - increased mucus production (≥15% BW) and increased protein release (≥30 µg/ml/g BW) and/or enzyme release = severely irritating	Endpoints: • Cell viability • Chemokines (IL-8, MIP, RANTES) • Cytokines (IL-1, IL-6, TNF-α) • Inflammatory mediators (PGs, VEGF, MPO) • Innate immunity mediators (defensins, SLP1, Lf, gp340) • Transcription factors (NF-kB, AP-1) • Others (IgG, IL-1ra, IP-10)	Endpoints: • Tissue viability • Cytokines (IL-1, IL-6, IL-8)	Endpoints: • Tissue viability • Cytokines • Inflammatory mediators
Advantages	• Whole organ reaction • Systemic component • Full-strength formulations can be tested	• Whole organ reaction • Systemic component • Full-strength formulation can be used • Relatively inexpensive and easy to handle • Proposed to predict human burning and itching associated with the use of vaginal formulations.	• Human origin • Relatively inexpensive • Easy to grow	• Organotypic morphology is relatively easy to achieve and is inexpensive • Human origin • Structurally similar to human vaginal tissue	• Tissue structures with full cell component (epithelial, connective, immune) • Pig explants: resemble human structurally and, to some extent, functionally, easy to obtain and inexpensive, no regulatory considerations
Disadvantages	• Animal welfare concerns • Need for Institutional Animal Care and Use Committee (IACUC) approval • Non-human tissues • Labor intensive • Not a good structural model for human vagina – partly stratified squamous epithelium and a larger area of columnar epithelium compared to human tissue	• Challenging for testing of rings, films, capsules, tablets	• No barrier structure • No systemic component	• Not all functional characteristics of tissue *in vivo* can be reproduced • Barrier tends to be more permeable than *in vivo* • No vascular component so absence of inflammatory response to challenges	• Limited number, variability • Institutional Review Board (IRB) approval • More technically demanding • Human explants: not easy to obtain, limited age range, fresh tissue is a potential transfer of infectious agents; maintains viability for 12 hours after tissue removal • Pig explants: no vascular system – limits functional response
References	Eckstein et al., 1969. Garg et al., 1993. Zaneveld et al., 2001. D'Cruz et al., 2002. Costin et al., 2011 (*in press*). **An extensive list of references will be available as hard copy during poster presentation.**	Adriaens et al., 2001. Adriaens et al., 2002. Adriaens et al., 2003. Dhondt et al., 2004. Dhondt et al., 2005. Adrianes et al., 2006.	Fichorova et al., 1997. Fichorova et al., 2001. Catalone et al., 2004. Costin et al., 2011 (*in press*).	Schaller et al., 2003. Schaller et al., 2005. Ayehunie et al., 2006. Canny et al., 2006. Fichorova et al., 2006. Trifonova et al., 2006. Ayehunie et al., 2011. Costin et al., 2011 (*in press*).	van der Bijl et al., 1997. Thompson et al., 2001. Hu et al., 2004. van Eyk et al., 2005. Gupta et al., 2006. Cummins et al., 2007. Squier et al., 2008. Richardson-Harman et al., 2009.

CONCLUSIONS

1. The *in vivo* test based on the rabbit (RVI) remains to date the only model recommended by the U.S. FDA for safety evaluation of vaginal products such as spermicides and microbicides (regulated as drugs) and menstrual tampons and pads (regulated as devices).
2. There are other types of products for intimate use that must be evaluated for safety for which the rabbit test is not specifically required, but is often used, such as baby diapers, incontinence products and cosmetics (feminine deodorants and moisturizers, moist toilet tissues, personal lubricants, bath and body washes).
3. The need to understand the irritating effects of therapeutic agents or cosmetic and personal care products on genital mucosae have led to the development of a variety of models using animals, cell culture, isolated tissue and organ culture.
4. Models are available, including 3-D (three dimensional) human reconstructed tissues, that show some promising early results and address many of the shortcomings of current animal and monolayer cell culture test systems. It is anticipated that these tissue models will be useful for preclinical irritation screening particularly during the early stages of spermicide, microbicide, and feminine-care product development.
5. One very significant driver for developing alternatives to animal testing is the Seventh Amendment to the EU Cosmetics Directive. This legislation significantly limits the use of animal testing for determining the safety of cosmetics products. Thus, an RVI assay cannot be used to assess the safety of a cosmetic product manufactured or sold in the EU. This means that until an *in vitro* assay is developed and proves reliable in predicting vaginal irritation, a human clinical study would be the only way to address the vaginal irritation potential of a cosmetic final product.
6. Currently, there is no alternative method validated and/or accepted by U.S. or European regulatory agencies for vaginal irritation assessment. The combined efforts of academic research, support from industry, and the drivers from the animal welfare community can lead to the development and use of efficient, reproducible and relevant models for assessing vaginal irritation of feminine-care and personal care products.



Human Vaginal Epithelium (20x)



Rabbit Vaginal Epithelium (20X)

FUTURE DIRECTIONS/FINAL REMARKS

Possible testing strategy

Cell-based models

Are useful as first-line screening tests to eliminate candidates that are significantly cytotoxic or cause the release of known biomarkers of inflammation.

Explants or 3-D reconstructed tissue models

Could provide the next screening (and is some cases, definitive) step, critical for assessment of preliminary formulations.

Animal-based models

Until *in vitro* methods become accepted by regulatory agencies, animal models are required for assessing full-strength formulations and would have to be used to provide information on whole-organ response.

Clinical studies

Clinical studies are performed for the formulations that advance through the pre-clinical tests.

Gaps

- Validation of existing models and biomarkers
- Identification of new biomarkers and models of microbicide/personal care (cosmetics)-induced mucosal alteration that correlate with relevant *in vivo* responses
- Need to organize and share data on markers and models and to assess their relative merits and limitations

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