Vaginal Irritation Models: The Current Status of Available Alternative and *In Vitro* Tests

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Summary — Mucosal surfaces, such as the vaginal epithelium, are natural barriers to infection that are constantly exposed to bacteria and viruses, and are therefore potential sites of entry for numerous pathogens. The vaginal epithelium can be damaged mechanically, e.g. by the incorrect use of objects such as tampons, and by chemicals that are irritating or corrosive. Consequently, this can lead to an increase in susceptibility to further damage or infection. Pharmaceutical, cosmetic and personal care products that are specifically formulated for application onto human external mucosae can occasionally induce undesirable local or systemic side-effects. Therefore, the compatibility of applied materials with this mucosal surface represents a key issue to be addressed by manufacturers. The most frequently used method for assessing vaginal mucosal irritation is the *in vivo* rabbit vaginal irritation test. However, the current emphasis in the field of toxicology is to use alternative *in vitro* methods that reduce, refine, and replace the use of animals, and which model and predict human, not animal, responses. Such an approach is of particular interest to the personal care and cosmetic industries in their effort to comply with European legislative measures, such as the 7th Amendment to the EU Cosmetics Directive that does not permit the marketing of cosmetic products if they, or their ingredients, have been tested for irritation responses in animals. The focus of this review is to provide an overview of the alternative and *in vitro* tests that are currently available for vaginal mucosal irritation assessment, and which are already used, or may become useful, to establish the safety of newly-designed products for human use.

Key words: *alternatives to animal testing*, in vitro *assays, vaginal irritation.*

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Introduction

The vaginal mucosa represents one of the body's host defence and immune surveillance components, providing an effective barrier against numerous pathogens. However, minor injuries may occur, following the use of feminine-care and cosmetic products, contraceptives or microbicides, which can induce irritation of the tissue and make the vaginal epithelium particularly susceptible to various types of infection. Therefore, it is important that the compatibility with the human mucosal surface of newly-developed cosmetic or personal care products, or of topically-applied drugs, is assessed before the product is launched.

Microbicides, for example, are designed to prevent infection by the human immunodeficiency virus (HIV) or other sexually transmitted infections. There are specific safety concerns regarding new microbicide formulations, in part due to the unexpected findings in recent clinical trials on the potential utility of nonoxynol-9 (N-9) as a microbicide (1). N-9 was originally designed as a spermicide, and has been in use for more than a quarter of a century. However, when it was recently tested for microbicidal potency in a large-scale phase III trial (1), the low dose of N-9 gel (3.5%) increased a woman's risk of HIV infection instead of reducing it, when used more than three and a half times per day. A major reason for this unexpected increase is thought to be the penetration of the virus into the vaginal epithelium resulting from vaginal irritation caused by N-9.

The current preclinical test for the assessment of vaginal irritation required by the US 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 (2). There are, however, other types of products for intimate use that must be evaluated for safety, but for which the RVI model is not appropriate. Examples include baby's nappies, incontinence products and cosmetics (e.g. feminine deodorants and moisturisers, moist toilet tissues, personal lubricants, and bath and body washes).

The efforts of the cosmetic and personal care product industry to replace animal procedures with alternative methods — such as those that use human-derived cells and tissues *in vitro*, physico-

chemical techniques, computer modelling, and volunteer studies — are driven by legislative considerations (e.g. the 7th Amendment to the EU Cosmetics Directive; 3), and scientific and ethical concerns. Thus, there exist both a cultural challenge requiring flexibility and openness to new ideas, and a scientific challenge that introduces the concept of a cross-disciplinary approach. While cosmetic and personal care product manufacturers are currently evaluating and adopting alternative and *in vitro* methods for eye irritation, skin irritation and corrosion, etc. (4–7), they do not currently benefit from an integrated programme focused on validating standardised tissue models or cellular assays for vaginal irritation assessment.

The purpose of building a complete set of alternative and *in vitro* safety testing strategies, including ones for vaginal irritation, is to offer the possibility of generating reliable and relevant safety data on the effects of human tissue exposure to chemicals, drugs and other appropriate personal care and cosmetic products. Here, 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 (rabbit, pig, mouse, monkey, etc.), and their role in the safety assessment strategy used for a wide array of active ingredients and final formulations.

The Human Vaginal Epithelium — Structure and Susceptibility to Irritation

The human vaginal mucosa consists of a thick, non-keratinising, stratified, squamous epithelium containing cells laden with glycogen, as well as a smaller number of cells of other types, such as macrophages and Langerhans' cells (LCs). Beneath the epithelium, there is a lamina propria, which is a dense connective tissue containing numerous elastic fibres, polymorphonuclear leukocytes, lymphocytes, and occasional lymph nodules (Figure 1a). Because the lamina propria also contains a dense network of blood vessels, the vaginal mucosa is an excellent route for delivering drugs for both local and systemic treatments (8–10).

The mucosal epithelium is important for host defence and immune surveillance, and represents the primary cell layer that initially encounters environmental microorganisms. The vaginal mucosa is commonly exposed to contraceptives, microbicides, feminine-care products, and drugs for the treatment of female-specific conditions, all of which can induce irritation of the tissue, thus making it more susceptible to infections. The currently-available detergent-type spermicides are cytotoxic to genital tract epithelial cells at spermicidal concentrations (11). For example, N-9 is known to promote inflammation of the vaginal tissue, and to cause irritation, ulceration, epithelial disruption and sloughing (12–14), which can make the tissue more sensitive to further interactions with pathogens or other topically-applied products. Clinical studies have confirmed that detergent-type spermicides that alter the normal vaginal flora lead to an increased risk of gynaecological infections or sexually transmitted diseases (15–17). Consequently, the development of safe, efficacious, non-detergent-type vaginal spermicidal microbicides has become the focal point in translational anti-HIV microbicide research (11), further emphasising the need for accurate safety testing of the new microbicide formulations.

In summary, there is a need for accurate and reproducible methods for vaginal irritation assessment for developers of products that are intended to be used in contact with the vaginal epithelium, and work is currently focused on replacing the existing *in vivo* tests with alternative and *in vitro* testing methods.

Testing Systems Used for Vaginal Irritation Assessment

Whole animal test systems

Testing protocols for the assessment of vaginal irritation have traditionally involved animals, and the safety testing of chemical substances and final products intended for use in or around the vagina has been based on both macroscopic observations of erythema, oedema and ulceration (18, 19), with histopathologic analysis of the tissues collected after exposure of the animals to the test materials. In addition to ethical issues related to the use of animals for the screening of human products, the large number of lead candidates and formulations generated by industry and academic institutions can require the use of a large number of animals, which can make product development very costly.

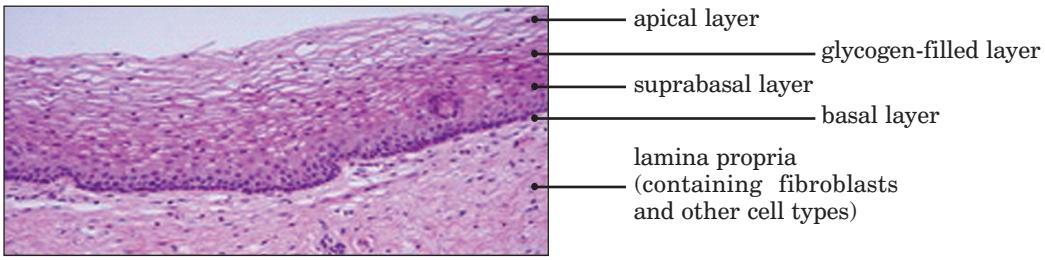
Several types of animals, including the rhesus monkey (20–22), pig-tailed macaque (23), pig (24), mouse (19), dog (25), rat (26–28) and slug (29, 30), have been used to assess the extent of vaginal irritation induced by microbicides and cosmetic products (Table 1). However, the animal test system currently used for the regulatory acceptance of new products remains the rabbit test, developed over 40 years ago at the Ortho Research Foundation, Raritan, NJ, USA (22).

The rabbit model

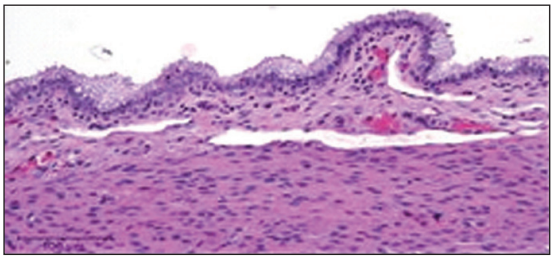
The *in vivo* RVI test has long been the preferred choice for vaginal toxicity studies (22). Briefly, the

Figure 1: Histology of the vaginal epithelium

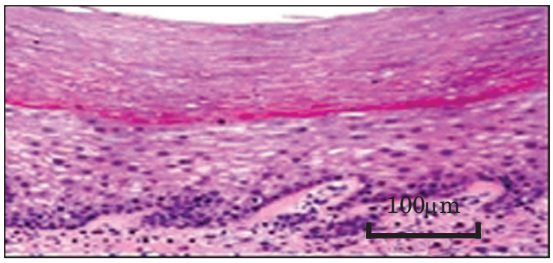
a) human



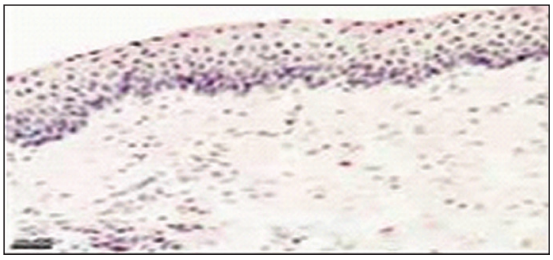
b) rabbit



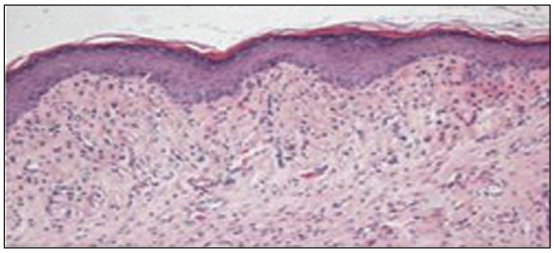
c) rhesus monkey



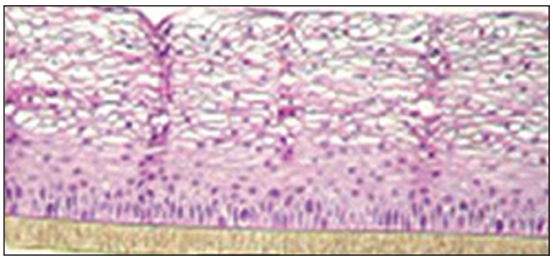
d) pig



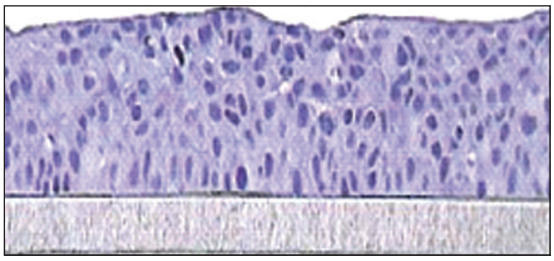
e) mouse



f) MatTek



g) SkinEthic



A comparison between H&E-stained vaginal epithelium of: a) human, b) rabbit, c) rhesus monkey, d) pig, e) mouse, and reconstituted human vaginal mucosa from f) MatTek Corporation (Ashland, MA, USA), and g) SkinEthic (Nice, France). The photographs are used only to illustrate the morphology of the tissues; exact magnification scales were not available for all tissues. Depending on the source of photographs, the tissues illustrated are either untreated or negative-control treated. A detailed legend of the tissue layers is provided only for the native human tissue.

Image a) was provided by Asterand, Detroit, MI, USA. Image c) is from Reference 86, with permission from the Society of Endocrinology. Image d) is from Reference 87, with permission from The American Association of Immunologists, Inc. Image e) is from Reference 88, with permission from the American Society for Microbiology. Images f) and g) were provided by the manufacturers, and are available online at http://www.mattek.com and http://www.skinethic.com, respectively.

Table 1: Whole animal test systems and endpoints used for vaginal irritation assessment

	Animal	Use (reference)	Endpoints
Mammals	Rabbit	Preclinical safety testing of microbicides (19) Testing of vaginal tolerance to spermicidal preparations (22) Testing of a long-lasting vaginal delivery system for contraceptives (33) Study on the contraceptive efficacy (84)	Histopathology Leucocyte number and phenotype Cytokines and soluble markers Epithelial damage Apoptosis Colposcopy Microflora
	Rhesus monkey	Safety testing of chemical agents/formulations for vaginal use (20–22)	
	Bonnet monkey	Study on the contraceptive efficacy (84)	
	Chinese rhesus macaque	Development of a device for sustained delivery of HIV microbicides (34)	
	Pig-tailed macaque	Development of a device for sustained delivery of HIV microbicides (34) Study of N-9 effect on vaginal microflora and chlamydial infection (23)	
	Stump-tailed macaque	Testing of a long-lasting vaginal delivery system for contraceptives (33)	
	Pig	Assessment of vaginal irritancy of microbicides and spermicides (24)	
	Mouse	Preclinical safety testing of microbicides (19)	
	Swiss Webster mouse	Preclinical assessment of microbicide toxicity (39, 43)	
	Rat	Safety testing of chemical agents/formulations for vaginal use (26–28)	
Invert-ebrate	*Arion lusitanicus*	Preclinical safety screening of new vaginal formulations (29, 30, 45, 47, 49, 51)	Amount of mucus produced Reduction of body weight Release of proteins and specific enzymes (LDH, ALP)

The most frequently used animal species are listed. ALP = alkaline phosphatase; HIV = human immunodeficiency virus; LDH = lactate dehydrogenase; N-9 = nonoxynol-9.

RVI test is performed as follows: 1ml of test material is inserted daily, for 10 days, through a lubricated catheter, or tuberculin syringe, into the vagina of each of three to four mature rabbits; the external genitalia are observed daily for any signs of erythema, oedema or discharge as a reaction to the exposure to the test materials. After specific time points (chosen to fit the objectives of the study), each rabbit is euthanised, and the vaginal tissue is removed and evaluated by a veterinary histopathologist. Usually, parts of the cervico-vagina, mid-vagina, and uro-vagina of each animal are fixed, paraffin-embedded, sectioned and stained with haematoxylin–eosin (H&E). Each of the three regions of the vagina is scored for epithelial ulceration, leukocyte infiltration, oedema and vascular congestion (22). An overall *individual* irritation score is assigned to each of the three regions, based on a semi-quantitative scoring system which takes into account the endpoints mentioned above (22), as follows: individual score 0 = no irritation; 1 = minimal; 2 = mild; 3 = moderate; and 4 = intense irritation. The scores for each region are combined, and the *total* irritation score is then related to human irritation potential as follows: scores of 0–8 are acceptable, scores of 9–10 indicate borderline irritation potential, and scores of 11 and above are indicative of significant irritation potential (Table 2). The International Organisation for Standardization (ISO) protocol 10993-10 is also used, which is based on the treatment of three rabbits for five days (31).

Some of the shortcomings of the RVI test may be due to the structural differences between rabbit and human vaginal tissue (Figure 1, Table 3). Two-thirds of the rabbit vagina is lined by columnar epithelium (Figure 1b), which is structurally distinct from the stratified squamous epithelium (8–12 cells thick) of the human vagina (Figure 1a), and is also highly sensitive to vaginal irritants when compared to its human counterpart (22). The RVI test features an extended contact time between the tissue and the

Table 2: Scoring systems for the assessment of human vaginal irritation in animal studies

Species	Endpoints and scoring	Ref.	Comments
Rabbit	**Endpoints:** Epithelial ulceration, leukocyte infiltration, oedema and vascular congestion **Individual irritation scoring:** 0 = no irritation 1 = minimal irritation 2 = mild irritation 3 = moderate irritation 4 = intense irritation	22	**Test duration:** 10 days The **total irritation 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
Monkey	A scoring system similar to that used for the rabbit model can also be employed for the monkey. It is more difficult to apply, mainly because of the minute size and frequent fragmentation of specimens and the occasional occurrence of odd or unclassifiable features such as epithelial hyperkeratosis, irregularity in either thickness, cornification or PAS-staining of the epithelium, etc.	22	**Test duration:** 60 days or two menstrual cycles
Pig	**Endpoints:** Epithelial ulceration, epithelial leukocyte influx, sub-epithelial leukocyte influx, sub-epithelial haemorrhage, vascular/perivascular haemorrhage, sub-epithelial oedema, vascular/perivascular oedema, vascular/perivascular congestion, muscle integrity and cell/tissue necrosis **Irritation scoring:** 0 = no abnormal findings 1 = minimal degree of severity 2 = mild degree of severity 3 = moderate degree of severity 4 = marked degree of severity	24	**Test duration:** four days **Cumulative range:** 0–40
Mouse	**Endpoints:** Epithelial integrity, epithelial vascular congestion, leukocyte infiltration and oedema **Irritation scoring:** A vaginal irritation grading system with scores from 0 (normal parameter or absent adverse effects) to 4 (most severe adverse findings) was used to score each test material for the above endpoints	43	**Test duration**: 10 days **Composite average scores grading:** 1–4 = vaginal irritation rating of 'minimal' 5–8 = vaginal irritation rating of 'mild' 9–11 = vaginal irritation rating of 'borderline' 12–16 = vaginal irritation rating of 'unacceptable' Formulations with vaginal irritation ratings between 1–8 are considered acceptable for vaginal application

The most frequent animal species used are listed. ALP = alkaline phosphatase; LDH = lactate dehydrogenase; PAS = periodic acid-Schiff.

Table 2: continued

Species	Endpoints and scoring	Ref.	Comments
Slug	**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% of the slug body weight), a low protein release, and no enzyme release = non-irritating No additional effect on the protein and enzyme release, but total mucus production of 15–20% of the slug body weight = mildly irritating Total mucus production of ≥ 20% of the slug body weight = moderately irritating Increased mucus production (≥ 15% of the slug body weight) and increased protein release (≥ 30μg/ml per g body weight) and/or enzyme release = severely irritating	30, 45	**Test duration:** five days (30 minutes exposure/day) This scoring system was used to test the effects of repeated use of vaginal gels
Clinical trials	**Endpoints:** Microscopic evidence of cervical, vaginal, or vulvar ulceration, abrasion, severe erythema, and/or oedema, colposcopy, physical examination including a Pap smear, wet mount, and culture, patient log of adverse events, serum test material levels (if applicable) **Irritation scoring:** Adverse events were defined as: Mild = minimal symptoms; transient or mild discomfort that lasted < 48 hours and did not require change in activity or medication Moderate = notable symptoms; required that the patient make some modification of activities and, possibly, medication, but did not result in loss of work or cancellation of social activities Severe = the patient had marked limitation in activities and usually required some assistance; patients required medication and/or bed rest, and symptoms sometimes resulted in loss of work or cancellation of social activities	53, 85	**Test duration:** 14 days (setup of the clinical trials may vary)

The most frequent animal species used are listed. ALP = alkaline phosphatase; LDH = lactate dehydrogenase; PAS = periodic acid-Schiff.

extracts applied to the vaginal mucosa, potentially exaggerating normal human exposure. Moreover, the rabbit lacks cyclic reproductive stages, vaginal *Lactobacilli* and acidity, cervical mucus production, and species-specific markers for inflammatory processes. It is also unresponsive to most human genital pathogens (32). However, despite the dissimilarities between rabbit and human vaginal tissues detailed above, the rabbit method is preferred and is widely used to determine the vaginal tolerance or mildness of topical spermicidal preparations, because it is a brief and economical test.

The monkey model

Monkeys have also been used to assess vaginal irritation induced by different agents, such as contraceptives (33) or microbicides (34; Table 1). Briefly, the vaginal tolerance test in rhesus monkeys uses a set amount of the test material, which is administered daily through a lubricated catheter into the vaginas of two cyclic rhesus monkeys for defined periods of time (60 days or two menstrual cycles; 22). The volume of the test material administered is chosen to combine maximal content of active ingredients — usually between five and 15 times the human dose on body weight basis — with the largest volume that can be conveniently accommodated by the average vagina and covers most of its surface (~1.5ml). Two biopsies from the lower vagina of the monkeys are usually taken, one at the beginning of the test and one at the end, and these are scored in a way similar to that for the rabbit model. Although providing a model that

Table 3: Some advantages and disadvantages of whole animal test systems used for vaginal irritation assessment

Species	Advantages	Disadvantages
Rabbit	**General**	**General**
Monkey	Whole organ reaction	Animal welfare concerns
Pig	Dynamic response	Need for IACUC approval
Mouse	Systemic component	Non-human tissues
Slug	Full-strength formulation can be used	Labour intensive
	Specific	**Specific**
	Rabbit and mouse: inexpensive and easy to maintain and handle.	Rabbit: not a good structural model for human vagina — partly stratified squamous epithelium and a larger area of columnar epithelium compared to human tissue.
	Monkey and pig: resemble human, both structurally and functionally.	Monkey: expensive to purchase and maintain, difficult to handle, increased risk of infections.
	Slug: relatively inexpensive and easy to handle; said to predict human burning and itching associated with the use of vaginal formulations.	Pig: can be expensive to maintain; as animals grow, they become difficult to handle.
		Mouse: not a good structural model for human vagina — keratinised tissue (during the oestrous phase of the cycle) tends to be less permeable and more resistant to damage.

The most frequently used animal species are listed. IACUC = Institutional Animal Care and Use Committee.

structurally and functionally resembles human tissue more closely than the rabbit (Figure 1c), monkeys are expensive to purchase and maintain, and carry the risk of transmissible infections (Table 3). In addition, their use for a purpose like vaginal irritation testing is extremely difficult to justify ethically.

The pig model

The similarity of porcine and human vaginas in terms of anatomical structure (Figure 1d), pH, vaginal secretion, cervical mucus production and inflammatory response induced by microbicides and spermicides, recently led D'Cruz *et al.* (24) to propose the pig as a model for studies of vaginal irritancy. Moreover, the pig is well-suited for colposcopic observations and for obtaining multiple biopsies, which can be used to evaluate the cervico-vaginal gene expression profile of inflammatory mediators expressed following exposure to topical agents. In addition, many physiological factors which influence ovulation, artificial insemination, fertilisation, early embryonic development and establishment of pregnancy in pigs, have been well documented (35–38).

D'Cruz *et al.* (24) have described the *in vivo* vaginal irritation assay performed on pigs in detail. Briefly, the animals are treated with 80ml of test material administered intra-vaginally via a catheter for four consecutive days. The pigs are observed daily for clinical signs such as genital oedema and erythema and vaginal discharge, including bleeding. On day 5, the genital tract is retrieved, and the vagina is excised and fixed in 10% (v/v) neutral-buffered formalin for microscopic evaluation. Numerous histological features are then observed and scored for irritation, as follows: 0 = no abnormal findings; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked degree of severity, with the cumulative range being 0–40 (Table 2).

Among the larger experimental animals, the pig has the advantage of being remarkably similar to humans in terms of anatomy, physiology, metabolism and pathology. Several disadvantages of testing on pigs are that they can be expensive to maintain, and can become difficult to handle as they grow (Table 3).

The mouse model

The Swiss Webster mouse model has been used by Catalone *et al.* (39) for the preclinical assessment of toxicity and inflammation associated with exposure to candidate topical microbicides. The model is relatively inexpensive compared to other animal models, and it can be used for the evaluation of cervico-vaginal toxicity and inflammation at the cellular and tissue levels (39). The test procedure based on the murine model provides information on inflammation and immune cell recruitment in response to microbicide application, and it can be used as a bridge between *in vitro* cytotoxicity assays and clinical trials. The mouse model has been used to establish the efficacy and/or safety of new microbicide and spermicide formulations (40–43). However, when deciding on the best testing strategy, it should be considered that the keratinised rodent vaginal tissue (during the oestrus phase of the cycle) is not a very good model for the

human tissue, since it tends to be less permeable and more resistant to damage (44; Figure 1e and Table 3).

The slug model

The Slug Mucosal Irritation (SMI) assay, with the terrestrial slug, *Arion lusitanicus*, used as the selected test organism, was developed at the Laboratory of Pharmaceutical Technology of the University of Ghent, Belgium. The body wall of the slug consists of a mucosal surface that contains cells with cilia and microvilli, as well as mucus-secreting cells, over a sub-epithelial connective tissue, which is similar to that of the human vaginal epithelium. Briefly, the rationale for the use of the assay is that slugs placed on an irritant substance produce mucus. In addition, if tissue damage occurs, it results in the release of proteins and specific enzymes.

The irritation potential of a test substance is evaluated by placing five slugs on the undiluted test material for contact periods of 30 minutes on five successive days, and then measuring the amount of mucus produced. After each 30-minute contact period, the amount of mucus produced, the reduction in body weight, and the release of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) enzymes from the body tissue, are quantified (45).

Several studies suggest that the SMI assay can be used as an early screening tool in the R&D phase of new pharmaceutical formulations, to evaluate their local tolerance without the use of vertebrates (30, 47–50). In general, the test materials that can be tested by using the SMI assay are solids, semi-solids or liquids, thus limiting the possibility of employing this assay for the screening of test materials in the form of films, rings or tablets. However, as with many *in vitro* assays, capsules or tablets can be crushed, and the resulting granules can be used in the SMI assay by following the same procedure as for solid materials.

Recently, Dhondt *et al.* (30) conducted a study aimed at optimising the SMI assay for the evaluation of the local tolerance of vaginal gels (Hydroxyethyl cellulose [HEC], Replens™, K-Y® jelly, Advantage S, Protectaid®, and Conceptrol®). They classified the formulations into four irritation categories, as follows: a) gels that induced, over a 5-day exposure period, a low total mucus production (< 15% of the slug body weight), a low protein release, and no enzyme release, were classified as non-irritating (e.g. HEC gel); b) gels that caused no additional effect on protein and enzyme release, but caused a total mucus production of 15–20% of the slug body weight, were classified as mildly irritating (e.g. Replens); c) gels that induced a total mucus production of ≥ 20% of the slug body weight were predicted as moderately irritating (e.g. K-Y jelly); and d) gels that caused a mucus production of ≥ 15% of the slug body weight, and an increase in protein release (≥ 30µg/ml per g body weight) and/or enzyme release, were classified as severely irritating gels (Advantage S, Protectaid, Conceptrol; Table 2). The study showed that the results obtained in the SMI test correlated with the *in vivo* data provided by various animal models (mouse, rabbit, macaque) and with clinical data for some of the gels tested (HEC, K-Y jelly and Advantage S).

The same group used the SMI test to evaluate the local tolerance of vaginal gels containing various concentrations of dapivirine, and compared the results with those from the RVI test (51). The authors concluded that they were comparable — the study showed that the placebo gel and the dapivirine-containing gels did not irritate the slug mucosa or the rabbit vaginal mucosal tissue. However, Conceptrol, which was used as a positive control, was irritating to both the slug mucosa and the rabbit vaginal tissue.

In addition, it has been proposed that the SMI assay can be used to predict genital burning and itching, which are limiting factors for products designed for vaginal application. The study by Adriaens (45), showed that an increase in the amount of mucus produced in the SMI assay is associated with an increase in the number of patients complaining of genital heat, itching or burning. This suggests that the slug test could be useful for screening new vaginal gel formulations for local tolerance before preclinical studies in vertebrates and clinical studies in humans are conducted, thus contributing to the reduction of the number of vertebrates used in preclinical studies (Table 3).

A summary of whole animal test systems

The use of whole animal test systems based on rabbits, rodents, primates or slugs (the latter could be described as a refinement) for the safety evaluation of pharmaceutical or cosmetic and personal care products has scientific, ethical and economic limitations. Except for non-human primates, the other animal models reviewed are limited in their ability to mimic the human vaginal inflammatory response, due to the differences in genital tract physiology and anatomy. Most importantly for frequently-used products, animal models are reportedly unable to provide a distinction between mild and ultra-mild formulations, because products in both classes will often induce macroscopic changes which are not discernible from each other. Furthermore, although human data should be the final standard against which the relevance of an alternative test is assessed,

comparison of the available data is difficult, due to differences in the experimental methods (exposure times, number of animals used, endpoints, etc.) used in the animal studies (Table 2). In addition, the interpretation of the available human clinical data can be challenging, since the clinical trials differ with respect to their purpose, frequency and duration of test material application, sample sizes, target populations, rules regarding intercourse, the means and time points for assessing safety outcomes, etc. (52–54).

The concept of *Reduction*, *Refinement* and *Replacement* (Three Rs) has stimulated the development of alternative methods such as those based on *in vitro* systems. Efforts are being made to develop alternative models that are more predictive of human experience, less time-consuming, and more cost-effective. Recent studies of vaginal *ex vivo* cultures, various cell lines and reconstituted tissue models, suggest that they have the potential to become replacements for the RVI model when used for cosmetic and personal care product screening.

In vitro systems

Cell culture systems

The use of cell culture systems for the *in vitro* toxicity screening of a wide category of compounds has increased significantly over several years. Cell cultures have consistently been used as a basis for alternative methods, particularly for the cosmetic and personal care industry, in the light of consumer concerns about animal testing and the stringent legal requirements in some countries that limit, or totally ban, the use of animal testing. Recently, large screening programmes and validation studies have been performed by using cell cultures, in order to provide information about many different toxicological endpoints. However, although significant efforts have been made in the area of ocular and dermal irritation, the *in vitro* assessment of vaginal irritation with cell culture systems has, regrettably, not yet become the subject of large-scale evaluation programmes. It is clear that research efforts in this area should be part of the future objectives of key players involved in the testing and regulatory acceptance process.

In 1979, Sobel *et al.* (55) published the first report on the development of vaginal epithelial cultures from healthy adults (Table 4a). Proliferating epithelial cells formed multilayers of stratified squamous epithelium that resembled vaginal epithelial tissue *in vivo*, but their proliferative activity decreased after 14 days. Continuous lines of epithelial cells were not obtained in this study, but the group achieved prolific outgrowth from explants, and concluded that this model could be used to investigate the effects of exogenous factors, such as hormones and vitamins, on the growth and differentiation pattern of vaginal epithelial cells, as well as the differences between outgrowths of normal tissues and those of tumour tissues.

In more-recent studies, monolayer cultures of immortalised and transformed cell lines derived from primary cultures of human vaginal and cervical epithelial cells, have been used to assess the toxicity of spermicides (56) or to study the interaction of pathogens with vaginal mucosal cells (57; Table 4a). Krebs *et al.* (56) assessed the sensitivity of primary human vaginal keratinocytes, isolated during reconstructive surgeries, to the topical vaginal microbicides, N-9, C31G (equimolar mixture of cetyl betaine and myristamine oxide in a hydroxyethyl cellulose gel) and sodium dodecyl sulphate (SDS). The resulting data showed that these cells had the potential to be used for the assessment of microbicide cytotoxicity. Rajan *et al.* (57) used an immortalised line of human vaginal cells to study the adherence to the vaginal mucosa of type 1 piliated *Escherichia coli*, a bacterium involved in the pathogenesis of ascending urinary tract infections in women. While this particular cell line represents a promising model for studying the mechanisms of bacterial adherence to vaginal epithelial cells, it has not yet been evaluated for screening the irritation potentials of microbicide candidates or of other compounds that might come into contact with the vaginal mucosa.

Several immortalised cell lines derived from human vaginal epithelium are commercially available from the American Type Culture Collection (ATCC, Manassas, VA, USA), for example CRL-2614 (Ect1/E6E7), CRL-2615 (End1/E6E7) and CRL-2616 (Vk2/E6E7) (58), and have been used in comparative studies to measure the toxicity of N-9 (17, 39; Table 4a). The phenotypes of these cell lines, during more than one year in continuous culture, were found to be similar to those of normal epithelial cells, thus making them a potential *in vitro* model for testing products intended for intravaginal application (58).

A study by Catalone *et al.* (39) showed that the endocervical cells (End1/E6E7) were consistently more sensitive to N-9-mediated cell death than the vaginal keratinocytes (Vk2/E6E7) during short-term and long-term exposures. Catalone *et al.* (39) also assessed and compared the cytotoxicity of N-9 to Vk2/E6E7 vaginal keratinocytes and End1/E6E7 endocervical keratinocytes with the results obtained in the Swiss Webster mouse model. The data obtained by using the cell lines correlated with the *in vivo* results provided by the mouse model, which showed that N-9 induced acute disruption of the cervical columnar epithelial cells and intense inflammatory infiltrates within the lamina propria, two hours after application.

Table 4: *In vitro* test systems used for vaginal irritation assessment

a) Cell culture systems

Description	Information		Reported/potential use	Endpoints
	Cell source	Ref.		
Human vaginal epithelial cells	Human biopsy	55	Study of the effect of exogenous factors on the growth and differentiation pattern of vaginal epithelial cells.	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-κB, AP-1) Others (IgG, IL-1ra, IP-10)
Primary human vaginal keratinocytes	Human biopsy	56, 60	Studies of the sensitivity of primary human vaginal keratinocytes, isolated during reconstructive surgeries, to topical vaginal microbicides.	
CRL-2614 (Ect1/E6E7) — immortalised epithelial cell line from human ectocervix CRL-2615 (End1/E6E7) — immortalised epithelial cell line from human endocervix CRL-2616 (Vk2/E6E7) — immortalised epithelial cell line from human vagina Cell lines available for research purposes; also available for safety assessment studies performed by industry entities after licensing from Brigham and Women's Hospital, Inc., Office of Corporate Sponsored Research and Licensing in Boston, MA, USA	ATCC	58	Studies on cervico-vaginal physiology.	
		17	Testing of pharmacological agents for intravaginal application.	
		39	Preclinical evaluation of topical vaginal microbicides.	
Immortalised human vaginal epithelial cell line	Human biopsy	57	Studies on the mechanisms of bacterial adherence to vaginal epithelial cells.	

3-D = three-dimensional; AP-1 = activator protein 1; ATCC = American Type Culture Collection; FT = full thickness; HIV-1 = human immunodeficiency virus-1; IgG = immunoglobulin G; IL = interleukin; IL-1ra = interleukin-1 receptor antagonist; IP-10 = interferon-inducible protein 10; LCs = Langerhans' cells; Lf = lactoferrin; MIP = macrophage inflammatory protein; MPO = myeloperoxidase; NF-κB = nuclear factor-κB; NHVC = normal human vaginal cells; PG = prostaglandin; RANTES = regulated upon activation, normal T-cell expressed and secreted; RHVE = reconstructed human vaginal epithelium; SLP1 = sphyngosine-1-phosphate lyase; TNF = tumour necrosis factor; VEC = vaginal-ectocervical cells; VEGF = vascular endothelial growth factor.

Table 4: ***continued***

b) Reconstructed tissues

System characteristics	Source of information: Company	Source of information: Ref.	Reported/potential use	Endpoints
Human reconstructed vaginal mucosa integrating LCs	—	62	Study of the infection of LCs by HIV. Toxicological, pharmacological, or cosmetological assays involving LCs.	Tissue viability Cytokines (IL-1, IL-6, IL-8)
Human reconstructed vaginal mucosa based on LCs, SiHa and NHVC	—	63	Study of the transepithelial route followed by pathogens. Safety evaluation of compounds applied on vaginal epithelium.	
Human reconstructed vaginal mucosa based on LC integrated within a pluristratified epithelium	—	65	Study of the early events in HIV transmission.	
3-D organotypic human vaginal epithelial cell model	—	66	Safety and efficacy of candidate microbicides.	
CellEstrous Species: rat The system models the vaginal epithelium during the oestrous cycle Commercially available	CELLnTEC	—	Studies on the oestrous cycle.	
VEC-100 Species: human Epithelial vaginal-ectocervical cells Commercially available	MatTek	—	Potential use for screening and assessment of the irritation, penetration, metabolism, or efficacy of active ingredients or final formulations for vaginal application.	
VLC-100 Species: human Epithelial tissue containing epithelial VEC and immunocompetent dendritic cells Commercially available, patented	MatTek	67	Toxicity studies of feminine hygiene, vaginal care, and microbicide products.	

3-D = three-dimensional; AP-1 = activator protein 1; ATCC = American Type Culture Collection; FT = full thickness; HIV-1 = human immunodeficiency virus-1; IgG = immunoglobulin G; IL = interleukin; IL-1ra = interleukin-1 receptor antagonist; IP-10 = interferon-inducible protein 10; LCs = Langerhans' cells; Lf = lactoferrin; MIP = macrophage inflammatory protein; MPO = myeloperoxidase; NF-κB = nuclear factor-κB; NHVC = normal human vaginal cells; PG = prostaglandin; RANTES = regulated upon activation, normal T-cell expressed and secreted; RHVE = reconstructed human vaginal epithelium; SLP1 = sphyngosine-1-phosphate lyase; TNF = tumour necrosis factor; VEC = vaginal-ectocervical cells; VEGF = vascular endothelial growth factor.

Table 4: ***continued***

b) Reconstructed tissues (continued)

System characteristics	Source of information: Company	Source of information: Ref.	Reported/potential use	Endpoints
VEC-100-FT Species: human Full thickness version of VEC-100 which includes epithelial cells and a fibroblast-containing lamina propria Commercially available	MatTek	68–71	Study of HIV-1 and other sexually transmitted infections.	Tissue viability Cytokines (IL-1, IL-6, IL-8)
VLC-100-FT Species: human Immuno-competent version of the VEC-100-FT which includes dendritic cells Commercially available, patented	MatTek	68–71	Study of HIV-1 and other sexually transmitted infections.	
RHVE Species: human Human vaginal epithelium reconstructed by using A431 cells derived from a vulval epidermoid carcinoma Commercially available	SkinEthic	—	Potential use for screening and assessment of the irritation, penetration, metabolism, or efficacy of active ingredients or final formulations for vaginal application.	
		72, 73	Studies on the expression and role of the *C. albicans* proteinases during infection and tissue damage of vaginal epithelium.	

c) Explants

System characteristics	Ref.	Reported/potential use	Endpoints
Human vaginal tissue	74–77	Safety evaluation and risk assessment of ingredients and finished products.	Tissue viability Cytokines Inflammatory mediators Infection
Porcine vaginal epithelium	79–82		

3-D = three-dimensional; AP-1 = activator protein 1; ATCC = American Type Culture Collection; FT = full thickness; HIV-1 = human immunodeficiency virus-1; IgG = immunoglobulin G; IL = interleukin; IL-1ra = interleukin-1 receptor antagonist; IP-10 = interferon-inducible protein 10; LCs = Langerhans' cells; Lf = lactoferrin; MIP = macrophage inflammatory protein; MPO = myeloperoxidase; NF-κB = nuclear factor-κB; NHVC = normal human vaginal cells; PG = prostaglandin; RANTES = regulated upon activation, normal T-cell expressed and secreted; RHVE = reconstructed human vaginal epithelium; SLP1 = sphyngosine-1-phosphate lyase; TNF = tumour necrosis factor; VEC = vaginal-ectocervical cells; VEGF = vascular endothelial growth factor.

These cell lines were also used to predict the mucosal toxicities of vaginal microbicidal contraceptives, based on cell viability and on cytokine release (Table 4a). The cells were grown to confluence and then incubated with the test materials for various times (from 10 minutes to 24 hours), after which the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed and/or the medium was collected for cytokine determination (17, 59). For example, a study by Fichorova *et al.* (59) with HPV16/E6E7-immortalised human vaginal (Vk2/E6E7) and endocervical (End1/E6E7) epithelial cell lines assessed interleukin (IL)-1, IL-6, and IL-8 release *in vitro* after treatment with four microbicide candidates (N-9, benzalkonium chloride, sodium dodecyl sulphate and sodium monolaurate). With regard to cytokine release after exposure to the microbicide candidates, the results obtained with the cell lines correlated well with those from vaginal secretions (i.e. cervico-vaginal lavages) collected during RVI tests. This implies that the measurement of cytokine release *in vitro* from several human vaginal-derived cell lines could provide important information concerning the pro-inflammatory potential and safety of various classes of spermicides and microbicides. However, these cell lines can be used in safety assessment studies by industry entities, only after licensing from Brigham and Women's Hospital, Inc., Office of Corporate Sponsored Research and Licensing in Boston, MA, USA.

Although the use of *in vitro* cell culture models avoids many of the ethical and regulatory issues that arise when working with humans and animals, such models do have technical limitations. For example, assays that use submerged monolayer cultures do not take into account the three-dimensional (3-D) differentiated structure of the vaginal epithelium. They lack the functional permeability barrier found in the epithelium, as well as other important cell types, such as dendritic cells and lymphocytes, which are also present *in vivo* (Table 5). In addition, different cell lines often differ in their sensitivities to the same agent (60). Other factors, such as differences in gene expression profiles between monolayer cultures and native vaginal tissue, and limitations on the testing of water insoluble products in a submerged cell culture environment, can ultimately limit their use in the study of vaginal irritation and toxicity.

Reconstructed tissues

Recent advances in tissue engineering and molecular and cellular biology have significantly contributed to the development of reliable 3-D tissue constructs for the safety and efficacy testing of pharmaceutical, personal care and cosmetic active ingredients and final formulations. The reconstructed tissues exhibit *in vivo*-like morphological and ultrastructural characteristics, and can be uniform in

Table 5: Some advantages and disadvantages of *in vitro* test systems used/potentially used for vaginal irritation assessment

In vitro system	Advantages	Disadvantages
Cell-based	**General** Relatively inexpensive Easy to grow	**General** No barrier function, no systemic component
Reconstructed tissue	**General** Organotypic morphology is relatively easy to achieve and is inexpensive	**General** 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
Tissue explants	**General** Tissue structures with full cell complement (epithelial, connective, immune) Better tolerance to formulations Parallel efficacy testing	**General** Limited number Variability IRB approval More technically demanding
	Specific Pig: resembles human structurally and, to some extent, functionally; easy to obtain and inexpensive; no regulatory considerations	**Specific** Human: not easy to obtain, limited age range, fresh tissue is a potential transfer of infectious agents; maintains viability for 12 hours after tissue resection Pig: no vascular system limits functional response

IRB = Institutional Review Board.

response from lot to lot, potentially providing a reproducible and consistent testing platform. To that end, it is worth pointing out that several commercially-available reconstructed human epidermis models have already been through a series of validation processes and have been shown to be valuable tools for assessing skin irritation (4–7, 61).

The microenvironment of the vaginal epithelium is very complex, and the macrophages, lymphocytes and LCs it contains are not easily included in the current reconstructed tissue models. However, several attempts have recently been made to resolve this issue. For example, Sivard *et al.* (62) made a significant advance by integrating LCs into an *in vitro* reconstructed vaginal mucosa (Table 4b). The reconstructed epithelium, consisting of 7–10 cell layers, was developed by using two cell types (human LCs and vaginal keratinocytes) and a sub-mucosa (a de-epidermised dermis). For the purpose of the experiments in the study, the model was cultured for 14 days. The model was proposed as a tool to study the role of LCs and other host factors in the physiopathology of HIV transmission in the female genital tract. In addition, this model might also be useful for studies of various mucosal infections (by viruses, bacteria, fungi, and parasites) and for toxicological or pharmacological assays involving LCs.

Cremel *et al.* (63) reported the incorporation of human LCs in a model based on the human vaginal epithelial cell line, SiHa, which displays numerous properties similar to those of normal human vaginal cells (NHVC) obtained from women undergoing routine hysterectomies (Table 4b). The addition of LCs to the SiHa multilayer demonstrated the ability of the immune system cells to integrate and remain viable inside the cell multilayer, in the same proportion as previously reported *in vivo,* for 96 hours (64). This could enhance the utility of the model for the evaluation of compounds that might influence the vaginal mucosal response, for studies on the transepithelial passage of pathogens, or for investigating epithelial cell–immune cell interactions through direct cell–cell contact (by using co-cultures).

Most recently, Bouschbacher *et al.* (65) reported an investigation on the early events in HIV transmission through a human reconstructed vaginal mucosa based on LCs integrated within a pluristratified epithelium. They proposed the model for use in the development of microbicidal products that prevent the entry of HIV into the genital tract.

A new 3-D tissue production system was recently introduced by Hjelm *et al.* (66). They reported the use of rotating-wall vessel bioreactor technology to generate a 3-D organotypic human vaginal epithelial cell model derived from the immortalised vaginal epithelial cell line, V191. The study showed that the 3-D vaginal cell aggregates were more resistant to N-9, as compared to the V191 monolayer culture. Cytokine analysis of the TNF-related apoptosis-inducing ligand (TRAIL) and IL-1α receptor antagonist (both markers of cervico-vaginal inflammation) showed that the dose-dependent production of these factors correlated to N-9 toxicity in the 3-D model. The authors concluded that the model could be used as a complementary tool for the safety and efficacy screening of microbicide candidate formulations.

The development of stratified, differentiated human vaginal epithelium cultures has the potential to overcome some of the disadvantages of cell monolayers, in that the former contain a barrier layer and permit the topical application of active ingredients and final formulations, including those that are not water-soluble. In addition, they avoid animal welfare and inter-species extrapolation issues, and the human reconstructed tissue models permit a distinction to be made between the very mild products to which animal models are insensitive (67). Several commercially available models are briefly described below and in Table 4b.

MatTek Corporation (Ashland, MA, USA) currently produces four vaginal epithelial models, all of which are multilayered, highly differentiated cultures that closely resemble native human tissues. They are based on normal, human-derived vaginal-ectocervical (VEC) epithelial cells and, in some cases, incorporate dendritic cells (DCs), as detailed in Table 4b. However, for the evaluation of the irritancy potential of ingredients and final formulations to vaginal tissue, it is likely that the addition of DCs or LCs to the model is not necessary. The VEC-100 model, shown in Figure 1f, is based on normal VEC epithelial cells, and contains differentiated basal, suprabasal, intermediate and superficial cell layers that are similar to those in *in vivo* tissues. The VEC models have been used in toxicity studies of feminine hygiene, vaginal care and microbicide products (67), and in research focused on HIV-1 and other sexually transmitted infections (68–70). In a recent study, Ayehunie *et al.* (71) tested six materials in two different VEC models and the RVI test. N-9 was found to be irritant in both the *in vitro* and the *in vivo* systems, at the same concentration. Benzalkonium chloride, known to be irritating to humans at 2% w/v (59), was predicted to be irritant by the VEC model at 0.125%, but not by the RVI test, indicating that the tissue model may be more sensitive than the RVI assay. Povidone–iodine was also predicted to be irritant by the VEC models, but not by the RVI assay, at the highest dose used in the assay (i.e. 20% w/v), while three other substances were negative in both assays. The fact that the VEC models, identified the single irritant material similarly identified in the RVI test seems encouraging, but more work will need to be done to determine whether the reconstructed tissue models have sufficient specificity.

The study by Ayehunie *et al.* (71) also introduced the possibility of employing biomarkers for inflammation and irritation in conjunction with the VEC models. The authors suggested that, among the cytokines analysed in their study, a combination of IL-1α, IL-1β and IL-8 release patterns may be useful as biomarkers for the hazard identification of topically-applied vaginal chemical formulations.

SkinEthic (Nice, France) markets a reconstructed human vaginal epithelium (RHVE; Figure 1g) based on the vulval epidermoid carcinoma cell line, A431. The model has been used for the safety and efficacy testing of topically-applied gynaecological compounds and products, particularly for studies on vaginal permeability and metabolism, bacterial and viral adhesion screening for antibiotics and antiviral compounds, and for products used in the treatment of vaginal candidosis (72, 73). No publications reporting on direct comparisons between irritation in the RHVE model and either the rabbit model or human clinical studies could be found.

CELLnTEC (Bern, Switzerland) provides a rat *in vitro* reconstructed system (CellEstrous), which is reported to model the complex and highly dynamic structure of the rat vaginal epithelium during the oestrous cycle. So far, there have been no reports on the use of this model in vaginal irritation studies.

In summary, the use of 3-D vaginal *in vitro* models shows some promising early results, which 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 (Table 5). However, many more substances will have to be evaluated. Although some level of animal testing may still be required to monitor systemic toxicological effects, it is anticipated that *in vitro* vaginal tissue models will play an increasingly important role in the initial screening of new products and formulations for their irritant effects.

Explants

— *Human explants*: Human cervical tissue explants have mostly been used for research into mechanisms of the early events in HIV infection (74), and as a bridge between the preclinical and clinical phase of microbicide candidate evaluation (75). Fresh cervical explants are usually obtained from women undergoing planned therapeutic hysterectomies. Several companies and repositories offer explants of human tissues: Asterand (Detroit, MI, USA) has a worldwide network of hospitals that provide fresh samples for research institutes and industry partners, and the US National Disease Research Interchange (NDRI; Philadelphia, PA, USA) also provides fresh tissues, upon request, to partners for use in research programmes. Although these tissue explants are available for research purposes, it is doubtful that sufficient tissue would be available for large-scale toxicity screening programmes.

Studies with human explants usually start by assessing tissue morphology, cellular distribution and permeability (76), which are relatively insensitive parameters of the suitability of the tissues for experimental studies. More important is the viability of the explants, which is usually assessed by the reduction of MTT, a method that is also employed to determine the toxicity of topically-applied microbicides or other formulations. The effect of each test material on tissue viability is determined by comparing the viability of treated explants to that of untreated controls.

A relatively-recent report described the use of previously frozen cervical tissues to test the antiviral activity of microbicides (77). The study showed that frozen-thawed cervical explants could provide an alternative model to screen topically-applied microbicide candidates for their ability to block sexual transmission of HIV-1. However, it is unlikely that the frozen-thawed tissue would retain sufficient viability to be useful for vaginal irritation studies.

A study by Richardson-Harman *et al.* (75) showed that the fresh human cervical explant model can produce reasonably consistent results (in intra-laboratory and inter-laboratory comparisons, $n = 5$), when used for microbicide testing. However, there are many issues to be addressed, if this explant system is to be optimised for routine use in consumer product safety studies. Anderson *et al.* (78) presented a thorough overview of the most common issues that can confound data interpretation between laboratories (such as unknown clinical history and varying hormonal status of the tissues), when human cervical explants are used for HIV and microbicide research. Although not all of these variables would affect product safety studies to the same extent that they would affect efficacy studies, it is likely that considerably more standardisation would be needed before explant tissues could be routinely used for regulatory purposes.

An additional confounding aspect is that, in general, human explants need to be used within 12 hours of resection, the period during which mucosal tissue remains vital and retains its barrier function (79; Table 5). This can be a serious technical challenge due to the shipping time involved and stress applied to the tissues, which can affect their integrity and responsiveness to test materials applied topically.

In conclusion, more work is needed in order to standardise the protocol for the use of human cervical explants for the safety and efficacy assess-

ment of microbicides or other topically-applied test materials intended for human use.

— *Porcine explants*: Porcine vaginal mucosa could be a useful *in vitro* alternative for the safety screening of personal care and cosmetic products, although *in vitro* studies with porcine explants do not overcome all the scientific concerns associated with animal testing. As previously mentioned, human and porcine vaginal tissues share important similarities, e.g. both types of mucosa consist of stratified squamous epithelium that is supported by connective tissue (Figures 1a and 1d). Structurally, the porcine vaginal epithelium resembles that of the human, including the organisation of the membrane-coated granules and intercellular lipid lamellae that make up the permeability barrier (80, 81). The tissue should be relatively easy for many laboratories to procure, since at least two companies, Sioux-Preme Pork Products (Sioux Center, IA, USA) and Pel-Freez (Rogers, AR, USA), provide fresh porcine tissue for biological studies (Table 4c), which is obtained from animals used for food production.

Data on the lipid composition of vaginal epithelium from pigs and humans indicate similar concentrations of lipids, including ceramides, glucosyl ceramides and cholesterol, which are key permeability barrier components (79). However, the study published by van Eyk *et al.* (82) showed that the permeability of explants of human and porcine vaginal mucosal tissues differed for several test substances. For example, when testing hydrophilic molecules, such as water or vasopressin, the porcine vaginal mucosa was an accurate *in vitro* permeability model of human tissue, but for molecules that were more lipid-soluble, such as oxytocin, the flux through porcine vaginal mucosa was 53% higher than the corresponding estimated value through the human vaginal mucosa. As with human explants, it is very likely that limitations of tissue viability to within the first few hours after resection will also be applicable to porcine explants. In conclusion, the use of porcine tissue is promising, but has limitations for accurately modelling human vaginal absorption.

Validation Efforts

If the routine use of *in vitro* models for vaginal irritation testing is to become a reality, then test methods must be standardised. Recently, a few small studies have been performed to address this need. For example, Beer *et al.* (83) reported on comparisons of intra-assay, inter-assay, and inter-laboratory reproducibility for N-9 toxicity and efficacy testing, when using 11 different cell lines, four primary cell types and vaginal explant tissue. In the authors' opinion, the *in vitro* toxicity data for both the tissue and cell lines were broadly consistent between laboratories (75% of the assays showed good inter-assay reproducibility), and with the outcome of phase III clinical trials. Richardson-Harman *et al.* (74) have also reported on the reproducibility of microbicide activity assays conducted in human primary explants from several different types of tissue. They found good inter-laboratory reproducibility by using a novel endpoint, a statistical evaluation of several parameters, which provided a single objective measurement of virus growth. A similar study design, i.e. employing a toxicity measure as the endpoint, would be helpful in standardising product safety studies. Intrinsically, 3-D models should be more reproducible than human biopsy materials, but only limited multi-laboratory studies of this type have been performed, and more are clearly necessary to move any of the proposed alternative vaginal irritation assays closer to validation.

It is hoped that the manufacturers of 3-D reconstructed vaginal tissue models, in collaboration with other interested parties, could begin efforts toward: a) establishing the inter-laboratory reproducibility of their models for the assessment of irritation potential, and b) providing more information on comparisons between existing RVI and human data and the results obtained by using the tissue models. This would be a significant step forward toward establishing a validated alternative method for vaginal irritation (71). Similar strategies to those used in the Beer *et al.* (83) study could be extended to the testing of personal care and cosmetic products intended to come into contact with the vaginal epithelium, in the quest of validating a protocol for such uses.

Conclusions

The need to understand the irritating effects of therapeutic agents and of cosmetic and personal care products on genital mucosae have led to the development of a variety of models that involve the use of animals, cell and organ cultures, and isolated tissues. Although the rabbit model has been predominantly relied on to provide this information, current scientific, ethical, economical and legal concerns are driving a search for alternative models. In this review, we have provided an update of the current knowledge regarding the different methods used for vaginal irritation assessment, their relative advantages and drawbacks, and their applications. The *in vivo* test based on the rabbit remains, to date, the only model recommended by the US FDA for the safety evaluation of vaginal products (2). However, the pig model is considered appropriate for studies on the inflammatory processes related to irritation induced by drugs or personal care and feminine-hygiene prod-

ucts. Among other animal test systems, studies with the SMI assay (a potential refinement) showed that the data obtained by using this alternative method correlated well with those produced with the classical RVI test.

The *in vitro* methods currently available have not yet been shown to fully reproduce many of the specific features of the human vagina. Early *in vitro* methods focused on monolayer cultures of human vaginal or cervical cell lines (sometimes transformed), and outgrowths from primary vaginal tissue explants. These monolayer models lack the complex 3-D structure and the variety of cell types present within the vaginal epithelium. In addition, it is well established that cells in monolayer culture generally do not exhibit the same differentiated state as cells in their normal 3-D milieu. Understandably, the field has moved toward alternative *in vitro* methods with a better predictive power of the human responses to certain classes of products designed for vaginal use. The 3-D models constructed from explanted tissue or grown from human cell strains have the potential to more-closely model the human vaginal epithelium than do the *in vivo* animal models. Several of these models are now commercially available, which represents a significant advantage, since the commercial cultures tend to be much more reproducible — an attribute that is almost mandatory when a long-term toxicity evaluation programme is being considered.

One very significant driver for developing alternatives to animal testing is the 7th Amendment to the EU Cosmetics Directive. This legislation significantly limits the use of animal testing for determining the safety of cosmetic products and their ingredients. Thus, the 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 is proven reliable for predicting vaginal irritation, a human clinical study would be the only way to address the vaginal irritation potential of a cosmetic final product. In the EU, the European Centre for the Validation of Alternative Methods (ECVAM) has the responsibility for coordinating the validation of alternative test methods and to promote their use. It seems reasonable that ECVAM needs to add the development of *in vitro* vaginal irritation models to its list of priorities.

Based on the knowledge thus far gained regarding the current status of vaginal irritation models and the available alternative and *in vitro* tests outlined herein, a testing strategy could be structured as follows. Cell-based models, especially those that use cells from the genital tract, could be used as first-line screening tests to eliminate candidates that are significantly cytotoxic and/or cause the release of known biomarkers of inflammation. Explant-based or reconstructed tissue models, could provide the next screening (or in some cases, definitive) step, critical for the assessment of preliminary formulations. Finally, until *in vitro* methods become accepted by regulatory agencies, animal-based models that are suitable for assessing full-strength formulations would have to be used, to provide information on whole-organ response. Some gaps in this proposed testing strategy hinder the efficient development of microbicide candidates or the high-throughput screening of personal care and cosmetic products. This situation warrants further research with respect to the validation of existing models and biomarkers, and the identification of new biomarkers and models of microbicide and personal care (cosmetic)-induced mucosal alterations that correlate with relevant *in vivo* properties. The development of tissue-engineered models of vaginal mucosal disease(s) will also contribute to a better understanding of disease processes and the discovery of new treatments, a particularly important task for the pharmaceutical industry.

In summary, we would like to emphasise that, at the moment, no alternative method has been validated and/or accepted by US or European regulatory agencies for vaginal irritation assessment. Because of earlier reports on the correlation between rabbits and humans with respect to the irritation potential of vaginal formulations, the rabbit remains the recommended model for the safety evaluation of vaginal products. Therefore, this review calls for combined efforts from all parties involved in supporting alternative methods, like those detailed herein, to work together toward the validation of *in vitro* methods that can be accepted by regulatory agencies for the vaginal irritation testing of pharmaceutical, personal care and cosmetic products.

The efforts of animal protection organisations, combined with academic research and industry support, can lead to efficient, reproducible and predictive models that will benefit all parties involved in the challenge of reducing animal testing and in finding appropriate testing techniques that can accurately predict the effects of vaginal products during the research and development process.

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