Research Article

Testing Vaginal Irritation with the Hen’s Egg Test-Chorioallantoic Membrane Assay

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Summary

The HET-CAM (Hen’s Egg Test-Chorioallantoic Membrane) assay is an in vitro alternative to the in vivo Draize Rabbit Eye test that mimics vascular changes in the chorioallantoic membrane. This qualitative method assesses the irritancy potential of chemicals. The CAM responds to injury with an inflammatory process similar to that in the rabbit eye’s conjunctival tissue. Regarding topical toxicity assessment of medical devices, ISO 10993-10 states that any skin or eye irritant material shall be directly labelled as a potential vaginal irritant without animal testing, suggesting that the irritancy potential of the eye and the vaginal epithelia is similar. The aim of this work was to apply the HET-CAM assay to test the irritancy potential of vaginal formulations. Vaginal semisolid medicines and lubricants currently marketed were tested along with the Universal Placebo formulation that has been shown to be clinically safe. Nonoxynol-9 (N-9), a known vaginal irritant, was enrolled as positive control (concentrations ranging from 0.001 to 100% (v/v)). The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 – 2010). Formulations were then classified according to irritation score (IS), using the analysis methods (A) and (B). The studied vaginal formulations showed low potential for irritation. N-9 was classified as a severe irritant at concentrations above 2%, which corroborates clinical data, envisaging a possible in vitro/in vivo correlation. IS (B) was considered a better classification output. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for vaginal irritancy potential in vitro studies.

Key words: vaginal irritation, HET-CAM, in vitro

1 Introduction

Topical toxicity has been a main topic of work within the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM). Having also a regulatory responsibility, the EU and associated laboratories, have been working on developing and validating toxicity test methods for eye irritation, phototoxicity, skin corrosion, irritation and sensitization. Furthermore, several in vitro Test Guidelines are already provided or being developed by the Organisation for Economic Co-operation and Development (OECD). In vitro studies have been valued and recommended as a first screening for toxicity evaluation, in detriment to in vivo models.

The assessment of the irritancy and toxicity of products for vaginal administration does not yet include in vitro methods validated and recognized by both the referred authorities. Currently, there are numerous medicines and medical devices marketed or under development for vaginal administration (Woolfson et al., 2000; Alexander et al., 2004; Hussain and Ahsan, 2005; Choudhury et al., 2011). Regarding topical toxicity assessment of medical devices, the ISO 10993-10 states that any skin or eye irritant material shall be directly labelled as a potential vaginal irritant without animal testing, suggesting that the irritation potential for the eye and the vaginal epithelia is similar (ISO 10993-10, 2010). The HET-CAM assay is already widely applied to ocular products, yet its application for the testing of vaginal formulations is an innovative proposal.

As a general assumption, maximum effectiveness with the least amount of adverse effects is always desired for vaginal formulations. Since these products are mostly intended for self-administration and aim to offer maximum comfort both at the moment of application and during the time of usage, the evaluation of effects such as local irritation become especially important. One of the methods most used to assess vaginal irritation is performed in vivo on rabbits (RVI) (OECD,
2012; Eckstein et al., 1969). However, the European Commission has undertaken several regulatory decisions in pursuance of the 3Rs policy (Replacement, Reduction, Refinement) such as the approval of the REACH (Registration, Evaluation and Authorization of Chemicals) legislation (applicable to the fields of raw materials, drug products and medical devices, for example) and the prohibition of the marketing of cosmetic products tested in animals (EC, 2003). The HET-CAM assay is one of the alternatives presented by EURL-ECVAM1 for the in vitro study of ocular irritancy (ICCVAM, 2007). Although various techniques for the characterization of vaginal formulations have been described in the literature (Garg et al., 2001, 2010; Adriaens and Remon, 2008; Cunha et al., 2014), most have been applied only to developing formulations with no correlation with in vivo results. The HET-CAM assay outcome consists on an Irritation Score (IS) that is a value calculated by different analysis methods (A and B), which is used to classify the irritancy potential of a test substance. The IS (A) analysis method takes into account the observation of endpoints at specified time points after application of the test substance (0.5, 2, and 5 min post exposure). At the time points, the presence of an endpoint is determined and a score assigned, in case it is present. The scores are calculated to yield an overall irritation score. Instead, when applying the IS (B) analysis method, the endpoints are monitored over the entire observation period after applying the test substance (typically 5 minutes). The time (in seconds) when an endpoint develops is registered and these values are used to yield an overall irritation score using a mathematical formula (U.S. Public Health Service, Department of Health and Human Services, 2006).

The aim of this study was to evaluate the possibility of using the HET-CAM assay as an in vitro alternative method to the in vivo vaginal irritation test. Vaginal semisolid medicines and lubricants were examined for their irritation potential considering two scoring categories, IS (A) and IS (B). Additionally, nonoxynol-9, a well-known vaginal irritant substance, was studied from concentrations ranging from 0.001 to 100% (v/v).

2 Materials and Methods

2.1 Chemicals and testing products

For the preparation of the assay controls, the following chemicals were used: sodium chloride (NaCl, JT Baker, USA), sodium dodecyl sulphate (SDS, Acros Organics, Belgium), nonoxynol-9/tergitol (N-9, Sigma, Germany), sodium hydroxide (NaOH, VWR Prolabo, Germany) and type I water (obtained in house through a Millipore System, Merck, USA).

The products included in this study were vaginal semisolids, available in the international market, intended both as therapeutics for several pathological conditions (drug products), and as lubricants for sexual and menopausal discomfort. The ten different therapeutic products were: Gino-Canesten® (Bayer), Sertopic® (Ferrer), Dermofix® (Azevedos Laboratories), Gyno-pevary® (Johnson & Johnson), Lomexin® (Jaba Recordati), Gino Travogen® (Bayer), Dalacin® (Eustapharm), Blissel® (ITF Medivida), Colpotrophine® (Teva Pharma). The lubricants tested were: Fillergyn® gel (BSDPharma), Gelofit® Classic gel (Laboratoires Effik), GelSea® gel (LDPISA), Ginix® gel (ISUS), Ginix® Plus gel (ISUS), Hyalo Gyn® gel (Fidia Farmaceutici), K-Y® Jelly (Johnson & Johnson), Phyto Soya® gel (Arkopharma Laboratoires Pharmaceutiques), Velastisa® Intim VG moisturizer gel cream (ISDIN) and Vidermina® gel (Istituto Ganassini). Both Replens® (Laboratoires Majorelle) and Universal Placebo (Tien et al., 2005) were used as controls, since their toxicity profiles are largely described on the literature (Nachtigall, 1994; Bygdeman and Swahn, 1996; Tien et al., 2005; Valenta, 2005; Schwartz et al., 2007; Adriaens and Remon, 2008; Acartürk, 2009; Garg et al., 2010; Clark et al., 2011; Caramella et al., 2015). Universal Placebo was prepared by dissolving 2.7g of Hydroxyethyl-cellulose (2000P) in 96.3g of water containing 0.85g of sodium chloride and 0.1g of sorbic acid. The final pH was adjusted to 4.4 by adding sodium hydroxide, and the gel was stored at 2–8°C. To evaluate the method sensitivity to vaginal irritants, nonoxynol-9 was used in concentrations ranging from 0.001 to 100% (v/v).

2.2 Eggs and incubation conditions

The test system consists on Fertile White Leghorn chicken eggs, fresh (not more than 7 days old), clean, weighing 45 to 65 g. Upon arrival to the lab, they were checked for damages in the shell: damaged eggs were discarded while the others were incubated, at 37.8±0.3°C in an atmosphere with a relative humidity of 58±2% and under automatic rotation, during 8 days (Corti AF-50 and Copele 30652, Spain). By the eighth day, eggs were inspected using a LED light to confirm the embryo formation. Non-embryonated eggs were rejected and the others were incubated for one more day in the same conditions but without egg rotation.

2.3 HET-CAM assay

The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 – 2010). At day 9, the eggs were taken out of incubator and were placed on an appropriate support with the larger part facing up. The shell was opened with the help of a scalpel and tweezers and the chorioallantoic membrane (CAM) was exposed. This membrane was then hydrated with NaCl 0.9% (w/v) for a maximum of 30 min. Afterwards, the solution was aspirated and the membrane was peeled off without damaging the blood vessels. For each product 0.3 mL were applied on the membrane and 3 eggs per products were used. Considering that all samples were semisolids, they were applied over the CAM covering around 50% of it, as defined by the protocol. The irritant effect of these products was evaluated on the remaining part of the CAM (except for non-opaque formulations which allowed the observation of the whole CAM), by monitoring the appearance of three endpoints, for 5 minutes: hemorrhage (vessel bleeding), lysis (vessels disintegration) and coagulation (protein denaturation intra and extra- vascular). In the present study, these endpoints were evaluated accordingly to two different analysis methods: Irritation Score (IS) A and B. While for criteria A the endpoints were checked at predetermined time intervals (0.5; 2 and 5 min), for criteria B these effects were monitored continuously during 5 minutes, and the time when the irritant response began was registered. This methodological difference conducts to different range of categories in the irritant

outcome (see Fehler! Verweisquelle konnte nicht gefunden werden.). Photographs were taken at the beginning and at the end of the assays. Calculation of the IS for each test product is represented as mean ± standard deviation (SD) of a total of three eggs.

**Tab. 1: Irritancy classification**

Classification on the *in vitro* HET-CAM assay concerning Irritation Score (IS) analysis methods A and B

<table>
<thead>
<tr>
<th>Analysis method A</th>
<th>Analysis method B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Irritation response</strong></td>
<td><strong>IS calculation method</strong></td>
</tr>
<tr>
<td>0 to 8: Non-severe irritant</td>
<td>Calculation of the IS is the sum of the scores attributed at each time point to the arising of the corresponding effect, as stated on the following scheme:</td>
</tr>
<tr>
<td>9 to 21: Severe Irritant</td>
<td>Endpoint</td>
</tr>
<tr>
<td>Lysis</td>
<td>5</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>7</td>
</tr>
<tr>
<td>Coagulation</td>
<td>9</td>
</tr>
<tr>
<td>0 to 0.9: Non-irritant</td>
<td>0 to 0.9: Non-irritant</td>
</tr>
<tr>
<td>1 to 4.9: Slight Irritant</td>
<td>1 to 4.9: Slight Irritant</td>
</tr>
<tr>
<td>5 to 8.9: Moderate Irritant</td>
<td>5 to 8.9: Moderate Irritant</td>
</tr>
<tr>
<td>9 to 21: Severe Irritant</td>
<td>9 to 21: Severe Irritant</td>
</tr>
</tbody>
</table>

Calculation of the IS by applying the following equation:

\[
\text{IS} = \left( \frac{301 - \text{Lysis time}}{300} \times 5 \right) + \left( \frac{301 - \text{Hemorrhage time}}{300} \times 7 \right) + \left( \frac{301 - \text{Coagulation time}}{300} \times 9 \right)
\]

Times should be replaced by the time (in seconds) at which each effect started.

### 3 Results

The irritation potential determined for N-9 using the HET-CAM assay was reasonably comparable when using both evaluation tools, IS (A) and (B), as can be observed in Fig. 1. Only at the concentration of 0.5% (v/v) the difference between these two testing criteria results was statistically different (two way-ANOVA, *p* < 0.05, Sidak’s multiple comparisons test), although it shall be noted that standard deviations were relatively high. This concentration seems to be the one that presents a borderline-type behavior, since its score (on criteria B) is in the limit between slight and moderate irritant. The same might be happening for concentration 1% which is in between the moderate and severe irritant categories, on the IS (B) scale. It can be also observed that concentrations above 2% corresponded to IS values higher than 9, meaning that at these concentrations N-9 exhibits severe irritation effect (IS (A) N-9 2% = 10.0±0; IS (B) N-9 2% = 10.8±0). Taking into consideration the IS (B) criteria, concentrations between 0.3 and 1% were classified as moderately irritants (IS (B) N-9 0.3% = 4.8±0.5; IS (B) N-9 1% = 8.7±0.4). Concentrations between 0.01 and 0.2% were regarded as non-irritants, having scores of 0.9±0.8 and 1.1±1.0, respectively. In what concerns to the IS (A) criteria, the non-severe irritant response was found for concentrations ranging from 0.001 and 1 with scores of 0.7±0.6 and 7.3±1.2, respectively. These results show that the most irritant N-9 concentration is found independently of the scale being used. Nonetheless, the IS (B) is able to discriminate the irritation response of a range of N-9 concentrations since it considers more irritation categories when compared to IS (A). Interestingly, a reduction of both scores (although more pronounced for IS(A)) was observed when N-9 was tested undiluted. This result may be due to the reduction of interactions between the molecules of this non-ionic surfactant and the membrane, in the absence of water, consequently reducing the irritant effect in the experimental setup.

![Fig. 1: Irritation Scores (IS) for N-9 (nonoxynol-9) according to the analysis methods A and B.](image)
Results are presented as mean values ± standard deviation (SD), n=3. * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, p < 0.05, Sidak’s multiple comparisons test).

The irritation scores determined for the vaginal formulations are represented on Fig. 2. Concerning the therapeutic products Fig. 2 (a), Universal Placebo, Replens®, Dermofix®, Sertopic®, Dalacin V®, Ovestin® and Blisrel® did not conduct to any irritant response, having been scored with 0±0. Gyno Pevaryl®, Gino Canesten® and Colpotrophine® had significantly different scores when evaluated with the two criteria. Concerning criteria A, they were all classified as non-severe irritants. However, when the criteria B was used, Gino Canesten®, Colpotrophine®, Gyno Pevaryl® were classified as slight irritants IS (B) Gino Canesten®= 3.3±0.3; IS (B) Colpotrophine®= 2.0±0.0; IS (B) Gyno Pevaryl®= 4.8±2.0. All except Gino Travogen®, had scores higher in scale B than on scale A IS (A) Gino Travogen®= 3.0±0.0; IS (B) Gino Travogen®= 2.0±0.6), although this difference was not statistically supported.

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Regarding the vaginal lubricants (Fig. 2 (b)), only Phyto Soya® had a score of zero. Hyalo Gyn®, Velastisa VG® and Gelsec®, which showed no significant difference when comparing both scales, also obtained the least irritant scores. The remaining products had significant differences between the evaluation through IS (A) and (B). The remaining products had significant differences between the evaluation of IS (A) and (B). All lubricants were classified as non-severe irritants by the IS (A) irritation criteria with Ginix® reaching the highest score in this scale (3.7±1.2). When the IS (B) was applied Hyalo Gyn® and Velastisa VG® were classified as non-irritants and all the others were classified as slight irritants. For all but Ginix®, the score obtained in scale B was higher than the one obtained in A. As it can be seen on Fig. 2 (b), when comparing both scales, they also obtained the least irritant scores. The remaining products had significant differences between the evaluation of IS (A) and (B). Fehler! Verweisquelle konnte nicht gefunden werden. details the score obtained and the classification attributed to all products tested in this study. Furthermore, a picture from the test end-time (5 min) is also shown for products and controls. As it can be observed, the IS (B) analysis method allowed to differentiate irritancy responses among the tested products (non-irritant or slight irritant) although they all fit the "non-severe irritant" classification of IS (A). In the case of medicines, Gyno Pevaryl®, Gino Canesten®, Lomexin®, Gino Travogen® and Colpotrophine®, were the ones to present a classification of slight irritants on IS (B) and non-severe irritants on IS (A). Regarding lubricants, KY Jelly®, Vidermina®, Ginix Plus®, Geliofil® and Fillergyn®, presented this behavior.

Fig. 2: Irritation Scores (IS) for therapeutic vaginal products (a) and vaginal lubricants (b) according to the analysis methods A and B
Results are presented as mean values ± standard deviation (SD), n=3. * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, p < 0.05, Sidak’s multiple comparisons test).

**Tab. 2: Irritation potential**
Irritation scores, IS (A) and (B) determined for vaginal semisolid medicines and lubricants
### Medicines

<table>
<thead>
<tr>
<th>Product</th>
<th>IS (A)</th>
<th>IS (B)</th>
<th>Product</th>
<th>IS (A)</th>
<th>IS (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Universal</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>KY Jelly®</td>
<td>Non-severe irritant (3)</td>
<td>Slight irritant (4.9)</td>
</tr>
<tr>
<td>Replens®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>Vidermina®</td>
<td>Non-severe irritant (3)</td>
<td>Slight irritant (4.7)</td>
</tr>
<tr>
<td>Dermofix®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>Hyalo Gyn®</td>
<td>Non-severe irritant (0.7)</td>
<td>Non-severe irritant (0.3)</td>
</tr>
<tr>
<td>Sertopic®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>Velastisa VG®</td>
<td>Non-severe irritant (0.3)</td>
<td>Non-severe irritant (0.1)</td>
</tr>
<tr>
<td>Gyno Pevaryl®</td>
<td>Non-severe irritant (2.3)</td>
<td>Slight irritant (4.5)</td>
<td>Ginix plus®</td>
<td>Non-severe irritant (3)</td>
<td>Slight irritant (4.3)</td>
</tr>
<tr>
<td>Dalacin V®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>Ginix®</td>
<td>Non-severe irritant (3.7)</td>
<td>Slight irritant (1.7)</td>
</tr>
<tr>
<td>Gino Canesten®</td>
<td>Non-severe irritant (1)</td>
<td>Slight irritant (3.3)</td>
<td>Geliofil®</td>
<td>Non-severe irritant (3)</td>
<td>Slight irritant (4.5)</td>
</tr>
<tr>
<td>Ovestin®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td>Gelsea®</td>
<td>Non-severe irritant (0.3)</td>
<td>Slight irritant (0.7)</td>
</tr>
<tr>
<td>Lomexin®</td>
<td>Non-severe irritant (1)</td>
<td>Slight irritant (2)</td>
<td>Phyto soya®</td>
<td>Non-severe irritant (0)</td>
<td>Slight irritant (0)</td>
</tr>
<tr>
<td>Gino Travogen®</td>
<td>Non-severe irritant (2)</td>
<td>Slight irritant (3)</td>
<td>Fillergyn®</td>
<td>Non-severe irritant (1)</td>
<td>Slight irritant (2.6)</td>
</tr>
<tr>
<td>Blissel®</td>
<td>Non-severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colpotrophine®</td>
<td>Non-severe irritant (1)</td>
<td>Slight irritant (3.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Assay controls

<table>
<thead>
<tr>
<th>Product</th>
<th>IS (A)</th>
<th>IS (B)</th>
<th>Product</th>
<th>IS (A)</th>
<th>IS (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-9 2% (w/v) (Positive control)</td>
<td>Severe irritant (10)</td>
<td>Severe irritant (10.8)</td>
<td>NaOH 0.1N (Positive control)</td>
<td>Severe irritant (19)</td>
<td>Severe irritant (19)</td>
</tr>
<tr>
<td>SDS 1% (w/v) (Positive control)</td>
<td>Severe irritant (10)</td>
<td>Severe irritant (10)</td>
<td>NaCl 0.9% (w/v) (Negative control)</td>
<td>Severe irritant (0)</td>
<td>Non-severe irritant (0)</td>
</tr>
</tbody>
</table>
Nowadays, the increasing number of new chemicals and products introduced into the market coming from several industries (cosmetic, pharmaceutical and medical devices) has generated a need to validate in vitro techniques able to screen potential irritation effects at the early stages of development.

The Hen’s Egg Test, or Hühner-Embryonen-Test (HET), firstly presented by Luepke, back in the 80’s, was developed to be a rapid, sensitive and inexpensive toxicity test able to provide information on embryotoxicity, teratogenicity, systemic, metabolic and immunopathological effects (Luepke, 1985). This method was designed to be applied in mucocutaneous membrane irritation testing, and the tool provided for score and classification was analogous to the Draize test. Since then, it has been confirmed that a good correlation between these two tests, i.e. in vitro/in vivo correlation, for plenty of chemicals does exist. The HET-CAM assay cannot totally replace the irritation tests in mammals, but can largely decrease the number of animals used, and limit the pain and injury inflicted to animals during experiments (Luepke, 1985). Later on, in the 90’s, Spielmann and Steiling have contributed to enlarge the application of this method by adapting it to be able to classify eye irritants. The procedure was published through the protocol number 47 from INVITTOX (Spielmann, 1992; Steiling et al., 1999).

The application of the HET-CAM assay to vaginal irritation testing comes in line with the referred compliance with the 3R’s policy. In the vaginal products field, there is no organotypic irritation test approved neither on the EU, nor the USA. In fact, there are some cellular and tissue models available for toxicity testing, however mainly comprise techniques for specific metabolic pathways, histological analysis and inflammatory response (Fichorova et al., 2004; Repetto et al., 2008; Gali et al., 2010; Costin et al., 2011). Our research group is focused in developing strategies for preclinical safety characterization of vaginal products using cellular and ex vivo tissues assays (paper submitted for publication). The usage of the HET-CAM assay for vaginal products testing comes to widen the safety assessment portfolio that can be applied to test substances or products in a quicker and more effective way on the first step of preclinical safety testing. In this study, several semisolid vaginal medicines and lubricant products were tested. Moreover, two analysis methods for the IS calculation were applied. It was concluded that when testing N-9, a pure substance, there was no statistical difference when applying one method or another. Actually, the concentration found to be the one that could trigger severe irritant effects, 2% (w/v), was the same that was shown to generate severe toxicity in clinical trials, being less safe than thought at preclinical evaluations (Van Damme et al., 2002). In that case, N-9 was being studied as a spermicide, but, because of its surfactant nature, it ended up being irritant, and even promoting the transmission of HIV infection (Stafford et al., 1998; Phillips et al., 2000; Dayal et al., 2003). Having this episode in mind, the need for more appropriate in vitro assays to predict in vivo safety issues, is even more highlighted. In our study, the surfactant nature of N-9 may explain the fact that the abrupt increase of its concentration did not result in a higher rate of irritation was observed (U.S. Public Health Service, Department of Health and Human Services, 2006). In this study a different behavior was observed when this substance was tested undiluted although it was expected to have a high score. In fact, reported data from a Draize test with undiluted N-9 indicate that this is severely irritating to the eyes of rabbits2. This result highlights the fact that, although most tests are performed with undiluted substances to assess their worst possible effect, it shall be considered that depending on the mechanism of action of each substance, some degree of dilution may be important (especially when the substance is intended to be use diluted) so that the response in the (dry) CAM may be similar to that observed in the moistened real environment. Universal placebo, on the other hand, was herein included because it is a vaginal gel that was wittingly designed to be a control formulation in clinical trials of vaginal microbicdes (Tien et al., 2005; Schwartz et al., 2007). Its safety profile, already confirmed by clinical trials, was once more confirmed in this study, having an IS of zero on the two scoring grids (A and B).

Concerning semisolid vaginal medicines, no severe irritant responses were observed, and this outcome was already expected. They are already commercialized and since they are classified by the competent authorities as medicines, they were not only subjected to preclinical evaluations, but also to extensive clinical trials. On the products’ available literature (Summary of Product Characteristics) provided by Pharmaceutical Companies to Health Professionals, all these vaginal products have been associated with adverse effects related to irritancy in clinical trials or post-marketing surveillance, although the frequency reported for these effects is generally either rare or unknown. Moreover, the products herein classified as non-severe irritants (IS (A)) and slight to moderate irritants (IS (B)), such as Gyno Pevary®, Gino Canesten®, Colpotrophine®, Gino Travogen® and Lomexin® were also tested by our research group using two in vitro cytotoxicity tests: 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) into uterine (HEC-1A), cervical (HeLa) and vaginal (VK2 E6/E7) cell lines; and, on an ex vivo porcine vaginal model using the MTT reduction assay and histological analysis; having also demonstrated toxicity profiles higher than the controls which can be due to the drug composition of these products. In fact, Gino Canesten® had one major impact on the vaginal ex vivo epithelia when histological analysis was performed (paper submitted for publication). Having this in mind, and concerning our cytotoxicity results, it was also herein demonstrated that these products could be considered borderline products, which means that some degree of irritancy could be revealed by the IS (B) method. Moreover, Gino Travogen® and Colpotrophine®, were also the ones that presented higher toxicities on a toxicity ex vivo model developed by our research group. These results, however, do not mean that these products are not adequate for the purpose of use since it should be kept in mind that most of the drug products are prescribed for short term use and this factor is obviously important for their benefit/risk assessment. This data coincidence might corroborate the need to perform several in vitro tests for a complete toxicity evaluation, since the combined results might be stronger predictors for in vivo toxicity, as the current mind-set in toxicology states (Samuel et al., 2016). Furthermore, some other technological parameters have also been shown to be associated to the safety of vaginal products. Osmolarity, in particular, it is considered a predictor for irritation (Adriaens and Remon, 2008).

Our research group has previously reported osmolarity results for the products herein tested (Machado, 2017) and found that Lomexin® and Colpotrophine® were highly hyperosmolal (144±620 and 1723±20 mOsmol/kg, respectively), above the upper limit recommended by the WHO (1200 mOsmol/kg) (WHO, 2012; Machado, 2017). In fact, osmolarity is well known to be an important technological parameter for safety of both ocular and vaginal products. Previously, we

performed a similar study on vaginal lubricants and found that Geliofil®, Vidermina®, K-Y® Jelly, the ones that showed higher IS in the HET-CAM assay, had extremely high osmolalities (3582±11; 3707±16 and 3631±13 mOsmol/kg, respectively). Besides, Ginix and Ginix Plus were also the ones that showed higher toxic profiles on HeLa cervical cells using the lactate dehydrogenase (LDH) colorimetric cytotoxicity assay (Cunha et al., 2014). Once again, K-Y® Jelly and Ginix Plus® that were considered as HET-CAM borderline products, had previously demonstrated higher cytotoxicities. These findings, strength the hypothesis that products osmolality and cellular and tissue toxicity could be highly predictive of irritation potential and, it can further suggest that these techniques (HET-CAM, osmolality, cell/tissue metabolic toxicity) should be applied concomitantly for a more robust clinical irritation response preview. The in vivo impact of this slight irritation result according to IS (B) is not known since, to our knowledge, clinical data referring to the adverse effects of these lubricants are not generally available.

The application of the two scoring analysis methods, IS (A) and (B), confirmed that IS (B), although being more difficult to perform and also requiring a more qualified operator, can be used to a more detailed classification output. Also, it leads, generally, to higher IS which means it could be scoring false positive irritants, rather than false negatives. In a safety perspective, this should be regarded as valuable in comparison to IS (A), being especially important for screening of prototypes during product development processes. Nevertheless, it is important to note that the current ICCVAM protocol suggests the use of IS (A) in prospective studies and the conversion of IS (B) in IS (A) regarding retrospective studies. This recommendation is based on the fact that, according to the currently available data, the purpose of this test method is to detect severe ocular irritants and not to differentiate the range of irritancy potential among products.

The suitability of the HET-CAM assay for vaginal irritation testing was demonstrated with this study, despite more assays and controls are needed to be enrolled to complete the validation process as well as inter-laboratory testing in order to confirm its reproducibility. Additionally, the improvements that were tested in the past for the HET-CAM applied to testing cosmetic ingredients eye toxicity, like a historical methodological analysis (Djabari et al., 2002) and the combination of two softwares (ImageJ® and Adobe Photoshop®) that allows live monitoring of the assay, reducing the subjectivity in the endpoints evaluation (McKenzie et al., 2015) shall be considered in this approach in order to assure more accurate results.

The HET-CAM assay has already been applied to test the irritation potential in other epithelia. In 1999, Lönroth et al. (1999) tested eight polymeric products to be used as dental restorative materials. In 2003, the CAM technique had also been applied to an angiogenesis model for tissue engineering. Fertilized White Leghorn eggs were incubated and after 3 days preadipocyte-seeded fibrin constructs were implanted on the surface of the chorioallantoic membrane. The endpoint evaluated in this case was the survival rate for embryos receiving transplanted constructs that was about 90%, confirmed by histology (Borges et al., 2003). Later, on 2007 other research group inquired the irritative potential of dental adhesive agents, and answered this question by performing the HET-CAM assay (Dahl, 2007). In the same year, Vargas et al. compiled the applications that had already been tested concerning the chick embryo and its CAM for the evaluation of drug delivery systems (Vargas et al., 2007). Furthermore, it has been applied to testing of skin irritation, for the anti-inflammatory response of plasma to treat chronic skin wounds (Bender et al., 2011), and also, to evaluate the irritation potential of topical antiesthetics (Marquardt et al., 2010). The HET-CAM has also been applied to test medicines for ocular application, for example compounded fluconazole and voriconazole eye drops prepared in an hospital pharmacy department to disclose potential eye irritation (Fernández-Ferreiro et al., 2014). Recently, Batista-Duharte et al. (2016) have used the HET-CAM assay to access the irritation potential of vaccine adjuvants for nasal and subcutaneous administration. Since this method was originally tested in this study, it was made a comparison with an in vivo local toxicity study in mice to compare irritation score in order to determine if there was a correlation between the two methods. It was concluded that the correlation was high (Pearson test; r=0.9034) and that the HET-CAM assay can be used as an alternative method to the animal test to study the local toxicity potential of vaccines’ adjuvants (Batista-Duharte et al., 2016).

Although using incubated Hen’s eggs for tests could represent a borderline case between in vivo and in vitro systems, it does not conflict with ethical and legal obligations, especially animal protection laws. It was already demonstrated that incubation up to day nine, the embryonic differentiation of the chicken central nervous system is sufficiently incomplete to avoid suffering and pain perception. Actually, the few sensory fibers present at day nine only develop after incubation during 11 to 14 days (Liebsch et al., 2011). Studies also suggested that the extra-embryonl vascular systems (e.g., yolk sac, CAM) are not sensitive to pain (Spielmann, 1995). Therefore, this test method can reduce the number of animals subjected to testing and reduce the pain and suffering of rabbits by their exclusion from the testing of corrosives and severe irritants (U.S. Public Health Service, Department of Health and Human Services (2006)).

Until now no single in vitro test has emerged as being completely acceptable for full replacement of in vivo tests. However, the Hen’s Egg Test Chorioallantoic Membrane has gained regulatory acceptance in various countries to classify severe eye irritants, and has potential to be applied to other mucosal/epithelial substrates such as the vaginal epithelia.

5 Conclusions

The HET-CAM assay was transposed and applied to vaginal irritation testing. This strategy represents an innovative approach for the preclinical safety assessment of vaginal products, being them classified as medicines, cosmetics, hygiene products or medical devices. The application of the two scoring analysis methods, IS (A) and (B), confirmed that IS (B) can conduct to a more detailed classification output and preferably should be chosen provided that this sensitivity is supported by in vivo studies. The studied vaginal formulations, comprising medicines and lubricants, showed, as expected, low potential for irritation. N-9 was considered as a severe irritant above 2% (v/v) concentrations, which corroborates clinical data from the literature, envisaging a possible in vitro/in vivo correlation. Comparisons with previous studies by our workgroup confirmed that HET-CAM can predict and/or confirm toxic profiles for products also tested for osmolality and cellular/tissue toxicity. Ideally, an integrative methodology should be designed to embrace all these preclinical tests, for a better in vivo safety.
preparation. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for vaginal irritancy potential in vitro studies.

References


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Conflict of interest
The authors declare that they have no conflicts of interest.

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