Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmaceutics and Biopharmaceutics 60 (2005) 419–425
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

The evaluation of the local tolerance of vaginal formulations containing dapivirine using the Slug Mucosal Irritation test and the rabbit vaginal irritation test

Marijke M.M. Dhondt[a], Els Adriaens[a], Jens Van Roey[b], Jean Paul Remon[a,*]

[a]*Laboratory of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium*
[b]*Tibotec BVBA, Mechelen, Belgium*

Received 3 August 2004; accepted in revised form 24 January 2005
Available online 8 April 2005

Abstract

The purpose of this study was to evaluate the local tolerance of vaginal gels (three gels containing dapivirine, the placebo gel, and Conceptrol®) with the Slug Mucosal Irritation test and to compare the results with those of the rabbit vaginal irritation test. The irritation potential on the slug mucosa was assessed by the mucus production caused by a repeated treatment for 5 successive days. Additionally, membrane damage was estimated by the protein and enzyme release. By means of a classification prediction model the formulations were classified into four irritation classes. The effect of a 10-day intravaginal application of the gels on the rabbit vaginal and cervical mucosa was evaluated by means of macroscopic and microscopic examination. The placebo and dapivirine gels induced no irritation of the slug mucosa (low mucus production and protein release, no enzyme release) and no vaginal or cervical irritation in rabbits. Conceptrol® caused severe irritation of the slug mucosa (increased mucus production, protein release, and enzyme release) and irritation of the rabbit vagina and cervix. The results obtained with the Slug Mucosal Irritation test were comparable to those of the rabbit vaginal irritation test.

Keywords: Local tolerance; Vaginal formulation; Mucosal irritation; Alternative test

1. Introduction

Frequent use of some vaginal formulations can induce mucosal irritation and damage of the epithelium, one of the natural protective barriers to disease [1,2]. Irritation of the vaginal mucosa might increase the susceptibility to sexually transmitted pathogens during sexual intercourse [3]. Therefore, pre-clinical studies that document the local tolerance of newly developed vaginal formulations are required [4–6].

It is generally recommended that vaginal irritation tests of both the active agent and the clinical vaginal formulation are carried out in rabbits for 10 consecutive days [4,5]. Various other in vivo models have been used to investigate the safety of vaginal products, such as mice [7,8], rats [9], and non-human primates [10]. However, rabbits are frequently used for vaginal irritation tests, because, they have a simple cuboidal or columnar epithelium that is highly sensitive to mucosal irritants when compared to the stratified squamous epithelium of the human vagina [11].

In general, the use of vertebrates to evaluate the safety has been widely criticised based on scientific and ethical grounds. The concept of the three Rs (refinement, replacement, and reduction) stimulates the development of alternative methods such as in vitro methods and the use of 'lower' organisms (invertebrates, plants, and micro-organisms) as test organisms [12]. In vitro assays utilizing cells of primary human origin or immortalized cervical and vaginal epithelial cell lines are developed to evaluate the cytotoxicity of vaginal formulations [13,14]. However, studies on animals can only be substituted by validated in vitro tests as described in the CPMP position paper 'Replacement of animal studies by in vitro models'. Before an in vitro test can be considered valid, the test must undergo a procedure aiming at establishing relevance

* Corresponding author. Laboratory of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Ghent University—UGent, Harelbekestraat 72, 9000 Ghent, Belgium. Tel.: +32 9 264 8056; fax: +32 9 222 8236.
E-mail address: jeanpaul.remon@UGent.be (J.P. Remon).

0939-6411/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2005.01.012

and reliability. The relevance of the alternative test has to be compared to the accepted in vivo standard methods [15]. Up to date, no in vitro reconstructed human vaginal mucosa model has undergone complete validation.

Adriaens and Remon developed an alternative test using invertebrates as a model organism [16]. The terrestrial slug *Arion lusitanicus* was selected as test organism. The body wall of slugs consists of a single-layered epithelium composed of epithelial cells and mucous gland cells overlying connective tissue. Slugs exposed to irritating substances produce mucus in order to protect the body wall. The amount of mucus produced by the slugs during a repeated contact period is a measure for irritation. Membrane damage can be estimated from the release of proteins, lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) from the body wall of the slugs.

The Slug Mucosal Irritation test enables to estimate the irritation potential of a repeated treatment with bio-adhesive powder formulations on nasal and buccal mucosal tissue [17,18]. Furthermore, the local tolerance of ocular minitablets can be evaluated by means of the Slug Mucosal Irritation test [19,20]. There was an excellent agreement between the Slug Mucosal Irritation test and the in vivo data on local tolerance. Recently, the procedure of the Slug Mucosal Irritation test was optimised for local tolerance testing of semi-solid vaginal formulations. As no list of reference standards exists for local tolerance screening of vaginal formulations, the relevance of the test procedure was assessed by means of a few commercially available gels with published human data on vaginal tolerance. The vaginal gels could be classified into four irritation categories (non, mild, moderate, and severe) and the results of the mucosal irritation test were comparable to the available in vivo data [21]. Although human data should be the final standard against which the relevance of an alternative test is assessed, comparing the available data was difficult. The studies differed with respect to their purpose, frequency and duration of use, sample sizes, target populations, comparison products, rules regarding intercourse, the means and time points for assessing safety outcomes.

In order to compare local tolerance data of vaginal gel formulations tested under the same conditions, the present study evaluated the local tolerance of some vaginal gels—planned for being tested by means of the standard rabbit vaginal irritation test—with the Slug Mucosal Irritation test. The vaginal gel formulations contained different concentrations of dapivirine. Dapivirine is a lipophilic non-nucleoside reverse transcriptase inhibitor, being tested as a potential HIV-specific vaginal microbicide. Vaginal gel formulations containing 2.25 μM, 22.5 μM or 225 μM dapivirine were reported to inhibit vaginal transmission of HIV, when the gels were administered to severe combined immunodeficient mice 15–20 min prior to a non-invasive vaginal challenge with HIV-1 infected peripheral blood lymphocytes [22].

With the exception of the varying concentrations of the active product, local tolerance testing was conducted with the formulation intended for further clinical testing. The gel containing the same ingredients except dapivirine was used as placebo gel. As a positive control, the slugs and the rabbits were treated with Conceptrol®, a spermicidal gel containing 4.0% nonoxynol-9, as recommended by the International Working Group on Vaginal Microbicides [4].

2. Materials and methods

2.1. Vaginal formulations

The placebo gel and the dapivirine gels were kindly provided by Tibotec BVBA (Mechelen, Belgium). The gels were made up of 2.0% hydroxyethyl cellulose (HEC) and contained 22.5 μM, 225 μM or 10 mM dapivirine. The placebo gel contained the same ingredients with the exception of dapivirine. Conceptrol® (Ortho-McNeil Pharmaceutical, Inc., Raritan, USA) is a commercially available over-the-counter vaginal contraceptive gel preparation containing 4.0% nonoxynol-9, sodium carboxymethylcellulose, propylene glycol, methylparaben, povidone, sorbic acid, sorbitol solution, lactic acid, and purified water.

2.2. Slug Mucosal Irritation test

The mucosal irritation test procedure described by Adriaens and Remon [16] was optimised for the evaluation of the local tolerance of vaginal gels and will be described briefly. The parental slugs of the *A. lusitanicus* were collected in local gardens along Varsenare and Aalter (Belgium). The slugs were bred in plastic containers in the laboratory at 18–22 °C and they were fed with lettuce, cucumber, carrots and commercial dog food. Slugs weighing between 3 and 6 g were isolated from the culture 2 days before the start of an experiment and were placed in a vented plastic box lined with a paper towel (moistened with phosphate buffered saline (PBS, pH 7.4)) at 18–22 °C.

The slugs were placed daily in a Petri dish on 100 mg gel formulation for 30 min. For each formulation five slugs were used. A 5% (w/w) HEC gel and Conceptrol® were used as negative and positive control gel, respectively. The amount of mucus produced during the contact period was measured by weighing the Petri dishes containing the formulation (without the slugs) before and after the contact period. The mucus production was expressed as percentage (w/w) of the body weight. After the contact period, the slugs were transferred to a fresh Petri dish and 1 ml PBS was added. The PBS was added samples were collected with a micropipette after 60 min. Then the slugs were placed in a fresh Petri dish and 1 ml PBS was added again. After 60 min, the PBS samples were collected again. The samples were immediately analysed for the presence of proteins, LDH, and ALP released from the body wall of the slugs. The slugs were

placed in a Petri dish on a membrane filter (cellulose acetate 0.45 μm, Sartorius AG, Goettingen, Germany) moistened with 2 ml PBS until the next contact period. This procedure was repeated on 5 successive days [21].

The protein concentration in the samples was determined with a NanoOrange® protein quantitation kit (Molecular Probes, Leiden, The Netherlands) and expressed as μg/ml per gram body weight. The NanoOrange reagent allows accurate detection of proteins in solution at concentrations between 10 ng/ml and 10 μg/ml. The fluorescence measurements were carried out on a fluorometer (Wallac 1420 multilabel counter, PerkinElmer, Turku, Finland) using excitation/emission wavelengths of 485/590 nm. Bovine serum albumin was used as a standard.

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) and alkaline phosphatase activity (ALP, EC 3.1.3.1) were measured with enzyme kits (DG 1340-UV and DG 1245-UV, respectively, Sigma Diagnostica, Bornem, Belgium) and expressed as U/l per gram body weight. The reagents measure the enzyme activity based on a modified optimised standard method recommended by the German Society for Clinical Chemistry [23]. The activity measurements were conducted on a Cobas Plus analyser (ABX, Brussels, Belgium) at 37 °C.

2.3. Rabbit vaginal irritation test

The rabbit vaginal irritation test was performed by a qualified Clinical Research Organisation in the field. The study was conducted in accordance with the requirements of current, internationally recognised Good Laboratory Practice Standards, and the applicable sections of the United Kingdom Animals (Scientific Procedures) Act 1986. At the start of the treatment, the female New Zealand white rabbits (Charles River UK Limited, Margate, UK) were 13–17 weeks old and weighed between 2.4 and 3.6 kg. The animals were housed singly in suspended cages in a room in which the temperature and the relative humidity were maintained within the range of 16–20 °C and 40–70%, respectively. A 12-h light/dark cycle was maintained.

The rabbits were treated once daily with 1 ml gel. The gel was inserted approximately 6 cm into the vagina via a soft rubber catheter that was wetted with water and attached to a syringe. For each formulation five rabbits were used. Prior to each administration, the animals were examined for clinical signs of vaginal or vulval irritation, discharge or bleeding from the vagina. The dosing procedure was repeated for 10 consecutive days.

On day 11, the rabbits were killed by an intravenous injection of pentobarbitone and were necropsied. The vaginal and cervical tissues of each animal were examined macroscopically, dissected out, fixed in 10% neutral buffered formalin, and embedded in paraffin. Transverse sections of the vagina and the cervix were taken approximately 5 mm above the entry point of the urethra and at the cervico-vaginal junction, respectively, and were stained with hematoxylin and eosin. The vaginal and cervical tissues were examined microscopically by an experienced pathologist for (1) the severity of epithelial loss and atrophy and (2) the presence of leukocyte infiltration. The severity of the findings was described by one of the following six grades: non, minimal, slight, moderate, marked or severe.

2.4. Data analysis

For each slug, the total mucus production, the mean protein release (without the data of the first day of treatment), the mean LDH release, and the mean ALP release were calculated and these data were used for the statistical analyses. Because all slugs released high protein amounts on the first day of treatment, a more defined distinction between non-irritating and irritating formulations was obtained by calculating the mean protein concentrations without the data of the first day. Statistically significant differences between repeated experiments or different treatments were determined using a one-way ANOVA. The data were tested for normal distribution with a Kolmogorov–Smirnov test. The homogeneity of variances was tested with the Levene's test. If the variances were found not to be equal the data were transformed to their logarithm. To further compare the effects of the different treatments a multiple comparison among pairs of means was performed using a Scheffé test. A probability value of $P < 0.05$ was considered as statistically significant. For all the statistical analyses, the computer program SPSS (version 11.0) was used.

3. Results

3.1. Slug Mucosal Irritation test

Table 1 shows the total amount of mucus produced by the slugs and the mean protein and enzyme release from the slug body wall caused by a repeated treatment with the gel formulations. HEC gel and Conceptrol® were used as negative and positive control gel, respectively. The placebo gel was tested independently on two separate occasions. ANOVA testing resulted in no significant differences between those repeated experiments for the total mucus production and the mean protein release ($P > 0.05$).

All the slugs treated with the placebo gel or the dapivirine gels survived the 5-day experiment. A daily treatment with Conceptrol® led to 20% mortality by day 5 (before the fifth treatment). The total mucus production of the slugs treated with the placebo gel, the 22.5 μM dapivirine gel, the 225 μM dapivirine gel or the 10 mM dapivirine gel was comparable to that of the negative control slugs ($P > 0.05$). The mucus secretions were colourless, which is normal. The slugs treated with Conceptrol® produced significantly larger amounts of (yellow up to

Table 1
Influence of a repeated treatment for 5 successive days with 100 mg gel on the end points of the Slug Mucosal Irritation test

	Total MP (%)	Mean protein release (μg/ml.g)	Mean LDH release (U/l.g)	Mean ALP release (U/l.g)	*n*
HEC gel	13.4±3.1[a]	10±4[a]	–	–	5
Placebo gel	13.3±2.1[a]	15±6[a]	–	–	10
22.5 μM Dapivirine gel	9.4±2.8[a]	26±23[a]	–	–	5
225 μM Dapivirine gel	10.0±2.2[a]	12±3[a]	–	–	5
10 mM Dapivirine gel	12.0±1.7[a]	11±4[a]	–	–	5
Conceptrol®	24.6±5.3[b]	233±69[b]	6.05±1.50	1.63±0.59	5

Data are presented as the mean±SD (n=5–10). MP, mucus production expressed as % (w/w) of the body weight.
[a] Data are not significantly different from negative control HEC gel ($P>0.05$, Scheffé test).
[b] Data are not significantly different from positive control gel Conceptrol® ($P>0.05$, Scheffé test).

orange coloured) mucus than the negative control slugs ($P<0.05$).

A repeated treatment of the slugs with the placebo gel, the 22.5 μM dapivirine gel, the 225 μM dapivirine gel or the 10 mM dapivirine gel resulted in a protein release comparable to that of the negative control slugs ($P>0.05$). The slugs treated with Conceptrol® released significantly higher concentrations of proteins than the negative control slugs ($P<0.05$). An increased release of the cytosolic enzyme LDH and the membrane-bound enzyme ALP from the mucosa of the slugs is an indication of severe membrane damage. For the slugs treated with the HEC gel, the placebo gel or the dapivirine gels, the release of these enzymes was below the detection limit. Enzyme release was only detected for the positive control slugs and increased with repeated treatment.

3.2. Rabbit vaginal irritation test

There were no mortalities in any of the groups during the 10-day application period. Clinical examination of the rabbits prior to each administration revealed no signs of vaginal or vulval irritation, discharge or bleeding from the vagina for all the rabbits of the placebo gel group and the 22.5 μM dapivirine gel group. One rabbit treated with the 225 μM dapivirine gel and one rabbit treated with the 10 mM dapivirine gel were reported to have creamy vaginal discharge on day 4 and 8, respectively. The isolated nature of these signs suggested that the discharge was not an adverse effect and possibly represented leaked gel. One animal treated with Conceptrol® was found to have a red vaginal discharge 2 h after the first treatment, but no evidence of bruising and no further discharge were apparent. This isolated finding was considered likely to have occurred as an initial response and therefore to be not treatment-related.

Macroscopic examination of the vaginas exposed to the placebo gel, the dapivirine gels or Conceptrol® for 10 consecutive days revealed the presence of abnormal contents in 2–3 rabbits per group. The abnormal contents were considered likely to be retained gel. One rabbit treated with the 22.5 μM dapivirine gel had a distended vagina, which appeared to contain accumulated test substance. One animal exposed to the 10 mM dapivirine gel was reported to have multiple slightly raised dark areas along the length of the vagina. The dark vagina of one rabbit treated with Conceptrol® and a dark depression on the mucosal surface of the vagina of another Conceptrol®-treated animal were considered to be treatment-related macroscopic observations.

Fig. 1A and B show representative rabbit vaginal sections following 10-day intravaginal administration of the placebo gel and the 10 mM dapivirine gel, respectively. Table 2 shows that intact vaginal and cervical epithelia without leukocyte influx were observed in the rabbits treated with the placebo gel or the dapivirine gels, with the exception of the vaginal tissue of one placebo-treated rabbit that was reported to be slightly infiltrated by leukocytes. Because the leukocyte influx was observed in just one single small area of the vagina and no other lesions were seen in the vaginas of the other rabbits treated with the placebo, the finding was considered to be caused by the administration procedure. The vaginas of the rabbits treated with the placebo gel or the dapivirine gels consisted of a single epithelial layer with many mucous cells, which is normal for the rabbit vagina. In contrast, all the rabbit vaginal tissues exposed to Conceptrol® showed marked to severe epithelial loss and atrophy accompanied by leukocyte infiltration (Fig. 1C, Table 2). The vaginal epithelial layer of the rabbits treated with Conceptrol® appeared very thin without mucous cells. In the cervical tissues of four rabbits treated with Conceptrol®, slight to marked epithelial loss and atrophy and leukocyte influx were seen.

4. Discussion

Pre-clinical safety studies are required to address the local tolerance of newly developed vaginal formulations [4,6]. It is generally recommended that vaginal irritation tests are carried out in rabbits. The vaginal irritation potential of vaginal formulations is evaluated by the macroscopic and microscopic examination of the exposed rabbit tissues after a daily administration for 10 consecutive

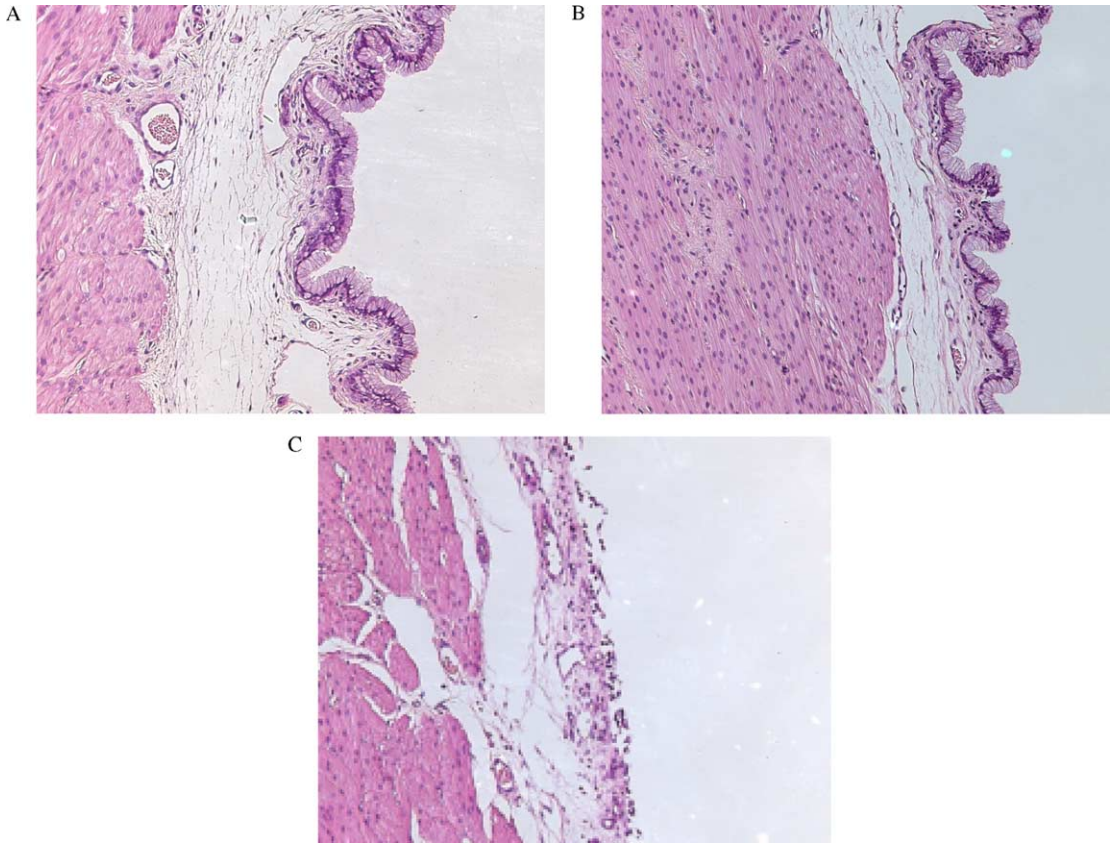

Fig. 1. Light microscopic images of hematoxylin and eosin stained sections (×10) of the vagina of rabbits treated intravaginally with (A) the placebo gel, (B) the gel containing 10 mM dapivirine or (C) Conceptrol® for 10 consecutive days. Note the intactness of the vaginal epithelium and the lack of leukocyte influx in the rabbits treated with the placebo gel or the 10 mM dapivirine gel versus the loss of epithelium and the influx of leukocytes in the rabbit vagina exposed to Conceptrol®.

days [4,5]. However, there is a tendency to reduce, refine, and replace the use of vertebrates for pre-clinical safety studies [12].

In an effort to reduce the number of laboratory vertebrate animals, the Slug Mucosal Irritation test was optimised for local tolerance testing of semi-solid vaginal formulations. The relevance of the test procedure was assessed by means of commercially available gels with published human data on vaginal tolerance [21]. However, direct comparison of the data was difficult to make given the different testing conditions. In the present study, the local tolerance of several vaginal gels (the vaginal gels containing 22.5 μM, 225 μM or 10 mM dapivirine, the placebo gel, and the positive control gel Conceptrol®) was evaluated by means of the Slug Mucosal Irritation test and the rabbit vaginal irritation test. The data obtained with both tests were compared.

The placebo gel was classified as non-irritating gel by means of the Slug Mucosal Irritation test, since a repeated treatment of the slugs with the placebo gel induced a low mucus production and protein release, no enzyme release, and no mortality. It is interesting to note that the placebo gel contains hydroxyethyl cellulose (HEC) just as the negative control gel for the Slug Mucosal Irritation test and that

Table 2
Number of rabbit vaginal and cervical tissues in which a certain grade of epithelial loss and atrophy and the presence of leukocyte influx were observed after treatment with the gel for 10 consecutive days

	Vagina				Cervix				*n*
	Epithelial loss and atrophy			Influx	Epithelial loss and atrophy			Influx	
	Non	Marked	Severe	Present	Non	Slight	Marked	Present	
Placebo gel	5	0	0	1	5	0	0	0	5
22.5 μM Dapivirine gel	5	0	0	0	5	0	0	0	5
225 μM Dapivirine gel	5	0	0	0	5	0	0	0	5
10 mM Dapivirine gel	5	0	0	0	5	0	0	0	5
Conceptrol®	0	2	3	5	1	1	3	4	5

the total mucus production and the mean protein release caused by these two gels were very similar. In addition, light microscopic examination of the vaginal and cervical tissues of the rabbits treated intravaginally for 10 consecutive days with the placebo gel revealed an intact epithelium and the lack of leukocyte influx. Consequently, it was concluded that intravaginal administration of the placebo gel to rabbits does not cause vaginal irritation. The results of the Slug Mucosal Irritation test and the rabbit vaginal irritation test corresponded with human vaginal tolerance data, since Ballagh et al. [24] reported that a single daily use of HEC gel for one week was acceptable.

The 22.5 μM, 225 μM and 10 mM dapivirine gels were classified as non-irritating gels by means of the Slug Mucosal Irritation test, since a repeated treatment with these gels resulted in a low total mucus production and a protein release comparable to those of the negative control slugs. No enzyme release and no mortality were detected. Furthermore, the rabbits treated with the 22.5 μM, 225 μM and 10 mM dapivirine gels showed a few macroscopic findings. However, these findings were considered to be incidental, since histopathological examination did not reveal any corresponding findings. Indeed, the vaginal and cervical tissues of all rabbits treated with the dapivirine gels were very similar to those of the animals treated with the placebo gel. Microscopic examination revealed an intact vaginal and cervical epithelium and no leukocyte infiltration in the vaginal and cervical tissues. Based on these results it was concluded that intravaginal administration of the dapivirine gels to rabbits does not cause vaginal irritation.

In contrast, a repeated treatment of the slugs with Conceptrol® resulted in a high mucus production, a high protein release, and an increased release of LDH and ALP, indicating severe damage of the slug mucosa. Moreover, intravaginal administration of the positive control gel Conceptrol® to rabbits resulted in macroscopic treatment-related changes of the vagina. Histopathological investigation revealed epithelial loss and atrophy and leukocyte infiltration in the vaginal and cervical tissues of those rabbits. The macroscopic and associated microscopic findings were considered to be a result of local irritation within the vagina and cervix. These results are in agreement with data from the literature. Several in vivo studies showed genital irritation after single or frequent use of Conceptrol® [7,10,25,26].

The results of this study clearly demonstrated that the placebo gel and the gels containing dapivirine do not irritate the slug mucosa and the rabbit vaginal mucosa. In contrast, Conceptrol® was irritating to the slug mucosa and the rabbit vaginal mucosa. The study indicated that the results obtained with the Slug Mucosal Irritation test and the rabbit vaginal irritation test were comparable. Additionally, a previous study showed that the results obtained with the Slug Mucosal Irritation test were comparable to human vaginal tolerance data. Furthermore, the previous study showed that the Slug Mucosal Irritation test can be used to evaluate the irritation potential of mildly and moderately irritating semi-solid substances. Indeed, Replens® and K-Y® jelly, two out of the six tested formulations, were, respectively, classified as mildly and moderately irritating formulations [21]. It is also interesting to note that the human vaginal and buccal epithelia are histologically similar [27,28]. Both the human vaginal epithelium and the buccal epithelium are not keratinized stratified squamous epithelia [20]. Previously the Slug Mucosal Irritation test was found to be useful to estimate the irritation potential of bio-adhesive formulations intended for e.g. buccal administration [18].

The Slug Mucosal Irritation test seems to be a promising alternative to screen new vaginal semi-solid formulations for local tolerance early in the development process. The use of the test can possibly contribute to the reduction of the number of vertebrates used in pre-clinical studies.

References

[1] R.E. Roddy, M. Cordero, C. Cordero, J.A. Fortney, A dosing study of nonoxynol-9 and genital irritation, Int. J. STD AIDS 4 (1993) 165–170.
[2] M.K. Stafford, H. Ward, A. Flanagan, I.J. Rosenstein, D. Taylor-Robinson, J.R. Smith, J. Weber, V.S. Kitchen, Safety study of nonoxynol-9 as a vaginal microbicide: evidence of adverse effects, J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 17 (1998) 327–331.
[3] L. Van Damme, G. Ramjee, M. Alary, B. Vuylsteke, V. Chandeying, H. Rees, P. Sirivongrangson, L. Mukenge-Tshibaka, V. Ettiegne-Traore, C. Uaheowitchai, S.S.A. Karim, B. Masse, J. Perriens, M. Laga, Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial, Lancet 360 (2002) 971–977.
[4] The International Working Group on Vaginal Microbicides, Recommendations for the development of vaginal microbicides, AIDS 10 (1996) UNAIDS1–UNAIDS6.
[5] Divisions of reproductive and urologic, antiinfective and antiviral drug products, Points to consider in the nonclinical pharmacology/toxicology development of topical drugs intended to prevent the transmission of sexually transmitted diseases (STD) and/or the development of drugs intended to act as vaginal contraceptives (1996), http://www.fda.gov/cder/guidance/ptcnonclinical.htm, Accessed on 7 January 2005.
[6] European Commission, guideline 3BS12a non-clinical local tolerance testing of medicinal products, In the rules governing medicinal products in the European Union 1998; 107–116.
[7] G.N. Milligan, K.L. Dudley, N. Bourne, A. Reece, L.R. Stanberry, Entry of inflammatory cells into the mouse vagina following application of candidate microbicides—comparison of detergent-based and sulfated polymer-based agents, Sex. Transm. Dis. 29 (2002) 597–605.
[8] S.L. Achilles, P.B. Shete, K.J. Whaley, T.R. Moench, R.A. Cone, Microbicide efficacy and toxicity tests in a mouse model for vaginal transmission of Chlamydia trachomatis, Sex. Transm. Dis. 29 (2002) 655–664.
[9] M. Chvapil, W. Droegemueller, J.A. Owen, C.D. Eskelson, K. Betts, Studies of nonoxynol-9.1. The effect on the vaginas of rabbits and rats, Fertil. Steril. 33 (1980) 445–450.
[10] D.L. Patton, G.G. Kidder, Y.C. Sweeney, L.K. Rabe, S.L. Hillier, Effects of multiple applications of benzalkonium chloride and

nonoxynol 9 on the vaginal epithelium in the pigtailed macaque (Macaca nemestrina), Am. J. Obstet. Gynecol. 180 (1999) 1080–1087.
[11] P. Eckstein, M.C.N. Jackson, N. Millman, A.J. Sobrero, Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys, J. Reprod. Fertil. 20 (1969) 85–93.
[12] M. Balls, A.M. Goldberg, J.H. Fentem, C.L. Broadhead, R.L. Burch, M.F.W. Festing, J.M. Frazier, C.F.M. Hendriksen, M. Jennings, M.D.O. vanderKamp, D.B. Morton, A.N. Rowan, C. Russell, W.M.S. Russell, H. Spielmann, M.L. Stephens, W.S. Stokes, D.W. Straughan, J.D. Yager, J. Zurlo, B.F.M. vanZutphen, The three Rs: the way forward—the report and recommendations of ECVAM workshop 11, ATLA 23 (1995) 838–866.
[13] R.N. Fichorova, L.D. Tucker, D.J. Anderson, The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission, J. Infect. Dis. 184 (2001) 418–428.
[14] R.A. Maguire, N. Bergman, D.M. Phillips, Comparison of microbicides for efficacy in protecting mice against vaginal challenge with herpes simplex virus type 2, cytotoxicity, antibacterial properties, and sperm immobilization, Sex. Transm. Dis. 28 (2001) 259–265.
[15] Committee for Proprietary Medicinal Products, Replacement of animal studies by in vitro models, CPMP/SWP/728/95 (1997). Available at: http://www.emea.eu.int/pdfs/human/swp/072895en.pdf, Accessed on 7 January 2005.
[16] E. Adriaens, J.P. Remon, Gastropods as an evaluation tool for screening the irritating potency of absorption enhancers and drugs, Pharm. Res. 16 (1999) 1240–1244.
[17] C. Callens, E. Adriaens, K. Dierckens, J.P. Remon, Toxicological evaluation of a bioadhesive nasal powder containing a starch and Carbopol® 974 P on rabbit nasal mucosa and slug mucosa, J. Control. Release 76 (2001) 81–91.
[18] E. Adriaens, D. Ameye, M.M.M. Dhondt, P. Foreman, J.P. Remon, Evaluation of the mucosal irritation potency of co-spray dried Amioca®/Poly(Acrylic Acid) and Amioca®/Carbopol® 974P mixtures, J. Control. Release 88 (2003) 393–399.
[19] J. Ceulemans, A. Vermeire, E. Adriaens, J.P. Remon, A. Ludwig, Evaluation of a mucoadhesive tablet for ocular use, J. Control. Release 77 (2001) 333–344.
[20] W. Weyenberg, A. Vermeire, M.M.M. Dhondt, E. Adriaens, P. Kestelyn, J.P. Remon, A. Ludwig, Ocular bioerodible minitablets as strategy for the management of microbial keratitis, Invest. Ophthalmol. Vis. Sci. 45 (2004) 3229–3233.
[21] M.M.M. Dhondt, E. Adriaens, J.P. Remon, The evaluation of the local tolerance of vaginal formulations, with or without nonoxynol-9, using the Slug Mucosal Irritation test, Sex.Transm. Dis. 31 (2004) 229–235.
[22] S. Di Fabio, J. Van Roey, G. Giannini, G. Van den Mooter, C. Lapenta, M. Spada, A. Binelli, E. Germinario, F. Belardelli, Vaginal transmission of cell-associated HIV-1 is inhibited in hu-SCID mice by a microbicide containing an NNRTI dapivirine (TMC120), Microbicides, Antwerp, Belgium, 2002.
[23] Deutsche Gesellschaft für Klinische Chemie, Standardisierung von methoden zur bestimmung von enzymaktivitäten in biologischen flüssigkeiten, Z. Klin. Chem. Klin. Biochem. 10 (1972) 182.
[24] S.A. Ballagh, J.M. Baker, D.M. Henry, D.F. Archer, Safety of single daily use for one week of C31G HEC gel in women, Contraception 66 (2002) 369–375.
[25] A.N. Poindexter, H. Levine, H. Sangi-Haghpeykar, M.L. Frank, A. Grear, K.O. Reeves, Comparison of spermicides on vulvar, vaginal, and cervical mucosa, Contraception 53 (1996) 147–153.
[26] C. Mauck, D.H. Weiner, S. Ballagh, M. Creinin, D.F. Archer, J. Schwartz, H. Pymar, J.J. Lai, M. Callahan, Single and multiple exposure tolerance study of cellulose sulfate gel: a phase I safety and colposcopy study, Contraception 64 (2001) 383–391.
[27] E.N. Marieb, Tissue: the living fabric in: R. Heyden, J. Schmid, E.M. Schaefer (Eds.) Human Anatomy and Physiology, The Benjamin/Cummings Publishing Company, Inc., Redwood City, USA, 1995, pp. 102–134.
[28] I.O.C. Thompson, P. van der Bijl, C.W. van Wyk, A.D. van Eyk, A comparative light-microscopic, electron-microscopic and chemical study of human vaginal and buccal epithelium, Arch. Oral Biol. 46 (2001) 1091–1098.