Reviewer’s report

Title: Decreased cervical epithelial sensitivity to nonoxynol-9 (N-9) after four daily applications in a murine model of topical vaginal microbicide safety

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Reviewer number: 2

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Overall, I think this paper is of interest to those in the microbicide field and it addresses the important issue of multiple applications of a product and the potential for “resistance” or tolerance to a drug when the tissue has been exposed multiple times. I appreciate that the authors have included multiple histology images and it is of interest to compare cytokines and markers of immune cells with timing of injury. My biggest criticisms center around microscopic descriptions and reporting of microscopic findings.

- Major Compulsory Revisions

The author must respond to these before a decision on publication can be reached. For example, additional necessary experiments or controls, statistical mistakes, errors in interpretation.

1. Results: Table 2

After each daily exposure to N-9, mice were sacrificed following the indicated post-exposure intervals. Each experiment evaluated three animals at each time point within each exposure group. Scores (as described in Table 1) were assigned after multiple fields from replicate mice were examined microscopically.

There are no statistics in Table 2. Since there were three mice per timepoint, and potentially several microscopic fields to review, there would be variation amongst the slides, and therefore there should be statistics used to describe the numerous samples. It appears, though, that each timepoint score was derived from looking at multiple microscopic images. This would not be ideal in that the reader would not be blinded since they are looking at all slides from the same timepoint together, and would not capture any variation between different mice. I would prefer to see each microscopic slide/image given a score and then the scores combined for each timepoint.

2. This paper is primarily about microscopic changes, however there appears to be deficiencies in the descriptions of the mouse epithelium. The authors would benefit from an expert pathologist contribution.

Results - 3rd paragraph
Although some indications of toxicity were apparent in the upper layers of the vagina at 2 h post-exposure, this damage was limited to isolated regions of the vaginal epithelium (Figure 2C).

Please expand more on the areas of the vagina that were damaged. Were these areas stratified squamous epithelium or the columnar (overlying basal cells) epithelium similar to the cervix? If the injury was in isolated regions of the vaginal epithelium, how was this taken into account in reporting in Table 2?

This reviewer has looked at hundreds of Depo-Provera treated mouse cervices and vaginas and the majority of the vagina, especially mid and upper vagina, looks similar to the cervix after Depo-Provera treatment, with a single columnar cell layer overlying 1-2 basal cell layers to make up the epithelium. The lower vagina, near the introitus, tends to remain stratified squamous. This is the type of vaginal epithelium in all of the authors’ figures, so it appears that the authors’ vaginal images come from the lower vagina and introitus with stratified squamous epithelium (keratinized, in fact). What did the mid-vagina or “upper layers” of the vagina look like? Was it affected by the medroxyprogesterone and thus was similar to the cervix, or was the entire vagina stratified squamous epithelium?

3. Results – 4th paragraph
At 8 h post-exposure, very little of the cervical epithelial layer remained intact, leaving large areas of the cervical basal layer almost completely exposed (Figure 2H).

This sounds like the cervical epithelial layer and cervical basal layer are two different things. The cervical basal layer is part of the cervical epithelium, which consists of a columnar cell layer overlying 1-2 basal cell layers. It would be less confusing to write “very little of the superficial columnar cell layer of the epithelium remained intact, leaving large areas of the deeper basal cell layer completely exposed”.

4. Discussion
Paragraph 4
Need to point out that the cervix had columnar epithelium overlying a basal layer and the vagina was primarily stratified squamous epithelium. This is in a Depo-Provera treated model, which is different from a normal cycling mouse. In this case, the differential response to N9 would be due to a microscopic difference between the response of columnar epithelium in the Depo Provera treated cervix when compared to the response of the stratified squamous epithelium of the vagina. The mouse cervix may mimic the human endocervix, but the mouse vagina more properly reflects the human ectocervix and vagina. So it is the type of epithelium, rather than the anatomic location, that determines the level of injury.

5. Discussion – 3rd to last paragraph
This apparent tolerance to N-9 exposure after multiple applications may likely be related to changes in cervical tissue morphology observed during these studies. After multiple N-9 exposures, the tissue appeared to be metaplastic, forming multiple cell layers instead of the single layered columnar organization of the cervical epithelium.

The cervical epithelium after Depo Provera treatment is not single layered columnar epithelium (or simple columnar as single layered columnar suggests); it is composed of several cell layers, with the superficial layer of columnar cells and the underlying 1-2 layers basal cells. This is apparent in Figure 1 c, e and the other figures that have the cervical epithelium. In the other figures, the superficial columnar layer has mostly been denuded, but it is intact in some places and the epithelium is still multilayered with columnar cells overlying the basal cells.

- Minor Essential Revisions
The author can be trusted to make these. For example, missing labels on figures, the wrong use of a term, spelling mistakes.

1. Methods – Animals 1st paragraph - - “prior to n9 applications, mice were anesthetized” - also should be prior to n9 and control (saline) applications since there were control mice as well
2. Methods: 1st paragraph - 3 animals per timepoint (4 timepoints per day for 4 days – 16 timepoints – 48 mice) – is there saline control at all timepoints as well?
3. Figure 1A – Appears to be missing the “red dots” indicating treatment with N9 on day 2 and 3 before euthanasia.
4. Figure 1A – caption - Says “treatment with N9” but also was treated with saline – should be “treatment with n9 (n=3 per timepoint) or saline (n=*** per timepoint)”

5. Results - All figures with microscopic images need magnification reported
6. Results – last paragraph
As was observed after a single N-9 exposure, few CD14+ cells were observed in the day 4 vaginal epithelium (Figure 7E). In the cervical epithelium (Figure 7F), CD14+ cells were present in greater numbers relative to cell numbers in the corresponding vaginal tissues. However, cell infiltration was clearly not as intense as was noted at 2 h on day 1. Furthermore, the intense cervical sub-surface staining seen on day 1 was not apparent on day 4; infiltrating CD14+ cells were only present deeper in the lamina propria. CD14+ cell staining in the cervix at 4 h and 24 h post-exposure was also reduced relative to levels noted in cervical tissues on day 1 (data not
How did the authors come to an objective conclusion that the staining was greater or lesser at a given timepoint? Was this quantified or just subjective? On how many mice per timepoint was this conclusion based?

7. Discussion - Paragraph 2

Second, this model also differs from the human female reproductive tract (FRT) in that the rabbit FRT (i) does not undergo cyclic reproductive stages, (ii) is not colonized by lactobacillus (resulting in a lack of acidity within the vaginal tract), (iii) lacks the production of cervicovaginal mucus, and (iv) is lined primarily with columnar epithelial cells (instead of stratified squamous epithelial tissue in the human vagina) [29-31].

The deficiencies and limitations of the RVI are as discussed, however the rabbit has columnar epithelium in the cervicovagina (upper vagina) and stratified squamous epithelium in the urovagina (lower vagina). (Philip E. Castle, Timothy E. Hoen, Kevin J. Whaley, and Richard A. Cone. Contraceptive Testing of Vaginal Agents in Rabbits. CONTRACEPTION 1998;58:51–60)

In addition, mice do not have lactobacilli either. This should be pointed out in the discussion, as this deficiency for the rabbit also exists in the mouse and most other models except the non-human primate model.

8. Discussion – The authors did not discuss any limitations. They should discuss their limitations, including limitations of the mouse model in general. Every model has similarities to humans, benefits, and limitations, and it should be pointed out, especially when the discussion points out limitations in other models.

- Discretionary Revisions

These are recommendations for improvement which the author can choose to ignore. For example clarifications, data that would be useful but not essential.

1. Intro - First paragraph: First sentence has redundancies and should be edited to be less cumbersome

The global human immunodeficiency virus type 1 (HIV-1) epidemic currently affects approximately 33 million HIV-1-infected people worldwide and includes a high incidence of HIV-1 infections (~23 million individuals) in Sub-Saharan Africa [1].

2. Methods - Not consistent in use of the terms lactated ringer’s saline solution vs lactated ringers solution

3. Discussion - Last sentence of paragraph 2 - Squameous should be spelled squamous.

4. Discussion - 3rd to last paragraph

A focus of future studies will be the amount of time required for repaired and
tolerant tissues to return to their normal structure and level of susceptibility to damage.
This is confusing – would read better “to return to their baseline structure and level of susceptibility to damage”

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.