Reviewer's report

Title: Quantitative assessment of microbicide-induced injury in the ovine vaginal epithelium using confocal microendoscopy

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Reviewer: Thomas Moench

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Vargas et al: Quantitative assessment of microbicide-induced injury in the ovine vaginal epithelium using confocal microendoscopy

This manuscript describes the application of a new technique, confocal microendoscopy, to assess microbicide safety in the sheep vagina. The data reported show the potential for this technique to improve on the sensitivity of the current clinical gold standard, colposcopy. Colposcopy has proved to be inadequate to detect toxicities that increase susceptibility to HIV and other STIs before prior candidates were advanced to clinical efficacy testing. Importantly, the authors also developed a data analysis algorithm that makes the technique quantitative, and allows the analysis to be automated, objective, and amenable to central reading when and if the technique is applied to clinical studies. The technique is already applicable to single observation ex vivo observations in animals, and is likely to be adaptable to in vivo and longitudinal observations in animals, and should be very useful for these applications. The feasibility of or obstacles to advancing the technique to the clinic are less clear, and merit further discussion in the manuscript. Likewise, the ability to detect toxicity of agents with minimal direct cytotoxicity has not yet been studied. The experiments are appropriate, well described, and the authors’ conclusions are well supported by the data.

MAJOR COMPULSORY REVISIONS:

1) Page 8, description of conversion to binary images: This approach is innovative and valuable, but I believe the terms “background” and “foreground” are inappropriate. First, the signal threshold is not a “background signal”, i.e., it is not due to non-specific binding of the dye. It is a baseline signal, a true signal caused (as the authors convincingly explain) by PI binding to nuclear DNA in dead cells in the ovine vaginal epithelium, similar to the dead layer of cells known to be present on the surface of the human vaginal epithelium. Second, the term “foreground” conventionally has a positional rather than an intensity meaning, which is not the meaning intended here, and is distracting and confusing. I would urge this terminology be changed, perhaps to “baseline” and “supra-baseline”, assigned to the values of 0 or 1 respectively.

2) Page 1: In the parts of the narrative sections of the manuscript (Abstract, Background, and Discussion) the writing is somewhat wordy and indirect. Many
places could be shortened and edited for clarity and directness. As an example, the first sentence of abstract could be changed to: “Improved methods are needed to detect microbicide disruption of epithelial barriers.” Careful re-editing of the manuscript could make it more effective in delivering its important points.

3) Page 1, Results, Sentence 2 implies that the images disclose a disrupted surface, but this is a conclusion based on inferences from the images, and from the correlated histology. Sticking to the observed findings and avoiding inference in the Results section, I would change to: “… those after BZK or N-9 showed heavily stained and disrupted nuclei, which increased in proportion to injury detected on histology.”

4) Page 4, 1st paragraph: It is not correct to say there have been no Phase III microbicide trials. For example, MDP 301 (PRO 2000) qualifies as a Phase III study (with a previous Phase II, and power adequate to definitively assess efficacy). Likewise, COL 1492, and the Cellulose Sulfate and C31G studies qualify.

5) Page 4: I think it is an overstatement to say that biopsy is “fundamentally incompatible with longitudinal studies”. Though difficult, at least one longitudinal study has been done with biopsy pre and post exposure. I would say instead that biopsy is cumbersome and raises safety issues when used clinically in high-risk populations.

6) Page 7, fourth to last line: This seems to indicate that the images are surface only, with minimal depth of field. An estimate of the depth of field being viewed should be provided. Is depth limited by the optical setup, or is it primarily limited by the penetration or exclusion of the PI dye? In conventional confocal microscopy, optical sectioning can be done, resulting in a z-stack of images. I am tentatively assuming this is not possible with the current instrumentation, and if so, this limitation should be mentioned. If optical sectioning IS feasible, the future possibility of using the technique to subsurface changes, such as inflammatory infiltrates, should be discussed, perhaps using a different dye. Since a significant limitation of colpo is detection only of surface features, alternatives are needed that would give more information about deeper structures. The authors’ prior publications on OCT provide one such method, but it would be interesting to learn whether confocal microendoscopy could provide an alternative subsurface imaging technique with higher resolution.

Further discussion of limitations:

7) These experiments were ex vivo, which is practical in single observations in animal models, and, as demonstrated by the results reported, has the potential to be very useful. Longitudinal studies in animals, and clinical studies in humans would further extend the usefulness of the technique, but would require in vivo observations. The endoscopic probe should make intravaginal and intra rectal/colonic observations possible. However, the additional requirement of a non-toxic dead-cell dye that can be used in humans complicates the translation of this technique to clinical use. The authors should discuss this, and comment on the prospect or difficulty of obtaining or developing dead-cell dyes for clinical
studies.

8) The sensitivity of the technique is impressive in that it disclosed damage from a subclinical concentration (0.02%) of the surfactant BZK, which I expect would not be detected by colposcopy. However, surfactants are no longer being actively considered for microbicides (with the possible exception of glycerol monolaurate). The authors should discuss the future potential to apply this technique to evaluate other (non-surfactant, not directly cytotoxic, or minimally cytotoxic) agents that have been shown to alter susceptibility in animal models. This will likely be necessary for the technique to have utility in evaluating agents in the microbicide pipeline, all of which are now prescreened for low in vitro cytotoxicity before being further pursued as microbicide candidates. One way this might be achieved, even with agents that are not directly cytotoxic, would be to adapt the technique to detect alterations in epithelial permeability to appropriate sized (virus-sized) fluorescent mucus penetrating nano-particles, or other permeability probes. If the authors believe the technique has sufficient depth of field to make such assessments, they should discuss this possibility, and/or other potential expanded applications of the technique.

MINOR ESSENTIAL REVISIONS:

9) Page 6: identify and reference HEC gel: it needs further identification (e.g., reference Tien D et al, AIDS Res Hum Retrovir 2003). It may be useful to refer to it once in the methods as the “universal placebo”: though this is something of an overstatement, this is how it is conventionally described.

10) Page 10: missing period after “…intensity)” third-to-last line.

DISCRETIONARY REVISIONS:

11) Page 2, Conclusions: An important advantage of the new technique over colposcopy is that the former is objective and the latter rather subjective and operator-dependent. Would thus add the word “objective” to the list of attributes enumerated for microendoscopy.

12) Page 4, 2nd paragraph: Toxicity is not necessarily beyond the detection capabilities of colpo (for example Roddy et al have detected epithelial damage after intensive N9), but more subtle lesions may also matter, and have resulted in altered susceptibility. For example COL1492 and Cellulose sulfate looked good in Phase I and Phase II studies with colposcopy as the main safety evaluation, yet resulted in increased susceptibility to HIV or a strong trend toward increased susceptibility). Would revise the beginning of the second sentence to something like “Epithelial changes below the detection threshold of colposcopy may yet increase susceptibility to infection [ref the van Damme COL 1492 NEJM paper], thus tools which aid…”

13) Page 4, 3rd paragraph: In pathology, gross examination refers to unmagnified visual examination of organs. Since colposcopy provides considerable magnification, I would drop the word “gross” from “only gross visual assessment of the tissue surface.” Surface yes, but gross no, in the sense that
the inspection is under magnification.

14) Page 4: Other disadvantages could be added in the list of colpo limitations: colpo training requirements are intensive, colpo does not easily allow for centralized reading (digital photos are inferior to on-the-ground observation) and thus interpretation is quite subjective and only modestly quantitative.

15) Page 7: The reported value for PI concentration (13.3 ug/mL) looks odd in its precision. Add or substitute that this is “20 micromolar”, which probably explains the 13.3 ug/mL concentration chosen.

16) Page 7, line 6: Please clarify “single channel”… resolve any ambiguity due to interpretation as electronic vs endoscope “channel”. If electronic, please give further explanation as to what it means.

17) Page 8, last sentence: Add the word “Matlab”, thus reading: “A Matlab connected component labeling operation (bwlabel)… [This would clarify what I assume is the case: that bwlabel is a function within the Matlab image analysis program.]”

18) Page 11: nuclei appearing to merge: It may be useful to explain or speculate that this may be due to cell lysis.

19) Page 11: I am struck by the fact that confocal microendoscopy assessment (Figure 2) appears to match the pattern across treatment groups also seen in the histological score (Fig 3a) and the epithelial thickness (Fig 3b). Thus far the authors only analyze across treatment groups within each test. Can they also analyze whether the correlations between the tests are significant, thus quantitatively supporting validation of the new technique by the gold standard of histology?

Level of interest: An article of outstanding merit and interest in its field

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.