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Pigtailed Macaque Model Refinement: Topical Microbicide Safety in the Presence of Coitus

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Abstract

Background—Inclusion of sexual activity in the macaque model for topical microbicide safety evaluation would more closely mimic human use of topical microbicides and provide a more rigorous safety assessment.

Methods—Initially male-female partners were monitored in co-housing arrangements to determine whether macaques would copulate ad libitum. The logistics of performing vaginal exams before and after coital visits were analyzed and optimized. Findings from cervicovaginal exams conducted before and after sexual activity were compared.

Results—Coital activity was reliably observed in the majority of co-housing sessions, representing all phases of the menstrual cycle. Female macaques were trained to be restrained while fully alert for pre-coital vaginal sampling. Post coital exams occur under general sedation. Post-coital exams reveal alterations to tissues, microbiology and pH compared to pre-coital visits.

Conclusions—This work clearly demonstrates that it is feasible to incorporate sexual activity in the macaque model for topical microbicide safety assessment.

Keywords

Macaca nemestrina; sexual activity; safety model

Introduction

Despite currently available prevention strategies, sexually transmitted infections (STI) continue to generate tremendous health and economic burden throughout the world. It is estimated that more than 340 million new cases of bacterial and protozoal STI occur each year (e.g. syphilis, gonorrhea, chlamydia, trichomoniasis), as well as many millions of viral STI (e.g. herpes, HPV, hepatitis B, HIV) (19). Currently there is an estimated 33.4 million people living with HIV around the world (18). In 2008, approximately 2 million HIV infected people died of AIDS, while 2.7 million new infections occurred. Most new infections are transmitted heterosexually, and global surveillance now shows that half of all people living with HIV are women (7). So while it has slowed in recent years, the HIV epidemic continues to grow, with redirected dynamics. Clearly there is a significant global need for safe, effective topical microbicide products.

Topical microbicides will offer a prophylactic mechanism to prevent disease acquisition via sexual transmission. An ideal vaginal microbicide would inactivate pathogens introduced into the vagina during sexual intercourse without adversely affecting the vaginal or cervical

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tissues. We have established and extensively utilized a standardized protocol for preclinical safety assessments of vaginally applied topical microbicide candidates in a nonhuman primate (*Macaca nemestrina*) model (11-14). Briefly, this safety protocol is designed to identify potentially adverse tissue and microbiologic responses to repeated daily exposures to topical microbicide products in development. To date, these studies have been completed in the absence of coitus, which is to say the safety profiles were determined for the simple presence of test microbicides in the vagina when delivered once daily for four days.

Sexual activity in and of itself may cause micro-abrasions to the mucosal tissues of the cervicovaginal environment. This altered environment may be particularly prone to pathogen uptake when exposed to infectious seminal fluid. Additionally, the introduction of semen, with increased pH and potential micro-organism populations, may affect the natural protective factors of the cervicovaginal ecosystem. Few studies have investigated tissue trauma, microbiologic or pH effects that may result from vaginal intercourse without condoms (5). However it has been shown that intercourse with non-lubricated condoms may increase the risk of urinary tract infection, perhaps by facilitating organism transport (6). It is important to distinguish tissue/environment responses due to sexual activity alone from those resulting from topical microbicide exposure prior to intercourse. Likewise, it is important to assess product safety in the presence of coitally induced changes to the cervicovaginal environment. To more closely mimic human use of topical microbicides and to provide a more rigorous assessment of topical microbicide safety it is imperative to know how the products are tolerated in the presence of coitus. Therefore, we explored the feasibility of refining the macaque model for topical microbicide safety assessments to include macaque coital visits. Our goal was to include sexual activity after product administration once, or multiple times during the standardized protocol timeline, to assess a product's safety with coital activity.

Three basic questions were established to guide our efforts toward determining the feasibility of this model refinement. First, can we expect *Macaca nemestrina* to engage in coitus ad libitum? Next, can we measure any alterations to the macaque's cervicovaginal environment resulting from coital activity? Finally, can we incorporate coital visits to the existing standardized topical microbicide safety protocol in the macaque model?

Materials and Methods

All studies were conducted in the Washington National Primate Research Center (WaNPRC) in Seattle, Washington. The project described was supported by Grant Number R21AI071939 from the National Institute of Allergy And Infectious Diseases. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

Animals

Sexually mature male and female *Macaca nemestrina* (pigtailed macaques) were obtained from a colony of animals at the WaNPRC. Prior approval for use of monkeys in this protocol was obtained from the Institutional Animal Care and Use Committee at the University of Washington. Animals were handled humanely, and experiments were performed within the National Institutes of Health's animal use guidelines, and in compliance with the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act. Animals were individually housed with visual, auditory, olfactory and limited tactile contact with other primates in the research colony when they were not taking part in timed co-housing sessions. A total of seven male and twelve female pigtailed macaques were used in the studies described herein.

Each female macaque's menstrual cycle was monitored by visual assessment of perineal tissue tumescence and detection of menstrual blood. Menstrual cycles were divided into four phases: follicular, ovulatory, luteal and menstruating.

Temperament Assessment

Suitable mating pairs were determined by a series of compatibility assessments which monitored social behaviors of male and female *Macaca nemestrina* when housed in adjacent cages. Initially, female macaques were housed in individual cages placed adjacent to that of one of the male macaques on the project. Because the males are larger animals, requiring larger caging, the females were always transported to the male macaque's home cage. We observed each macaque's behavior for signs of aggression or acceptance (3,16). If temperaments were compatible, the small mesh cage dividers were replaced by wide mesh dividers which allow for finger tip access (first stage of tactile contact). Following positive compatible observations with wide mesh, animals were allowed further contact through grooming bars which allow arms and legs to reach into the neighboring cage. Following successful grooming contact observations, all cage dividers were opened to allow for a timed co-housing session of male and female macaques. At the end of each timed session, cage dividers were closed and the female was returned to her home cage. If aggressive behavior was noted to dominate a compatibility session, the session was ended and each animal returned to their separate home cage.

Co-housing

Initial co-housing sessions were observed for thirty to forty minutes, and later reduced to fifteen minutes. Incidents of mutual grooming, females presenting and male mountings (coitus) were documented and timed. Visual evidence of male ejaculation was also noted, as was the female's menstrual cycle phase. At the conclusion of each timed session, the female macaque was separated from the male and sedated for post-coital vaginal exam. Over two years, each macaque took part in multiple co-housing sessions.

Post-coital Cervicovaginal Assessments

Colposcopy was performed 15-30 minutes after coital visits. Colposcopic exam requires ketamine sedation of the pigtailed macaque for speculum placement and to ensure a motionless pelvis for cervicovaginal exam. Standardized assessments were evaluated by a team of three cross-trained individuals. Vaginal and ectocervical mucosal surfaces were evaluated for erythema, edema, ecchymosis, friability, and tissue abrasions. The presence or absence of a copulatory plug (coagulated semen) was documented on post-coital examination forms and by digital photography. If present, copulatory plugs were removed to allow for unobstructed colposcopic evaluation.

Vaginal pH was determined by rolling a swab on the mucosal surface of the vaginal wall, then onto a pH indicator strip (resolution 0.5 units). Similarly, a swab was rolled on the surface of five removed copulatory plugs to establish the pH of coagulated semen.

Swabs for vaginal microbiologic assessment were placed individually in a Port-A-Cul transport tube (Becton-Dickinson, Sparks, MD) and transported on ice to Magee-Womens Research Institute within 24 hours of collection for semi-quantitative culture analysis. This transport system has been shown to maintain the viability and quantity of organisms for up to 48 hours (17). The swabs were removed from the transport gel and immediately used to inoculate a Columbia sheep blood agar, Brucella sheep blood agar, laked-blood kanamycin agar (PML Microbiologicals, Wilsonville, OR), two human bi-layer Tween agar (HBT) (Becton-Dickinson, Sparks, MD), and Rogosa agar (prepared in-house). The Columbia blood agar and one HBT plate were incubated at 37°C in 6% CO₂ for 48 hours. The

remaining media was incubated at 37°C within an anaerobic chamber (Sheldon Laboratories, Cornelius, OR) for a minimum of four days. The aerobic and anaerobic organisms were identified to the genus, species, or groups of bacteria using phenotypic tests, Gram stain and colony morphology, and aerotolerance as described in the Manual of Clinical Microbiology (8). *Lactobacillus* and viridans *Streptococcus* were tested for hydrogen peroxide production in a qualitative assay on tetramethylbenzidine agar plates (15). Species belonging to the genera *Bacteroides*, *Porphyromonas*, and *Prevotella* were grouped together as either anaerobic Gram-negative rods (nonpigmented) or black Gram-negative rods. Results were reported semi-quantitatively, graded on a scale of zero to four (0 = not detected, 4 = growth on all quadrants of a plate).

Incorporating to standardized protocol: Table top restraint device

It is vital to the standardized safety protocol that the vagina be accessible for test product application and for vaginal environment assessment immediately prior to coitus for comparison to post-coital measures. However, it is not feasible to sedate female macaques for pre-coital vaginal assessments immediately prior to co-housing sessions because they require several hours to fully recover from the effects of ketamine sedation. While in this “recovery phase” it is not safe to house the previously sedated animal with a fully alert male. Therefore incorporating coital visits into the existing standardized safety protocol necessitates the utilization of a table top restraint device (TTRD) to immobilize fully alert female macaques for pre-coital vaginal swabs. Intravaginal product delivery prior to co-housing can also be accomplished with use of the TTRD. Pre-coital colposcopic exams are conducted 24 hours prior to co-housing sessions, because they require animal sedation. After co-housing sessions end, females are returned to their home cage where they are sedated for post-coital exam including colposcopy.

The prototype TTRD is an IACUC approved, humane restraint device commonly used at the Wisconsin National Primate Research Center. The Bioengineering Machine Shop at the WaNPRC manufactured a slightly modified TTRD and transfer box to accommodate our research needs. The TTRD unit is attached to a stainless steel table. [Figure 1] The transfer box is specially designed to lock onto the restraint device. When introducing an animal to the TTRD, a fully alert female macaque is coaxed out of her home cage into the transfer box. The transfer box is set beside the TTRD, on the tabletop, and secured to one side door of the TTRD. To give the animal access to the TTRD, both the door on the transfer box and the door on the adjoining end of the TTRD are opened. With training, the animal will hunch or lie down in the TTRD, then is restrained from movement by closing the clamshell door (length of the unit) and locking it in place. After procedures are carried out, the restraint is released, the animal returns to the transfer box and is returned to her home cage. In accordance with the *Guide for the Care and Use of Laboratory Animals* all macaques were conditioned by positive reinforcement to the use of the TTRD prior to initiation of the research study, and restraint time is minimized to less than five minutes per session.

Results

Coital activity ad libitum

The initial feasibility studies utilized four female and two male pigtailed macaques. A third male macaque was removed from this cohort due to his lack of copulatory participation. Suitable mating pairs were established by observation of non-aggressive behaviors while housed side by side, in grooming contact and while co-housed for mating sessions. Within established mating pairs, coital activity was documented during each stage of each female's menstrual cycle. Generally, multiple episodes of mounting occur, with the majority of activity occurring within fifteen minutes of co-housing the animals. On average five mounts

per co-housing session were documented (range 0 to 19), and 78% of all co-housing sessions were considered successful in that coital activity was observed. These observations confirm that compatible *Macaca nemestrina* macaques will copulate during any phase of the female's menstrual cycle, and that co-housing sessions of fifteen minutes are sufficient for reliable mounting to occur.

After confirming the feasibility of conducting coital studies, an increased number of macaques, twelve females and seven males, underwent temperament and co-housing assessments. In these studies, aggressive behavior was noted in less than 10% of all male-female introductions. Observation of aggression between any two animals ruled them out as mating partners. In total, six of twelve female macaques became pregnant as a result of these cohousing studies. Interestingly, though not germane to these studies, one gave birth to twins (4). Four of seven male macaques have remained on this program for further coital studies, and with NIH (sponsor) and IACUC approval, all have undergone vasectomy. To date, each of the vasectomized males continues to reliably mount females during co-housing sessions.

Post-coital cervicovaginal environment

From the original cohort of four female and three male *Macaca nemestrina*, a total of 48 pre-coital (baseline) vaginal exams conducted 24 to 48 hours prior to co-housing, were paired with 48 vaginal exams conducted 15-30 minutes after documented coital activity. Measures collected for comparison include colposcopic observations, vaginal microbiologic assessments and vaginal pH values.

Colposcopy—Upon initial placement of the colposcope, the presence or absence of a copulatory plug was immediately noted. In 18 of 48 post-coital exams, semen in the form of a copulatory plug was observed and removed from the vagina so the colposcopic exam could continue. Hematoxylin and eosin staining of semen samples collected before and after vasectomy confirm the presence and absence of sperm in the copulatory plugs [Figure 2].

Results of 48 paired colposcopic exams are summarized in Table 1. Cervical and vaginal ecchymosis was noted at least once in post-coital visits in each of the four macaques included in these exams. Every observation of tissue friability and abrasion occurred in a single animal, though they occurred at multiple visits. We anticipate that sexual activity (mounting) will likely result in increased incidence of petechiae and ecchymosis on the macaque tissues, and less frequently cause tissue friability and/or abrasions. The occurrence of friable tissue and abrasions underscores the importance of testing topical microbicides in the presence of coitus.

Vaginal Microbiology—Microbiologic assessment of vaginal swabs prior to and after coital visits revealed very similar components and prevalence of vaginal microflora.[Figure 3] Notably, *Gardnerella vaginalis* was not present in any of the pre-coitus specimens, but detected in 10% of the post-coital samples. The presence of aerobic Gram positive cocci (including *Aerococcus* species, *Facklamia* species, or unknown genus) also increased after coital visits. Additional species that were assessed but found in 5% or fewer samples include: bacillus, proteus, micrococcus, non-pigmented anaerobic Gram negative rods, enterococcus, *E. coli*, group B, C and F streptococci, and non-groupable beta streptococci.

pH measures—Vaginal pH increased by about one half of a pH unit following coital activity.[Table 2] Post-coital vaginal pH was similar whether or not semen was documented in the vagina. In five instances we measured the pH of removed copulatory plugs, providing a mean pH value of 7.4.

Integrating coital visits with the safety protocol

Since we cannot sedate females immediately prior to coital visits, we intend to use the previous day's colposcopy for comparison to the post-coital colposcopy. That is to say, for the eight day safety evaluation, females will be sedated on days one and three to perform pre-coital safety assessments including colposcopy, and collection of vaginal swabs for microflora and pH measures, followed by intravaginal product delivery. [Table 3] After thirty minutes a second set of vaginal swabs will be collected for microflora and pH measurements. On days two and four, the females will be gently restrained in the TTRD for vaginal swab collection and intravaginal product delivery, then co-housed with their established male partner for fifteen minutes and separated. The female will then be sedated to perform post-coital safety measurements including colposcopy, microflora and pH assessments and documentation of the presence or absence of a seminal plug. Days five and eight will involve sedation for cervicovaginal assessments including colposcopy as follow up visits. This modified safety protocol allows for two co-housing sessions (days two and four) during the eight-day safety protocol.

Discussion

The overarching goal of this study was to establish a macaque model for coital activity which could be used to assess the safety of topical microbicide products. In order to do so, we had to determine that pigtailed macaques could be expected to engage in coitus (mounting) independent of the female's menstrual cycle. This fact alone would enable us to conduct coital studies without having to hormonally treat females or otherwise monitor their menstrual cycles for timed experimentation. Male rhesus and pigtailed macaques have been observed to mount females with regularity(2); although in individually housed captivity such as ours, mating times are purposefully scheduled to coincide with ovulation to optimize breeding success (1,10). Because macaques naturally exist in hierarchical groups where dominance plays an important role in mating activity, we had to determine whether our male/female pairs were compatible before granting them access to one another. For screening purposes, we individually housed each female in close proximity to each individually housed male. When compatible pairs were identified, the female was slowly introduced to her male partner by gradually increasing access to each other, until animals were allowed full access to each other for mating contact. In order to determine whether individually housed pigtailed macaques would reliably mount ad libitum during brief co-housing sessions, we monitored the menstrual cycle of several females, while establishing compatible male partnerships, and repeatedly allowed the animals full access to each other during brief, monitored sessions throughout the female's cycle.

While co-housed, paired macaques were visually monitored. The male pigtailed macaque has been described as a "multiple mount ejaculator", meaning ejaculation is generally preceded by a series of intromissive mounts (9,10). The period of time elapsed before initial mounting, between subsequent mountings and total number of mountings were documented. Initially, animals were co-housed for up to forty minutes but the time was reduced after we determined the majority of observed coital activity took place within the first 15 minutes of open access. With this phase of the study, we indeed determined that compatible pigtailed macaque partners will engage in coital activity ad libitum.

The pigtailed macaque vaginal safety model is under NIH contract (N01-AI-70013) to provide standardized preclinical safety data for topical microbicide products in development. In this model, measures of product safety include cervicovaginal colposcopy, vaginal microbiologic evaluation, and vaginal pH monitoring. These measures are collected twice daily for four consecutive days, once prior to product exposure, and again 30 minutes after administration. Follow up measures are collected again on the fifth and eighth day. Through

colposcopic, microbiologic and pH evaluations, this model characterizes the vaginal environment's response to repeated topical product applications in the absence of the exogenous factors of intercourse and potentially infectious ejaculate. While our preclinical evaluations of topical microbicide products have been well-rounded in many aspects, we had yet to investigate the effects of sexual intercourse on the cervicovaginal environment. It is reasonable to hypothesize that mucosal perturbation, including potential micro-trauma in the form of epithelial abrasions, are likely to result from sexual activity. Additionally, the effect that semen may have on constituents of vaginal microflora and on the environment's pH level had not yet received due attention.

By comparing cervicovaginal measures before and after coital activity in a small subset of animals, we have noted increased tissue perturbation by colposcopy, a slight shift in vaginal microbiology, and an increase in vaginal pH. These findings lead us to believe that the standardized topical microbicide safety protocol currently followed by this laboratory for NIH contracted studies will indeed be sensitive enough to measure coitally-induced alterations to the cervicovaginal environment. Studies designed to assess a larger pool of macaques using the standardized protocol with the inclusion of two coital visits are now underway.

In the standardized safety protocol, cervicovaginal measures are collected immediately prior to product exposure, and 30 minutes later. The thirty minute time point was selected to approximate the time of potential vaginal/cervical exposure to infectious pathogens if a test product was intended for coitally-dependent usage. Comparing the measures from product-naïve to product-exposed tissues allows us to better understand short term effects that a test product may induce to the target environment. When expanding the safety model to include coital measures, we were faced with the difficulty of collecting pre-coital measures from animals that would need to be fully alert immediately after vaginal exam. The hurdle of pre-coital sedations was overcome by the introduction of the table-top restraining device and habituating the animals to its use. While the TTRD has a long history of use in other Primate Centers and with other macaque species, this was a novel approach in our hands. It has proven to be a successful alternative to sedating macaques for minimally intrusive procedures. We cannot however perform colposcopic exams on fully alert, restrained macaques. Therefore, we modified the safety protocol to accommodate this limitation. Rather than conducting colposcopy during the pre-coital exam for comparison to the post-coital colposcopy, we compare the post-coital colposcopy to that conducted the previous day. With this design, we can now implement an eight day safety study with two coital visits occurring on days two and four. All of the standardized safety measures are retained, with the additional notation regarding the presence or absence of a copulatory plug as an indicator of semen in the vaginal environment. The revised protocol is poised to assess topical microbicide safety alone (days one and three), with coital activity (days two and four), interacting with semen components (if documented as present), and after repeated daily applications (follow up days five and eight). The addition of sexual activity in this model better mimics intended human use of topical microbicides.

In conclusion, the pigtailed macaque model for assessment of topical microbicide safety has been refined to include sexual activity throughout the menstrual cycle allowing for a more rigorous assessment of topical microbicide safety in the presence of coitus. The post-coital model now more closely mimics intended human use of the products. Baseline data is being collected in the absence of any intravaginal product and in the presence of a HEC placebo gel to provide for comparisons in subsequent topical microbicide product testing.

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Figure 1. Table-top Restraint Device (TTRD)

- a. Transfer box with doors removed
- b. Animal holding area of TTRD. Clamshell hinge is deployed to restrain the macaque
- c. Transfer box attached to TTRD.

Figure 2. Documented presence of semen

a. Copulatory plug(coagulated semen) *in situ*; **b.** copulatory plug removed from vagina; **c.** Hematoxylin and eosin (H&E) staining documents the presence of sperm in the pre-vasectomy ejaculate; **d.** H&E stain confirms absence of sperm in post-vasectomy ejaculate.

Figure 3. Percent prevalence of vaginal microflora pre- and post-coitus: Repeat visits among 4 female macaques

Bars indicate the prevalence (semi-quantitative score 1-4) of each noted microorganism detected from vaginal swabs collected from 48 paired visits (pre- and post-coitus), representing 4 female macaques. Pre-coital samples were collected 24 to 48 hours prior to macaque co-housing. Post-coital samples were collected 15 to 30 minutes after the completion of the co-housing session.

Table 1
Pre- and Post-Coital Colposcopic findings: Repeat visits among 4 female macaques

N=48 paired exams		Pre-coital (n=48)	Post-coital (n=48)		Pre-coital (n=48)	Post-coital (n=48)
Cervix	Erythema	0	0	Vagina	Erythema	0
	Petechiae	27	29 ¹		Petechiae	19
	Ecchymosis	2	18 ²		Ecchymosis	7
	Friable	0	2 ³		Friable	1 ³
	Abrasion	0	4 ³		Abrasion	1 ³

¹ 22 of which had petechiae noted at pre-coital exam

² one of which had ecchymosis noted at pre-coital exam

³ all observed in the same macaque

⁴ 18 of which had petechiae noted at pre-coital exam.

Table 2
Macaque pH measures: Repeat visits among 4 female macaques

	Pre-coital exam		Post-coital exam	
	mean	sd	mean	sd
Vagina n=48	7.0	±0.7	7.6	±0.4
Vagina: copulatory plug noted n=18			7.4	±0.3
Vagina: no copulatory plug noted n=30			7.7	±0.5
Semen: copulatory plug material n=5			7.4	±0.1

Table 3
Topical Microbicide Safety Assessment with Coital Activity: Protocol Design

			Day 1	Day 2	Day 3	Day 4	Day 5	Day 8
			Sedate	Restrain	Sedate	Restrain	F/U	F/U
Time-0	Colposcopy	Vaginal swab	X		X		X	X
			X	X	X	X	X	X
		Microflora pH	X	X	X	X	X	X
Time-30min			No coitus Wait 30 min	Co-housed 15 min then sedated	No coitus Wait 30 min	Co-housed 15 min then sedated		
	Colposcopy	Vaginal swab		X		X		
			X	X	X	X		
		Microflora pH	X	X	X	X		