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Evaluation of vaginal drug levels and safety of a locally administered glycerol monolaurate cream in rhesus macaques

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Abstract

The human immunodeficiency virus (HIV) epidemic affects millions of people worldwide. As women are more vulnerable to infection, female-controlled interventions can help control the spread of the disease significantly. Glycerol monolaurate (GML), an inexpensive and safe compound, has been shown to protect against simian immunodeficiency virus infection when applied vaginally. However, due to its low aqueous solubility, fabrication of high dose formulations of GML has proven difficult. We describe the development of a vaginal cream that could be loaded with up to 35% GML. Vaginal drug levels and safety of three formulations containing increasing concentrations of GML (5, 15 and 35% w/w) were tested in rhesus macaques following vaginal administration. GML concentration in the vaginal tissue increased as the drug concentration in the cream increased, with 35% GML cream resulting in tissue concentration of ~0.5 mg/g, albeit with high inter-individual variability. Compared to the vehicle control, none of the GML creams had any significant effect on the vaginal flora and cytokine (MIP3 α and IL-8) levels, suggesting that high dose GML formulations do not induce local adverse effects. In summary, we describe the development of a highly-loaded vaginal cream of GML, and vaginal drug levels and safety following local administration in macaques.

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Introduction

Human immunodeficiency virus (HIV) infection remains a major epidemic with ~2.1 million new cases and ~1.5 million acquired immunodeficiency syndrome (AIDS)-related deaths reported in 2013.¹ Women are disproportionately burdened by HIV in many regions,^{2,3} including in Sub-Saharan Africa, which is home to over 70% of the world's HIV infected population.⁴ Therefore, effective female controlled HIV prevention options are clearly needed. Although antiretroviral (ARV) containing vaginal products for HIV prevention have had encouraging results,^{5–7} adherence to these products has proven to be a major issue.^{6,8,9} The presence of pre-existing inflammation in the female reproductive tract (FRT) has also been shown to decrease the efficacy of vaginal microbicides.¹⁰ Therefore, a vaginal product that is highly acceptable to women and also capable of modulating inflammation in the FRT has the potential to be more effective than previously studied formulations.

Glycerol monolaurate (GML), a fatty acid monoester commonly used in the cosmetic and food industries, has been shown to block the expression of pro-inflammatory cytokines and chemokines expressed in the FRT in response to HIV.^{11,12} Blocking these signaling pathways inhibits the recruitment of CD4⁺ target cells to the site of initial infection, which in turn prevents the local expansion of HIV that ultimately leads to systemic dissemination of infection.^{11,13} When applied vaginally, a gel containing 5% GML has been shown to protect against repeated high dose vaginal challenge of simian immunodeficiency virus (SIV) in rhesus macaques.¹¹ Furthermore, the vaginal delivery of GML has been shown to be safe for repeated use and to have minor effects on the vaginal flora.^{12,14}

While its mechanism of action, efficacy and safety profile makes GML an attractive microbicide candidate, the physicochemical properties of the gel used in studies to date are not optimal. Because of GML's hydrophobicity, KY warming gel has been used as the vehicle rather than hydroxyethyl cellulose (HEC)-based aqueous gel, the preferred vehicle for vaginal microbicides.¹⁵ Furthermore, given the solubility issues of GML, the maximum concentration of the drug achievable in the original formulation was only 5%. Finally, the original 5% GML/KY warming gel formulation has very low viscosity and is associated with leakage when applied vaginally, a characteristic that may impact acceptability.¹⁶ Therefore, we set out to develop and rheologically characterize an HEC-based cream of GML and to evaluate the safety and local concentrations of this cream formulation in a rhesus macaque model.

Materials and methods

Materials

Tween® 80, glycerol, N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA), pyridine and heptadecanoic acid were obtained from Sigma Aldrich (MO, USA). HyClone™ cell culture grade water was purchased from Thermo Scientific (MA, USA). GML was obtained from BASF Corporation (NJ, USA). Natrocellulose® HEC was obtained from Ashland Inc. (KY, USA).

Preparation of GML cream

To prepare GML cream, GML and Tween® 80 were weighed and transferred to a glass vial. HEC, glycerol and HyClone™ cell culture grade water were mixed in a separate glass vial. Both mixtures were heated to 70°C for 10 minutes in a water bath. The cream was formed by adding the oil phase to the aqueous phase and mixing thoroughly.

For viscosity measurements, the cream was stored overnight at room temperature. For animal studies, the hot emulsion was filled into 1 mL needleless tuberculin syringes and allowed to cool *in situ*. The composition of the cream used for animal studies is listed in Table 1.

Rheology of GML creams

To determine the influence of HEC on the viscosity of the cream, the quantity of the polymer was varied from 0.5–2.5 % w/w while maintaining the concentration of GML constant (15 % w/w). To determine the influence of GML concentration on the viscosity, the quantity of GML was varied from 5–15 % w/w at a HEC concentration of 2.5% w/w.

Rheometry was performed using an Ares-G2 parallel plate rheometer (plate diameter 25 mm, 25°C). An appropriate amount of cream was loaded on to the plate using a spatula. The shear rate was increased as a step function and viscosity was measured at each shear rate. Power law was used to model the relationship between viscosity and shear rate.¹⁷

$$\eta = K * \dot{\gamma}^{n-1}$$

η = viscosity

$\dot{\gamma}$ = shear rate

K= flow consistency index

n= flow behavior index

The values of K and n were used to determine the intrinsic viscosity and pseudoplastic behavior of the system, respectively.

Rhesus macaques, product administration and collection of samples

We followed the National Research Council Guide for the Care and Use of Laboratory Animals and received approval from the University of Wisconsin Institutional Animal Care and Use Committee (IACUC) for this study. Twelve female rhesus macaques (5 to 12 years old) were randomly assigned to one of the four study groups: HEC/Tween® 80, 5% GML, 15% GML or 35% GML. Macaques were housed in pairs in standard stainless steel primate cages (Suburban Surgical, Chicago, IL). Care of the animals was performed as previously described.¹² For product administration and collection of swabs and biopsies, animals were transferred to a table top restraint device using a transfer box and were gently restrained. Prior to sample collection or dosing, the genital region of each animal was wiped with dilute chlorhexidine solution in a single motion from vagina to anus followed by a clean gauze

wipe in the same direction. The vagina was manually opened slightly, and a 1 mL syringe without a needle was inserted atraumatically into the vagina until approximately the 0.4 mL mark. A dose of administered agent was delivered into the vagina and the syringe was removed; animals were dosed once a day, between 7:00 a.m. and 8:00 a.m. Control animals received 1 mL of vehicle control gel, and GML animals received 1 mL of GML containing gel. For collection of vaginal swabs for microbial and cytokine studies, swabs remained in the vagina for approximately 1 min before they were carefully removed and placed in appropriate containers. Colposcopy was performed prior to biopsy to evaluate for gross inflammation. Findings were graded as previously described.¹² Animals were anesthetized for cervical biopsies, which were performed as previously described. Cervical biopsy samples for drug analysis were placed in 1.5 mL Eppendorf tubes, and shipped priority overnight on dry ice to the University of Minnesota, Minneapolis, MN. Schedule and timing of sample collection is detailed in Figure 1.

Determination of GML concentrations in tissue biopsies and swabs

GML was extracted from tissue biopsies and swabs using dichloromethane for 2 h at room temperature. A fraction of the dichloromethane extract was transferred to vial inserts (Sigma Aldrich, USA) and spiked with the internal standard (heptadecanoic acid dissolved in dichloromethane). The solvent was evaporated under nitrogen gas for 30 minutes at room temperature. The samples were reconstituted in a mixture of BSTFA and pyridine (5:1 v/v) and placed in a pre-heated oven at 70°C for 30 minutes. The samples were cooled to room temperature and analyzed by GC/MS.

For GC/MS analysis, 2 µL of sample was injected onto a DB5 column (30 m × 0.25 mm ID, 0.25 µm film thickness). The temperatures of the injector and detector were maintained at 300°C. The initial column temperature was 100°C. After 1 min, the column temperature was increased to 150°C at a rate of 10°C/min, and then increased to 210°C at a rate of 25°C/min. Finally, it was increased to 320°C at a rate of 35°C/min and held there for 7.1 minutes. The total run time was 18.64 minutes.

Ions were generated using electron impact ionization. Two ions were monitored – GML (m/z 315.2) and heptadecanoic acid (m/z 327.2). The retention times were 9.42 min and 9.22 min respectively.

Cytokine analysis

Concentrations of interleukin 8 (IL-8) and macrophage inflammatory protein 3α (MIP3α) in cervical vaginal fluid (CVF) were measured at two points prior to product administration (day 7 minus 7 and day 0) and at weeks 1, 8, 10 and 12 during the period of daily product administration (Figure 1). CVF was collected using weighed cotton swabs that consistently and reproducibly absorbed 0.1 mL of CVF. CVF was always collected before administration of the vaginal product. After collection, swabs were placed at 4°C and shipped from the Wisconsin National Primate Research Center to the University of Minnesota. Upon arrival, swabs were diluted in 0.9 mL of phosphate buffered saline and concentration of MIP3α and IL-8 was measured by ELISA according to the manufacturer's directions (R&D Systems, Minneapolis, MN).

Vaginal microflora determination

CVF for microflora determination was collected on swabs and shipped to the University of Minnesota as described above for cytokine samples. Samples were obtained 7 days prior to product administration, the day of treatment initiation and weeks 1, 2, 4, 6, 8, 10 and 12 during the period of daily product administration. Swabs were also collected 1 and 2 weeks after the last dose of vaginal products were administered (Figure 1). Upon arrival to the University of Minnesota, swabs were placed in 0.9 mL pre-cooled Todd-Hewitt broth at 4°C and then serially diluted in additional broth for quantitative counts on chocolate agar plates that were incubated aerobically at 37°C in a 7% CO₂ incubator. Lactobacilli, staphylococci, streptococci, gram-negative rods and yeasts were identified using methods previously described.¹²

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and post-hoc Bonferroni test. Differences were considered statistically significant if $p < 0.05$.

Results

Rheology of GML creams

The viscosity of GML creams was analyzed using a plate rheometer. Figure 2A shows the effect of concentration of GML on the viscosity of the cream. All formulations displayed a shear thinning behavior. The flow consistency index increased with an increase in GML concentration. The flow behavior index decreased slightly with an increase in concentration of GML.

The effect of different concentrations of HEC on the rheology of the cream is shown in Figure 2B. As noted with increasing concentrations of GML, with an increase in concentration of HEC there was an increase in the flow consistency index. This indicated that the creams with higher levels of GML and HEC had a higher viscosity. However, the flow behavior index was unaffected by changes in the concentration of HEC. This indicated that the pseudoplastic behavior of the cream was unaffected by HEC concentration.

The effect of GML and HEC concentrations on the rheology of GML creams is summarized in Table 2.

Vaginal levels of GML in rhesus macaques following local delivery

We monitored the levels of GML in tissue biopsies and swabs 4 h post dose at various times during the treatment cycle (Figure 3A). As expected, GML concentrations on swabs were highest in animals treated with 35% GML cream. On days 0 and 1, the levels of GML on the swabs were higher than those observed on the later days. The levels of GML remained constant over the rest of the study. We also monitored the levels of GML on the swabs at day 7 and 14 after discontinuation of the treatment. GML was undetectable in all the treatment groups.

We measured the concentration of GML in vaginal tissue biopsies during the treatment cycle. Similar to our results with swabs, with an increase in concentration of GML in the cream there was an increase in GML concentration in the tissue biopsies (Figure 3B). However, no detectable levels of GML were observed at 7 and 14 days after the treatment was stopped.

Analysis of the GML levels on swabs and in biopsies with the different formulations is shown in Table 3.

To determine the clearance kinetics of GML from the vaginal cavity, we monitored amount of GML on swabs obtained 4, 8 and 12 h post dose. These results are shown in Figure 3C. The elimination of GML from the vaginal cavity followed a first order rate. A curve describing first order kinetics was fit to the data to estimate the rate constant of elimination of GML. The elimination rate constant was found to independent of the concentration of GML in the cream ($0.25\text{--}0.3\text{ h}^{-1}$). These results are summarized in Table 4.

Cytokine analysis

Overall, there was no difference in concentration of IL-8 in CVF in GML treated groups compared to HEC treated animals (Figure 4A). However, the HEC treated group had a higher level of IL-8 at baseline (day 0; prior to administration of study products) than GML treated animals ($p<0.05$). No statistically significant difference was seen at any other time point. Although concentrations of MIP3 α were quite variable in all groups throughout the study period, there was no overall difference between HEC and GML treated groups and no difference in MIP3 α concentration at any single time point (Figure 4B).

Vaginal microflora determination

In all animals, *Lactobacillus* was the most commonly isolated organism. Number of *Lactobacilli* colonies remained relatively constant over the entire study period in all animals (Figure 5). There were fewer *Lactobacilli* isolated from HEC treated animals compared to GML treated animals one week after completion of the vaginal product treatment course ($p<0.05$) but no difference at any other time point and no overall difference between GML treated and HEC treated animals. Except for two time points (week 10 and 12) in animals treated with 35% GML cream, there were no significant differences in the levels of other organisms (Supplementary Figure S1).

Colposcopy

Colposcopy was performed prior to the collection of biopsies. No abnormalities were identified in any of the animals assigned to the HEC/Tween or 5% GML groups. One of animals receiving 15% GML was noted to have mild epithelial disruption and cervical hyperemia at week 12 but no abnormal findings before or after. The other 2 animals in this treatment arm had normal exams at all time points. In the 35% GML treatment arm, one animal had mild inflammation, cervical hyperemia and epithelial disruption at week 8 only. The other 2 animals had no abnormalities.

Significant gel leakage was noted in 2 of the HEC treated animals and 2 of the 5% GML treated animals. Mild intermittent leakage was noted in 2 of the 15% GML treated animals and in none of the 35% treated animals.

Discussion

HIV infection remains a major health concern, particularly in many African countries. The use of vaginal products as a preventive for HIV transmission has great potential for containing the spread of this disease.¹⁸ GML is an affordable and safe anti-microbial that has been shown to be highly effective at preventing the vaginal transmission of SIV. However, the GML formulation that has used in microbicide studies to date is not optimal from a pharmacologic standpoint given issues obtaining concentrations of GML over 5%, the need to use a hydrophobic vehicle and its low viscosity which contributes to significant leakiness. Our goal was to develop an acceptable vaginal formulation of GML capable of delivering high doses in an aqueous vehicle.

HEC gels are considered the ‘universal placebo’ for vaginal microbicide trials given their stability, lack of epithelial toxicity and lack of anti-HIV activity.^{15,19} However, aqueous gels are not suitable for the delivery of hydrophobic molecules. This is particularly problematic for high dose drugs such as GML. To overcome this issue, we developed an emulsion-based method to incorporate GML in HEC gels. This technology allowed for the incorporation of up to 35 %w/w of GML in the formulation. The formulation consisted of all FDA-approved excipients and involved simple manufacturing conditions.

Further, we hypothesized that higher viscosity of a cream formulation will enable a longer residence time for GML. Administering GML in highly loaded GML creams helped achieve higher local concentrations of the cream in the vaginal tract. The concentration of GML achieved with 35% cream remained higher than the initial concentration achieved by 5% creams for at least 24 hours. This indicates that 35% GML creams may provide protection similar to the 5% creams for a longer period of time, thus decreasing the frequency of dosing. Reducing dosing frequency may positively impact patient adherence to the product.²⁰ It should be noted however that this effect is manifested by higher dose administered and not by a lower clearance. The elimination kinetics for the drug remained identical across the various concentrations in the cream. The residence time of the gel was generally limited by the turnover of fluids in the vaginal cavity.²¹ It is possible that a certain minimum viscosity is required to prolong the *in vivo* residence of creams, and the viscosities of these formulations do not exceed the minimum threshold. Understanding the effect of viscosity on the *in vivo* residence time of creams will be important for further formulation development. Moreover, the use of mucoadhesive excipients may provide a means to further improve residence times.

In a blinded *in vivo* study, we found that higher concentrations of GML in creams were associated with higher levels of the drug on swabs and in tissue biopsies. The amount of GML in tissue swabs and biopsies in animals treated with 15% GML creams were ~3-fold higher than in animals treated with 5% GML. In contrast, amount of GML on vaginal swabs in animals treated with 35% GML creams was ~20–50-fold higher than that in animals

treated with 5% GML. The elimination rate constant for the drug was independent of drug loading in the cream (Figure 3C). It is likely that the higher viscosity of 35% GML creams aids greater adhesion of the cream to the vaginal tract. This could explain why the initial concentration of GML achieved at the time of administration is disproportionately higher for the 35% GML cream (Table 4).

Previous studies have elaborated on the mechanism of action of GML.^{11,22} Exposure of the endocervical epithelium to simian immunodeficiency virus (SIV) initiates innate immune response. This provides the virus with a founder population of immune cells, which enables its systemic dissemination. GML blocks this initial signaling originating from the endocervical epithelium, thereby reducing the number of immune cells available to the virus. Thus, high local concentrations of GML are required for its activity. In our study, high local tissue concentrations were achieved by increasing the loading of the drug in the cream. The mechanism and extent of GML absorption into epithelial cells, and its efficacy when delivered in this formulation are yet to be studied. Future work understanding the mechanism of absorption of GML may aid in further improving the tissue penetration of GML. Moreover, testing the efficacy of the highly loaded GML creams in protecting against SIV infection will be important for the further translation.

Despite significant standardization in our methods of collection of swabs and biopsies, there was significant inter-individual variability in GML levels. Clinical studies with vaginal rings point to differences in variable drug levels in different parts of the vaginal tract.²³ Consequently, small variations in the site of biopsy could affect the observed tissue concentration. Additionally, differences in the turnover rate of vaginal fluids could significantly affect elimination rates. Due to high inter-individual variability, differences in concentrations observed with various formulations of GML were not statistically significant. A better powered study may be required to establish these differences.

An additional issue with vaginal gel or cream formulations is the lack of patient adherence.²⁴ This may stem from poor physical properties of these formulations. For example, women using vaginal gels have reported leakage from the vaginal cavity and messiness associated with the use of these products.^{25–27} Additionally, due to the high turnover rate of vaginal fluids, the active drug has a low residence time at the site of administration and hence requires daily dosing.²⁵ We reasoned that increasing the viscosity of these formulations, while maintaining pseudoplastic behavior, might help overcome both these issues. Incorporation of GML, a waxy hydrophobic compound, significantly increased the viscosity of the formulation, while minimally affecting its shear thinning properties. In fact, we noted significantly less leakiness in the new formulations compared to the original GML/KY warming formulation and, among the new formulations, leakiness improved as GML concentration increased. We expect that these favorable rheological properties will improve patient acceptance of these formulations.

Effective clinical translation of this strategy may require chronic dosing of high amounts of GML. Hence, safety and tolerability of the formulation are of primary concern. Local delivery of GML achieved with our formulation does ensure that highest concentrations of GML are achieved at the site of action. The levels of two cytokines, MIP3 α and IL-8,

markers of inflammation, remained unaltered during the entire study. Additionally, these levels were comparable to the levels obtained with placebo treatment. Moreover, GML did not impact the levels of healthy lactobacilli in the CVF and no persistent signs of vaginal or cervical inflammation were seen in any of study animals.

In summary, we described here the formulation, rheological analysis and vaginal concentrations of a novel, high-dose formulation of GML. The creams displayed pseudoplastic behavior. Topical application of the cream led to high concentrations in vaginal tissue biopsies (15–400 µg/g) with no detectable signs of inflammation or alteration of healthy vaginal microflora. Future studies will investigate the efficacy of these formulations of GML in preventing HIV transmission.

Conclusion

We reported here the formulation of a highly loaded HEC based cream of GML. Administration of the cream in the vaginal cavity was found to be safe and resulted in high GML concentrations in the vaginal cavity.

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Figure 1. Time line of treatment administration, colposcopy, and swab and biopsy collection
 Twelve rhesus macaques were randomly assigned to four treatment groups: HEC/Tween 80, 5% GML, 15% GML and 35% GML. Animals were treated with one dose of the various formulations each day for 12 weeks. Vaginal swabs were collected at various time intervals for analysis of cytokine levels, vaginal flora and drug concentration. Tissue biopsies were taken for analysis of drug concentration.

Figure 2. Analysis of viscosity of GML creams

GML creams with different concentrations of (A) GML and (B) HEC were prepared and their viscosity was measured using a parallel plate viscometer. A curve describing the power law was fitted to the data. The flow consistency index increased with an increase in concentration of GML and HEC.

Figure 3. Vaginal concentrations of GML in rhesus macaques following local administration Rhesus macaques were treated with three formulations of GML cream containing 5, 15 and 35% GML for 12 weeks. (A) Vaginal swabs and (B) tissue biopsies were collected at various times during the treatment period and drug levels were assessed using GC/MS. Data represented as mean $\pm$ S.D., n=3, *indicates $p < 0.05$, 35% vs. 5%, one-way ANOVA, post-hoc Bonferroni test. (C) Drug concentrations in vaginal swabs after the completion of 12-week treatment period were measured to determine elimination kinetics of GML. Data is represented as an average of mean $\pm$ S.D., n=3.

Figure 4. Evaluation of cytokine levels in CVF

The concentrations of **(A)** IL-8 and **(B)** MIP3α in CVF were analyzed using ELISA. In **(A)** data is represented as mean $\pm$ S.D., $n=3$, *indicates $p<0.05$, HEC/Tween 80 vs. each treatment group, one-way ANOVA, post-hoc Bonferroni test. In **(B)** each line indicates a single animal, for several animals levels of MIP3α were below the lower limit of quantification.

Figure 5. Effect of GML cream on vaginal lactobacilli

CVF was collected using on swabs and then cultured on chocolate agar plates, and the number of lactobacilli colonies were counted. Data represented mean $\pm$ S.D., $n=3$. *indicates $p<0.05$, one-way ANOVA. Post-hoc Bonferroni tests showed that differences between placebo and treatment groups were statistically insignificant.

Table 1

Formulation of GML cream

Ingredient	Quantity (%w/w)
Glycerol monolaurate	5–35
Tween 80	2
Hydroxyethyl cellulose	1
Glycerol	20
Water	q.s.

Table 2

Effect of GML and HEC concentration on the rheological properties of GML cream

Concentration of GML (%w/w)	Concentration of HEC (%w/w)	Flow consistency index, K (Pa.s)	Flow behavior index, n
5	2.5	288.56	0.352
10	2.5	321.08	0.313
15	2.5	561.1	0.27
15	1.5	255.25	0.282
15	0.5	102.65	0.269

Table 3

Analysis of vaginal uptake of GML

	Formulations		
	5% GML	15% GML	35% GML
Swabs			
Maximum observed amount (μg)	128 ± 113	495 ± 206	5705 ± 3774
Time at maximum amount (days)	0	0	0
$AUC_0^{84} (\mu\text{g} * \text{days})$	696	6641	78018
Biopsies			
Maximum observed concentration ($\mu\text{g/g}$)	39 ± 63	109 ± 76	518 ± 581
Time at maximum concentration (days)	84	84	28
$AUC_0^{84} (\mu\text{g} * \text{days/g})$	1823	6169	38574

Table 4

Elimination kinetics of GML delivered in various formulations

Concentration of GML (%w/w)	Elimination rate constant (h^{-1})	Amount at time 0 (μg)
5	0.31	5.3
15	0.25	66.5
35	0.25	5477