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Glycerol Monolaurate Inhibits *Candida* and *Gardnerella vaginalis In Vitro* and *In Vivo* but Not *Lactobacillus*▽

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We investigated the effects of glycerol monolaurate (GML) on *Lactobacillus*, *Candida*, and *Gardnerella vaginalis* human vaginal microflora. Our previous work demonstrated that 6 months of GML treatment vaginally does not alter lactobacillus counts in monkeys. *Candida* and *G. vaginalis* are commonly associated with vaginal infections in women, many becoming chronic or recurrent. *In vitro* growth inhibition studies determined the effects of GML (0 to 500 μg/ml) against multiple *Candida* species and *G. vaginalis*. A randomized, double-blind study investigated the effects of GML on vaginal microflora *Lactobacillus*, *Candida*, and *G. vaginalis* in colonized or infected women ($n = 36$). Women self-administered intravaginal gels containing 0% ($n = 14$), 0.5% ($n = 13$), or 5% ($n = 9$) GML every 12 h for 2 days. Vaginal swabs were collected before and immediately after the first gel administration and 12 h after the final gel administration. Swabs were tested for *Lactobacillus*, *Candida*, *G. vaginalis*, and GML. *In vitro* GML concentrations of 500 μg/ml were candicidal for all species tested, while a concentration of 10 μg/ml was bactericidal for *G. vaginalis*. Control and GML gels applied vaginally in women did not alter vaginal pH or *Lactobacillus* counts. Control gels reduced *G. vaginalis* counts but not *Candida* counts, whereas GML gels reduced both *Candida* and *G. vaginalis*. No adverse events were reported by participating women. GML is antimicrobial for *Candida* and *G. vaginalis in vitro*. Vaginal GML gels in women do not affect *Lactobacillus* negatively but significantly reduce *Candida* and *G. vaginalis*.

The human vagina is colonized by microbes, and infections occur when the balance is disturbed. Under healthy conditions, vaginal flora is dominated by lactobacilli, which maintain acidic pH through production of organic acids at times other than menstruation (1, 8, 13, 30). Disruptions of vaginal pH or lactobacilli may allow potentially pathogenic microorganisms to grow and dominate.

Bacterial vaginosis (BV) is a common chronic infection characterized by complex vaginal flora changes, which include elevations of vaginal pH and, when symptomatic, malodorous discharge and inflammation (2, 5, 11). BV is associated with preterm delivery, increased risk of HIV transmission, and risk of other infections (17). The prevalences of BV range from 4 to 40% of women, with the highest prevalence among patients at sexually transmitted infection clinics (25). During BV infection, there are reductions in lactobacilli and increases in bacterial groups such as the Gram-negative bacterium *Gardnerella vaginalis* (3, 10, 18, 25). Additional bacterial groups that are associated with BV include *Bacteroides fragilis* and *Peptostreptococcus* (12, 28). Current treatment recommendations for BV include metronidazole and clindamycin (4).

Vulvovaginal candidiasis (VVC) is also a common infection. VVC is caused by *Candida* species, most often *Candida albicans* (7, 26). It is estimated that 70 to 75% of women experience VVC at least once during their reproductive years (14), and 5 to 8% have recurrent VVC (9). *C. albicans* is isolated from the vaginas of 85 to 95% of women (17, 27). Due to the propensity of *C. albicans* to colonize, up to 30% of women develop VVC as a posttreatment complication of BV (6). Current recommendations for VVC treatment include topical azole agents or oral fluconazole for uncomplicated vaginitis; recurrent VVC should be managed with fluconazole (19). The high recurrence rates of BV and VVC indicate the limitation of current antimicrobial therapy and the need for better therapeutics.

Glycerol monolaurate (GML) is a naturally occurring monoglyceride that is generally recognized as safe for oral use by the FDA (Title 21, Code of Federal Regulations [CFR], Part 184) and has been used extensively in the food and cosmetic industries. GML has bactericidal properties for Gram-positive organisms (22, 23) and inhibits signal transduction at microbial plasma membranes, thereby inhibiting transcription of Gram-positive exotoxins (20, 22, 29). A critical exception is that lactobacilli are insensitive to GML. Long-term *in vivo* studies of monkeys show that 50 mg/ml of GML in intravaginal gels does not inhibit lactobacilli and is not proinflammatory (24). Gram-negative bacteria, such as *Enterobacteriaceae*, with intact lipopolysaccharide (LPS) are not susceptible to GML (23).

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We conducted this study to assess the effect of GML on *Candida* and *G. vaginalis*, associated with VVC and BV, respectively. GML was microbicidal for both organisms *in vitro*. GML gels (0.5% and 5%) applied vaginally in women reduced both organisms but did not reduce vaginal lactobacilli.

MATERIALS AND METHODS

Microorganisms. A clinical isolate of *G. vaginalis* was provided by Fairview University Medical Center Microbiology. Four clinical isolates of *C. albicans*, the laboratory *C. albicans* strain SC5314, and one isolate each of *Candida glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. pseudotropicalis* (*kefyr*) were generously provided by Judith Berman, University of Minnesota (UMN).

***In vitro* experiments.** Growth inhibition studies investigated the effects of GML on *Candida* (*C. albicans*, *C. glabrata*, *C. pseudotropicalis [kefyr]*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) and *G. vaginalis*. The organisms were grown for 24 h in the presence of GML (0, 10, 50, 100, and 500 μg/ml) in Todd Hewitt (TH) broth at 37°C with shaking (200 rpm; Becton, Dickinson and Co., Sparks, MD) in 25 ml of medium in 125-ml Erlenmeyer flasks. Numbers of microbial CFU at various time points were determined by serial dilutions and plate counts.

Human study design. We conducted a single-center, double-blind, randomized study, approved by the UMN Institutional Review Board (IRB), to determine the effects of an intravaginal gel containing GML on *Lactobacillus*, *Candida*, and *G. vaginalis*.

Women were recruited by flyers posted at the UMN, Twin Cities, campus. Written informed consent was obtained prior to participation. Women between the ages of 18 and 50 who suspected they might have VVC and/or BV were eligible. Women with acute systemic infection or those using vaginal or perineal antimicrobial, immunosuppressant, antihistamine, or anti-inflammatory medication (except oral nonsteroidal anti-inflammatory medication) within 2 weeks prior to participating were excluded. Women were not allowed to participate during menstruation. Pregnant women were excluded. Women were asked to refrain from sexual intercourse during participation. When CFU counts of *Lactobacillus*, *Candida*, and *G. vaginalis* were analyzed individually, subjects were omitted from analysis if they did not have the microbe being analyzed during at least at one of the two visits (<100 CFU/ml). Study participants were examined by a gynecologist (June LaValleur), who also confirmed the presence of yeast and/or BV before the women received intravaginal gels (except the first dose) and upon study completion. Participants maintained a diary of their experiences with the gels.

Thirty-nine women were randomized in that they used intravaginal GML gels (0.5% or 5%) or no GML for 2 days (total of 4 applications; 1 application every 12 h). Swabs of each woman's vaginal secretions were collected at the initial visit (visit 1) (before gel insertion) and at the final visit (visit 2, 12 h after the final gel application) and were analyzed for microbes. Our studies have shown that vaginal swabs consistently absorb 0.1 ml of fluid, thus allowing quantification of microorganisms. Briefly, during each clinical visit, vaginal swabs were collected and placed directly into 1 ml of TH broth. These were then serially diluted, plated onto chocolate agar plates, and allowed to grow at 37°C in 7% CO_2 for up to 3 days. Plates were analyzed for quantities of *Lactobacillus*, *Candida*, and *G. vaginalis* on the basis of expected microbial characteristics. Numbers of CFU per milliliter of vaginal secretions were determined, and numbers from visit 1 versus those from visit 2 were compared. The minimum level of detection of microbes in our analyses was 10^2 CFU/ml.

By IRB requirements, one subject was not allowed to complete the study, due to the presence of *Trichomonas vaginalis* in the sample collected before the first administration of gel; this subject was enrolled in the study and received her first gel treatment (determined to be 0.5% GML gel after the blind was broken). The subject was advised to seek treatment from her regular physician; she later called the investigators to report that she showed no further evidence *T. vaginalis* infection, as determined by her physician, even though she had received no additional therapy. Data from two subjects, one in the control group and one in the 5% GML group, were excluded from analyses because of unknown material present vaginally during the visit 2 examination.

Swabs were also collected at the initial visit after gel insertion and at the final visit for determination of GML content through use of gas chromatography-mass spectrometry (GC-MS) at the UMN, College of Pharmacy. The lower limit of GML detection was 0.7 μg/ml.

Gel formulation. GML (Cognis, Cincinnati, OH) was dissolved in gels made at the Fairview Compounding Pharmacy, Minneapolis, MN, by a pharmacist (M. L. Peterson) to mimic K-Y warming gel to achieve final concentrations of 0.5% or 5% and pHs of 4 to 4.5 (16, 24). Vaginal applicators (Exacta-Med vaginal dispensers; Baxa Corporation, Englewood, CO) were filled with 5 ml of gels. Applicators containing gels were given to the Fairview Investigational Drug Studies Pharmacy, which distributed filled applicators to study participants so that treatment and data analyses remained blinded to the investigators during experimentation.

The vehicle control for preparing GML gels, mimicking K-Y warming, was chosen because the intravaginal gel is an approved class II medical device for use in women, and GML is highly soluble in the gel. The combination of the intravaginal gel and GML is also regarded as a class II medical device, as determined by the UMN IRB. Medical devices are subject not to Investigational New Drug (IND) exemptions for clinical studies but rather to Investigational Device Exemption (IDE) procedures. In the case of a device that is considered to be a "nonsignificant risk" device, as this one is, the IDE is an "abbreviated IDE," involving review by an IRB rather than by the FDA itself. This device and the research study were reviewed and approved by the UMN IRB, and this approval can be considered to be the "abbreviated IDE."

Data analyses. Differences in number of CFU/ml of vaginal *Lactobacillus*, *Candida*, and *G. vaginalis* were calculated for the initial samples (visit 1), collected prior to the use of intravaginal gels, and the final samples (visit 2), collected upon study completion. Significant differences ($P < 0.05$) by the paired Student's t test or trends toward significance ($P < 0.2$) in number of CFU/ml were regarded as evidence of GML effects. McNemar's test was used to compare the prevalences of microorganisms in visit 1 and visit 2. The analyses were performed using GraphPad Prism version 5.01 (GraphPad software, La Jolla, CA).

RESULTS

***In vitro* studies.** Dose-related effects of GML on *C. albicans* strain SC5314 and five other species of *Candida in vitro* are shown in Fig. 1A to F. GML was fungicidal, as defined by a ≥3-log drop in number of CFU/ml for 24 h, for all *Candida* species at GML concentrations of 500 μg/ml GML within 4 to 8 h and for *C. albicans* and *C. pseudotropicalis* (*kefyr*) also at 100 μg/ml. Four additional clinical isolates of *C. albicans* showed susceptibilities to GML similar to those observed for the laboratory strain. GML (10 μg/ml) was bactericidal, as defined by a ≥3-log drop in number of CFU/ml for 24 h, for *G. vaginalis* (Fig. 2).

***In vivo* studies.** A total of 36 women were analyzed in this study. Most women exhibited vaginal discharge during visit 1, prior to receiving vaginal gel. A few also showed vulvar erythema and edema. One woman was determined to have BV associated with anaerobic streptococci (presumed to be *Peptostreptococcus*) but not *G. vaginalis*; this woman also had a documented *Candida* infection and was included in the study. A Gram stain of this participant's vaginal discharge collected at visit 1 revealed yeasts and high numbers of Gram-positive anaerobic cocci; these organisms were absent in visit 2. This subject was in the 0.5% GML treatment group. After the blind was broken, 9 analyzable subjects received 5% (50 mg/ml) GML gel, 13 received 0.5% (5 mg/ml) GML gel, and 14 received control gel without GML. No adverse events were reported by any of the women.

GML was detected vaginally in the women immediately after initial application of the GML gels (data not shown); GML was not detected (<0.7 μg/ml) in women who received control gels. GML was undetectable in women during the second visit, regardless of gel treatment, indicating that GML did not persist in vaginal secretions for the 12 h between treatments.

Table 1 summarizes the prevalences of the three study microorganisms, comparing visit 1 to visit 2. Because of the small

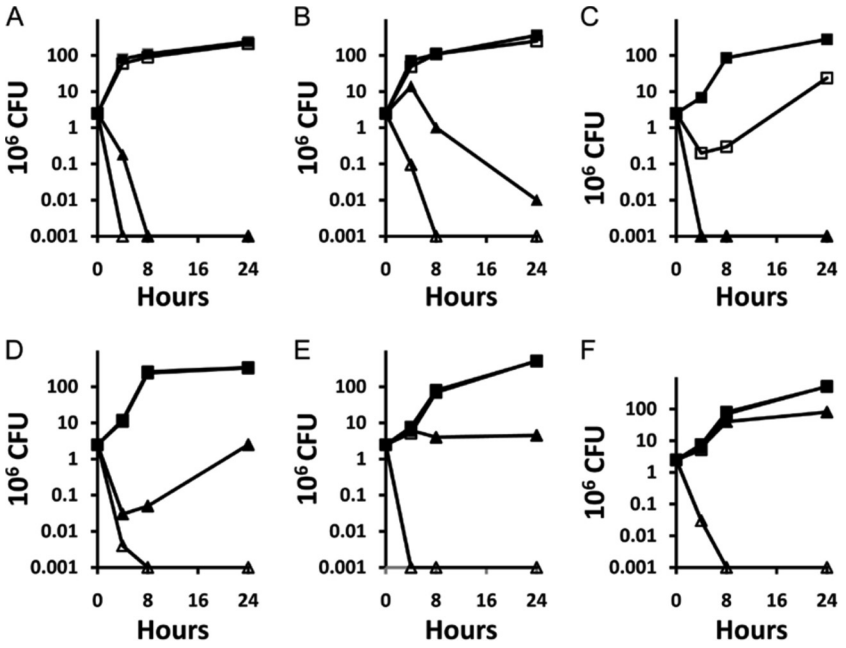

FIG. 1. *In vitro* effect of GML on the growth of *Candida* species: *C. albicans* (A), *C. glabrata* (B), *C. pseudotropicalis* (*kefyr*) (C), *C. parapsilosis* (D), *C. tropicalis* (E), and *C. krusei* (F). *Candida* strains were incubated for 24 h in the presence of various concentrations of GML at 37°C. Samples were removed at the indicated times for determination of CFU counts. The GML concentrations were 0 μg/ml (■), 50 μg/ml (□), 100 μg/ml (▲), and 500 μg/ml (△).

numbers of study participants, data for GML (0.5% and 5%) were evaluated individually but also pooled for determination of significant differences between visits. The prevalences of *Lactobacillus* between visit 1 and visit 2 for all treatment groups were not significantly different, consistent with the idea that the gels with or without GML have no effect on lactobacilli (24). *Candida* prevalences between visit 1 and visit 2 were not different in women given control gels ($P = 0.45$) but were significantly reduced by treatment with GML gels ($P = 0.003$).

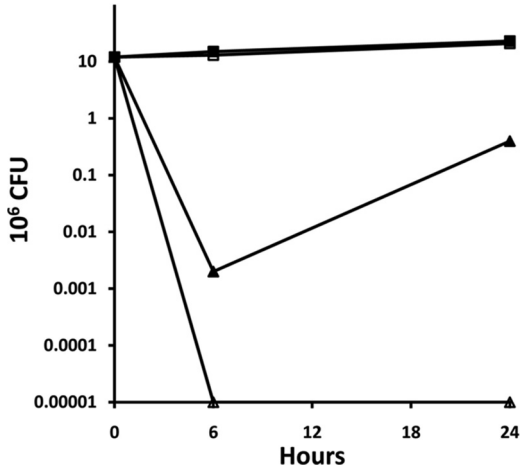

FIG. 2. *In vitro* effect of GML on the growth of *G. vaginalis*. *G. vaginalis* was cultured for 24 h in the presence of various concentrations of GML at 37°C. Samples were removed at the indicated times for determination of CFU counts. The GML concentrations included 0 μg/ml (■), 1 μg/ml (□), 5 μg/ml (▲), and 10 μg/ml (△).

G. vaginalis prevalences between visit 1 and visit 2 were also not significantly different in women given control gels but were significantly lower at visit 2 than at visit 1 for the combined GML treatment groups.

Table 2 summarizes quantitative colony count data for the three microorganisms in the treatment groups. The control and GML gels did not inhibit *Lactobacillus* between visit 1 and visit 2. Three study participants had significant increases in *Lactobacillus* counts, from 10^2 to 10^8 CFU/ml ($P = 0.007$). Since *Lactobacillus* is important for establishing acidic pH in the vagina, pH was measured at visits 1 and 2. No significant difference was seen in vaginal pH between visits 1 and 2, suggesting that none of the gels altered vaginal pH (data not shown).

No significant differences in *Candida* counts were seen between visits 1 and 2 in women who received control gels (Table 2). In contrast, women who received the 0.5% GML gels showed significant reductions in *Candida* CFU at visit 2 in comparison to the level for visit 1 ($P = 0.001$). Women who received the 5% GML gel treatment also showed reductions in *Candida* CFU from visit 1 to visit 2, but this reduction was not statistically significant ($P = 0.15$). When the *Candida* counts for subjects who received either of the GML-containing gels were combined for analysis, thereby increasing the sample size, highly significant reductions from visit 1 to visit 2 were found ($P = 0.001$) (Table 2). The reduction of *Candida* in the GML gel-treated women who showed reductions between visits 1 and 2 was nearly at the minimum level of detection in our analyses (10^2 CFU).

Significant reductions were seen in *G. vaginalis* counts between visits 1 and 2 for women who received the control

TABLE 1. Prevalences of vaginal *Lactobacillus*, *Candida*, and *G. vaginalis* during clinic visits 1 and 2

GML treatment (no. of subjects)	*Lactobacillus*			*Candida*			*G. vaginalis*		
	No. of women (%) positive		P^a	No. of women (%) positive		P^a	No. of women (%) positive		P^a
	Visit 1	Visit 2		Visit 1	Visit 2		Visit 1	Visit 2	
0% (14)	8 (57)	8 (57)	1	9 (64)	6 (43)	0.45	10 (71)	5 (36)	0.07
0.5% (13)	9 (69)	11 (85)	0.48	10 (77)	3 (23)	0.02	7 (54)	4 (31)	0.25
5% (9)	6 (67)	7 (78)	1	6 (67)	2 (22)	0.13	6 (67)	3 (33)	0.25
0.5% and 5% combined (22)	15 (68)	18 (82)	0.25	16 (73)	5 (23)	0.003	13 (59)	7 (32)	0.04

[a] *P* values determined by McNemar's test.

gel ($P = 0.006$) (Table 2). Women who received the 0.5% and 5% GML gel treatments did not individually show significant reductions in *G. vaginalis* levels between visit 1 and visit 2 ($P = 0.13$ and $P = 0.07$, respectively). When data from the 0.5% and 5% GML gel treatment groups were combined and analyzed, the counts of *G. vaginalis* at visit 2 were found to be significantly lower than those observed at visit 1 ($P = 0.015$) (Table 2).

DISCUSSION

Our studies demonstrate that *Candida* and the Gram-negative bacterium *G. vaginalis* are killed by GML *in vitro* and in women by intravaginal GML gels; however, *G. vaginalis in vivo* is also killed by gels lacking GML. Both *Peptostreptococcus* and *T. vaginalis* may be killed *in vivo* by GML gels.

Previous studies demonstrate that GML inhibits growth and production of exotoxins of various Gram-positive bacterial groups but does not inhibit the growth or metabolism of lactobacilli (23, 24). GML does not inhibit *Enterobacteriaceae* unless LPS mutants, such as Re, are present (23). *G. vaginalis* is a Gram-negative bacterium that contains a lipooligosaccharide in its outer membrane and, like Re mutants, is sensitive to growth inhibition by GML (23). We confirmed, and significantly extended, previous reports that GML inhibits *Candida* (15). These yeasts are killed *in vitro* by GML (100 to 500 μg/ml).

Our *in vivo* studies investigated the effects of GML gel on the vaginal microflora in 36 women with VVC and/or BV organisms. In blinded fashion, we evaluated gels containing 0%, 0.5% (5,000 μg/ml), or 5% (50,000 μg/ml) GML for effects on *Lactobacillus*, *Candida*, and *G. vaginalis* over 2 days; both of the GML concentrations are in excess of the *in vitro* concentrations necessary to exert microbicidal activity against *Candida* and *Gardnerella.* When the *Lactobacillus* levels in the women were monitored, we observed that neither the 0.5% nor the 5% GML gels reduced *Lactobacillus* counts or altered vaginal pH; in three instances, *Lactobacillus* counts increased dramatically. In contrast, GML inhibited the growth of *Candida*, in many cases below the limit of our detection. When the data from both the 0.5% and the 5% GML treatment groups were analyzed together, the reduction in *Candida* counts was found to be highly significant compared to the level for the control group. Both control and GML gels inhibited the growth of *G. vaginalis*.

Collectively, our studies show that GML gels simultaneously inhibit the growth of both VVC and BV organisms. (Although not presented, GML *in vitro* inhibits *Bacteroides fragilis*, another Gram-negative bacterium associated with BV.) GML gels may be the first agents that allow simultaneous management of both VVC and BV.

GML may be considered a dual-acting agent for interference with vaginal microorganisms. GML directly kills both *Candida* and *Gardnerella.* However, we recently demonstrated that 5% GML gels, applied vaginally to monkeys, prevented simian immunodeficiency virus (SIV) transmission (16, 24) by interference with epithelial cell production of proinflammatory cytokines that attract CD4 T cells into cervical/vaginal tissues. These data agree with prior studies that demonstrate that GML exerts membrane-stabilizing anti-inflammatory effects (21). In our study, GML anti-inflammatory effects are also likely to contribute to interference with VVC and BV.

It is important to note that this is a small pilot study of GML effects on vaginal microflora. Clinical studies are required to confirm and extend our findings, particularly to assess the effectiveness of GML gel in treating vaginal infections.

TABLE 2. Average log numbers of CFU/ml of *Lactobacillus*, *Candida*, and *G. vaginalis* during clinic visits 1 and 2

GML treatment group	*Lactobacillus*			*Candida*			*G. vaginalis*		
	Avg ± SD		P^a	Avg ± SD		P	Avg ± SD		P
	Visit 1	Visit 2		Visit 1	Visit 2		Visit 1	Visit 2	
0%	5.3 ± 3.2	5.3 ± 3.2	0.45	3.4 ± 1.7	3.0 ± 1.6	0.16	4.5 ± 1.9	3.1 ± 1.9	0.006
0.5%	6.1 ± 2.8	6.9 ± 2.3	0.24	4.6 ± 1.9	2.5 ± 1.2	0.001	3.9 ± 2.1	3.1 ± 2.0	0.13
5%	5.5 ± 3.0	6.1 ± 2.8	0.32	2.9 ± 1.2	2.3 ± 0.6	0.15	4.1 ± 2.1	2.7 ± 1.3	0.07
0.5% and 5% combined	5.8 ± 2.9	6.6 ± 2.5	0.12	3.9 ± 1.8	2.4 ± 1.0	0.001	4.0 ± 2.0	2.9 ± 1.7	0.015

[a] *P* values determined by Student's paired *t* test.

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