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Directed shift of vaginal microbiota induced by vaginal application of sucrose gel in rhesus macaques

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SUMMARY

Objectives: Sucrose gel was used to treat bacterial vaginosis in a phase III clinical trial. However, the changes of vaginal flora after treatment were only examined by Nugent score in that clinical trial, While the vaginal microbiota of rhesus macaques is characterized by anaerobic, Gram-negative bacteria, few lactobacilli, and pH levels above 4.6, similar to the microbiota of patients with bacterial vaginosis. This study is aimed to investigate the change of the vaginal microbiota of rehsus macaques after topical use of sucrose gel to reveal more precisely the bacterial population shift after the topical application of sucrose gel.

Methods: Sixteen rhesus macaques were treated with 0.5 g sucrose gel vaginally and three with 0.5 g of placebo gel. Vaginal swabs were collected daily following treatment. Vaginal pH levels and Nugent scores were recorded. The composition of the vaginal micotbiota was tested by V3∼V4 16S rDNA metagenomic sequencing. Dynamic changes in the *Lactobacillus* genus were analyzed by qPCR.

Results: The vaginal microbiota of rhesus macaques are dominated by anaerobic Gram-negative bacteria, with few lactobacilli and high pH levels above 4.6. After five days' treatment with topical sucrose gel, the component percentage of *Lactobacillus* in vaginal microbiota increased from 1.31% to 81.59%, while the component percentage of *Porphyromonas* decreased from 18.60% to 0.43%, *Sneathia* decreased from 15.09% to 0.89%, *Mobiluncus* decreased from 8.23% to 0.12%, etc.. The average vaginal pH values of 16 rhesus macaques of the sucrose gel group decreased from 5.4 to 3.89. There were no significant changes in microbiota and vaginal pH observed in the placebo group.

Conclusions: Rhesus macaques can be used as animal models of bacterial vaginosis to develop drugs and test treatment efficacy. Furthermore, the topical application of sucrose gel induced the shifting of vaginal flora of rhesus macaques from a BV kind of flora to a lactobacilli-dominating flora.

1. Introduction

Sucrose gel has proven its efficacy in a phase III clinical trial as a novel option for a non-antibiotic-based treatment of bacterial vaginosis (BV). Sucrose gel restores the normal vaginal flora in BV patients by promoting growth of protective lactobacilli and suppressing the replication of pathogenic bacteria.[1] Similar to other clinical studies on BV treatment, the clinical study on the use of sucrose gel was conducted based on grading determined by microscopic examination. Whether the *Lactobacillus* morphotypes belonged to *Lactobacillus* or not was unconfirmed. The influence of sucrose on the vaginal mcirobiota requires further study.

BV has a high rate of recurrence[2] and threatens the health of over 30% of women of childbearing age.[3–5] The vaginal flora of BV patients is characterized by a lack of lactobacilli and a predominance of anaerobic pathogens such as *Gardnerella vaginalis*, *Atopobium vaginae* and *Prevotella* spp., and often presents a high vaginal pH above 4.8. Recent studies have provided robust evidence demonstrating that the vaginal microbiota of rhesus macaques is similar to that of human BV patients and might be

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used as an animal model.[6] In this study, we investigated the impact of sucrose gel on the vaginal microbiota of rhesus macaques to elucidate the mechanism of sucrose gel in the treatment of BV patients.

2. Materials and Methods

2.1. Intervention, animal model and sample collection

The sucrose and placebo gels were produced as previously reported.[1] Briefly, the main ingredient of the placebo gel was xanthan gum, and the sucrose gel was prepared by adding 9% sucrose to the placebo gel. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), Nanshan Hospital, Guangdong Medical College. Female rhesus macaques were provided by West China-Frontier PharmaTech Co. (WCFP)/National Chengdu Center for Safety Evaluation of Drugs (NCCSED), SPF (specific pathogen free), and were approximately four years of age. Female rhesus macaques were included in the study when vaginal conditions satisfied the criteria of human BV diagnosis, including a baseline Nugent score >4, a high vaginal fluid pH (>4.8), few or lack of (1) leukocytes, (2) lactobacilli, (3) *Candida*. After eliminating any data collected from menstruating rhesus macaques, the sucrose gel group contained 16 monkeys, and the placebo gel group contained three. Female rhesus macaques in the sucrose gel group were administered with the gel by syringe to the fornix of the vagina for five consecutive days while the placebo gel group was administered with the placebo gel. On study days one to six, vaginal samples were collected daily to evaluate the Nugent score and measure pH levels. Vaginal smears were observed by microscopy. Separate vaginal swabs were collected for pH measurements and microscopy grading examination.

2.2. Swab samples and DNA extraction

On study days one and six, quadruplicate vaginal swab specimens were obtained and stored at -80 °C for 16S rDNA V3∼V4 metagenomic sequencing and qPCR analysis. The head of the sterile cotton swab collected for each specimen was cut and placed into an eppendorf tube containing 1 mL PB. The tubes were centrifuged for 10 min at 10,000 g to collect bacterial cells, and the supernatant was discarded. Genomic DNA extraction was performed using the QIAamp DNA mini kit (Qiagen, Germany) as described.[7]

2.3. Illumina MiSeq sequencing, sequence processing and classification

The concentration of total DNA extracted from the vaginal swabs was measured using a High Sensitivity Qubit (Life Technologies Corporation, Carlsbad, CA). For each sample, three 50ul PCR mixes were prepared. The V3∼V4 region of the 16S rDNA was then amplified through a polymerase chain reaction (PCR) as previously reported.[8,9] The samples and the negative template control were then analyzed on an agarose gel (1% w/v in TAE buffer) for quality control. The three PCR reactions were pooled and 45 μL of each pooled PCR reaction was purified using Agencourt AMPure beads (Beckman Coulter, CA) according to the manufacturer's instructions, with a final elution in 15 μL of 10× TE buffer. DNA concentration was measured using a Qubit kit (Invitrogen, California, USA). The sample concentrations were adjusted and the same total amount of each sample was used to perform Illumina MiSeq sequencing with a standard amplicon kit according to the 300PE MiSeq protocol.[9] 300PE reads were further processed, including denoising by clustering sequences with less than 3% dissimilarity using USEARCH[10] and de novo chimera detection, conducted with UCHIME v5.1.[11] Taxonomic ranks were assigned to each sequence using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier v.2.2[12] trained on the Greengenes database (Oct, 2012 version),[13] using 0.8 confidence values as the cutoff. Taxonomic classification results for vaginal swabs are shown in Figures 1 and 2 for 300PE MiSeq runs.

2.4. Quantitative Lactobacillus genus-specific real-time PCR Assays

The dynamic changes of the *Lactobacillus* genus as influenced by sucrose were examined by quantitative PCR using a *Lactobacillus* genus-specific forward primer (5′-TGGAAACAGRTGCTAATACCG-3′) and reverse primer (5′-GTCCATTGTGGAAGATTCCC-3′) as previously reported to quantify the copy number of *Lactobacillus* 16S rDNA in each sample.[14] DNA was extracted as described above. Each SybrGreen qPCR reaction mixture contained SybrGreen qPCR Master Mix (2×; ABI, USA), 12.5 μL (10 μM) each of forward and reverse primers, and 1 μL of template DNA in a total volume of 25 μL. The thermocycling conditions for SybrGreen qPCR assays were 95 °C for 10 min, followed by 25 cycles of 95 °C for 10 sec and 60 °C for 40 sec, with a fluorescence read at 60 °C followed by a melting curve analysis. The entire process was conducted on an ABI StepOnePlus Real-Time PCR instrument. For the relative quantification of *Lactobacillus* genus, genomic DNA from *L. fermentum* was extracted and quantified using a Qubit Kit (Invitrogen). The PCR product from *L. fermentum* was used as positive control after gel purification and DNA sequencing verification. Standard curves were generated using five 10-fold dilutions of *L. fermentum* DNA which ranged from 2.58×10^4 to 2.58×10^8 copies/μL. Assay results were expressed as genome/gene lg10 copy numbers per 1 μL of DNA. Negative controls were included in each PCR run.

2.5. Statistical analysis

Data were analyzed using IBM statistical SPSS 2.0 software. Differences in bacteria scores (Table S1-S3) before and after treatment were compared using the Mann-Whitney U Test. Differences in the proportion of *Lactobacillus* DNA in all sequences and the FQPCR assay result of the number of *Lactobacillus* DNA lg copies before and after the treatment were compared using a paired T test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Vaginal pH detection and microscopy grading

The vaginal pHs of animals treated with sucrose gel decreased to below 4.6 in 14/16 animals by day three and by day five the decrease was observed in all treated animals. In contrast, the vaginal pHs of the placebo gel group rhesus macaques stayed above 4.8 following treatment. Prior to treatment, the relative abundance of non-lactobacilli bacteria (*G.vaginalis*, *bacteroides* and *Mobiluncus* spp. morphotypes) dominated the vaginal flora of all rhesus macaques based on Nugent scores, suggesting a depletion of lactobacilli in these models. By day five of sucrose gel treatment, however, the number of lactobacilli gradually increased and dominated the vaginal flora (Fig 3A). Furthermore, the lactobacilli score of the sucrose gel group was significantly lower than before treatment ($P < 0.001$, Table S1), and the *G. vaginalis/bacteroides* and *Mobiluncus* score before treatment was higher than after treatment ($P < 0.001$ and $P < 0.05$, respectively, Table S2 and S3). Based on the Nugent scoring system, 93.8% (15/16) of the vaginal flora of the sucrose gel group became "normal" (Nugent score < 4) according to the human criteria, suggesting that sucrose facilitates the