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Tea Extract Vaginal Ovule for Intermediate Flora: Randomized Blinded Vehicle-Controlled Multicenter Pilot Clinical Trial with Microbiome Analysis

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Abstract: Although tea contains antioxidant and antibacterial compounds, it has not yet been clinically tested in the context of abnormal vaginal flora. Our aim was to determine the safety and efficacy of vaginal ovule with 2 mg tea extract once-daily for 5-7 days for symptoms of intermediate vaginal flora. This was randomized blinded vehicle-controlled multicenter clinical trial with composite primary endpoint (drop in the Nugent score (NS), vaginal pH, or improvement of subjective symptoms) compared between the active and vehicle arm using Fisher´s exact test at α=0.05. Wet mounts were taken from 274 participants from 14 centres, 97 + 95 participants fulfilled the inclusion criteria. In the active and vehicle arm, composite scores improved in 51.8 % (43/83) and 38.3 % (31/81) participants, respectively, representing an odds ratio = 1.73 (CI 0.93-3.23, p=0.082). In all 274 participants, 7 mild adverse reactions were recorded of which 6 occurred in the passive arm. Intention-to-treat analysis showed more frequent improvement of subjective symptoms (p=0.047) and reduction in burning (p=0.006) in the active arm. The results suggest that as little as 2 mg of tea extract have the capacity to relieve subjective symptoms, notably the perception of genital burning. Tolerability of the tea ovule seems at least as good as that of the vehicle alone. Tea extract deserves further evaluation in larger doses.

Key words: Antioxidant; vaginosis; catechin; Nugent score; vaginal pH; vaginal flora.

Introduction

Bacterial vaginosis (BV) is the most common vaginal infection among women of reproductive age [1]. It is characterized by a milky, homogeneous, malodorous vaginal discharge. Generating vulvovaginal discomfort, irritation, and sexual dysfunction [2], BV may also lead to frustration, anxiety, and various psychosocial issues [2]. Even un-

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complicated BV has a moderate to severe impact on a woman's quality of life [3].

Urogenital infection is the major factor associated with difficult conception [4], and consequent *in vitro* fertilization may be unsuccessful due to the bacterial biofilm reaching the endometrium [5]. In addition, pregnant women with BV are twice as likely to experience a preterm birth [2].

Both abnormal flora and symptomatic BV are associated with a higher risk of HPV-mediated cervical carcinogenesis [6,7], pelvic inflammatory disease [2], and sexually transmitted infections [8,9]. Low *Lactobacillus* populations and highly diverse mucosal colonization can trigger vaginal recruitment of CD4+ T-cells [10], which are known to be responsible for initial HIV dissemination [11]. As a consequence, women with BV have 3 to 4-fold higher odds of acquiring and transmitting HIV [2,9,11]. The risk increases with the severity of symptoms [12,13], moreover, advanced pathogenic microflora can degrade prophylactic tenofovir *in situ* [14,15].

The general prevalence of BV ranges from 23 % to 29 % across regions. In Europe and Central Asia, it is estimated to be around 23 % [1]. Due to its high prevalence, BV imposes a large economic burden on society. A recent estimate of the global annual direct costs was €4.2 billion (95 % CI: 3.2, 5.3). When the costs of associated preterm births and incident HIV cases are included, the economic burden nearly triples [1]. Although vaginal metronidazole and clinda-mycin are widely prescribed as the gold standard, the recurrence of BV reaches 50 %, not to mention the risk of resistance. Moreover, neither clindamycin [16], nor metronidazole [17] improve the pregnancy outcome when used to treat BV or even asymptomatic abnormal microflora [18]. Taken together, the treatment alternatives for BV are few and they fail in large portion of patients, hence, exploring durable strategies for normalization of vaginal environment and prophylaxis of BV are of interest.

It was previously shown that redox imbalance significantly correlates with the Nugent score (NS), and the concentration of pro-oxidants is predictive of BV [19]. Tea contains a wide range of strong antioxidant molecules and it can inhibit mucosal adhesion of various pathogenic strains without the development of resistance [20,21]. Quite surprisingly, it has never been tested to treat BV. With respect to a series of promising results from our *in vitro* and animal models, we decided to investigate its potential in clinical setting.

Materials and methods

We conducted a randomized, blinded vehicle-controlled multicenter clinical trial (MAT072017) to determine the effect of vaginal ovules containing 2 mg of the tea extract on the subjective symptoms (5-point scale pruritus (itching), burning , rubor (redness), fluor (discharge), and fetor (odor)), and objective symptoms (pH, NS) of BV. For more details, see clinicaltrials.gov record NCT04171947.

Disturbances of vaginal microflora can be evaluated using the NS [22]. Intermediate vaginal microflora is indicated by a NS of 4-6, whereas a score of 7 or higher is considered indicative of bacterial vaginosis [22]. It has been previously shown that roughly one-third of women with intermediate flora (i.e., NS 4-6) progresses to BV [23,24]. In addition, they often have vaginal complaints and insist on receiving treatment. However, when there are no clinical signs of BV or other infection, antibacterial therapy is usually not indicated. Moreover, resistance to antibiotics is considered one of the greatest threats to public health. Thus, there is an apparent unmet medical need in this population of women since they are not suitable for antibiotic therapy and require alternative treatment. Because of these factors, the trial was designed to enroll patients with NS below 6.

An ovule containing only the vehicle (polyethylene glycol 3000 S) was used as a control. The pH of both tea and placebo ovule were identical.

Primary outcome measures

Correction of the vaginal environment after 7 days of once-daily application of the ovule was defined as: a drop-in NS to < 4 and/or a drop in vaginal pH to < 4.5, or a subjective improvement in at least 2 symptoms by at least 2 points on a 5-point scale in patients whose baseline pH was < 4.5 and NS < 4 (composite end-point).

Secondary outcome measures

(1) Correction of the vaginal environment (as

previously defined) 7 days after the completion of the prescribed 7-day regimen (day 14); (2) change in each parameter separately; (3) change in individual subjective symptoms, (4) exploratory analysis of the microbial environment at each subject visit.

Statistical considerations and the primary hypothesis

Kilmarx *et al.* 2003 have highlighted the unavailability of a true placebo in trials investigating vaginal formulations [25] and Ardolino *et al.* 2016 showed that chemically intact polymers could be used as a mucoadhesive barrier reducing colonization of vaginal mucosa by pathogenic strains [26,27]. Anticipating some vehicle efficacy, we pressumed maximum of 20 % of the subjects in the placebo arm would achieve the primary endpoint compared to at least 40 % in the active arm. Setting the power at 90 % and the significance level at 5 %, and using the Fisher´s exact test, 109 participants per treatment arm were required. With assumed 20 % drop-out, therefore we enrolled 274 participants and randomized them 1:1. The confirmatory analyses were conducted in the per-protocol population defined by inclusion/exclusion criteria. To complement the result, we present estimates from intention-to-treat (ITT) population, that is all patients randomized (137 vs. 137).

STATA 13.1 software (StataCorp LP, USA) was used to calculate the sample size. R software version 3.4.0 (R Foundation, Austria) and Excel (Microsoft, USA) were used for data cleaning and the final analysis.

Eligibility criteria

The inclusion criteria were 1) premenopausal participants with 2) a disturbed vaginal environment 3) who were not in need of antibiotic or antimycotic treatment. The participants were 4) citizens of the Czech Republic 5) between 18 and 55 years who had 6) either a vaginal pH > 4.5 or/and vaginal discomfort and 7) were able to follow the prescribed regimen. All participants 8) signed informed consents prior to any intervention, including diagnostic procedures.

The exclusion criteria were 1) known sensitivity to tea or polyethylene glycol, 2) women with a positive pregnancy test (sensitivity 10 mlU/ml; GSMamatest10; GS; Czech Republic) or 3) who were breastfeeding, 4) women with vaginal bleeding at inclusion, 5) women who had used any type of antibiotic 30 days prior to inclusion or 6) had used any other products, devices or supplements with similar indications, 7) participants with acute urogenital infection, 8) diabetes mellitus, and those 9) who had recently participated in another clinical investigation. During the follow-up, we additionally excluded 10) participants who used less than 5 out of the 7 prescribed ovules and participants with an NS > 6.

Allocation and blinding

A random sequence, in blocks of 4 participants, was generated by an external statistician using STATA 13.1 software (StataCorp LP, USA). Placebo ovules and tea ovules were distinguished by "L" and "K", respectively, at the end of a 9-digit serial code on both the primary and secondary packaging. The ovules were allocated chronologically according to the serial code. Neither the doctor nor patient knew which arm of the study the patient was in. The wet mounts were sent to a central laboratory under a 4-digit code that identified the patient but had no information regarding the study arm. Microscopic analyses were also blinded. The final statistical analysis was blinded from the perspective of the statistician.

Interventions

At the first visit, patients signed informed consent and consent for the processing of personal information (The General Data Protection Regulation (EU) 2016/679). Patients then filled in questionnaires, underwent an initial general examination, had an assessment of their medical history, pH measurements were taken, and wet mounts were prepared. Ovules for a 7-day treatment (7 ovules) were distributed to the participants during the first visit. The time of application, the presence of vaginal bleeding, coitus, subjective evaluations, or adverse events (AE) were recorded daily by the patient in a diary. Physicians contacted participants by phone between the first and the second appointment to verify the correct application of the ovules, note any adverse events, and to organize an immediate appointment with partici-

pants whose laboratory findings showed an NS > 6 or other infection determined based on the mounts taken at baseline prior to treatment initiation. Additionally, adherence and AE were verified by the physician during the follow-up visit. On the second and third appointment, patients underwent the same procedures as during the baseline assessment. All procedures were performed by the patient´s personal gynecologist. All interventions were discussed with the patients and approved by the patients. Patients had the right to withdraw at any time for any reason. For ethical reasons, patients were not rewarded financially.

Microscopy, nugent score and pH

In all patients, pH was determined using uniform gynecology test strips (MColorpHast, Merck, Germany) according to a predefined procedure. Wet mounts were prepared from the posterior vaginal wall using a cotton swab. Fixed and blinded slides (4-digit code) from all study centers were transported to a central accredited laboratory (Private laboratory AeskuLab k.s, Prague, Czech Republic). One slide was stained using Giemsa-Romanowsky and the second using Gram stain. Slides were assessed using an oil immersion lens, providing 1000 x total magnification. The following elements were quantified by a microbiologist: squamous epithelial cells; clue cells; mixed flora; yeast as pseudomycelia; Gram neg. *diplococci*; fibrous *Lactobacillus*; *Gardnerella*; spirochetes; parabasal epithelial cells; leukocytes; yeast as blastospores; Gram pos. *cocci* in chains; *Lactobacillus*; *Mobilincus*; *Leptotrichia*, and *Trichomonas vaginalis*. To describe the vaginal microenvironment, we employed a semiquantitative rating of the number of elements per field (Figure 3). Pointwise semiquantitative rating was performed according to the laboratory standardized procedure. Briefly, the number of elements per field were categorized into 4 points. For Mobiluncus and Gardnerella, 0= none; 1~tenths; 2~hundreds, 3~thousands; and 4 means abnormal infection. For Lactobacillus, 0= none; 1~10 to 15; 2~15 to 50, 3~50 to 100; and 4 means over 100 bacteria per field. For squamous epithelial cells, 0= none; 1~0 to 5; 2~10 to 15, 3~more than 20 elements per field. For leukocytes, 0= none; 1~0 to 5; 2~15 to 25, 3~more than 30 elements per field. For mixed flora, 0= none; 1~0 to 50; 2~100 to 200, 3~more than 300 elements per field. For mixed flora, 0= none; 1~0 to 50; 2~100 to 200, 3~more than 300 elements per field. All ratings were done by the same microscopists. The NS was determined, according to Nugent *et al.* in 1991 [22]. Unclear findings were resolved by 2 independent experts.

The ethics committee, the competent authority, and quality standards

Protocol MAT072017 version 2.0 dated 10-Oct-2017 was approved by the multicenter ethics committee of the Faculty Hospital Hradec Kralové and the State Institute for Drug Control for the Czech Republic. The protocol was registered before trial initiation with EUDAMED reference number CIV-17-09-021504. The final report is available through EUDAMED. The study was designed and organized in collaboration with members of the Czech Gynecological and Obstetric Society. There were 12 remote data check-ups and 3 to 4 onsite monitoring visits carried out in each study center. A total of 49 error reports were recorded. We report in line with CONSORT statement [28].

Characterization of the extract

Tea extract is prepared from pharmaceutical grade *Camellia sinensis* (L.) Kuntze black-tea dried extract by maceration in sodium hydroxide aqueous solution with pH ~ 10 and temperature ~ 60°C for ~24 hours. After pH correction, the extract is dried. The final decaffeinated extract is in crystalline form with water solubility of ~ 6 W/w %. In the amount used for each globule (2 mg), the contribution to the globule pH is negligible.

Used polyethylene glycol 3000 has pH 4 to 7 at 100 g/L, melting point 55 to 58°C, aqueous solubility of 550 g/L at 20°C and corresponds to requirements of Ph. Eur.

Black tea extract was standardized using oxygen radical absorbance capacity assay (ORAC [29]), total phenolic compound (TPC [30]) and mass spectrometry coupled ultra-high-performance liquid chromatography (UPLC-MS). Briefly, the samples of crystalline tea extract were homogenized, dissolved in demineralized water to final concentration of 1 mg/mL and filtered on 0.2 μm membrane. The insoluble portion was removed

by centrifugation, and the ORAC values were determined in the supernatant in the presence of free radicals generated by heating 2,2'-azobis-2-methyl-propanimidamide as the net area under the curve of fluorescein fluorescence at 480 nm. Tea extract has an antioxidant capacity of 1268.5 ± 207.7 µmol trolox equivalent/g (mean ± standard deviation from 3 independent replicates). The TPC values were also determined in the supernatant spectrophotometrically at 750 nm using Folin-Ciocalteu reagent. Tea extract has a total phenolic content of 127 ± 11 mg of gallic acid equivalents/g (mean ± standard deviation from 3 independent replicates).

The caffeine content was analyzed using standardized reference method of UPLC with detection at 270 nm and binary gradient acetonitrile/5mM formic acid. Semi-quantitative determination was performed by comparing the areas of the caffeine peak with a sample of known caffeine concentration (original ph. quality tea extract). The tea extract contains <0.015% caffeine.

The extraction process induces polyphenolic condensation leading to formation of oligomeric structures that are not analyzable by conventional LC. Apart from these structures, smaller molecules appear in the mixture. The most abundant low-molecular substances of the extract were separated using dialysis via 3kDa membrane and analyzed MS-UHPLC. The most abundant low-molecular substance was protocatechuic acid and its derivatives.

In vitro cytotoxicity test

As part of preclinical evaluation of biocompatibility, *in vitro* cytotoxicity tests were performed according to ISO 10993-5:2010. The extract was not cytotoxic in Balb/c 3T3-L1 cell line compared to a positive control (dodecyl sulphate) and negative control (poly(2-hydroxyethyl) methacrylate). The standardized method was performed by an accredited laboratory of the National Institute of Public Health accredited by the Czech Accreditation Institute.

Potential for skin irritation

Skin irritation tests were performed preciously according to ISO 10993-10:2010. 0 out of 30 volunteers (aged 24 to 66) responded to the extract when applied for 15 min, 30 min, 1 hour, 2 hours, 3 hours and 4 hours. Under the test conditions, the extract was not a significant skin irritant. The standardized method was performed by an accredited laboratory of the National Institute of Public Health accredited by the Czech Accreditation Institute and respecting CIOMS 2002 guideline. The experiment was approved by the local Ethics committee.

Sensitization

Skin sensitization tests and local lymph node assay was performed according to ISO 10993-10:2014 and Commission Regulation (EC) 440/2008. The extract was dispersed in a vehicle (acetone + cottonseed oil 4:1) and applied on day 1,2,3 and 7. On the 8th day the animals were sacrificed. Simulation index calculated based on result obtained via autopsy of the local lymph nodes in 4 tested mice, 4 mice in the positive control group (1-Chloro-2,4-dinitrobenzene) and 4 mice in the negative control group (vehicle alone) shows that the extract has no potential for sensitization under the test conditions. The animals showed no signs of systemic toxicity. No redness or skin irritation was observed at the application site. The overall assessment shows that the extract shows no signs of systemic toxicity and is not irritating. The standardized method was performed by an accredited laboratory of the National Institute of Public Health accredited by the Czech Accreditation Institute.

Bactericidal activity

Since the vaginal globules were designed for an intermediate vaginal environment where antibiotic treatment is not indicated, the aim was to verify that the extract would not present bacteriostatic effect. In addition, non-specific bacteriostatic effects could lead to a reduction in symbiotic strains and disturbance of the microbial balance. Bactericidal activity of the extract as well as of the globule vehicle (polyethelene glycol 3000s) were tested in samples of pathogenic strains of *S. aureus, P. aeruginosa, E. coli* and *P. hauserii* and symbiotic strains *L. delbrueckii, L. plantarum, L. paracasei* and *L. acidophillus*. The minimum inhibitory concentration, the minimum

inhibitory concentration with the neutralizer, the minimum bactericidal concentration at protein load and the minimum inhibitory concentration in aqueous solution were determined where the total number of viable cells decreased by at least 5 logs. Tea extract did not show bactericidal activity in any of the strains at a concentration of 6 W/w t % at an incubation time of 32 minutes and for 10 minutes under protein loading in a suspension experiment. Polyethylene glycol 3000s did not show bactericidal activity in any of the strains at a concentration of 1 g/1 ml at an incubation time of 32 minutes for 10 minutes in protein load in a suspension experiment. The standardized method was performed by an accredited laboratory of the National Institute of Public Health accredited by the Czech Accreditation Institute as per CSN EN ISO / IEC 17025:2005.

Results

Between 4-Dec-2017 and 9-May-2018, we included and randomized 274 participants in 14 outpatient gynecology clinics evenly distributed across the Czech Republic. An unforeseen drop-out was caused by a high number of patients with a baseline NS > 6 (15.0%) and the non-compliance of one center, which had to be prematurely closed and excluded from the final analysis of efficacy (8.8%). Thus, data from only 97 + 95 participants were available for the evaluation of the primary endpoint (2nd visit) and 89 + 85 participants for the 3rd visit (see Figure 1). All 274

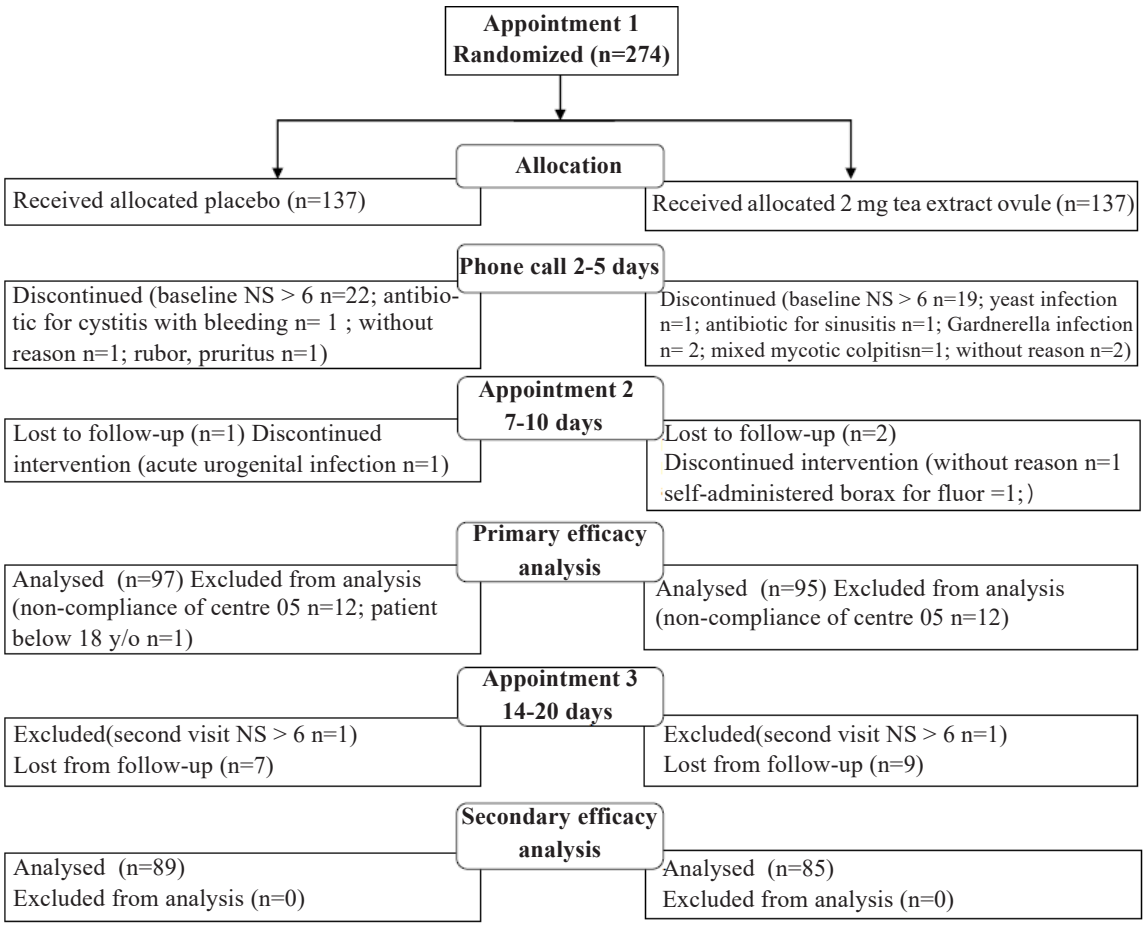


Figure 1. Subject flow-chart

An unexpected drop-out was caused by a high number of patients with a baseline NS > 6 (15.0%) and the non-compliance of Center 05, which had to be completely excluded from the final analysis (8.8%). The data from 97 + 95 participants were available for the evaluation of the primary endpoint. The analysis of the 3rd visit was done for 89 + 85 participants, respectively.

randomized patients entered the safety analysis.

Patient characteristics, anamnesis, and risk factors were collected from patient records, patient questionnaires, and during interviews with physicians. A detailed description of the baseline population (see Table 1) shows that both arms had comparable characteristics. Interestingly, the most common risk factors were sexual activity before 18 years of age, previous pregnancy, and the use of tampons. The dominant symptom of BV was fluor. Thus, if we want to portray an archetypal patient suffering from BV-related discomfort, it is a sexually active woman with a previous pregnancy and using tampons. Vaginal discomfort burdens this patient for more than 3 years, recurring at least every six months and lasting for more than one month. Additionally, she had unsuccessfully used antimycotics/antibiotic/probiotics and is currently not being treated.

At the first visit, 150 participants had a pH > 4.5, 26 participants had an NS between 4 and 6, and 57 participants rated at least two subjective symptoms to be ≥ medium (i.e., 2) on the scale 0-4 corresponding to "none-mild-medium-strong-very strong."

At the second visit (7.7 ± 1.3 days) after the completion of the regimen (1 intravaginal ovule once-daily for 5-7 days), the pH dropped below 4.5 in 36/76 (47.4 %) and 25/74 participants (33.8 %) in the active and vehicle arm, respectively. Intermediate NS (4-6) dropped to normal (NS < 4) in 9/13 (69.2 %) and 10/13 participants (76.9 %; see Figure 2). A 2-point improvement in at least 2 symptoms (i.e., 40 % improvement in each symptom) was noted in 23/32 participants (71.9 %) and 12/25 participants (48.0 %), respectively. Composite scores (see Methods) improved in 43/83 participants (51.8 %) and 31/81 participants (38.3 %), representing an odds ratio (OR) = 1.73 (0.93-3.23) and the number needed to treat (NNT)

Table 1. Anamnestic details, baseline characteristics and risk factors in both arms*

Characteristic	Placebo ovule (N = 97)	Tea ovule (N = 95)
Age (years)	33.7 ± 7.9	33.6 ± 8.4
Body-mass index †	23.7 ± 6.2	23.1 ± 3.6
Caucasian	97 (100 %)	95 (100 %)
Nugent score (0-10)	1.2 ± 1.6	1.1 ± 1.6
pH	5.0 ± 0.5	5.0 ± 0.6
Menstruation during first 7 days	13 (13.4 %)	7 (7.4 %)
Coitus during first 7 days	7 (7.2 %)	6 (6.3 %)
Interval to second visit (days)	7.7 ± 1.4	7.7 ± 1.2
Interval to third visit (days)	16.1 ± 3.2	15.8 ± 2.1
Baseline pruritus ‡ (0 to 4)	0.8 ± 1.0	0.8 ± 0.9
Baseline burning ‡ (0 to 4)	0.7 ± 0.9	0.8 ± 1.0
Baseline rubor ‡ (0 to 4)	0.5 ± 0.7	0.5 ± 0.8
Baseline fluor ‡ (0 to 4)	1.3 ± 0.9	1.3 ± 0.8
Baseline fetor ‡ (0 to 4)	0.4 ± 0.6	0.5 ± 0.8
Tobacco use	15 (15.5 %)	14 (14.7 %)
Psychological difficulties (depression, death in the family, end of a relationship, troubles at work, etc.)	24 (24.7 %)	23 (24.2 %)
More than 3 male sexual partners in the previous 12 months	11 (11.3 %)	14 (14.7 %)
Female sexual partner in the previous 12 months	2 (2.1 %)	1 (1.1 %)
Sexual activity before 18 years of age	68 (70.1 %)	70 (73.7 %)
Previous pregnancy	43 (44.3 %)	49 (51.6 %)
Vaginal douching during previous 3 months	5 (5.2 %)	16 (16.8 %)
Personal hygiene products without low pH	27 (27.8 %)	36 (37.9 %)

table 1. (continued).

Characteristic	Placebo ovule (N = 97)	Tea ovule (N = 95)
Use of tampons	61 (62.9 %)	56 (60.0 %)
Decreased immunity	19 (19.6 %)	19 (20.0 %)
Allergy to condoms, pessary, lubricants or personal hygiene products	7 (7.2 %)	7 (7.4 %)
Previous unsuccessful treatment of colpitis		
Antibiotic	29 (29.9 %)	25 (26.3 %)
Antimycotic	38 (39.2 %)	45 (47.4 %)
Probiotic per vaginam	33 (34.0 %)	30 (31.6 %)
Probiotic per oral	13 (13.4 %)	10 (10.5 %)
Hormonal contraception	33 (34.0 %)	29 (30.5 %)
Other hormonal treatment	7 (7.2 %)	5 (5.3 %)
Antibiotic/antimycotic treatment during previous 3 months	20 (20.6 %)	27 (28.4 %)
Probiotics or other OTC products during the previous 3 months	19 (19.6 %)	24 (25.3 %)
Corticosteroids during the previous 3 months	2 (2.1 %)	3 (3.2 %)
First episode of abnormal vaginal environment	35 (36.1 %)	26 (27.4 %)
Recurrent discomfort	23 (23.7 %)	32 (33.7 %)
Time from the first episode of the abnormal vaginal environment (years)	4.0 ± 5.7	3.5 ± 4.3
Duration of the current episode (days)	35.3 ± 94.8	40.7 ± 121.3
Recurrence once per 6 months	11 (11.3 %)	18 (19.0 %)
Recurrence once per month	7 (7.2 %)	10 (10.5%)
Elements of the vaginal microenvironment (0 to 3)§		
Squamous epithelial cells	2.2	2.2
Clue cells	0.0	0.0
Mixed flora	1.4	1.3
Yeast as pseudomycelia	0.1	0.1
Gram neg. diplococci	0.0	0.0
Fibrous Lactobacillus	0.0	0.0
Gardnerella	0.1	0.1
Spirochetes	0.0	0.0
Parabasal epithelial cells	0.1	0.1
Leukocytes	1.3	1.4
Yeast as blastospores	0.1	0.1
Gram pos. cocci in chains	0.0	0.1
Lactobacillus	1.9	1.9
Mobilincus	0.0	0.0
Leptotrichia	0.0	0.0
Trichomonas vaginalis	0.0	0.0

*Plus-minus values are means ± SD. There were no significant differences between the two randomized groups. † The body mass index is the weight in kilograms divided by the square of the height in meters. ‡ Subjective symptoms rated on a scale 0–4 corresponding to “0-none, 1-mild, 2-medium, 3- strong, and 4-very strong. § Semiquantitative rating according to laboratory standardized procedure

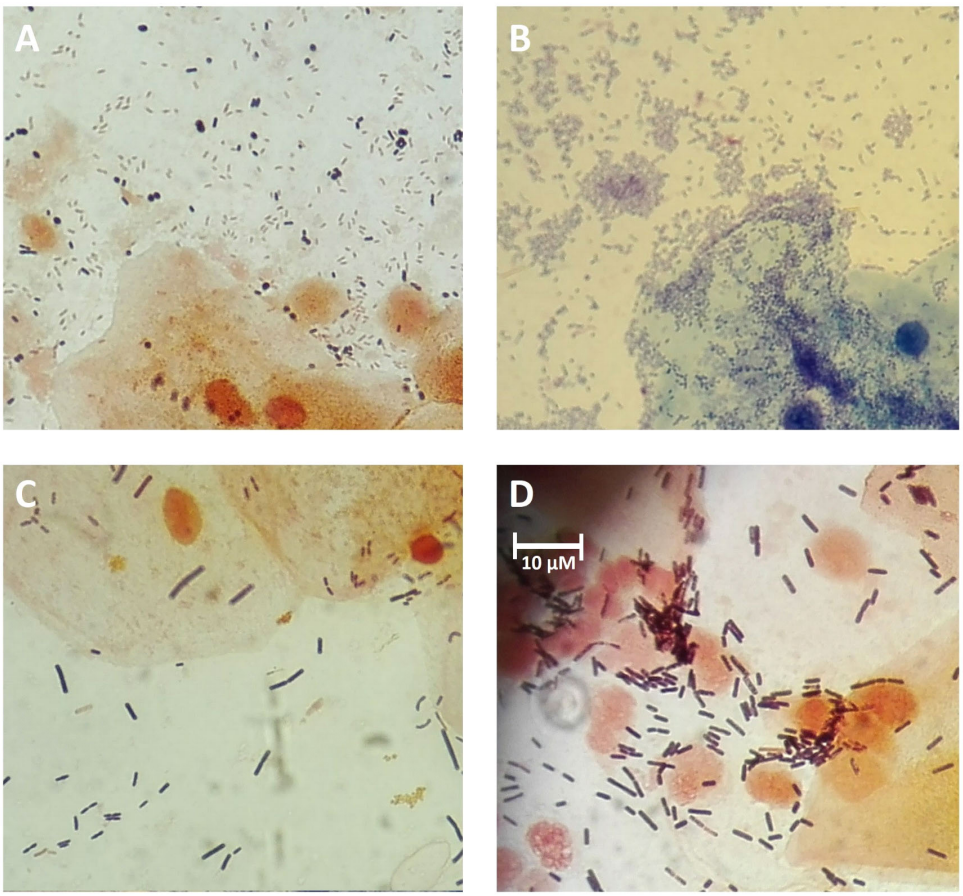


Figure 2. Representative micrograms of the vaginal environment

For each patient, one slide was stained using Giemsa-Romanowsky and the second using Gram stain. Slides were assessed using an oil immersion lens, providing 1000X total magnification. The following elements were quantified by a microbiologist: squamous epithelial cells, clue cells, mixed flora, yeast as pseudomycelia, Gram neg. *diplococci*, fibrous *Lactobacillus*, *Gardnerella*, spirochetes, parabasal epithelial cells, leukocytes, yeast as blastospores, Gram pos. *cocci* in chains, *Lactobacillus*, *Mobilincus*, *Leptotrichia*, and *Trichomonas vaginalis*. Nugent scores (NS) were determined according to Nugent et al., 1991 (46). Panels A and B represent a microscopic image of bacterial vaginosis. Panels C and D show a normalized environment. Panel A: NS = 10 (2+, 4+, 3+); Panel B: NS = 8 (0,4+,0) is a typical *Gardnerella* infection; Panel C: NS = 1 (3+,0,0); Panel D: NS = 0 (4+,0,0) with frequent rod-like *Lactobacilli*.

~ 7 (see Table 2). The difference between tea extract + vehicle and the vehicle alone reached a significance level of p = 0.082 (Fisher´s exact test) meaning that the primary endpoint was not reached. Of note, the total number of patients at risk (83 and 81) was 25 % lower than presumed by the statistical plan. Moreover, the efficacy of the vehicle was about two-fold higher than expected.

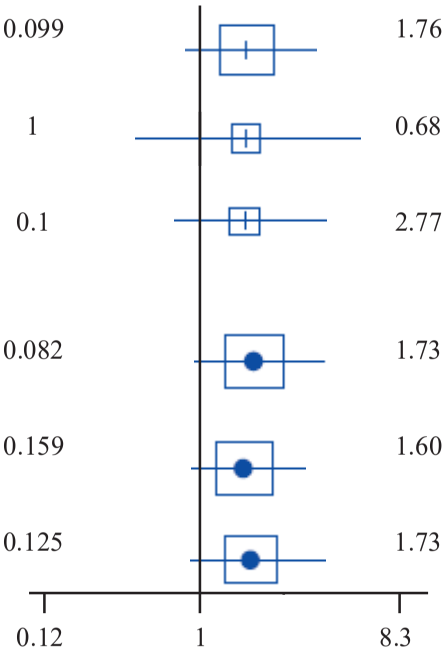
Similar results were observed on the third visit (15.9 ± 2.7 days since the first visit and ~ 8 days after the last application), where 39/74 (52.7 %) and 30/73 (41.1 %) patients achieved the composite endpoint (Table 2).

Worsening of symptoms

Subjective symptoms, NS, and pH worsened in 1.1 % vs. 10.5 %, 10.5 % vs. 15.5 %, and 1.1 % vs 3.1 % participants on the 2nd visit (tea extract ovule vs. placebo), respectively, and in 7.4 % vs. 6.3 %, 13.7 % vs. 18.6 %, and 2.1 % vs. 5.2 % participants on the 3rd visit, respectively.

Table 2. Improvement in individual parameters on the 2nd and 3rd visits

	Parameter	Shift	Matuzalem	Placebo	Fisher´s exact p-value	Odds ratio (95 % CI)	Odds ratio (95 % CI)	Approx. NNT
Visit 2	pH	>4.5 to <4.5 Did not improve	36 (47.4%) 40 (52.6%)	25 (33.8%) 49 (66.2%)	0.099		1.76 (0.91-3.41)	7
	Nugent score	4 to <4 Did not improve	9 (69.2%) 4 (30.8%)	10 (76.9 %) 3 (23.1%)	1		0.68 (0.19-3.87)	–
	2 subjective symptoms changed by at least 2 points	Improved Did not improve	23 (71.9%) 9 28.1%)	12 (48.0%) 13 (52.0%)	0.1		2.77 (0.92-8.32)	4
	Composite primary endpoint	Improved Did not improve	43 (51.8%) 40 (48.2%)	31 (38.3%) 50 (61.7%)	0.082		1.73 (0.93-3.23)	7
Visit 3	Composite secondary endpoint	Improved Did not improve	39 (52.7%) 35 (47.3%)	30 (41.1%) 43 (58.9%)	0.159		1.60 (0.83-3.07)	9
Visit 2 & 3	Both composite endpoints	Improved Did not improve	28 (37.8%) 46 (62.2%)	19 (26.0%) 54 (74.0%)	0.125		1.73 (0.86-3.50)	8



Exploratory analysis of subjective symptoms

Improvement in all subjective symptoms was not significant in the per protocol analysis (Table 2) but reached a marginal significance in the ITT analysis (p=0.047, Table 3). A breakdown to individual subjective symptoms showed that, frequently experienced at baseline, genital burning was reduced in the active arm on the 2nd visit. PP OR = 3.43 (95 % CI 1.30-10.07; χ^2 p-value = 0.006, Table 4).

Table 3 Effect estimates from the ITT population

	Parameter		Matuzalem	Placebo	χ^2 p-value	Odds ratio (95 % CI)
Visit 2	pH	>4.5 to <4.5	36	25	0.110	1.60 (0.86-3.00)
		All randomized-improved	101	112		
	Nugent score	≥ 4 to <4	9	10	0.812	0.89 (0.31-2.54)
		All randomized-improved	128	127		
	2 subjective symptoms by at least 2 points	Improved	23	12	0.047	2.10 (0.95-4.85)
		All randomized-improved	114	125		
	Composite primary endpoint	Improved	43	31	0.103	1.56 (0.88-2.79)
		All randomized-improved	94	106		
Visit 3	Composite secondary endpoint	Improved	39	30	0.210	1.42 (0.79-2.56)
		All randomized-improved	98	107		
Visit 2 & 3	Both composite endpoints	Improved	28	19	0.149	1.60 (0.81-3.20)
		All randomized-improved	109	118		

Table 4. Improvement in individual symptoms in the PP population

	Parameter		Matuzalem	Placebo	χ^2 p-value	Odds ratio (95 % CI)
Visit 2	Pruritus	Improved by at least 2 points	12	11	0.783	1.13 (0.43-3.00)
		Did not improve + baseline below 2 points	83	86		
	Burning	Improved by at least 2 points	20	7	0.006	3.43 (1.30-10.07)
		Did not improve + baseline below 2 points	75	90		
	Rubor	Improved by at least 2 points	12	4	0.033	3.36 (0.96-14.77)
		Did not improve + baseline below 2 points	83	93		
	Fluor	Improved by at least 2 points	20	21	0.920	0.97 (0.46-2.04)
		Did not improve + baseline below 2 points	75	76		
	Fetor	Improved by at least 2 points	7	4	0.333	1.85 (0.45-8.90)
		Did not improve + baseline below 2 points	88	93		

Exploratory analysis of the vaginal microbiota

To describe vaginal microenvironments, we employed a semiquantitative rating of the number of elements per field. We noted an apparent shift in only 4 elements. The differences between the baseline and 2nd or 3rd visit were expressed via histogram (Figure 3). Mixed flora became less frequent in the active arm in both follow-up visits compared to baseline. Regarding leukocytes and *Lactobacilli*, their abundance decreased on the 2nd visit. In the active arm only, there was a clear trend towards recolonization on the 3rd visit (the peak around 0 covers 90 % of all observations).

Safety

The most frequent AEs at MedDRA LLT were genital burning, genital itching, and vaginal discharge. Investigators assessed a positive causality in 1 vs. 6 cases; hence, the only adverse reaction reported for tea ovule was genital burning in one patient. In the active arm, 66.3 % participants and 76.9 % of investigators were very satisfied or satisfied; only 1 subject reported being unsatisfied.

Discussion

Principal findings

A numerical trend suggests that as little as 2 mg of tea extract in a PEG ovule used for 5 to 7 days has the capacity to reduce both objective and subjective symptoms of intermediate vaginal flora in one-half of the participants while the effect remained evident in about one-third of patients after ~ 8 days without application. The effect seems to be driven by a decrease in vaginal pH and relief from subjective symptoms. Despite the fact that women with persisting abnormal vaginal flora have an increased risk of developing BV [23,24], with the tea ovule, only 1 (1 %) subject experienced worsening of the pH or subjective symptoms while in 10 (10.5 %) participants the NS had increased. Results of the safety assessment show that adverse reactions were slightly more frequent in the passive arm, which is consistent with the trends observed in the efficacy analysis.

The primary hypothesis was not confirmed at the prespecified significance level. The trial did not achieve the sample size determined by the power analysis and the effect could not be detected with the desired degree of confidence. In-

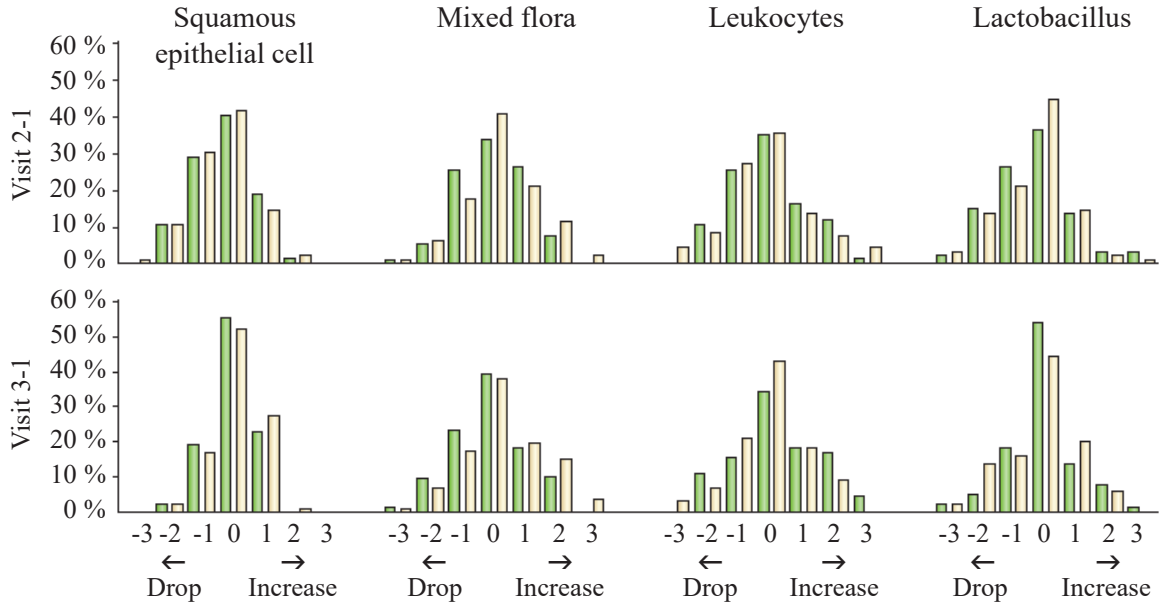


Figure 3. Shifts in vaginal epithelial cells, mixed flora, leukocytes, and Lactobacilli – tea (green) and placebo (white)

To describe the vaginal microenvironment, we employed a semiquantitative rating of the number of elements per field (as per the laboratory standardized procedure see Methods). We noted an apparent shift between the 1st and the 2nd or the 3rd visit in only 4 elements

terestingly, comparable results were obtained earlier during original metronidazole testing. Only after meta-analysis was prepared became the effect significant [31].

Clinical implications

Tea extract PEG ovule seems to be viable alternative for women not indicated to antibiotic who seek a safe complementary therapy. It should not, however, be considered as alternative to antibiotic treatment for BV.

Strengths and limitations

We did not identify a vehicle which could be considered and true placebo. The combination of research-participation & placebo effect & natural course of the disease along with the real efficacy of PEG resulted in about one-third of participants experiencing relief in the passive arm. This finding confirms previous observations [26] and recent interim analysis of other trial testing polycarbophil and lauryl glucoside for BV [27,27].

Another limitation is the patients enrolled were exclusively caucasian which somehow limits generalization of the results to other populations.

Research Implications

Tea extracts were shown to downregulate transduction via interleukin-1β [32–34], a cytokine involved in BV-mediated HIV integration [10,11]. Vaginal formulations based on tea catechins were tested as candidate substance to prevent HIV transmission [35–37]. This trial was not designed with the goal of screening individual cytokines. Thus, it could not explain the previously postulated anti-inflammatory properties of tea extracts that should be explored in relation to other infectious diseases.

We also did not identify enough participants with an intermediate NS to provide conclusive evidence regarding the shifts in bacterial microflora. Microscopic description of individual elements of the vaginal environment advocates an immediate reduction of mixed flora, *lactobacilli*, and leukocytes during ovule application. In BV, there is a wide range of anaerobic bacteria that overgrows symbiotic *Lactobacilli* (e.g., *Peptostreptococcus, Peptococcus, Eubacterium spp, Prevotella bivia, P. disiens, P. melaninogenica, and Porphyromonas*). There were signs of recolonization by *Lactobacilli* but not the mixed flora after the completion of the treatment. Such observation is promising and deserves further attention, notably with respect to risk factor such as smoking [38]. Larger prospective studies should investigate the efficacy of tea extract in a) larger doses, b) patients with NS above 4, c) longer treatment periods and d) other than Caucasian populations.

Conclusions

Half of the patients found relief using once-daily tea ovules for 5 to 7 days. The numerical trend strongly suggests that as little as 2 mg of tea extract daily has the capacity to stabilize low vaginal pH and relieve subjective symptoms, we show a significant effect on burning sensation. Along with good tolerability, tea ovules appear as good candidate for complementary therapy helping restore balanced vaginal environment. The tea extract deserves further exploration at both the laboratory and clinical levels.

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Conflict of interest and author contribution

J.T., B.F., J.J., H.D., and B.D. are employees of Value Outcomes, consultancy and research organization that designed, monitored, conducted and analyzed the trial on behalf of the sponsor Matuzalem.com-cz s.r.o. J.T. had the ultimate responsibility for trial conduct, ethical aspects and integrity of the research including this manuscript, T.D., H.D., J.T., B.F., J.J. and Z.C.L. designed the trial, B.F. and J.J. were responsible for data management, J.J., T.D. and J.T. designed and

performed the statistical analysis, H.D. and J.T, were responsible for regulatory aspects, J.T., B.F. and P.G. performed on-site and centralized monitoring, T.D. and B.D. were safety physicians and performed medical review of the protocol and this manuscript, T.D. is the director of Value Outcomes. J.M. designed and managed all laboratory analyses, Z.C.L. and V.D. performed medical review of the trial design, J.K. prepared the investigated tea extract and affiliates to Matuzalem.com-cz Ltd. Authors Z.C.L., V.D., V.K., H.K. and Z.A. were principal investigators in respective outpatient gynecology clinics. All authors participated on the creation and review of this manuscript.

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