Combining a synthetic spermicide with a natural trichomonacide for safe, prophylactic contraception

Ashish Jain¹, Lokesh Kumar¹, Bhavana Kushwaha¹, Monika Sharma¹, Aastha Pandey¹, Vikas Verma¹, Vikas Sharma¹, Vishal Singh¹, Tara Rawat², Vishnu L. Sharma², Jagdamba P. Maikhuri¹, and Gopal Gupta¹,*

¹Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow 226031, India ²Division of Medicinal & Process Chemistry, CSIR-Central Drug Research Institute, Lucknow 226031, India

*Correspondence address. Division of Endocrinology, CSIR-Central Drug Research Institute, Jankipuram Extension, Lucknow 226031. India. E-mail: g_gupta@cdri.res.in

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STUDY QUESTION: Can a specifically acting synthetic spermicide (DSE-37) be combined with a natural microbicide (saponins) for safe, prophylactic contraception?

SUMMARY ANSWER: A 1:1 (w/w) combination of DSE-37 and Sapindus saponins can target sperm and *Trichomonas vaginalis* precisely without any noticeable off-target effects on somatic cells at effective concentrations.

WHAT IS KNOWN ALREADY: Broad-spectrum vaginal agents like nonoxynol-9 (N-9) and cellulose sulfate have failed clinically as microbicides due to non-specific off-target effects, whereas agents that specifically target retroviruses have shown promise in clinical trials. DSE-37 and Sapindus saponins, respectively, specifically target human sperm and *T. vaginalis* in vitro.

STUDY DESIGN, SIZE, DURATION: A comprehensive study of efficacy and safety was undertaken using in vitro (human cells) and in vivo (rabbit) models. The 1:1 combination of DSE-37 and Sapindus saponins was based on the in vitro spermicidal and anti-Trichomonal activities of the two components. N-9, the spermicide in clinical use, served as reference control. Free sperm thiols were fluorescently glinted to reveal differences in the targets of the test agents.

PARTICIPANTS/MATERIALS, SETTING, METHODS: On/off-target effects were evaluated in vitro against human sperm, *T. vaginalis*, HeLa, Vk2/E6E7, End1/E6E7 and *Lactobacillus jensenii*, using standard assays of drug susceptibility, cell viability, flow cytometric assessment of cell apoptosis and qPCR for expression of pro-inflammatory cytokine mRNAs. The spermicidal effect was also recorded live and free thiols on sperm were fluorescently visualized using a commercial kit. In vivo contraceptive efficacy (pregnancy/fertility rates) and safety (vaginal histopathology and in situ immune-labeling of inflammation markers VCAM-1, E-selectin and NFkB) were evaluated in rabbits.

MAIN RESULTS AND THE ROLE OF CHANCE: A 0.003% drug ‘combination’ containing 0.0015% each of DSE-37 and Sapindus saponins in physiological saline irreversibly immobilized 100% human sperm in ~30 s and eliminated 100% *T. vaginalis* in 24 h, without causing any detectable toxicity to human cervical (HeLa) cells and *Lactobacillus* in 48 h. N-9 at 0.003% exhibited lower microbicidal activity against *Trichomonas* but failed in spermicidal assays while causing severe toxicity to HeLa cells and *Lactobacilli* in 24–48 h. The ‘combination’ of DSE-37 and Sapindus saponins completely prevented pregnancy in rabbits at a vaginal dose of 20 mg (1% in K-Y Jelly), while application of 5% ‘combination’ in K-Y Jelly for 4 consecutive days caused negligible alterations in epithelial lining of rabbit vagina with only minor changes in levels of inflammation markers. N-9 at a 20 mg vaginal dose prevented pregnancy in 33% animals and a 4-day repeat application of 2% N-9 gel caused severe local toxicity to vaginal epithelium with molecular expression of acute inflammation markers.

LIMITATIONS, REASONS FOR CAUTION: The number of animals used for the in vivo efficacy study was limited by the approval of the animal ethics committee.
WIDER IMPLICATIONS OF THE FINDINGS: Anti-Trichomonal contraceptives with specifically acting synthetic component and clinically-proven safe natural component may define a new concept in empowering women to control their fertility and reproductive health.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by CSIR-Network Project ‘PROGRAM’ (BSC0101) and partly by the Ministry of Health and Family Welfare, Government of India (GAP0001). The funding agencies did not play any role in this study and none of the authors had any competing interest(s).

Key words: vaginal contraception / Trichomonas vaginalis / spermicide / DSE-37 / Sapindus saponins

Introduction

High rates of unintended pregnancies and sexually transmitted infections seriously jeopardize the reproductive health mission of the United Nations, which seeks to ensure that every child is wanted, every birth is safe and every person is free of STI and HIV (UNFPA, 2013). An estimated 25% of all pregnancies worldwide end in abortions (Sibbald, 2004; Haddad, 2009) and an annual 499 million new cases of curable sexually transmitted infections (WHO, 2008) exponentially increase the risk of HIV and other viral STDs. Impoverished women and sexually active adolescents form the most vulnerable group. The unmet need for women-controlled dual protection contraceptives is quickly growing, particularly since women often find it difficult to negotiate condom-use with their partners. The prophylactic approach of vaginal microbicides offers a low-cost, user-friendly option for less-educated and low-income people, and by being virtually impermeable and women-controlled, they may have an advantage over condoms. In appropriate formulation, these microbicides may also reduce friction-associated trauma during coitus (Dezzutti et al., 2012a,b) and thus further inhibit STD/HIV transmission.

Attempts to use a single broad-spectrum agent to kill sperm and STI pathogens (including HIV) simultaneously in the vagina during coitus have proved futile, plausibly due to the associated off-target toxic effects of the product (Obiero et al., 2012). On the other hand, the recent success of specifically acting agents and their combinations have renewed hope in microbicides (Dezzutti et al., 2012a,b; Cost et al., 2012; Moss et al., 2012). Hence a combination of two or more specific agents may provide safer protection from unwanted pregnancy and/or STDs than non-specific agents.

We have reported the designed synthesis and efficacy of DSE-37 (Jain et al., 2007, 2009), a potent, specifically acting spermicicide that targets thiol, which are in several-fold excess on sperm than on somatic cells (Mercado et al., 1976). However, the compound proved to be a weak microbicid against Trichomonas vaginalis, the most prevalent non-viral STD pathogen that increases susceptibility to viral STDs and HIV (Upcroft and Upcroft, 2001). Concurrently, potent anti-Trichomonal activity was detected in Sapindus saponins (Tiwari et al., 2008), a plant product which has cleared a Phase III clinical trial in India for vaginal use in women (Gupta, 2005). The microbicidal effects of saponins were at extremely low, non-spermicidal, non-surfactant concentrations, devoid of any general non-specific action. Here we have made an attempt to design a dual-protection contraceptive by rationally combining the two agents together and assessing their activity and safety using various in vitro and in vivo models. This includes the first live report of the robustness of spermicidal action required for effective contraception.

Materials and Methods

Chemicals and reagent

DSE-37 was synthesized as water-soluble ditaurate salt and fully characterized by the authors (T.R. and V.L.S.). A standardized sample of Sapindus saponins was procured from Dr.A.K. Dwivedi, Chief Scientist at the Pharmaceuticals Division of this Institute. Nonoxynol-9 was purchased from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ, USA). The LDH release assay kit was purchased from Roche, USA. Keratinocyte serum-free medium was purchased from Invitrogen Life Technologies. Rogosa SL agar and broth media were purchased from Hi-Media, Mumbai, India. Mouse anti-rabbit monoclonal antibodies used were against E-selectin (Sigma-Aldrich) and vascular adhesion molecule-1 (VCAM)-1 (Biosource, Camarillo, CA, USA), Nuclear factor (NF)-kβ/p65 (Chemicon, Temecula, CA, USA). The Dead End Fluorometric TUNEL system was from Promega (USA). All other chemicals, culture media and other reagents were from Sigma-Aldrich, USA.

Human sperm and spermicidal assays

Freshly ejaculated human semen was obtained by masturbation from healthy, young, adult volunteers, and collected directly into a sterile plastic tube and transported immediately into the laboratory. Prior informed consent was obtained from the donors for this study. The samples were allowed to liquefy at 37°C for 45 min and semen characteristics and analysis were performed according to the normal criteria as per World Health Organization guidelines (WHO Manual, 2010). Sperm count and motility analysis were performed manually as well as in a Computer Automated Semen Analyzer system using a small drop of liquefied semen placed on a ‘Makler’ counting chamber (Sefy’ Medica, Hafia, Israel) pre-warmed to 37°C. The LDH release assay was performed as detailed earlier (Jain et al., 2007, 2009), a potent, specifically acting spermicide that targets thiol, which are in several-fold excess on sperm than on somatic cells (Mercado et al., 1976). However, the compound proved to be a weak microbicid against Trichomonas vaginalis, the most prevalent non-viral STD pathogen that increases susceptibility to viral STDs and HIV (Upcroft and Upcroft, 2001). Concurrently, potent anti-Trichomonal activity was detected in Sapindus saponins (Tiwari et al., 2008), a plant product which has cleared a Phase III clinical trial in India for vaginal use in women (Gupta, 2005). The microbicidal effects of saponins were at extremely low, non-spermicidal, non-surfactant concentrations, devoid of any general non-specific action. Here we have made an attempt to design a dual-protection contraceptive by rationally combining the two agents together and assessing their activity and safety using various in vitro and in vivo models. This includes the first live report of the robustness of spermicidal action required for effective contraception.

Live spermicidal action: combination versus N-9

The combination (DSE-37 and Sapindus saponins) and N-9 were prepared at a spermicidal concentration in 5% Ficoll (in saline) to match the viscosity of semen. About 15 μL of this solution was placed close to a drop (~15 μL) of fresh liquefied semen on a glass slide and gently covered with a cover glass so that the two drops coalesced. The interface was examined under a phase contrast microscope and an AVI video was recorded using a 5-megapixel digital camera for live display of spermicidal action.

Trichomonas vaginalis cultures and drug susceptibility assays

Clinical isolates of metronidazole-susceptible T. vaginalis were obtained from the laboratory of Divya Singh (Tiwari et al., 2008), and a
metronidazole-resistant strain of T. vaginalis (CDC085 [ATCC 50143]) was procured from the American Type Culture Collection (ATCC). Both strains were cultured under a partial anaerobic condition in TYM medium (0.1% K2HPO4, 0.06% KH2PO4, 0.5% NaCl, 0.5% glucose, 2.0% yeast extract, 0.2% L-lysine, 0.15% tryptone [pH 6.8]) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 2% vitamin mixture, 100 U of penicillin/ml and 100 μg/ml streptomycin solution in 15-ml screw-cap sterile tubes, followed by incubation at 37°C. The drug susceptibility assays were performed as detailed by us earlier (Jain et al., 2011).

Measurement of surface tension for surfactant potency
The surface tension (γ) of water, saline and solutions of N-9, saponins and DSE-37 (in saline) was measured at 25°C with a stalgameter (to measure the surfactant potency of solutions) using the equation

$$\gamma = \gamma_{H_2O} - \frac{m}{V_{H_2O}} \cdot \rho = \gamma_{H_2O} - \frac{m}{V_{H_2O}} \cdot \rho$$

The γ of water was taken as 71.97 dynes/cm, at 25°C.

Cell lines
HeLa cells were procured from the National Centre for Cell Sciences (NCCS), Pune, India; Human papilloma virus type 16/E6E7-immortalized endocervical (End1/E6E7) and vaginal (VK2/E6E7) epithelial cell lines were obtained as a kind gift from Dr. S.N. Kabir, CSIR-IICB, Kolkata, India, but were originally sourced from Dr. Raina Fichorova (Harvard Medical School, Boston). VK2 and End1 cells were maintained in keratinocyte serum-free medium supplemented with bovine pituitary extract (50 μg/ml), epidermal growth factor (0.01 ng/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was further supplemented with CaCl2 to a final concentration of 0.4 mM. The cultures were maintained in KSF medium in a CO2 incubator at 37°C in a 5% CO2–95% air atmosphere. The Lactobacillus jensenii (ATCC 25258, strain 62G) were procured from the ATCC and grown in 6% Rogosa SL broth medium containing 0.132% acetic acid, at 37°C.

LDH release cytotoxicity assay
Cells (HeLa) at a density of 1 × 10⁶ per well were seeded in 96-well plates and maintained for 24 h at 37°C in 5% CO2/95% air atmosphere. After 24 h, culture medium was replaced with fresh medium containing dilutions of test compounds and incubated for another 24 h. Thereafter, LDH released from non-viable cells was determined using kit manufacturer’s protocol (Roche Applied Science). The absorbance was determined at a wavelength of 490 nm.

Broth and plate cultures of L. jensenii and compatibility studies
Broth culture
Briefly, Rogosa SL broth medium was prepared in Milli-Q water, boiled for 2–3 min and distributed in 48-well plates (500 μl/well). Serial dilutions of test compounds were added to experimental wells, and vehicle was added to control wells in triplicate. Approximately 1000 cfu of L. jensenii were inoculated into each well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24 h. At the end of the experiment, cultures were mixed thoroughly and 100 μl from each well was transferred to the corresponding well of a 96-well plate. The numbers of Lactobacilli were mixed thoroughly and 100 μl from each well was transferred to the corresponding well of a 96-well plate. The numbers of Lactobacilli were estimated by measuring the turbidity (OD610) in a microplate reader.

Plate culture
Rogosa SL Agar medium (7.5% in distilled water) containing 0.132% acetic acid was boiled at 100°C for 2–3 min, poured in petri plates and allowed to gel. These were inoculated with an equal number of L. jensenii to the broth culture (control and treated) and incubated at 37°C in 5% CO2 for 48 h for the growth of colonies.

Fluorescent labeling of sperm thiosl
Free thios on human sperm (after treatment with vehicle, combination, N-9 and saponins) were examined and imaged using a fluorescence microscope, after labeling with the fluorometric thiol detector using a thiol-detection assay kit (Cyanam). The semen sample (0.5 ml) was treated with 2.5 ml of either saline or 0.003% combination or 0.015% N-9 (in saline) and incubated for 15 min at room temperature. After incubation, sperm were pelleted at 700 × g for 10 min at 4°C and washed 2–3 times with PBS. To pelleted sperm in 50 μl PBS, 50 μl fluorometric thiol detector (pre-diluted 100× with dilution buffer) was added and incubated for 5 min in the dark. A cover of glass and imaging used the UV/IA filter on a Nikon Eclipse 80i microscope equipped with epifluorescence illumination. Exposure times were the same for all samples.

AnnexinV-FITC/propidium iodide labeling
Dual fluorescent labeling with AnnexinV-FITC and propidium iodide (PI) was used to study the apoptotic and late apoptotic/necrotic cells, respectively. HeLa cells, as the control (in 0.05% DMSO) or treated with combination (in DSE-37 + Saponins, 15 + 15 μg/ml), Staurosporine (in 1 μg/ml) or N-9 (in 30 μg/ml), were incubated for 24 h. Staurosporine was used as a positive control for apoptosis. After incubation, cells were processed using the Annexin-V FITC apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA) by following the manufacturer’s instructions and detected for positive Annexin-V and PI labeling using a flow cytometer (Model FACS Calibur, BD biosciences, USA) equipped with an argon laser (488 nm) for excitation.

Mitochondrial transmembrane potential (Ψm)
The loss of ΔΨm (an early marker for cell apoptosis) was quantified by flow cytometry using the lipophilic cationic dye JC-1. HeLa cells, as the control (in 0.05% DMSO) or treated with combination (in DSE-37 + Saponins, 15 + 15 μg/ml) or CCCP (in Carbonyl cyanide 3-chlorophenylhydrazone; 20 μM), were incubated for 24 h. CCCP was used as positive control. JC-1 (5 μg/ml) was added from stock in DMSO (1 mg/ml) to the cell cultures and incubated for an additional 10 min and thereafter cells were analyzed by flow cytometry for JC-1-specific fluorescence. The excitation was at 488 nm, and the emissions for the green and red/orange fluorescence were at 530 and 570 nm, respectively.

Expression of pro-inflammatory cytokine genes by real-time PCR
Extraction of total RNA from HeLa cells (treated with vehicle or ‘combination’ or N-9 for 12 h) was performed using the Tru-reagent (Invitrogen). The quantity and quality of purified RNA was evaluated by spectrophotometry. cDNA was synthesized from 1.0 μg of total RNA using ‘Revert Aid H Minus first strand’ cDNA synthesis kit (Fermentas Life Sciences, Canada). Real-time reactions were set up in a final volume of 20 μl, containing 1 μl of cDNA sample, 0.5 μM of forward primer, 0.5 μM of reverse primer, 2× ready-to-use reaction mix including Taq DNA polymerase, reaction buffer and deoxyribonucleotide triphosphate mix. PCR was performed using the following program: initial denaturation at 95°C for 10 s, denaturation at 95°C for 20 s, annealing at 58–60°C for 30 s and elongation at 72°C for 30 s. Real-time detection of PCR amplification was based on the change in fluorescence reading employing ‘LightCycler 480’ real-time system (Roche). Changes in the cycle threshold (Ct) across PCR reactions were
plotted to calculate differences in the amount of the template between reactions. Expression levels of the investigated genes were compared with the steady expression of GAPDH mRNA expression of each gene was analyzed at least three times using the same cDNA preparation. The primer sequences are provided as supplementary data (Supplementary data, Table SI).

**DAPI and phalloidin staining of HeLa for enumeration of apoptotic nuclei and integrity of cytoskeleton**

HeLa cells were grown on glass cover-slips and treated with test compounds as detailed above and then fixed in PBS containing 4% formaldehyde for 30 min and subsequently permeabilized for 25 min in PBS containing 4% formaldehyde and 0.1% Triton X-100. For DAPI staining, fixed cells were washed with PBS (pH 7.4) and incubated with 1 μg/ml DAPI solution (Travert et al., 2006). DAPI-stained DNA was examined under a fluorescence microscope (Nikon Eclipse 80i). Normal DNA of viable cells was visibly fluorescent throughout the nucleus, whereas apoptotic cells exhibited a bright condensation of chromatin (magnification, ×200). For Phalloidin staining, fixed cells were stained with 1 μg/ml FITC-conjugated phalloidin (Sigma-Aldrich) in PBS for 2 h, in the dark. Finally, the samples were rinsed three times with PBS, washed for 15 min (in PBS), mounted in anti-fading mounting medium and viewed using a fluorescence microscope.

**Animal experiments**

Virgin nulliparous female Belgian rabbits (6 in each group; \( n = 6 \)) were primed with pregnant mare serum globulin (PMSG, 200 i.u.; i.p.; 96 h prior to testing) to induce ovulation. The test compounds were incorporated in K-Y-Jelly (Johnson & Johnson) through geometrical dilution. Pure K-Y-Jelly was used in control groups. Two microliters of test/control jelly was instilled 10–12 cm deep into the vagina of each rabbit with a catheter attached to the gavage needle of a syringe with the animal held in supine position and released after 5 min. Treated females were hand-mated once with a proven buck. Each buck was allowed one mating. To ensure ovulation, 100 i.u. of human chorionic gonadotrophin was administered through the marginal ear vein of each participating doe. The vaginal lavage of the mated doe was examined under a microscope for the presence of spermatozoa. The mated does were kept in separate cages and allowed to complete gestation and the number of pups delivered was recorded. The mean value (number) of the pups was calculated to determine the reduction in fertility rate. For the vaginal irritation study, female rabbits (three in each group, \( n = 3 \)) were administered once intravaginally with 2.0 ml of Vehicle (K-Y Jelly) or N-9 (2%) or combination DSE-37 + Sapindus (5%) for 4 consecutive days. Animals were euthanized on Day 5 and the cervico-vagina, mid-vagina and uro-vagina of each animal were dissected out and fixed in 10% neutral-buffered formalin. Thereafter, the fixed vaginal tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Stained sections were examined by light microscopy.

**Immunohistochemistry and in situ TUNEL labeling**

For in situ localization of NF-κB, E-selectin, vascular adhesion molecule (VCAM)-1 by immunohistochemistry and for TUNEL labeling, deparaffinized tissue sections (5 μm) were rehydrated gradually through graded alcohol and incubated in 3% H₂O₂ for 20 min to block endogenous peroxidase activity. The antigens were retrieved by boiling the slides in 20 mM citrate buffer (pH 6.0) for 15 min. After blocking non-specific background with 10% BSA (Sigma-Aldrich) for 60 min at room temperature, the slides were incubated separately with mouse anti-NF-κB (p65 subunit) or E-selectin or VCAM antibody, overnight at 4°C. Subsequently, the slides were incubated in appropriate biotinylated secondary antibody followed by incubation in ABC staining complex (Santa Cruz). The sections were finally counterstained with hematoxylin. For in situ end labeling (TUNEL) of DNA fragments, the tissue sections were processed according to manufacturer’s instructions (Promega, USA). The slides were examined under a microscope (Nikon 80i, Japan) and images were captured digitally with the NIS-Elements F 3.0 camera (Nikon, Japan).

**Data analysis**

All experiments were repeated three times and the results were analyzed by one-way analysis of variance (ANOVA) using the GraphPad Prism software (Version 3.0). \( P \)-values of \( < 0.05 \) were considered statistically significant. The IC₅₀ values were calculated by computer-based curve fitting using the ‘CompuSyn’ software.

**Results**

**Efficacy and in vitro safety of microbicidal contraceptive drug ‘combination’**

A 0.003% drug ‘combination’ containing 0.0015% (15 μg/ml) each of DSE-37 and Sapindus saponins in physiological saline/culture medium irreversibly immobilized 100% of human sperm in ~30 s and eliminated 100% T. vaginalis trophozoites in 24 h, without causing any detectable toxicity to human cervical (HeLa) cells in 24–48 h in vitro. Nonoxynol-9 at 0.003% (30 μg/ml) exhibited lower microbicidal activity against Trichomonas but failed in spermicidal assays and caused severe toxicity to HeLa cells in 12–24 h (Table I). N-9-treated HeLa showed distinct apoptotic

**Table I The spermicidal and anti-Trichomonal activities of DSE-37, Sapindus saponins, nonoxynol-9, ‘combination’ and metronidazole (Mean ± SE).**

<table>
<thead>
<tr>
<th>Compounds/combinations</th>
<th>Spermicidal MEC (μg/ml)</th>
<th>Anti-Trichomonal MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>DSE-37 (DSE)</td>
<td>15 – 20</td>
<td>250 ± 25</td>
</tr>
<tr>
<td>Sapindus saponins (Sap)</td>
<td>500</td>
<td>15 ± 2.5</td>
</tr>
<tr>
<td>Nonoxynol-9 (N-9)</td>
<td>150</td>
<td>43 ± 3.5</td>
</tr>
<tr>
<td>Combination (DSE + Sap, 1:1)</td>
<td>30</td>
<td>30 ± 2.5</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>Inactive</td>
<td>2.0 ± 0.25</td>
</tr>
</tbody>
</table>

MEC, minimal effective concentration; MIC, minimal inhibitory concentration.
nuclei in a significant number of cells after fluorescent staining of DNA with DAPI (Supplementary data, Fig. S1A), while fluorescent staining of actin with FITC-conjugated phalloidin indicated grossly damaged cellular cytoskeletons with formation of large apoptotic/necrotic pores (Supplementary data, Fig. S1B). However, no such damage was visible in ‘combination’ treated HeLa, and they appeared largely like control cells. Further in vitro assessment of toxicity of DSE-37, Saponins and combination (DSE + Sap) versus N-9 using MTT and LDH release assays revealed that among the test agents used, DSE-37 was the safest followed by Sap and the ‘combination’, while N-9 was most toxic (Supplementary data, Fig. S1C and D). In comparison to N-9, the combination was also much safer against human vaginal epithelial (VK-2/E6E7) and endocervical (End-1/E6E7) cell lines (Supplementary data, Fig. S1E and F).

**Surfactant nature of test agents**

The surface tension (γ) of saline (79 dynes/cm) was ~10% more than that of water (72 dyn/cm). The ‘combination’ (DSE-37 + Sap, 15 + 15 μg/ml in saline) had a γ of 71 ± 2.8 dynes/cm while individually saponins and DSE-37 at 15 μg/ml exhibited surface tension values of 69 and 77 dyn/cm in saline, respectively. On the other hand, N-9 solutions at spermicidal (150 μg/ml in saline) and anti-Trichomonal (45 μg/ml in saline) concentrations exhibited lower surface tension of 31 (P < 0.001 versus H2O) and 43 (P < 0.001 versus H2O) dyn/cm, respectively. Similarly saponins at spermicidal MEC (500 μg/ml in saline) also exhibited a lower surface tension value of 52 dyn/cm in saline (P < 0.001 versus water) (Fig. 1).

**Spermicidal action: live effects**

The potency of spermicidal effects was recorded live by creating a sharp interface between fresh human semen and spermicide solution (with matched viscosity). Vigorously and progressively motile sperm in semen crossing the semen-spermicide interface instantly lost motility and could not progress any further in the spermicidal solution. This was true for both N-9 and the ‘combination’. However, the after about 20 s, the combination seemed to diffuse into semen, broadening the area of action, while N-9 action remained limited largely at the sharp interface (Supplementary data, Movies S1 http://youtu.be/2iOvEhYPDfM and S2 http://youtu.be/qf–AKj9S6k).

**In vitro eco-safety of the new drug combination towards vaginal flora**

Using broth cultures, DSE-37 was found to be safest towards the normal vaginal flora (L. jensenii) with an IC50 = 282 μg/ml, followed by combination and saponins, which had similar compatibilities with Lactobacilli and exhibited IC50 values of 123 and 99 μg/ml, respectively. However, N-9 was least compatible with Lactobacilli and exhibited an IC50 of 21 μg/ml (Supplementary data, Fig. 2B). This difference in compatibilities of the new drug combination and N-9 with Lactobacilli was clearly visible in plate cultures (Supplementary data, Fig. S2A).

**Fluorescent detection of free sulfhydryls on motile and immobilized human sperm**

The free –SH groups were localized by fluorescence detection on sperm that were either motile (control) or immobilized permanently by ‘combination’ or N-9/saponin treatment, and digitally imaged for qualitative assessment. The motile sperm had strikingly higher numbers of free thiols (Fig. 2A) when compared with sperm immobilized by ‘combination’ treatment (Fig. 2B). This became clearly evident by visual assessment of fluorescence intensities. Although the difference was evident throughout the structure of sperm, it was prominently marked in the tail region (principal piece). However sperm immobilized by N-9 exhibited fluorescence intensities comparable with control, motile sperm (Fig. 2C), which reflected the difference in the spermicidal mechanisms of the ‘combination’ and N-9. Similarly, sperm treated with saponins alone (15 μg/ml in saline) had fluorescence intensities higher than those treated with the ‘combination’, and were basically comparable with the controls (Fig. 2D).

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**Figure 1** The surface tension of spermicidal/microbicidal solutions of the ‘combination’, its components (DSE-37 and saponins) and nonoxynol-9. Spermicidal concentrations (μg/ml): N-9 (150); Sap (500); DSE (15); anti-Trichomonal concentrations: N-9 (45); Sap (15); dual activity: DSE + Sap (15 + 15). (Mean ± SE; *P < 0.05; ***P < 0.001.)
Induction of apoptosis in HeLa by the ‘combination’ and N-9

Fluorescent staining with FITC-AnnexinV/propidium iodide followed by flow cytometry revealed that the number of apoptotic HeLa cells (in right-lower and right-upper quadrants with externalized phosphatidylserine and permeabilized membrane) in control cells (15.0 ± 2.34) increased marginally by ~1% to 16.0 ± 0.92 after treatment with the new contraceptive ‘combination’ at 30 μg/ml. However, the apoptotic cell number rose significantly to 50.5 ± 2.38 (P, 0.001) and 61.0 ± 2.31 (P < 0.001) after treatments with N-9 (30 μg/ml) and staurosporine (1.0 μg/ml; positive control), respectively (Fig.3A and B). Similarly at 30 μg/ml, the number of cells with compromised mitochondrial membrane potential (measured by flow cytometry after staining with JC-1) increased marginally after treatment with the ‘combination’, and significantly after treatment with N-9 (P < 0.001). N-9 was again equipotent to the positive control compound, CCCP (Fig. 3C and D).

Induction of pro-inflammatory cytokines by combination and N-9

The mRNA levels of pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-6 and IL-8 did not change significantly in HeLa cells after treatment with DSE + Sap (combination) at 30 μg/ml for 12 h. However, parallel treatment of HeLa cells with 30 μg/ml of N-9 significantly increased the transcript levels of TNF-α (P < 0.05), IL-1α (P < 0.01), IL-1β (P < 0.001), IL-6 (P < 0.001) and IL-8 (P < 0.001), in 12 h (Fig. 4).

In vivo contraceptive efficacy

Female rabbits that received vaginal instillation of 2.0 ml of 0, 0.5, 1.0 and 2.0% ‘combination’ (DSE + Sap) in K-Y Jelly before mating exhibited 0, 50, 100 and 100% reduction in pregnancy rates with 6/6, 3/6, 0/6 and 0/6 rabbits becoming pregnant in these groups, respectively. The average litter size was reduced from 9.5 ± 1.8 in control (0%) group to 2.8 ± 3.1, 0 and 0 in the 0.5, 1.0 and 2.0% experimental groups, respectively, thus reducing the average fertility rates by 71, 100 and 100% in these rabbits. N-9 at 1.0% vaginal dose (in K-Y jelly) prevented fertility in 33% of animals with a 53% reduction in average fertility rate (Table II).

Vaginal toxicity in rabbits: histopathological evaluation

A 4-day daily application of 5% combination gel (in K-Y jelly) caused minimal alterations in the vaginal histopathology of rabbits, as seen in Fig. 5. The epithelial architecture of cervico-vagina, mid-vagina and urovagina of rabbits treated with the ‘combination’ was largely comparable with the controls. In contrast, a parallel 4-day application of 2% N-9 (in K-Y jelly) caused distinct erosion of the vaginal epithelial lining with

Figure 2 Fluorescence due to free thiols on human sperm treated with (A) control, (B) ‘combination’ 30 μg/ml, (C) nonoxynol-9 150 μg/ml or (D) saponins 15 μg/ml.
conspicuous ‘washing-off’ effects, plausibly due to the detergent action of N-9. This was accompanied by marked leukocyte infiltration, erosion, hemorrhage, decreased epithelial cell-height and necrosis of the vaginal epithelium. The damage was more evident in the mid- and uro-vaginae than in the cervico-vagina (Fig. 5).

Vaginal toxicity in rabbits: induction of epithelial cell apoptosis

The induction of apoptosis in epithelial lining of the rabbit cervico-vagina was assessed by in situ TUNEL assay. The cervico-vagina obtained from rabbits treated for 4 consecutive days with 5% combination or 2% N-9 was sectioned and stained with DAPI and FITC-TUNEL as detailed. Mild and scant TUNEL labeling in ‘combination’ treated vagina (Fig. 6A-II) was largely like the control (Fig. 6A-I) while distinct TUNEL labeling throughout the epithelial lining was seen in the epithelium of N-9-treated rabbit vagina (Fig. 6A-III). This indicated a marked increase in the number of apoptotic cells in the vaginal epithelium of rabbits that
received instillation of 2% N-9 against negligible effects of parallel treatment with 5% ‘combination’.

In situ localization of inflammatory markers, NFκB, E-selectin and VCAM-1

NFκB, VCAM-1 and E-selectin were localized immunologically to the stroma of rabbit vagina (Fig. 6). Mild expression of NFκB was visible in combination-treated animal tissues (Fig. 6B-II) while significant expression was evident in N-9-treated tissues (Fig. 6B-III), in comparison with vehicle-treated controls (Fig. 6B-I). Likewise, VCAM-1 staining was barely evident in control (Fig. 6D-I) and ‘combination’ (Fig. 6D-II) treated rabbit vagina, but severely concentrated in N-9 treated vaginal tissues (Fig. 6D-III). Similarly, E-selectin expression was negligible in vehicle (Fig. 6E-I) and combination-treated rabbit vagina (Fig. 6E-II), but very intense in N-9 treated vaginal tissues (Fig. 6E-III).

Discussion

Trichomonas vaginalis causes punctate hemorrhages giving HIV open access to its target T-lymphocytes. These hemorrhages leak the virus and cause them to concentrate in the infected area (Sorvillo et al., 2001), which increase HIV transmission by 2- to 3-fold. Local antitrichomonal agents could be an effective method of controlling sexual transmission of Trichomoniasis, which in turn could significantly limit the number of new HIV infections. Furthermore, during 2003–2012 there has been a shift from sterilization to barrier methods for pregnancy protection with the unmet need for modern contraceptives remaining high in sub-Saharan Africa (60%), west Asia (50%) and south Asia

Table II  The in vivo contraceptive efficacy of ‘combination’ (DSE + Sap) in the rabbit model.

<table>
<thead>
<tr>
<th>Dose (intravaginal in 2.0 ml K-Y jelly)</th>
<th>Number mated</th>
<th>Number pregnant</th>
<th>Pups born</th>
<th>Pups/animal (Mean ± SD)</th>
<th>Fertility reduction (%)</th>
<th>Contraceptive efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (0 mg)</td>
<td>06</td>
<td>06</td>
<td>57</td>
<td>9.5 ± 1.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DSE + Sap [1:1] 0.5% (10 mg)</td>
<td>06</td>
<td>03</td>
<td>17</td>
<td>2.8 ± 3.1</td>
<td>70.17</td>
<td>50</td>
</tr>
<tr>
<td>DSE + Sap [1:1] 1.0% (20 mg)</td>
<td>06</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DSE + Sap [1:1] 2.0% (40 mg)</td>
<td>06</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N-9 1.0% (20 mg)</td>
<td>06</td>
<td>04</td>
<td>27</td>
<td>4.5 ± 3.5</td>
<td>52.63</td>
<td>33.33</td>
</tr>
</tbody>
</table>

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The anti-Trichomonal saponins used in this study were a fully standardized mixture of six sapindosides (sapindosides A, B, C, D and mukurozisaponins E1 and Y1), with sapindoside B as the major constituent. These were isolated from the fruit pericarp of *Sapindus mukorossi* (family Sapindaceae) by n-butanol extraction of its ethanolic extract, and identified by liquid chromatography-mass spectrometry (Dwivedi et al., 1990; Saxena et al., 2004). These saponins kill human sperm in vitro at 0.05% and comprise the active ingredient (2.5%) of the herbal contraceptive cream named ‘ConSap’. Repeated intravaginal application of ConSap in rabbits (2.5, 5 or 10% for 60 days) or rhesus monkeys (5, 10 and 25% for 90 days) does not cause any local irritation/pathological lesions in the vagina or other parts of the genital tract (Tiwari et al., 2008). ConSap has cleared Phase III clinical trials in India for vaginal use and has been licensed to M/s HLL Lifecare Ltd, Thiruvananthapuram, for marketing (Gupta, 2005; Tiwari et al., 2008).

In the ‘combination’, saponins were used at its microbicidal minimal inhibitory concentration (MIC) of 0.0015%, at which it displayed no detectable effect on sperm viability/motility. Saponins, being a mixture of sapindosides, were used at percent concentrations. Nevertheless, Sapindoside-B, the major component in saponin mixture, has a molecular weight (MW) of 883, which is similar to that of DSE-37 (MW = 874, ditartrate salt) and N-9 (MW = 616). Hence the percent concentrations (μg/ml) used in the present study largely represented their molar concentrations. It is pertinent to note here that N-9 is also a mixture of oligomers (Walter and Digenis, 1991), but for all practical purposes its MW is considered as 616, which is that of its major component. Since both nonoxynol-9 and saponins exhibit spermicidal activity by the surfactant mechanism and N-9 is >3 times as active as saponins, the 33% reduction in pregnancy rate of rabbits by 1% N-9 indicates that saponins may have only a very minor role in the 100% pregnancy protection by the 1% ‘combination’ containing 0.5% saponins. However, this minor contribution of saponins to the in vivo contraceptive activity of ‘combination’ cannot be ruled out.

All surfactants, as a general property, reduce the surface tension of water, which provides a measure of their surfactant potency. The surface tension of saline is about 10% higher than that of pure water (Paz and Rubin, 1970) as also observed in the present study. We experimentally demonstrate for the first time that N-9 exhibits very significant surfactant activity at both spermicidal and trichomonacidal concentrations, which has been appropriately considered as its most plausible mechanism of action. However, it is pertinent to note that saponins exhibit significant surfactant activity only at spermicidal concentration (500 μg/ml) and not at an anti-trichomonadal concentration (15 μg/ml). DSE-37 and the ‘combination’ were devoid of any significant surfactant activity at spermicidal/microbicidal concentrations. Saponins exhibit a specific mechanism-based anti-Trichomonadal activity without affecting host cell viability (Tiwari et al., 2008). Similarly, DSE-37 exerts very specific spermicidal activity at very low concentrations (15–20 μg/ml) by targeting free ‘reactive’ thiols available on sperm surface (Jain et al., 2007), which according to an estimate, are up to 30 times more common on sperm than on somatic cells (Mercado et al., 1976). Accordingly, it was hypothesized that a 1:1 combination of DSE-37 and Saponins would specifically combine microbicidal and contraceptive activities for dual protection. Mechanistically, N-9 permeates the sperm cell membrane and thereby affects cell viability while DSE-37 attenuates sperm by inactivating free thiols on cell surface, which are crucial for redox reactions that initialize energy metabolism (Nakamura et al., 2008), maintain membrane fluidity (Nivsarkar et al., 2001) and guide redox signaling (Miranda-Vizuete et al., 2004). The fluorescence of sperm thiols indicated that only DSE-37, the sperm immobilizing constituent of ‘combination’, attenuated sperm thiols while the saponin content of ‘combination’ remained largely inert in this mechanism. On the other hand, the spermicidal mechanism of N-9 was independent of thiol inactivation. Live spermicidal effects were recorded to indicate the rapidity and robustness of the sperm immobilizing potential of vaginal contraceptive candidates, which is mandatory for optimum effects. As evident in case of both the combination and N-9, not even a single spermatozoon could cross the semen/spermicide interface in spite of vigorous progressive motility. However, the quick diffusion of the ‘combination’ into semen may indicate its quicker and larger area of action than N-9. This is the first live display report of spermicidal action.

Figure 6 Immunohistochemical localization of pro-inflammatory/toxicity markers in cervico-vagina of rabbits treated intravaginally with (I) vehicle, (II) 5% combination, (III) 2% nonoxynol-9, for 4 consecutive days. (A) Terminal UDP nick end labeling (TUNEL-FITC); (B and C) nuclear factor-kB and DAPI staining of DNA; (D) vascular adhesion molecule-1 (VCAM-1); (E) E-selectin.

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The present comprehensive study utilized several in vitro and in vivo experiments to demonstrate that a 1:1 combination of DSE-37 and Sapindus saponins shows promise as an effective and safe vaginal contraceptive with anti-Trichomonal activity. The in vitro safety studies have indicated that DSE-37 is safer than the DSE + Sap combination, which in turn was safer than saponins. However, at its MEC, the ‘combination’ was almost as safe as the vehicle (control). The in vitro safety studies were mostly conducted in human cervical (HeLa) cells and not in vaginal epithelial (Vk2/E6E7) and endocervical (End1/E6E7) cell lines due to the extreme sensitivity of the latter cell lines to N-9 (IC50 = 21.46 and 17.33 μg/ml, respectively) than to the ‘combination’ (IC50 > 100 μg/ml). Since the mechanistic studies were conducted at the MEC of the combination (30 μg/ml), and N-9 was used at the same concentration (for comparison), very few cells (<20%) of Vk2 and End1 cell lines could survive the N-9 treatment at 30 μg/ml for effective assessment of comparative toxicity; hence HeLa was used for cytotoxicity experiments.

Vaginal inflammation due to vaginally applied products and/or pathogens is a major risk factor for acquisition of STDs, including HIV (Cone et al., 2006). The expression of pro-inflammatory cytokines like IL-1, IL-6 and IL-8 is considered as accurate predictor of mucosal toxicity of vaginal products (Fichorova et al., 2004) since they recruit CD4+ cells at the mucosal port of HIV entry and amplify viral replication in infected cells (Royce et al., 1997). Their expression by cervicovaginal cells is significantly induced via NF-κB mediated signal transduction pathways in response to pro-inflammatory stimulus (Tak and Firestein, 2001) as seen in the case of N-9 (Fichorova et al., 2001). Apparently, and in contrast to N-9, the inflammatory cascade remained unperturbed in DSE + Sap-treated HeLa cells, indicating the innocuous nature of the ‘combination’ to the vaginal environment. This became convincingly apparent through the in vivo studies where the in situ display of the expression of pro-inflammatory factors like NF-κB, VCAM-1 and E-selectin in the vagina of treated rabbits demonstrated the extreme innocuousness of ‘combination’ in comparison with N-9. In the human female genital tract, VCAM-1 and E-selectin are abundantly expressed in acute inflammatory conditions like cancer, but rarely and weakly expressed in the normal cervix and vagina (Trifonova et al., 2007).

Lactobacilli, which widely populate the healthy female vaginal mucosa (Vásquez et al., 2002; Zhou et al., 2004), provide natural prophylaxis by producing lactic acid (to maintain a low vaginal pH) and other antimicrobial substances, and perhaps also by countering inflammation (Fichorova et al., 2011; Rose et al., 2012). Sapindus saponins and DSE-37 are independently much more compatible with Lactobacillus acidophilus than N-9 (Ojha et al., 2003; Jain et al., 2007), and accordingly the ‘combination’ exhibited negligible detrimental effects on L. jensenii while N-9 was incompatible with this vaginal microflora at comparable concentration. To further prove the safety of the ‘combination’, the topical intravaginal effects were evaluated in rabbits at its 5% concentration, which was >1500 times more than its in vitro effective microbicidal/spermicidal concentration against human sperm/infection and 5 times the effective in vivo contraceptive dose in rabbits. In comparison, N-9 was used at 2% concentration, which was ~133 times the in vitro spermicidal dose against human sperm and the minimum concentration of N-9 used in any vaginal product for human use. The data clearly establish the higher safety and negligible inflammatory reaction of ‘combination’ over N-9.

The present study indicates that combining synthetic spermicide DSE-37 with natural saponins may present a promising recipe for microbicidal vaginal contraception, worthy of further consideration.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
A.J., L.K., B.K., M.S., A.P., V.V., V.Sh. and V.Si. performed all the biological experiments and acquired the data. V.L.S. conceptualized the spermicide DSE-37, designed its synthesis and undertook its chemical characterization, and T.R. synthesized DSE-37. A.J. and J.P.M. performed the experiments with rabbits and analyzed the data. G.G. conceived the study, planned and designed the experiments, obtained the funding, analyzed the data and wrote the manuscript draft. All authors were involved in revising the manuscript and approval of the final version.

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Conflict of interest
None of the authors had any competing interest(s).

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