

Novel disulphide esters of carbothioic acid as potent, non-detergent spermicides with low toxicity to *Lactobacillus* and HeLa cells *in vitro*

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BACKGROUND: The design, synthesis, characterization and evaluation of a novel series of non-detergent spermicides has led to the discovery of two unique molecules (DSE-36 and DSE-37) that were ~25 times more potent spermicides than nonoxynol-9 (N-9). **METHODS:** Normal human spermatozoa were used to assess the spermicidal activity (Sander–Cramer Assay), the effect on sperm-membrane integrity [hypo-osmotic swelling test (HOST)], supravital staining and scanning electron microscopy (SEM) and the induction of apoptosis [fluorescein isothiocyanate (FITC) Annexin-V and JC-1 labelling using flow cytometry] by the new class of compounds. HeLa and *Lactobacillus* cultures were used to assess the cytotoxicity of compounds and their compatibility to normal vaginal flora, respectively. **RESULTS:** Compounds DSE-36 and DSE-37 exhibited a strong spermicidal activity [minimum effective concentration (MEC) = 0.002%], which was ~25 times more potent than that of N-9 and Sapindus saponins (MEC = 0.05%). As compared with surfactants, DSE-36 and DSE-37 were found to be safer at MEC towards the growth and survival of *Lactobacilli* and HeLa cells *in vitro* and to have a milder effect on sperm plasma membrane. At EC₅₀ both induced apoptosis in sperm cells as characterized by increased labelling with Annexin-V and decreased polarization of sperm mitochondria. **CONCLUSION:** Preliminary studies have revealed that in sharp contrast to the non-specific surfactant action of N-9, DSE-36 and DSE-37 have a highly potent, mechanism-based, detrimental action on human sperm. The unique ability of these non-detergent molecules to selectively kill sperm and spare *Lactobacilli* and HeLa cells at MEC values much lower than that required for N-9 indicates their potential as superior ingredients for formulation into microbicial contraceptives.

Key words: contraception/non-detergent spermicides/spermicidal activity/selective toxicity

Introduction

The continued high rates of unintended pregnancies and the relentless expansion of the sexually transmitted disease/human immunodeficiency virus (STD/HIV) epidemic, especially in less-developed countries, warrant the development of novel strategies to help individuals avoid these risks (Doncel, 2006). Condoms can provide adequate protection against pregnancy as well as STDs and HIV but is not woman-controlled. In addition to the risk of unwanted pregnancy, women are also at a higher risk than men of acquiring HIV after unprotected sex (Saracco *et al.*, 1993). Despite this, female partners in many relationships do not control pregnancy or STD risk and may benefit from discrete methods other than condoms (Howett and Kuhl, 2005). Microbicial contraceptives offer a suitable alternative to condoms as the most viable, women-controlled method for dual protection. However, recent clinical trials have shown

that nonoxynol-9 (N-9), the ‘most promising’ spermicidal-microbicide, not only failed to offer any protection against STDs and HIV but actually increased the incidence of these diseases in the users (Stephenson, 2000; Van Damme *et al.*, 2002). This was mainly attributed to the surfactant (detergent) nature of the compound that caused vaginal inflammation/lesions after repeated use resulting in increased susceptibility to STDs, including HIV (Fichorova *et al.*, 2001). The WHO has cautioned the users of N-9 containing vaginal preparations especially those who are at high risk of acquiring HIV (WHO/CONRAD, 2001). Thus, an urgent need has emerged to identify a suitable, safe, non-detergent and potent spermicide that can replace N-9 in vaginal contraceptive preparations/devices. This need is also augmented by the fact that 80–90% of new HIV infections are caused by heterosexual contacts (Catalone *et al.*, 2005), especially ‘those’ contacts in which protective

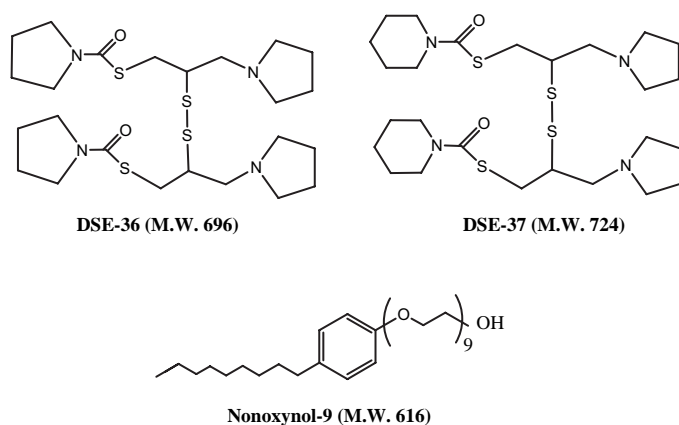


Figure 1. Molecular structures and weights of DSE-36, DSE-37 (non-detergent) and N-9 (detergent) spermicides.

measures (if any employed) are limited only to pregnancy protection. Hence, unwanted pregnancy may be a greater concern than STDs and HIV during 'such' contacts (Gupta *et al.*, 2005). Consequently, vaginal preparations with potent spermicidal as well as microbicidal properties may be used with more compliance in such relationships than pure microbicides. Therefore, a potent contraceptive activity needs to be integrated essentially in most of the microbicidal preparations so as to ensure its consistent use during the majority of coital acts (Gupta, 2005).

Keeping in view the ill effects of N-9, the design, synthesis and evaluation of many non-detergent spermicidal/microbicidal molecules was taken up at the Central Drug Research Institute (Srivastava *et al.*, 1999; Kumaria *et al.*, 2002; Maikhuri *et al.*, 2003; Gupta *et al.*, 2005; Kiran Kumar *et al.*, 2006a,b). Recently, a novel series of non-detergent disulphide esters of dialkylaminocarbothioic acid was discovered and yielded several very promising spermicidal molecules (Dwivedi *et al.*, unpublished). Some of these can potentially replace N-9 in contraceptive preparations. We report here the biological activity, preliminary safety data and basic mechanism of action of two very potent and promising molecules from this series (Figure 1). These compounds have been studied in comparison with N-9 and Sapindus saponins to bring out the salient advantages of these molecules over surfactant spermicides.

Materials and methods

Human spermatozoa

Fresh human semen samples obtained by masturbation into a sterile vial from healthy, young, fertile donors were liquefied for 45 min at 37°C and used for *in vitro* spermicidal and mechanism of action assays. Samples having $>65 \times 10^6/\text{ml}$ sperm count with $>70\%$ motility and normal sperm morphology were used in the study. The ethical approval for this study was obtained from the Institute's ethics committee.

DSE-36 and -37

DSE-36 and -37 were prepared as tartarate salts by the chemist authors (V.L.S., S.T.V.S.K.K. and A.K.D.) in the Process and Medicinal Chemistry Division of the Institute, as per the molecular structures shown in Figure 1. All compounds were $>99.5\%$ pure [analytical

high-performance liquid chromatography (HPLC)] and were characterized by mass, infrared and nuclear magnetic resonance (NMR) spectroscopy and also by elemental analysis. The compounds were hygroscopic and extremely soluble in water (saline).

Spermicidal test

The test compounds were dissolved in physiological saline to make a 1.0% (10 mg/ml) solution and diluted serially up to 0.001%. A spermicidal test was performed with each dilution starting from 1.0% till the minimum effective concentration (MEC) was arrived at, following the modified method of Sander and Cramer (1941). Briefly, 0.05 ml of human semen was added to 0.25 ml of spermicidal compound solution and vortexed for 10 s. A drop was immediately placed on a microscope slide, covered with a cover glass and examined under a phase-contrast microscope. The result was scored positive if 100% spermatozoa became completely immotile within 20 s. The MEC was determined in three individual semen samples from different donors. EC_{50} values (concentration killing ~50% of sperm *in vitro*) for the spermicides were determined by a Computer Assisted Sperm Analyzer (CASA) system (Model HTM-IVOS, Hamilton Thorn Research, MA, USA). Semen was mixed with spermicide solution (serially diluted from MEC) in the ratio of 1:5 (as indicated above) and analysed immediately for percentage motility with the help of CASA using phase-contrast optics and an acquisition rate of 30 frames/s in a pre-warmed Makler (Sefi Medical Instruments, Haifa, Israel) chamber at 37°C. The data were acquired in ~60 s. A concentration-versus-percentage motility curve was plotted to determine the approximate EC_{50} . This value was used as a reference to prepare finer dilutions of the compounds, and the final EC_{50} value was determined manually through visual scoring under a phase-contrast microscope by two independent workers in three semen samples from different donors, employing the modified Sander-Cramer assay protocol. The most agreeable data were selected.

Cytotoxicity towards human cervical (HeLa) cell line by lactate dehydrogenase-release assay

A colorimetric assay for lactate dehydrogenase (LDH) release was used for the evaluation of the cytotoxicity of spermicidal compounds against the HeLa cell line. Exponentially growing HeLa cells were seeded into 96-well tissue culture plates at a density of 2×10^4 cells per well (in triplicate). After 24-h incubation in a CO_2 incubator at 37°C in 5% CO_2 , 95% air atmosphere, the culture medium [Dulbecco's modified Eagle's medium (DMEM)] was replaced with 100 μl of fresh medium containing serially diluted spermicidal compounds. Control wells contained the medium only. Culture plates were incubated for another 5 h, and then 50 μl of the supernatant from each well of the assay plate was pipetted into the corresponding well of a flat-bottom 96-well plate. Colour reaction for LDH assay and IC_{50} measurement for cytotoxicity were performed using CytoTox-96 kit (Promega, Madison, WI, USA) by following the instructions of the manufacturer. Optical densities at 490 nm were measured in a microplate reader (μQuant , Bio-Tek, USA).

Effect on *Lactobacillus acidophilus* in vitro

The effect of compounds exhibiting potent spermicidal activity on *Lactobacillus acidophilus* was determined by following the method published earlier from this laboratory (Ojha *et al.*, 2003). Briefly, Rogosa SL agar plates (7.5%; containing 0.132% acetic acid), prepared with (experimental) or without (control) the addition of spermicidal compounds, were inoculated with *L. acidophilus* (~70 spores/ 10 cm^2) and incubated at 37°C in 5% CO_2 and 95% air for 72 h. The number and size of colonies were recorded at the end of the experiment. The

average colony size (% of control) was multiplied by the colony number and divided by 100 to arrive at the data presented. The average colony size for the control was taken as 100%.

Effect on sperm plasma membrane integrity

Supravital staining with fluorescent dye [propidium iodide (PI)] and the hypo-osmotic swelling test (HOST) were used to assess the effect of new spermicides on sperm-membrane permeability, using three different semen samples. Samples of 0.2 ml liquefied semen were treated with 1.0 ml of spermicide solution at the MEC (1.0 ml of buffer in control tubes) and incubated for 1 min at 37°C. The spermatozoa were pelleted (500 g for 5 min) and 0.001% PI solution was added to the pellet, mixed and incubated for 15 min at 37°C. A wet mount for each compound was observed first in 'normal' light and then in 'blue' light [from a mercury lamp using the B2A (Nikon) filter], and the total number of sperm as well as sperm with fluorescent (red) heads was recorded, respectively. The same was repeated for other fields of view. The HOST of Jayendran *et al.* (1984) was used to determine the effect on the physiological integrity of the sperm membrane. Human spermatozoa treated with spermicide solution (as in the supravital staining experiment) were pelleted, treated with hypo-osmotic solution and mixed gently. The suspension was incubated for 30 min at 37°C. A wet mount was prepared for each compound solution and observed under a phase-contrast microscope, and spermatozoa with and without tail curling were counted and recorded.

Flow cytometric assays for induction of apoptosis

The induction of sperm cell apoptosis by spermicidal compounds was studied by labelling with fluorescein isothiocyanate (FITC)–Annexin-V (changes at sperm surface) and with the fluorescent probe JC-1 (changes in sperm mitochondrial transmembrane potential, $\Delta\Psi_m$) using flow cytometry.

Changes at sperm membrane by FITC–Annexin-V labelling

Dual fluorescent labelling with FITC–Annexin-V and PI was used to study the expression of phosphatidylserine on sperm surface (apoptotic cells) and the sperm-membrane permeability (necrotic cells), respectively. Aliquots of 1.0 ml of highly motile sperm (10^7) in triplicate were incubated in BWW-0.3% bovine serum albumin (BSA) at 37°C for 3 h with EC_{50} concentrations of spermicide solution (experimental) or buffer only (control) or 50 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP, positive control). After incubation, sperm were washed in 1% BSA in Tyrode's buffer (TBSA) and labelled with fluorescent probes using the Annexin-V–FITC apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA) by following the manufacturer's instructions. The percentages of sperm with positive Annexin-V and PI labelling were determined in a flow cytometer (Model FACS Calibur, BD Biosciences, USA) equipped with an argon laser (488 nm) for excitation, using the threshold signals for membrane-intact motile sperm. Three separate experiments were performed using sperm from different donors. Four populations of sperm cells (unstained, FITC, FITC + PI and PI) were identified (Anzar *et al.*, 2002) and designated as viable, apoptotic, necrotic and dead, respectively.

Changes in sperm mitochondrial transmembrane potential using JC-1 fluorescent probe

Mitochondrial transmembrane potential ($\Delta\Psi_m$) maintains the polarized state of mitochondria, and a loss of $\Delta\Psi_m$ is an early marker of apoptosis. The lipophilic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanine iodide) selectively enters the mitochondria and depending on the membrane potential forms J-aggregates that are associated with a large shift in emission spectra. The colour of the dye

changes reversibly from green to greenish orange as the $\Delta\Psi_m$ becomes more polarized. Highly motile sperm (10^7) in triplicate were incubated in BWW-0.3% BSA at 37°C for 3 h with EC_{50} concentrations of spermicide solution (experimental) or buffer only (control) or 50 μ M CCCP (positive control). After incubation, 10 μ g/ml JC-1 (Sigma-Aldrich) was added from a stock solution of 1 mg/ml in dimethylsulphoxide (DMSO) and incubated for another 10 min. Finally, sperm were washed with Tyrode's salt solution and analysed in a flow cytometer (Model FACS Calibur, BD Biosciences) using an argon laser (488 nm) for excitation, and emissions at 530 (green) and 575 (red/orange) were quantitated using the threshold signal for JC-1-labelled intact motile sperm.

High resolution scanning electron microscopy

High resolution scanning electron microscopy (HR-SEM) was utilized for topographical imaging of membrane domains over the sperm head. Briefly, sperm suspensions were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C. After washing in cacodylate buffer, suspensions were placed on 0.1% poly-L-lysine-coated glass chips and allowed to adhere for 1 h at room temperature. Samples were post-fixed in 1% osmium tetroxide for 1 h at room temperature and subsequently dehydrated through an ascending series of ethanol, critical point dried and coated with Au–Pd (80:20) using a sputter coater (Polaron E5000). All samples were examined in a FEI XL 30 (Philips FEI, The Netherlands) SEM at an accelerating voltage of 30 kV. Micrographs were taken at a magnification of $\times 10\,000$. For each sample, at least 150 sperm were scanned for intactness of the sperm acrosomal region.

Results

Spermicidal potential

Compounds DSE-36 and -37 killed 100% human sperm in 20 s at a minimum concentration of 20 μ g/ml (0.002%), whereas N-9 and Sapindus saponins did the same at 500 μ g/ml (0.05%). The EC_{50} for these compounds was 12, 10, 80 and 360 μ g/ml, respectively (Table I).

LDH-release assays for cytotoxicity

At MEC, DSE-36 and -37 did not exhibit any detectable cytotoxicity towards the human cervical (HeLa) cell line. The IC_{50} for cytotoxicity of DSE-36, DSE-37 and N-9 towards HeLa cells was 0.359, 0.690 and 0.0135 mM, respectively. The safety index of the compounds was calculated as a ratio of cytotoxicity IC_{50} to spermicidal EC_{50} . DSE-36 and -37 exhibited a safety index of 21.11 and 49.28, respectively, both much higher than that of N-9 (0.105). Thus, the cytotoxicity assay

Table I. The spermicidal potential of detergent and new non-detergent spermicides

Spermicidal compounds	MEC (%)	MEC (mM) (mean \pm SE)	EC_{50} (%)	EC_{50} (mM) (mean \pm SE)
DSE-36	0.002	0.0242 \pm 0.00083	0.0012	0.0166 \pm 0.0006
DSE-37	0.002	0.0225 \pm 0.00144	0.001	0.0140 \pm 0.0012
Nonoxynol-9	0.05	0.811 \pm 0.00167	0.008	0.129 \pm 0.0068
Sapindus saponins	0.05	–	0.036	–

Minimum effective concentration (MEC) determined by the Sander–Cramer Assay, and EC_{50} determined by visual scoring with the assistance of a Computer Assisted Sperm Analyzer (CASA).

Table II. Cytotoxicity and safety index of DSE-36 and -37 and N-9 towards human cervical (HeLa) cells *in vitro*, as quantitated by LDH-release assay

Treatments	Spermicidal EC ₅₀ (mM)	Cytotoxicity IC ₅₀ (mM)	Safety index ^a	Safety versus Nonoxynol-9
DSE-36	0.017	0.359	21.11	201
DSE-37	0.014	0.690	49.28	469
Nonoxynol-9	0.129	0.0135	0.105	1

^aThe ratio of cytotoxicity IC₅₀ to spermicidal EC₅₀ is denoted as safety index.

revealed a massive 201- and 469-fold higher level of safety for DSE-36 and -37, respectively, compared with N-9 (Table II). Sapindus saponins were almost equitoxic to N-9 on the basis of percentage (w/v) concentrations. Because saponins are a mixture of compounds, their millimolar solution could not be made, and hence the data has not been included in Table II.

Scanning electron microscopy

Compounds DSE-36 and -37 did not cause any visibly significant damage to the sperm plasma or acrosomal membrane structure at MEC (Figure 2A and B). However, N-9 acutely damaged the sperm membrane with complete loss of the acrosomal structure in most of the sperm cells (Figure 2C). Sapindus saponins also caused visibly severe damage to sperm cells by dissolving plasma/acrosomal membranes at several points and causing perforations (Figure 2D). It was noticed that the sperm cells treated with DSE-36 appeared plasmolysed and shrunk especially in the mid-piece region (Figure 2B), whereas those treated with DSE-37 were normal or slightly swollen (Figure 2A) as compared with control (Figure 2E).

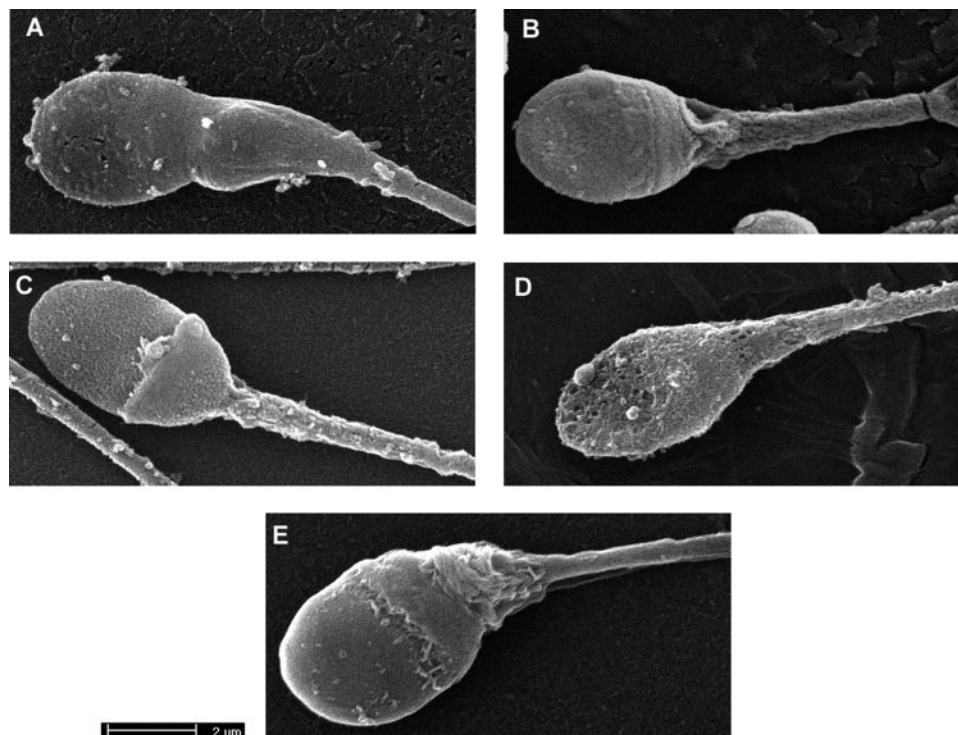


Figure 2. High resolution scanning electron micrographs ($\times 10\,000$) of human sperm treated with detergent and non-detergent spermicides at minimum effective concentration (MEC). (A) DSE-37, (B) DSE-36, (C) N-9, (D) Sapindus saponins and (E) control; bar = 2 μm .

Effect on normal vaginal flora (*Lactobacillus*) *in vitro*

DSE-36 and -37 did not significantly affect the size and number of *Lactobacillus* colonies at MEC and $\times 2$ MEC concentrations. However, at 10- and 20-fold higher concentrations over MEC, *Lactobacillus* growth was inhibited by ~ 20 and $\sim 60\%$ with DSE-36 and by ~ 27 and $\sim 40\%$ with DSE-37, respectively. These changes were statistically significant. On the contrary, N-9 inhibited the growth of *Lactobacillus* colonies significantly by $\sim 84\%$ at MEC and 100% at $\times 2$ MEC. In comparison with N-9, a lower but significant inhibition of *Lactobacillus* growth by ~ 24 , ~ 44 and $\sim 82\%$ (at MEC, $\times 2$ MEC and $\times 5$ MEC, respectively) was encountered with Sapindus saponins (Figure 3).

Supravital staining and HOST

A complete loss of structural membrane integrity in total sperm cells (100% PI stained) was accompanied by an anticipated total loss of sperm-membrane physiology (100% HOST-negative), after treatment with N-9 and saponins at MEC (Figure 4). The sperm-membrane permeability was also affected by the non-detergent spermicides at MEC, but the effect was much more mild in comparison with N-9 and saponins. The number of HOST-negative sperm increased from $\sim 27\%$ in the control to ~ 73 and $\sim 68\%$ in DSE-36- and DSE-37-treated samples, respectively. Similarly, the number of PI stained sperm also increased from $\sim 25\%$ in the control to ~ 55 and $\sim 51\%$ in DSE-36- and DSE-37-treated samples, respectively (Figure 4). Thus, the physiological integrity of sperm membrane appeared more vulnerable to treatment by the new spermicides than the structural integrity.

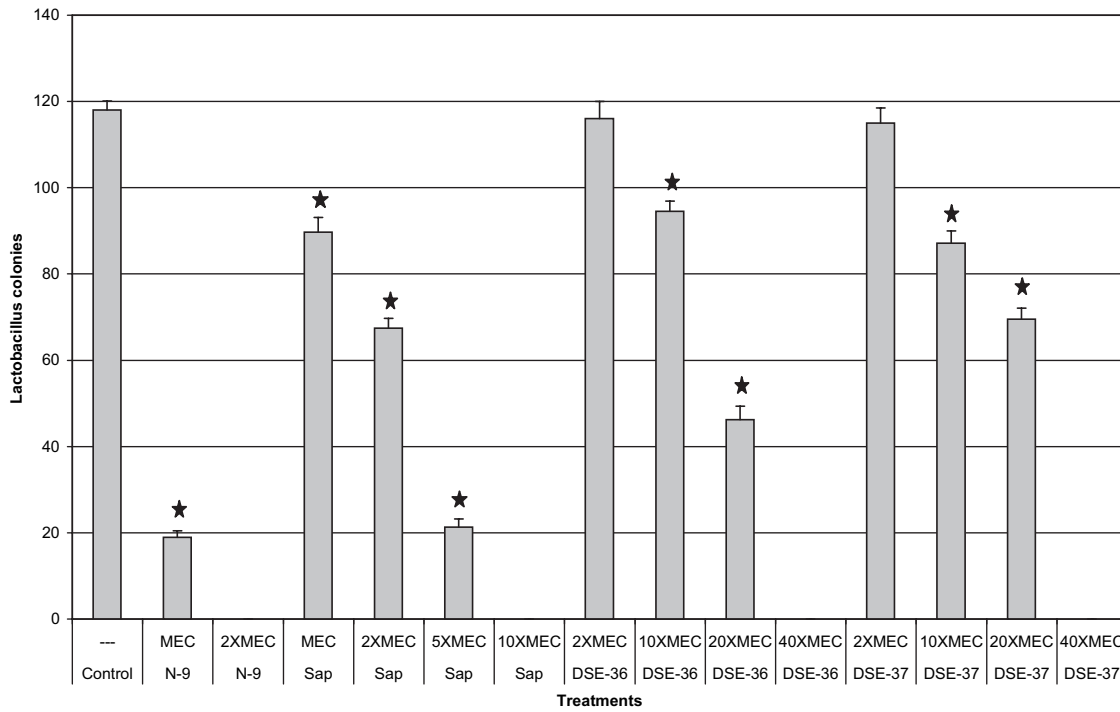


Figure 3. Effect of spermicides on growth and survival of *Lactobacillus acidophilus* colonies *in vitro*. No effect was seen in case of DSE-36 and -37 at minimum effective concentration (MEC) (data not included). Mean \pm SE of 3 values; * $P < 0.01$.

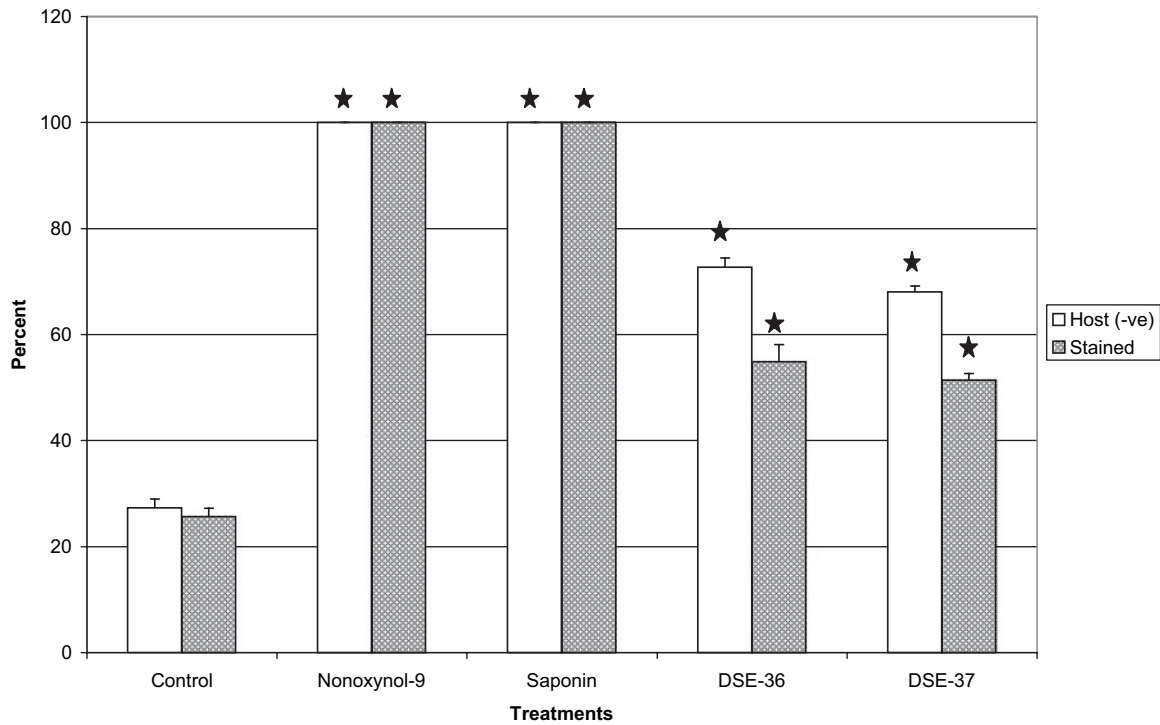


Figure 4. Effect of spermicides at minimum effective concentration (MEC) on structural [propidium iodide (PI) staining] and physiological [hypo-osmotic swelling test (HOST)] integrity of human sperm membrane. Mean \pm SE of 3 values; * $P < 0.01$.

Annexin-V-FITC labelling

The data from the dual fluorescent labelling with Annexin-V-FITC and PI showed that the control sample contained about 56% viable, 16% apoptotic, 26% necrotic and 1% dead sperm.

After treatment with N-9 at EC₅₀, the number of necrotic cells rose sharply and significantly to ~87% with a concomitant reduction in the populations of viable (~1%) and apoptotic (~10%) sperm cells (Figure 5). In the case of saponin treatment,

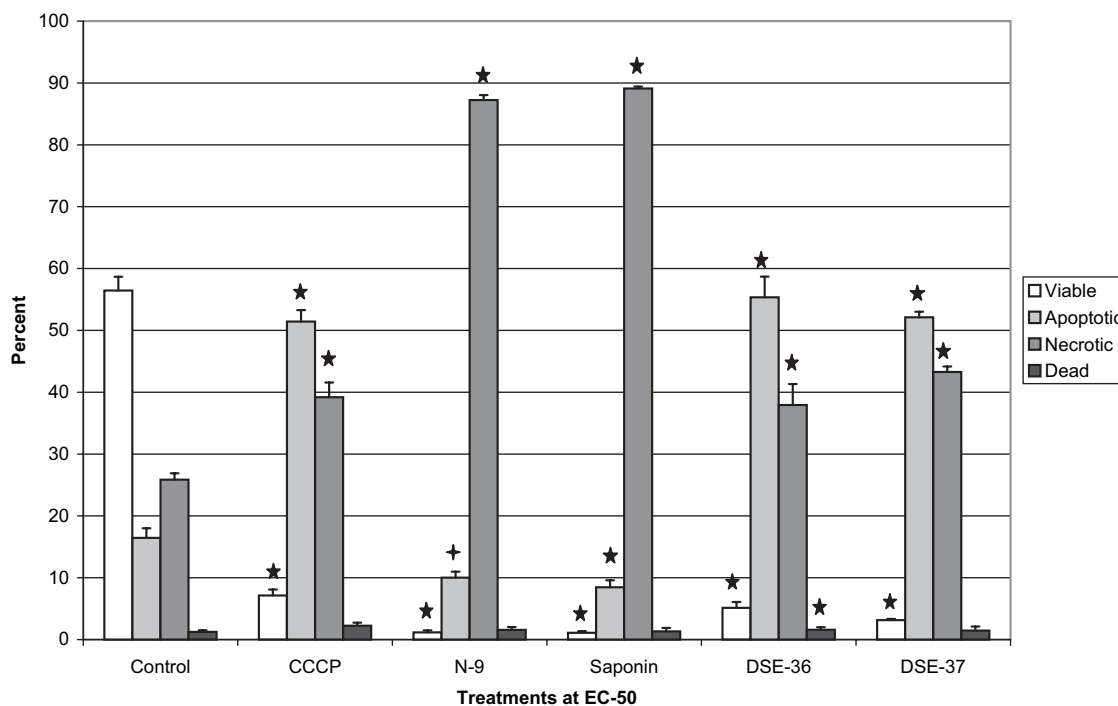


Figure 5. Flow cytometric assessment of FITC–Annexin-V/propidium iodide labelling of human sperm after treatment with spermicides at EC₅₀. Mean ± SE of 3 values; +*P* < 0.05; **P* < 0.01.

the necrotic cell number rose to ~89% with a simultaneous reduction in viable (~1%) and apoptotic (~8.5%) sperm numbers. Treatment with DSE-36 and -37 at EC₅₀ resulted in significant increases in the populations of both apoptotic and necrotic cells, with former being the predominant cell type after treatment. The apoptotic and necrotic cell populations were increased to ~55 and ~38%, respectively, after treatment with DSE-36 (Figure 5). Similarly, the apoptotic and necrotic cell populations were increased to ~52 and ~43% after treatment with DSE-37. The action of new spermicides was very much similar to that of the positive control compound CCCP (~51% apoptotic and 39% necrotic), which is well known for inducing apoptosis in living cells.

JC-1 labelling

There was a significant decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) of sperm cells treated with spermicides as indicated by an increase in number of sperm exhibiting green fluorescence for JC-1. However, the decline was greater in case of DSE-36 (28%), DSE-37 (20%) and CCCP (50%) than in case of N-9 (5%). Surprisingly, the $\Delta\Psi_m$ decline for saponins (47%) was more similar to that for CCCP than for N-9 (Figure 6).

Discussion

The role of sulphhydryl and disulphide groups in maintaining the membrane fluidity (Yelinova *et al.*, 1996; Nivsarkar *et al.*, 2001) and consequently the sperm motility/viability (Sinha *et al.*, 1993; Nivsarkar *et al.*, 1998) is well documented. Thus, molecules interacting with these groups are likely to affect

sperm viability. Several sulphhydryl-binding agents such as *N*-ethylmaleimide (Solanki *et al.*, 2005), SSRI antidepressants (Kiran Kumar *et al.*, 2006a), benzenepropanamine analogues (Kiran Kumar *et al.*, 2006b) and sodium dialkyl dithiocarbamates (Holzaepfel *et al.*, 1959; Tripathi *et al.*, 1996) have been reported to possess spermicidal activity. Taking a clue from these studies, molecules having a disulphide as well as a thiocarbamate group were designed with a notion to obtain non-detergent structures with potent spermicidal activity. Several molecules exhibiting varying levels of spermicidal efficacy were discovered, with two molecules (DSE-36 and -37) displaying exceptionally strong spermicidal potential (Dwivedi *et al.*, unpublished). A close examination of the chemical structures of DSE-36 and -37 and that of N-9 clearly indicates the non-detergent versus detergent type of structural characteristics. N-9 structure comprises distinct lipophilic (lengthy alkyl chain) and hydrophilic (long carbon chain with several ether linkages) domains, which is the primary feature of a detergent. Compounds DSE-36 and -37 have no such structural features and therefore are devoid of non-specific, surfactant types of cytotoxic action towards human cervical (HeLa) cells and *Lactobacillus in vitro*. On the contrary, disulphide and thiocarbamate groups contribute powerful spermicidal activity into these non-detergent compounds, making them perhaps the most potent spermicides reported thus far. The two properties (potent spermicidal and non-surfactant nature) together make these molecules very promising replacements for N-9 in vaginal contraceptive preparations. It would be relevant to mention here that the surfactant nature of N-9 is not solely responsible for the adverse effects on vaginal and cervical epithelium; its rather moderate spermicidal potential, that requires the compound

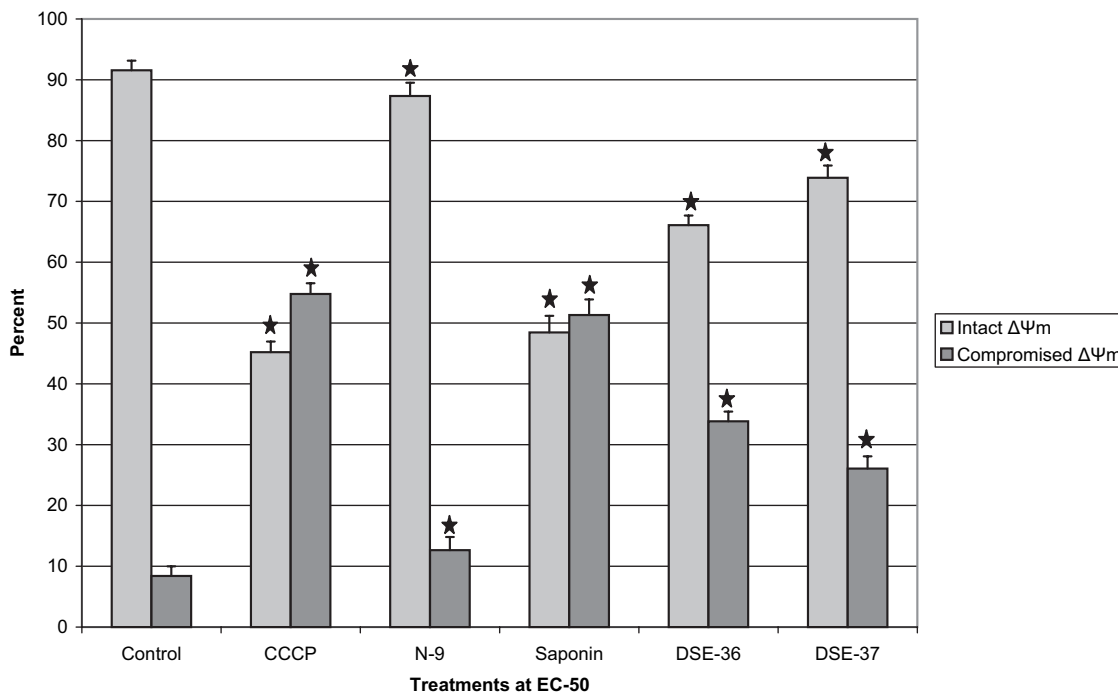


Figure 6. Flow cytometric assessment of human sperm mitochondrial transmembrane potential after treatment with spermicides at EC_{50} , as indicated by labelling with JC-1. Mean \pm SE of 3 values; * $P < 0.01$.

to be used at very high concentrations (5.0–12.5%) in local contraceptive preparations (McGroarty *et al.*, 1990), also results in increased toxicity. On the contrary, 25 times higher spermicidal activity of DSE-36 and -37 (over N-9) would enable the compound to be formulated at much lower concentrations than N-9 in vaginal contraceptive preparations, thus ensuring better safety. Because the concentration of the active ingredient in contraceptive preparations/formulations is based on its *in vitro* spermicidal MEC, the new compounds appear to have a major advantage over the existing surfactant spermicides. It is pertinent to note that although the new compounds effectively immobilized 100% human sperm in 20 s at MEC (0.002%) in the Sander–Cramer assay, they did not inhibit the growth of *Lactobacillus* colonies at the same concentration, during 72-h incubation. This was in sharp contrast to the action of N-9 that significantly inhibited both sperm motility and *Lactobacillus* viability at MEC. Additionally, the IC_{50} concentrations for cytotoxicity towards HeLa cells were much lower for N-9 than that for DSE-36 and -37. These indicate that, in comparison with detergent spermicides, the new non-detergent spermicides specifically and selectively target sperm cells, which provides a safety index of orders of magnitude in their favour. Inhibition of *Lactobacillus* colonies by N-9 is well known (Krebs *et al.*, 2002; Ojha *et al.*, 2003). Because disruption of the cervicovaginal epithelium by spermicides or microbicides may increase the susceptibility to HIV-1 infection by providing a direct portal of entry for the virus to subcutaneous tissues (Catalone *et al.*, 2005), spermicides with specific, mechanism-based action would be preferred over non-specific cell toxicants. Here, the action of new non-detergent spermicides appears to be quite specific and mechanism-based in comparison with the general cytotoxic effect of surfactant spermicides.

The distinct action of compounds DSE-36 and -37 from surfactant spermicides is also seen in supravital staining, HOST and SEM experiments. A complete disruption of structural (supravital staining, SEM) and physiological (HOST) integrity of sperm plasma membrane by surfactant action was in distinct contrast to the action of the new non-detergent spermicides, which was characterized by the impairment of predominantly the physiological rather than the structural sperm-membrane integrity. Recently, some other (di)thiocarbamate compounds were designed and reported for spermicidal activity against human sperm *in vitro* (D'Cruz *et al.*, 1998a; D'Cruz and Uckun, 2005). Although they have the advantages of being non-detergent in nature, still their spermicidal action appears to be weaker than N-9.

The finer aspects of spermicidal action on sperm cell were studied at EC_{50} concentration using flow cytometry. The compounds DSE-36 and -37 apparently induce apoptosis in sperm cells, which was characterized by an increase in FITC–Annexin-V labelling (green fluorescence) of sperm. Induction of apoptosis by increased Annexin-V labelling in human sperm has been demonstrated in case of some other non-detergent spermicides (D'Cruz *et al.*, 1998b). The surfactant spermicides on the contrary induced necrosis as revealed by increased FITC–Annexin-V as well as PI labelling (green and red/orange fluorescence) of sperm cells at EC_{50} concentration. This further indicates a mechanism-based action of DSE-36 and -37 on human sperm that is different from the general dissolution of cell membrane lipid bilayer by surfactants.

Mitochondrial transmembrane potential maintains the integrity of mitochondrial polarization for normal energy generation and dissipation (Bains *et al.*, 2006), and a significant drop in this potential indicates initiation of apoptotic process (Chaoui *et al.*,

2006). The depolarization of sperm mitochondria and induction of apoptosis by the non-detergent compounds, and not by N-9, once again reflects different mechanisms of action. Conversely, the saponin action here was similar to pro-apoptotic compound CCCP and not N-9. This may indicate that the action of plant saponins may differ from synthetic detergents at EC₅₀.

A very careful evaluation of SEM data indicates that the two new non-detergent spermicides with similar molecular structures and biological activities may in fact have a different action on sperm physiology. The mid-piece region of sperm treated with DSE-36 appears constricted, whereas that of sperm treated with DSE-37 looks normal or slightly swollen. This may indicate the two compounds affect the water channels on sperm cell in a contrasting manner, and this is being investigated in detail. However, this study draws attention to two unique non-detergent, disulphide esters of carbothioic acid that possess a specific and potent action on human sperm cell viability and therefore hold promise as safer options for local contraception. These compounds can suitably replace N-9 in vaginal preparations to make them more innocuous for repeated use without the apprehension of increasing the susceptibility of users to HIV and other STD infections. On the contrary, microbicidal agents supplemented with contraceptive activity are of particular interest because consumer preference studies suggest that most women worldwide prefer a vaginal prophylactic product to be both antimicrobial and contraceptive (Hardy *et al.*, 1998). However, owing to cytotoxicity and formulation problems, N-9 cannot be formulated with a potent microbicidal agent (Zaneveld *et al.*, 2002). Thus, the new molecules may find application for formulation into most of the future microbicidal preparations to incorporate potent contraceptive activity without increasing toxicity.

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