Chapter II

Establishing the safety of the formulation(s) for topical intravaginal application

Introduction

The normal healthy human vagina is quite effectively resistant to HIV infection resulting in very low odds for its heterosexual transmission during contact with HIV-positive male partners, which is estimated to about 0.1% of all sexual contacts and 2% of contacts during the highly infectious acute phase (Cone et al., 2006). The multilayered stratified epithelium in the vagina and ectocervix and the tight-junction columnar epithelium in the endocervix in unison with the normal vaginal microflora maintain an efficient barrier against HIV-1 (Fichorova et al., 2005). In spite of this ~5 million people are added every year to the 40 million people living with HIV, half of which are women (Lederman et al., 2006). This may not only indicate an excessive load of HIV infection in heterosexual contacts but also a rise in number of females with compromised vaginal/cervical integrity, induced by vaginally applied chemical products or pathogens. N-9, widely used in local contraceptives, barrier devices and sexual lubricants, has been shown to induce inflammatory response in vagina (Galen et al., 2007) resulting in increased susceptibility of users to STDs and HIV. At the present time, hundreds of spermicidal products have been marketed. Most of them contain a detergent as an active ingredient, such as nonoxynol-9 (N-9), and benzalkonium chlorides. Some of these detergents have also exhibited microbicidal activity in vitro. The spermicidal activities of these surfactants are associated with their structural affinity to the lipid membranes. However, numerous laboratory studies have now shown that detergent spermicides do not provide any protection against STD, and their effect in preventing human immunodeficiency virus (HIV) transmission remains controversial. The major drawback of using N-9 or other currently used surfactants is their detergent-like action on epithelial cells and normal vaginal flora. The repeated use of a surfactant as a vaginal contraceptive/microbicide has been associated with an increased risk of vaginal or cervical infection and irritation or ulceration,
detergent-type spermicides alter vaginal bacterial flora, and such disturbance of the vaginal microbial milieu can lead to opportunistic infections, which in turn increases the chance of HIV/STI transmission (Zairi et al., 2009). On the other hand, the most prevalent vaginal pathogen *Trichomonas vaginalis* immensely increases the susceptibility to HIV. Consequently, vaginal health has to be conferred the top priority in designing of new spermicides/microbicides and molecules specifically targeting sperm/pathogens have to be preferred over general cytotoxicants like N-9. In the previous chapter, we presented data to indicate that the new contraceptive combination (DSE+Sap) was safer than N-9 at multiple contraceptive doses in rats, in vivo. In the present chapter we have used in vitro human cervico-vaginal cell-lines to establish the ultimate selectivity of action of new microbicidal contraceptive in comparison to N-9. In the present study all *in vitro* experiments were performed at the therapeutic concentration of combination that is 30 g/ml (15+15 g/ml of DSE+Sap) because at this concentration 100% elimination of sperm and *Trichomonas* takes place in vitro, and we wanted to study the extent of apoptosis induction in HeLa cells at the same concentration to assess the specificity of action (which also indicates its safety). Since cervico-vaginal epithelium in unison with vaginal microflora (*Lactobacillus*) critically preserve the natural resistance to pathogens and HIV (Fichorova et al., 2005), the safety of the new molecules towards these two important components of the female reproductive tract was studied *in vitro*. Human cervical (HeLa) cells treated with the compounds were studied for structural changes in cell surface topography by FITC-conjugated phalloidin staining, cell viability by MTT cyto-toxicity assay and membrane integrity by LDH release assay. Inhibition of growth and proliferation of *Lactobacillus jensenii* (the normal vaginal flora) in plate and broth cultures was studied by colony counts and turbidimetry methods, respectively. Induction of apoptosis in HeLa cells due to chemical stress was evaluated by estimating the externalization of phosphatidyl serine (annexin-V/propidium iodide labeling), depolarization of mitochondrial membrane potential (JC-1 fluorescence) and ROS generation (fluorescence shift of DCFDA). Number of apoptotic nuclei was counted by fluorescent nuclear probe DAPI. Safety was also evaluated on two more human
vaginal (Vk2/E6E7) and cervical (End1/ E6E7) cell lines by MTT assay in vitro. N-9 and metronidazole were used as reference controls in all these experiments.

Materials and methodology

Chemicals and Reagents
DSE-37 and saponins were synthesized in Medicinal and Process Chemistry (MPC) division of the institute. Metronidazole, MTT was purchased by Sigma-Aldrich. N-9 was purchased from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ). LDH release assay kit was purchased from Roche, USA. Fetal bovine serum was obtained from Hyclone. Keratinocyte serum-free medium was purchased from Invitrogen Life Technologies. Rogosa SL agar and broth medium were purchased from Hi-Media. All culture media and other reagents were purchased from Sigma-Aldrich, USA.

Biological Materials

HeLa Cells
HeLa cells procured from National Centre for Cell Sciences (NCCS), Pune, India, were grown in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with fetal bovine serum (10%), and antibiotics (a penicillin streptomycin mixture [100 U/ml]). Cells at 80 to 90% confluence were split by trypsin (0.25% in phosphate-buffered saline [PBS]; pH 7.4), and the medium was changed at 24-h intervals. The cultures were maintained in a CO₂ incubator at 37°C in a 5% CO₂-95% air atmosphere.

Lactobacillus jensenii
Spores of Lactobacillus jensenii (ATCC 25258, strain 62G) were procured from the ATCC and grown in 6% Rogosa SL broth medium (Hi Media, India) containing 0.132% acetic acid at 37°C.
Human immortalize cervical (End1E6/E7) and vaginal (Vk2/E6E7) cells

Human papillomavirus 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 (IICB Kolkata), which were originally developed by and sourced from Dr. Raina Fichorova (Harvard Medical School, Boston). VK2 and End1 cells were maintained in keratinocyte serum-free medium (Invitrogen Life Technologies) supplemented with bovine pituitary extract (50 μg/ml), epidermal growth factor (0.01ng/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.

Cytotoxicity assays

Cytotoxicity of compounds toward human cervical (HeLa) cells by MTT assay

HeLa cell monolayers were used as an in vitro model of human cervicovaginal epithelium (Rojas, et al., 2004) for assessing the cytotoxicity of the new compounds. MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay for evaluation of the cytotoxicity of drug formulations against the human cervical cell line (HeLa) was used (D’Cruz and Uckun, 1999). Cells seeded at a density of 5 X 10^4 per well in 96-well plates were incubated in culture medium (DMEM supplemented with 10% fetal calf serum) for 24 h at 37°C in a 5% CO2-95% air atmosphere. After 24 h, the culture medium was replaced with fresh medium containing dilutions of test compounds in experimental wells and 0.05% DMSO in culture medium in control wells. After incubation for another 24 h, 5 μl of MTT solution (5 mg/ml in PBS [pH 7.4]) was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO, and the optical density at 540 nm (OD540) was recorded in a microplate reader (Microquant; BioTek USA). The percent viability was calculated according to formula given below:

% viability of cells = \{(OD of treatment)/OD of Vehicle control\} x 100
Cytotoxicity of compounds toward human cervical (HeLa) cells by LDH release assay (permeability of cell membrane)

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the culture supernatant. LDH activity is determined by a colorimetric assay. In the first step, NAD\(^+\) is reduced to NADH/H\(^+\) by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, a catalyst included in the reaction mixture (diaphorase) transfers H/H\(^+\) from NADH/H\(^+\) to the tetrazolium salt INT, which is reduced to a formazan dye (Fig 2A). An increase in the number of dead or plasma membrane-damaged cells leads to increased LDH activity in the culture supernatant, which directly correlates with the amount of formazan produced in a defined time period. Therefore, the amount of dye produced is proportional to the number of lysed (dead or plasma membrane-damaged) cells.

![Diagram of LDH enzyme reaction](image)

**Fig 2A:** A two step enzymatic reaction quantifies cell lysis and cell death

*HeLa* cells seeded at a density of 1 X 10\(^4\) per well in 96 well plates were incubated in culture medium (DMEM with 10% FCS) for 24 hours at 37°C in 5%CO\(_2\)/95% air atmosphere. After 24 hours, culture medium was replaced with fresh medium containing dilutions of test compounds. Positive control wells contained 1% triton X-100 in medium whereas control wells contained only medium. In case of DMSO soluble compounds, 10% DMSO was used as control. After an incubation of 24 hours, 100 l of culture supernatant was removed and delivered into flat bottom microtiter plates. Subsequently, 50 l of the reaction reagent (Roche Applied Science) was added to each well. After incubation at room temperature for 45 minutes, stopping reagent (50 l 1N HCl) was added to the wells. Within 90 minutes, absorbance at a wavelength of
490nm in each well was determined in a microplate reader. The LDH activity of supernatant was expressed as the percentage of total activity in supernatant. The amount of color formed in the assay is proportional to the number of lysed cells.

**Cytotoxicity of compounds towards human immortalized endocervical (End1/E6E7), and vaginal (Vk2/E6E7) epithelial cells**

Endocervical and vaginal keratinocyte cell lines were assessed for their sensitivity to N-9 and combination. These cell lines were selected as representative of the cell types found in genital tissues that would be exposed to topical vaginal microbicide in vivo. An MTT (3-[4, 5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay for evaluation of the cytotoxicity of drug formulations against the human immortalized endocervical (End1/E6E7), and vaginal (Vk2/E6E7) epithelial cells was used. Cells seeded at a density of 5 X 10^4 per well in 96-well plates were incubated in culture (KSF) medium for 24 h at 37°C in a 5% CO2–95% air atmosphere. After 24 h, the culture medium was replaced with fresh medium containing dilutions of test compounds in experimental wells and 0.05% DMSO in control wells. After incubation for 12 h, 5 μl of MTT solution (5 mg/ml in PBS, pH 7.4) was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO, and the optical density at 540 nm (OD_{540}) was recorded in a microplate reader (Microquant; BioTek).

**Cytotoxicity of compounds towards normal vaginal flora**

*Lactobacillus jensenii* (Broth culture)

The effect of test compounds on *L. jensenii* was determined by the method published earlier (Jain *et al*, 2009) with slight modification. Briefly, Rogosa SL broth medium was prepared in Milli-Q water, boiled for 2 to 3 min, and distributed in a 48-well plate (500 l/well). Serial dilutions of test compounds were added to experimental wells, and vehicle was added to control wells in triplicate. Approximately 1,000 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 estimated by measuring the turbidity (OD₆₁₀) in a microplate reader. The percent viability was calculated according to the formula given below:

\[
\% \text{ viability of cells} = \left( \frac{\text{OD}_{610} \text{ of treatment}}{\text{OD}_{610} \text{ of Vehicle control}} \right) \times 100
\]

**Cytotoxicity of compounds towards normal vaginal flora**

*Lactobacillus jensenii* (Plate culture)

To confirm the results obtained by turbidimetry, colony forming units (cfu) of *Lactobacillus* was determined on Rogosa agar using plate culture. Rogosa SL Agar medium was prepared by dissolving 7.5% powdered medium in distilled water. Glacial acetic acid was added to a final concentration of 0.132%. Medium was boiled at 100°C for 2-3 minutes to dissolve it completely, poured in petri plates and left undisturbed for solidification. A small sample of *Lactobacillus* in broth culture (with treatments) was suitably diluted and a measured amount was spread on Petri plates. Plates were incubated at 37°C in 5% CO₂ and 95% air for 48 h. After 48 h white, rounded colonies of *Lactobacilli* appear on the surface of medium. Each colony represents a single cfu.

**Apoptotic potential of test compounds**

*Annexin V-FITC/Propidium Iodide Labeling*

Dual fluorescent labeling with AnnexinV-FITC and propidium iodide (PI) was used to study the externalization of phosphatidylserine on *HeLa* cell surface (apoptotic cells) and membrane permeability (necrotic cells), respectively. *HeLa* cells (10x10⁴/ml) were cultured in 12 well plates in DMEM medium with 10% FCS and incubated for 24 h at 37°C/5%CO₂. Culture medium was replaced with fresh medium with (experimental) or without (control) test compounds; control (0.05% DMSO), DSE-37+Saponin (15+15=30 μg/ml), Staurosporine (1 g/ml) and N-9 (30 g/ml) for 24 hours and incubated again. Staurosporine was used as positive marker of apoptosis. After incubation, cells were detached via
trypsinization and washed with PBS and labeled with fluorescent probes using the AnnexinV-FITC apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA) by following the manufacturer’s instructions. The percentages of cells with positive Annexin-V and PI labeling were determined in a flow cytometer (Model FACS Calibur, BD biosciences, USA) equipped with an argon laser (488 nm) for excitation. Four populations of cells (unstained, FITC, FITC+PI and PI labeled) were identified and designated as viable, apoptotic, necrotic and dead, respectively.

Mitochondrial Transmembrane Potential (ΔΨm)
The loss of ΔΨm (an early marker for cell apoptosis) was quantified by flow cytometry using the lipophilic cationic dye JC-1. HeLa cells (10×10^4/ml) were cultured in 12 well plates in DMEM medium with 10% FCS and incubated for 24 h at 37°C in 5% CO₂. Thereafter, culture medium was replaced with fresh medium with (experimental) or without (control) test compounds control (0.05% DMSO), DSE-37+Saponin (30 g/ml), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 20 M) and N-9 (30 g/ml) and incubated for another 24 hours. CCCP (20μM) was used as positive control. 5μg/ml JC-1 was added from a stock solution in DMSO (1mg/ml) to the cell culture and incubated for an additional 10 min and then cells were detached via trypsinization and washed with and re-suspended in PBS and analyzed by flow cytometry for JC-1 specific fluorescence. Fluorescence was examined by FACS (Model FACS Calibur, BD biosciences, USA). The excitation was at 488nm, and the emissions for the green and red/orange fluorescence were at 530 nm and 570nm, respectively.

DAPI staining of HeLa for enumeration of apoptotic nuclei
HeLa cells were grown on glass cover-slips and treated with test compounds ie; control (0.05% DMSO), Combination (30 g/ml) and N-9 (30 g/ml) for a time period of 24 h 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. Each well was washed with PBS (pH 7.4) and incubated with 1 g/mL DAPI solution (Travert et al., 2006). Staining of DNA with DAPI
was examined under a microscope (Nikon 80i) with a fluorescent attachment. Normal DNA of viable cells was visibly fluorescent throughout the nucleus, whereas apoptotic cells exhibited a bright condensation of chromatin (magnification, 200X). Apoptotic cells were counted manually in 5 different fields of view.

**Effect on the integrity of HeLa cell cytoskeleton**

After a 24h treatment with compounds as stated above, HeLa cells were fixed in PBS containing 4% formaldehyde for 30 min and subsequently permeabilized for 25 min in PBS containing 4% formaldehyde plus 0.1% Triton X-100. After three washes in PBS for 5 min each, the cells were stained with 1 μg/ml FITC-conjugated phalloidin (Sigma Chemical Co., USA) 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 Nikon fluorescence microscope.

**Effect on Reactive oxygen species (ROS) generation in HeLa cells**

HeLa cells were seeded in 12-well plates (1x10^4/well) and grown for 24 h at 37°C in 5% CO_2. Culture medium was replaced with fresh medium containing test compounds ie; control (0.05% DMSO), Combination (30 g/ml), N-9 (30 g/ml) and of H_2O_2 (400µM) and incubated for another 24 h. H_2O_2 (400 µM) was used as positive control and was added 19 h after the start of the experiment to be incubated with cells for only 5 h. Thirty minutes before completion of the total incubation period, 10 mM dichlorofluorescein diacetate (DCFDA) was added to each well. Finally, cells were harvested, washed twice and re-suspended in PBS. Fluorescence was measured in a fluorescence microplate reader (FLx800, Bio-Tek) at 488 nm for excitation, and emission at 530 nm (green) was recorded.

**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 < 0.05 were considered statistically significant. The IC50 values were calculated by computer-based curve fitting using the ‘CompuSyn’ software.

**Results**

**Cytotoxicity assays**

**Cytotoxicity of compounds toward human cervical (HeLa) cells by MTT assay**

![Cells under phase contrast microscope](image)

**Fig. 2.1:** Effect of compounds on morphology and number of HeLa cells after 12h, 24h and 48h of treatment as seen under phase contrast microscope. Treatments: [I] Control (0.05% DMSO), [II] DSE-37+ Saponin (30 g/ml) and [III] N-9 (30 g/ml). The control and DSE-37+Sap treated cells were well adhered, displaying the normal morphology of HeLa cells. In contrast, abundant cytoplasmic vacuoles were observed in cells treated with N-9. Moreover, vacuolization in cytoplasm progressively became larger and denser with time period. The majority of HeLa cells treated with N-9 at the concentration of 30 g/mL became round and shrunken and could not be affixed to the wall and suspended in culture medium. [Magnification 200X. Upper left images 400X].
Fig. 2.2: Cell viabilities of HeLa cells: Cytotoxicity assessments by MTT-assay using HeLa cell line after treatment with promising agents DSE-37, Sap, DSE+Sap and N-9 at varying concentration (20-160 μg/ml) for 24h. The two molecules (DSE-37 and Sap), applied singly and in combination for 24 h, show safety toward HeLa cells when compared with N-9. Values are mean ± SE of three independent experiments.

After treatment with different compounds, HeLa cells observed under phase contrast microscope, the vehicle control (0.05% DMSO) and DSE-37+Sap (30 μg/ml) treated cells were firmly attached to the surface and showed the normal morphology. On the other hand, cytoplasmic vacuoles were observed in the cytoplasm of HeLa cells treated with N-9. Moreover, these vacuoles progressively became larger and denser with increasing the time period of treatment upto 48 h. The majority of HeLa cells treated with N-9 at the concentration of 30 g/ml became round and shrunken and could not be affixed to the wall and suspended in culture medium (Fig 2.1). The cytotoxicity towards human cervical (HeLa) cells in vitro as evaluated by the MTT assay and indicated as IC_{50} (concentration of compound which reduces the viability of cells by 50%) which was calculated from the concentration versus viability (or OD at 540) curve (Fig 2.2). With IC_{50} of 95.96 g/ml towards HeLa cells in vitro, combination of (DSE-37 & Saponins) was found ~ 3 times safer than N-9 (IC_{50}=34.6 g/ml). Individually, the IC_{50} of DSE-37 was 65.5 g/ml while that for saponins was 106.26 g/ml.
Cytotoxicity of compounds toward human cervical (HeLa) cells by LDH release assay (permeability of cell membrane)

![Graph showing LDH release in HeLa cells](image)

**Fig. 2.3**: Cytotoxicity assessments by LDH release assay using HeLa cell line after treatment with promising agents DSE-37, Sap, DSE+Sap and N-9 at varying concentration (0-160 μg/ml) for 24h. Values are mean ± SE of three independent experiments.

In order to confirm the toxicity results obtained by the MTT assay, the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the extracellular medium was measured, which evaluates plasma membrane integrity. HeLa cells were exposed for 24h to test compounds, as describe for the MTT assay. At the end of the experiment the culture medium was collected and the LDH activity of the incubation medium was determined. After treatment with different compounds cytotoxicity/membrane permeability of human cervical (HeLa) cells was evaluated by determining % LDH release. As revealed by LDH release assay, N-9 evoked 80% LDH release at a concentration of 40 μg/ml which is much lower than its effective spermicidal concentration. On the other hand, DSE-37, saponins and a combination of both showed cyto-compatibility even at 80 μg/ml, a concentration much higher than their effective spermicidal/microbicidal concentration, with comparatively insignificant release of 10%, 9% and 19% LDH, respectively. The cell viability results obtained with the MTT assay (Figure) correlated well with the observed release of LDH from the cells (Fig 2.3).
Cytotoxicity of compounds towards vaginal and cervical cells

**Fig. 2.4:** Cytotoxicity assessments by MTT assay using normal human vaginal Vk2 E6/E7 cell line after treatment with Combination or N-9 at varying concentration for 12h. Values are mean ± SE of three independent experiments.

**Fig. 2.5:** Cytotoxicity assessments by MTT assay using normal human endocervical End1 E6/E7 cell line after treatment with Combination or N-9 at varying concentration for 12h. Values are mean ± SE of three independent experiments.

Combination of DSE-37 and Saponin (1:1 ratio) exhibited greater safety toward cultured vaginal (Vk2/E6E7) epithelial cells (IC$_{50}$=100.53 µg/ml) and endocervical (End1/E6E7) cells (IC$_{50}$=112.66 µg/ml) than N-9 (IC$_{50}$=21.46 & 17.33 µg/ml, respectively) after 12 h treatment and therefore appeared to be much safer for vaginal use (Fig 2.4 & Fig 2.5).
Compatibility of compounds with normal vaginal flora

**Lactobacillus**

**Fig. 2.6:** *Lactobacillus jensenii* grown on agar medium *in vitro* after treatment with promising agents/combinations. Blank, Control (0.05% DMSO), Combination [DSE-37+Saponin (360 μg/ml)] and N-9 (40 μg/ml).

**Fig. 2.7:** Effect of promising agents/combinations after treatment with promising agents DSE-37, Sap, DSE+Sap and N-9 at varying concentration at varying concentration on the growth of *Lactobacillus* in broth culture *in vitro*. Values are mean ± SE of three independent experiments.

For evaluation the toxicity of compounds towards *Lactobacillus jensenii in vitro* a turbidimetric method was employed. The IC_{50} (concentration inhibiting *Lactobacillus* growth by 50% in culture) values indicated a high safety of DSE-37 and combination (IC_{50} = 282 and 123 g/ml, respectively). However the IC_{50} value of saponins was comparatively lower 99.3 g/ml, but it still appeared safer...
than N-9 (IC\textsubscript{50} = 20.67 μg/ml) (Fig 2.7). The overall safety of DSE-37+saponin towards \textit{Lactobacillus} was about 6 times higher than that of N-9. The number of colonies of \textit{Lactobacilli} in case of new drug combination (up to 12 X MEC) was nearly equal to that in control plate. However, there was a significant decrease in number of colonies in case of Nonoxynol-9 even at a low concentration of 40 g/ml (Fig 2.6).

\textbf{Apoptotic potential of test compounds towards HeLa cells}

\textit{AnnexinV-FITC/Propidium Iodide Labeling}

\textbf{Fig. 2.8}: Representative cytogram for flow cytometric detection of annexin-V FITC/PI labeling with statistical data (P value vs. control) and percent cell populations in four quadrants identified as: lower left, live; lower right, apoptotic; upper right, late apoptotic; upper left, necrotic. HeLa cells treatment: Control (0.05% DMSO), DSE-37+Saponin (15+15=30 g/ml), Staurosporine (1 g/ml) and N-9 (30 g/ml) for 24 hours.
Annexin V-FITC/PI double-labelling was applied to detect the apoptosis of HeLa cells treated with DSE-37+ Sap and N-9 at 30 μg/ml concentrations 24 h. The Annexin V-FITC-positive and PI-negative cells are early apoptotic cells and the Annexin V-FITC and PI-positive cells are late apoptotic cells. The total apoptotic rate is the sum of the early and late apoptotic cells. It has been proposed that high concentrations of surfactants cause necrosis whereas low concentrations induce apoptosis (Perani et al 2001). The data from the dual fluorescent labeling with Annexin V–FITC and PI showed that the normal (control) HeLa population contained about 15±2.34% apoptotic cells. After treatment with N-9 at 30 μg/ml, the number of apoptotic cells rose sharply and significantly (P<0.001) to 50.5±2.38% HeLa cells (Fig 2.9). A similar significant increase in apoptotic cell numbers 61±2.31% was also seen in case of treatment with positive control staurosporine (P<0.001). Treatment of HeLa cells with DSE-37 + Saponins at 30μg/ml (the effective concentration for dual protection) for 24 hours did not result in a significant change in the populations of apoptotic HeLa cell numbers over control (15± 2.34% to 16±0.92%. %). In this experiment, the reference control (N-9) was used at a much lower concentration than its spermicidal MEC since the HeLa cells could not survive at its MEC.

![Histogram showing AnnexinV-FITC/PI labeling in HeLa cells after treatment with different compounds: Vehical (0.05%DMSO); Comb (DSE+Sap); N-9 (nonoxynol-9); Sta (staurosporine). Values are mean ± SE of three independent experiments. Significance of difference from vehicle control (0.05% DMSO) is denoted by ‘P<0.001.](image)

**Fig. 2.9:** Histogram showing AnnexinV-FITC/PI labeling in HeLa cells after treatment with different compounds: Vehical (0.05%DMSO); Comb (DSE+Sap); N-9 (nonoxynol-9); Sta (staurosporine). Values are mean ± SE of three independent experiments. Significance of difference from vehicle control (0.05% DMSO) is denoted by ‘P<0.001.
**Mitochondrial Transmembrane Potential (Ψm)**

![Flowcytometric assessment of mitochondrial transmembrane potential](image)

*Fig. 2.10:* Flowcytometric assessment of mitochondrial transmembrane potential (Ψm) via JC-1 labeling of HeLa cell, after treatment with compounds for 24 hrs at 37°C in 5% CO₂. Control (0.05% DMSO), DSE-37+Saponin (30 g/ml), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 20 M) and N-9 (30 g/ml). JCL-1 fluorescence was determined on dual parameter histograms of FL1H (JC-1 monomers) vs FL2 H (JC-1 aggregates) by flow cytometry. Data depicts flow cytometry histograms that are representational of three independent experiments.

The control HeLa cell population had ~80% cells with intact mitochondrial transmembrane potential (Ψm). There was a significant (P<0.001) decrease in Ψm of HeLa cells treated for 24 hours with either the reference control N-9 (30 μg/ml,) or the positive control CCCP (20μM), as indicated by an decrease in number of cells exhibiting red fluorescence for JC-1. However, the decline was greater in case of CCCP (red fluorescence =17.6±0.065%) than in case of N-9 (red fluorescence = 28.35±1.66 %). On the other hand, no significant change in Ψm was observed in HeLa cells treated with DSE-37+Saponins for 24 hours at 30 μg/ml (red fluorescence 73.63±2.85%). Marginal change in Ψm after treatment with combination of DSE-37 and Saponins was statistically non-significant from vehicle control (red fluorescence 79.49±2.96) (Fig 2.10 & 2.11). In this experiment, the combination of DSE-37 and saponin was used at MEC
(30 g/ml) while N-9 was used at a similar concentration (30 g/ml) that was much lower than its spermicidal MEC, since HeLa cells did not survive at MEC.

**Fig. 2.11:** Histogram showing flowcytometric assessment of JC-1 labelling in HeLa cell. Mitochondrial depolarization (i.e., loss of $\Psi_m$) is indicated by a decrease in the red/green fluorescence ratio. Control (0.05% DMSO); Combination (DSE+Sap); N-9 (Nonoxynol-9); CCCP. Treatment of cells with N-9 resulted in a marked decrease in $\Psi_m$ whereas in Combination treated cells does not show significant change from control cells in $\Psi_m$. Values are mean ± SE of three independent experiments. Significance of difference from control is denoted by $^cP<0.001$.

**DAPI staining for enumeration of apoptotic nuclei**

**Fig. 2.12:** DAPI staining of HeLa cells after treatment with compounds ie (A) Control (0.05% DMSO), (B) Combination (30 g/ml) and (C) N-9 (30 g/ml) for 24 h. Apoptosis induced in N-9 treated cells. In the combination group, the apoptosis rates were marginally higher as compared with the control but significantly lower than N-9 treated group. [Red arrows indicate apoptotic nuclei and yellow arrows indicate normal nuclei].
Fig. 2.13: Effect of compounds on the apoptosis of HeLa cells. After treatment for 24h HeLa cells were stained with DAPI and detected using a fluorescence microscope. Percentage apoptotic cells after treatment with different compounds ie; Control (0.05% DMSO), Combination (30 g/ml) and N-9 (30 g/ml). (Mean±SE of 3 estimations aP< 0.05, bP< 0.001. In the control and combination group, cells showed normal cell nuclear morphology. Following treatment with N-9 for 24 h, a number of apoptotic bodies showing fragmented or condensed nuclei were detected.

DAPI staining was used to detect changes in nuclear morphology in HeLa cells treated with DSE-37+Sap and N-9 for 24 h. Fluorescence microscopic analysis of DAPI stained HeLa cell nuclei revealed changes in nuclear integrity after treatment with compounds. HeLa cells treated with DSE-37 in combination with Saponins (1:1) at 30 g/ml exhibited slightly more fluorescent (apoptotic) nuclei and than control (P<0.05). On the other hand, cells treated with 30 µg/ml N-9 exhibited a much significant increase in cells with chromatin condensation and formation of apoptotic bodies (P<0.001) (Fig 2.13). Normal and DSE-37+Sap treated HeLa cells had intact plasma membranes and ordered chromatin folding. Following treatment with N-9 for 24 h, apoptotic bodies were found to be present in the HeLa cells, in which the chromatin became condensed, the nuclear envelopes were lytic and the cytoplasm had decreased in size (Fig. 2.12).

Effect on the integrity of host (HeLa) cell cytoskeleton

Fluorescence microscopic analysis of cells with FITC-conjugated phalloidin revealed that there were no visible morphological changes in HeLa cytoskeleton
when treated with DSE-37 and its combination with Saponins at 30 μg/ml concentration. However, cells treated with N-9 exhibited an increase in the percentage of elongated cells with characteristic morphological changes in cytoskeleton indicating apoptosis/necrosis with membrane blebbing and pore formation (Fig 2.14).

![Fig 2.14: Effect of compounds on the cytoskeleton integrity of HeLa cells. After treatment for 24h HeLa cells were stained with FITC-conjugated phalloidin and detected using a fluorescence microscope. Untreated control (0.05% DMSO) and Combination (30 μg/ml) treated cells exhibit integrity in cytoskeleton but and N-9 (30 μg/ml) cause pore formation and necrosis like symptoms in HeLa after 24 h of treatment. [Yellow arrows indicate intact morphology of HeLa and red arrows show damage in HeLa cytoskeleton.]

**Effect on Reactive oxygen species (ROS) generation in HeLa cells**

The reactive oxygen species (ROS) generation in HeLa cells was studied after incubation in 30 μg/ml of either Comb (DSE-37+SAP, 1:1) or N-9 for 24 hours in comparison to those incubated in 400μM of H₂O₂ (positive control) for 5 hrs. There was a very significant increase in ROS production in HeLa cells incubated in H₂O₂ (2128±108) and N-9 (2136±43) (p<0.001). On the other hand, Comb (DSE-37+SAP) did not induce any significant ROS generation in HeLa cells (716±14) as compared to vehicle control (571±19) at its effective spermicidal/microbicidal concentration of 30μM (Fig 2.15).
Fig. 2.15: Histogram showing fluorimetric assessment of reactive oxygen species (ROS) generation in HeLa cell after treatment (24h) with compounds ie: Control (0.05% DMSO), Combination (30 g/ml), N-9 (30 g/ml) and of H$_2$O$_2$ (400μM). ROS accumulation contributes to the cytotoxicity induced by N-9 in HeLa cells. Values are mean ± SE of three independent experiments. Significance of difference from control is denoted by $^C$P<0.001.

**Discussion**

Within the cervicovaginal environment, natural barriers to infection by human immunodeficiency virus type 1 (HIV-1) includes a low pH (Kempf *et al.*, 1991), the presence of lactobacilli (Klebanoff and Coombs, 1991), a continuous epithelial surface, and the mucosal immune system of the genital tract, which may get compromised by the vaginal application of topical compounds that have less-than-ideal safety profile. Application of such compounds may disrupt the natural barriers to infection, resulting in a direct portal of entry for HIV-1 or other STD pathogens to the submucosa, or may cause the recruitment of HIV-1-susceptible immune cells to the epithelial surface. The ideal topical contraceptive would not only be effective at preventing pregnancy (by effectively and efficiently killing sperm) but would also be safe for cervicovaginal application, preserving the inherent defenses of the genital tract, including the natural vaginal microflora (*Lactobacillus*). The vaginal mucosa of healthy women of childbearing age is populated with commensal bacteria typically dominated by H$_2$O$_2$ producing *Lactobacillus* species, which play a protective role in preventing urogenital infections (Redondo-Lopez, 1990). Depletion of vaginal lactobacilli is
associated with establishment of opportunistic infections and increased risk of acquiring HIV and herpes simplex virus type-2 in women (Cherpes et al., 2005; Cohn et al., 2001). The present study utilized human cervical (HeLa) cell monolayers as an in vitro model of cervico-vaginal epithelium. The HeLa cell line has been used extensively in evaluation of safety of spermicidal/microbicidal compounds intended for intravaginal use (Catalone et al., 2005; Krebs et al., 2005). Nonoxynol-9 (N-9) was used as a reference control since it is the most commonly employed spermicidal compound that has a clinical-use record of over 3 decades. Recently it has come under critical appraisal due to concerns about its safety in recent clinical trials where its users experienced higher susceptibility to HIV and STDs than placebo (D'Cruz and Uckun, 2004). Using fluorescence labeling of HeLa with phalloidin-FITC and phase contrast microscopy, we tried to study the gross morphological disruptions in the cell surface topography of HeLa cells that had been incubated with either the new spermicide/microbicide combination (DSE-37+Sap) or N-9 at 30µg/ml (the effective concentration of combination for dual protection) for 12, 24 & 48 hours. While N-9 caused severe damage to HeLa cells causing lysis of nearly 50% of population at 30µg/ml, the picture of HeLa cells were nearly comparable to controls when incubated at the same concentration as of the new drug combination (DSE-37+Sap). This became more distinctly evident by the cyto-toxicity data. DSE-37 (15 g/ml), Saponins (15 g/ml) and combination (30 g/ml) did not inhibit HeLa cell viability at their effective spermicidal/microbicidal concentrations. Even at much higher concentrations, these compounds maintained HeLa cell viability more than N-9 at 30µg/ml. It is pertinent to mention here that N-9 was tested at a concentration much lower than its effective spermicidal MEC (150 g/ml) while the combination was tested at MEC and above. Endocervical and vaginal keratinocyte cell lines were assessed for their sensitivity to N-9 and the new drug combination. Since the IC₅₀ of N-9 for Vk2 E6/E7 and End1E6/E7cells was lower than 30µg/ml, i.e. 21.4 and 17.3 g/ml respectively, nearly 80% of cells were wiped off at 30µg/ml, which is the therapeutic concentration of DSE37+Sap. Hence for well-controlled experiments employing the positive control molecule N-9, HeLa cells were considered as more appropriate model than Vk2 E6/E7 and End1E6/E7cells. The new drug
combination was also extremely safe towards the normal vaginal flora (*Lactobacillus*) (even at multiple MEC doses), which further implies that it is most likely to be vaginally eco-friendly and therefore much safer as a topical contraceptive agent than N-9 that was more toxic towards *Lactobacillus* than combination as well as its individual ingredients (DSE-37 and Saponins).

Apoptosis can occur when a cell is damaged beyond repair, infected with a virus, or undergoing stress conditions such as starvation, chemical-toxicity etc. Promising compounds/combination were evaluated for their potential to cause apoptotic changes in HeLa cells. It is remarkable to observe that a concentration of 30µg/ml, the combination (DSE-37+Sap) that kills 100% sperm and *Trichomonas* did not induce any significant apoptosis in HeLa cells, in 24 hours. On the other hand, a very significant number of HeLa cells underwent either apoptosis or necrosis/late apoptosis in 24 hours after incubation with N-9 at 30µg/ml. The effect of N-9 on HeLa cells was even more severe than that of positive control compound (staurosporine) that is a well known agent for inducing apoptosis (and not necrosis/late apoptosis).

In addition to being a source of energy that supports life under aerobic conditions, mitochondria can also be the source of signals that initiate apoptotic cell death. Mitochondria contain key regulators of caspases; a family of proteases that are major factors in many apoptotic processes. Mitochondria utilize oxidizable substrates to produce a membrane potential in the form of a proton gradient across the mitochondrial inner membrane. Starvation and chemical insult may lead to a decline in mitochondrial trans-membrane potential and cells may ultimately undergo apoptosis that is initiated by cytochrome c release from mitochondria (Gottlieb *et al.*, 2003). The release of cytochrome c from the mitochondrial intermembrane space induces the assembly of the apoptosome that is required for activating downstream caspases. In the present study, we examined changes in mitochondrial trans-membrane potential in HeLa cells, in response to chemical insult by new spermicide/microbicide combination. The mitochondrial trans-membrane potential of HeLa cells was almost unaffected and remained comparable to that of control cells after treatment with
the combination of DSE-37+Sap at 30µg/ml for 24 hours. This showed the compatibility of the new vaginal agent with cervical cells in vitro at their effective spermicidal concentration. On the other hand, N-9 sharply reduced the mitochondrial transmembrane potential of HeLa cells at 30µg/ml in 24 hours and the action was similar to that of positive control. This further confirmed the safety of new agent in comparison to the marketed products containing N-9.

Molecular oxygen is essential for many biological events associated with aerobic existence. However, the thirst for electrons also results in the constant formation of reactive oxygen species (ROS) which are potent regulators of cell functions and can also lead to oxidative damages when the antioxidant capacity of the cell is overwhelmed, resulting in apoptotic or necrotic cell death. Oxidative stress is frequently observed when eukaryotic cells or tissues are exposed to agents such as peroxides, glutathione-depriving drugs, toxins, radiation, and inflammatory cytokines (Arrigo et al., 2005). In the present study, N-9 was shown to induce apoptosis in HeLa cells while combination of DSE-37+Sap did not do so. On the other hand, N-9 also induced significant ROS generation in HeLa cells. Here N-9 was apparently equitoxic to the potent ROS generator H$_2$O$_2$ that was used as a positive control. These results further support the non-toxic nature of novel agents/combination than the currently used products towards human cervical (HeLa) cells in vitro.

Repeated use of currently available microbicidal/contraceptive formulations has been shown to be associated with vaginal/cervical irritation or even ulceration and disturbance of the normal vaginal microflora that facilitates microbial infection and renders the subject susceptible to sexually transmitted diseases (Minnis & Padian, 2005). Therefore, development of safer vaginal preparations is a high priority. Vaginal spermicides/microbicides should protect against pregnancy/infection without disrupting the mucosal environment or its mediators of host defense (Keller et al; 2007). The continuous mucosal epithelium of the vagina and cervix plays an important role, both as a physical barrier and as an immunological barrier, in preventing entry of bacterial and viral pathogens into host tissues. The disruption of the integrity of epithelial barrier and the presence of genital tract inflammation are risk factors associated with an increased
incidence of HIV-1 infection as well as other STDs. The present investigation has provided sufficient clues to indicate that a combination of DSE-37+Sap could be far safer than N-9 to be used as an active ingredient in future prophylactic vaginal contraceptives. The higher spermicidal potential of this new drug combination (previous chapter) would also help in bringing down the failure rate of current vaginal contraceptives utilizing N-9, which warrants further development of this drug combination as a new vaginal contraceptive.