Chapter V

A product design utilizing the most promising combination of active ingredients and assessment of *in vivo* efficacies

Introduction

New and improved forms of “contraception on demand” for simultaneous protection against pregnancy as well as the risk of STD infection may comply with the unmet contraceptive needs and consumer expectations. In the past few years several new products for barrier contraceptives have arrived in market, which include spermicidal creams, gels, films, pessaries and condoms. These have revolutionized the futuristic approach for development of new contraceptives formulations that are user friendly, well-tolerated, low-cost and efficacious. Novel vaginal contraceptive formulations like creams, gels, mucoadhesive films, pessaries and medicated condoms are emerging as most promising, user-friendly contraceptive methods and devices.

Contraceptive microbicides offer women-controlled contraception and prevention of STI/HIV infections. Nonoxynol-9, a surfactant microbicide that has been used as a spermicide for nearly half a century, acts by disrupting the cell membrane of sperm as well as those of some sexually transmitted viral and bacterial pathogens (Jain, 2005). Unfortunately, serious public health concerns were raised following a few clinical studies including a study of N-9 in African sex workers that reported an increased rate of HIV infection when N-9 was used frequently (Stephenson *et al.*, 2000; VanDamme *et al.*, 2002). Topical applications (such as vaginal gels) with microbicidal and spermicidal activity, which can be used by women without the need for consent of a male partner (Stone & Jiang, 2006) are one possible answer to the problem (Inacio *et al.*, 2011). In the context of developing a safe contraceptive microbicide, therefore, safer molecules were preferred over potent synthetic detergents.

In the present study, the potent contraceptive activity of DSE-37 was combined with effectual microbicidal (anti-Trichomonal) activity of Sapindus saponins in
1:1(w/w) ratio and incorporated in KY jelly for establishing the *in-vivo* contraceptive efficacy using the rabbit model. Local safety of formulated compounds was evaluated in a rabbit vaginal irritation (RVI) model by performing the hematoxylin/eosin (HE) staining of vaginal tissues of rabbits exposed to this formulation. Cyto-toxic potential of formulation on rabbit cervicovaginal tissue was evaluated by studying its effect to induce apoptosis in vaginal epithelial cells through terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. Antigenic and inflammatory potential of formulation was evaluated by immunohistochemistry of rabbit vaginal tissues through detection of the level of specific markers like the nuclear factor kappa B (NFκB), E-selectin and VCAM-1.

**Materials and methodology**

**Chemicals and Reagent**

The following mouse anti-human monoclonal antibodies (with cross-reactivity to rabbit antigens) were used in this study, as reported earlier (Trifonova et al., 2007): anti E-selectin purchased from Sigma-Aldrich (St Louis, MO, USA), anti-vascular adhesion molecule-1 (VCAM)-1 from Biosource (Camarillo, CA, USA), anti-nuclear factor (NF)-κB/p65 from Chemicon (Temecula, CA, USA). [The mouse anti-human antibodies are raised in mouse against human antigens. These antibodies enable precise detection of antigens by cross-reacting with both rabbit-antigens as well as the biotinylated mouse-secondary antibody]. Dead End Fluorometric TUNEL system was purchased from Promega, USA. All culture media and other reagents were from Sigma-Aldrich, USA.

**Animals and Housing**

The laboratory animals were obtained from and kept at the Laboratory Animal Division of Central Drug Research Institute, in sterile cages housed in an atmosphere at 24±2°C with 55-60 percent relative humidity and 12:12 hour circadian rhythm. The rabbits were kept in stainless steel cages of appropriate sizes. The animals were allowed sufficient space and other provisions in their cages as per the recommendations of the Committee for the Purpose of Control
and Supervision of Experimentation on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India. Balanced diet and purified drinking water were provided \textit{ad libitum} to all animals.

**Contraceptive efficacy of the new microbicidal spermicide formulation following intravaginal application in rabbits**

Since the rabbit provides a standard animal model for testing vaginal agents for antifertility activity (Castle \textit{et al.}, 1998), we tested the ability of the new intravaginal drug combination to prevent pregnancy in rabbits. For this \textit{in vivo} contraceptive study, healthy 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 hrs prior to testing) to induce ovulation. The test compounds were incorporated in KY-jelly (a water-based, sterile, lubricant product of Johnson & Johnson) through geometrical dilution and used as test material. Pure KY-jelly was used in control group. KY Jelly also increases longevity of test compounds in the vaginal tract. Two ml of test/control jelly was instilled 10-12 cm deep into the vagina of each rabbit with a catheter attached to the gavaging needle of a syringe. The animal was held in supine position for about five minutes and then hand-mated by selected proven bucks. The buck was allowed one-time mating. To ensure ovulation 100 i.u. of human chorionic gonadotrophin (hCG) was administered through the marginal ear vein of each participating doe. The vaginal lavage of the mated doe was examined under a microscope and the presence of spermatozoa in the lavage confirmed the mating. The mated does were kept in separate cages and allowed to complete their gestation period. The number of pups delivered was recorded. The mean value (number) of the pups was calculated to determine the reduction in fertility. The results of intravaginal application of new microbicidal spermicide have been summarized in Table 5.1.

**In vivo safety studies in Rabbit Model**

Establishing \textit{in vivo} safety of combination/compounds for topical use
For the vaginal irritation study, female rabbits (3 in each group, n=3) were administered intravaginally with 1.0 ml of Vehicle (saline) or N-9 (2%) or DSE-37+Saponin (5%), for four 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.

**Hematoxylin and Eosin staining**

At the end of experiment, explants were fixed in 10% formalin solution overnight and paraffin blocks were prepared. Five micron thick sections were cut by using a microtome (RM2125RT, Leica, Germany) for hematoxyline & eosin (H&E) staining and the following procedures were adopted:

- Deparaffinized sections, 3 changes of xylene, 5 minutes each.
- Re-hydrated in 2 changes of absolute alcohol, 5 minutes each.
- Rehydrated in 90% alcohol for 5 min, 80% alcohol for 5 min and 70% alcohol for 5 min.
- Washed in distilled water.
- Stained with hematoxylin solution for 1-2 minutes.
- Washed in running tap water for 5 minutes.
- Counterstained with eosinY (Sigma Aldrich) solution for 30 seconds to 1 minute.
- Dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
- Cleared in 3 changes of xylene, 5 minutes each.
- Mounted in mounting medium (DPX).

Slides were finally examined under a light microscope (Eclipse 80i, Nikon Corporation, Japan).

**In situ Apoptosis Detection in vaginal tissue**

Apoptosis in the rabbit vaginal tissue after application of vaginal formulations was analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP
nick end-labeling (TUNEL), using the in situ Apoptosis Detection Kit (DeadEnd® Fluorometric TUNEL System, Promega, USA). TUNEL assay was performed using the manufacturer’s instructions. Briefly, tissue sections were washed in PBS three times for 5 min and equilibrated with an equilibration buffer for 10-15 min and finally incubated in a wet chamber at 37°C for 1 h with rTdT (in a reaction buffer) to label the 3’-OH ends of DNA with FITC-labeled nucleotides. The enzyme reaction was stopped with a stop/wash buffer (2X SSC) for 10 min at room temperature. After counterstaining with 1µg/ml DAPI (Sigma-Aldrich) the sections were mounted and analyzed by confocal microscopy. Cells were defined as apoptosis positive when the entire nuclear area was fluoroscently labelled with FITC or when apoptotic bodies could be observed.

**Determination of proinflammatory markers in vaginal tissue**

**Nuclear factor kappa B (NF-κB) activation at the epithelial level**

The effect of vaginal application of new drug formulation on expression of NF-κB in vaginal tissues was analysed by immuno-histochemistry. Briefly, after deparaffinization and dehydration antigen retrieval was performed in citrate buffer in a micro wave. Blocking was performed with bovine serum albumin. The tissue sections were incubated overnight at 4°C with mouse anti- NF-κB p65 subunit monoclonal antibody (Millipore MAB3026) and thereafter sections were probed with FITC-labelled secondary anti-mouse antibody. Nuclei were counterstained with 1 g/ml DAPI. The tissue sections were analyzed by a fluorescence microscope (Nikon).

**Immunolocalization of Inflammatory marker E-selectin and VCAM-1 protein in rabbit vaginal tissue**

**Deparaffinization**

Paraffin sections of 5 μm thickness were baked at 50°C for 10 min. Prior to immunostaining, deparaffinization and hydration was done in xylene and graded
ethanol, up to distilled water. During hydration, a 5 min. blocking for endogeneous peroxidase was done in 0.03% H₂O₂ in 95% ethanol.

**Standard Antigen Retrieval Method**

The standard antigen retrieval method was Heat Induced Epitope Retrieval (HIER) in retrieval buffer (20 mM citrate, pH 6) for 15 min.

**Incubation with E-selectin/VCAM antibody**

All reagents were applied at a volume of 200 μl per slide. After blocking nonspecific background with 10% BSA (Sigma-Aldrich) for 60 min at room temperature, the slides were incubated in E-selectin/VCAM antibody overnight at 4°C in a humid chamber. 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 and followed by dehydration. Slides were mounted and examined in light microscope (Eclipse 80i, Nikon Corporation, Japan).

**Reversibility of contraceptive effect on withdrawal and Effects of contraceptive failure – general phenotypic parameters of pups**

The female rabbits which were treated with contraceptive vaginal dose of new drug combination at ≥ 20 mg per application and exhibited 100% contraceptive effects were mated again after a week without the application of drug and observed for their fertility performance.

Female rabbits were also treated at subspermicidal dose (IC₅₀) of new drug combination (DSE-37+Sap), mated with fertile bucks and allowed to deliver pups to assess the adverse effects of new contraceptive drug combination on general phenotypic parameters of newborns.

**Reversibility of contraceptive effect on withdrawal of treatment**

The female rabbits which were treated with contraceptive vaginal dose of new drug combination at ≥ 20 mg per application and exhibited 100% contraceptive
effects were mated again after a week without the application of drug. These animals showed totally normal reproductive performance and delivered healthy pups.

**Data analysis**

All experiments were repeated three times and the results were analyzed by one way analysis of variance (ANOVA) using the Prism GraphPad software. *In vivo* data represents average of 6 animals.

**Results**

**Contraceptive efficacy of the new microbicidal spermicide formulation following intravaginal application in rabbits**

<table>
<thead>
<tr>
<th>Dose (Intravaginal in KY jelly)</th>
<th>Number Mated (n)</th>
<th>Number Pregnant (n)</th>
<th>Pups born (n)</th>
<th>Pups Born/animal (mean ±SD)</th>
<th>Fertility reduction (%)</th>
<th>Contraceptive efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (0mg)</td>
<td>06</td>
<td>06</td>
<td>57</td>
<td>9.5±1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DSE-37 +Sap [1:1] (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>N-9 (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>

**Table 5.1:** In vivo topical contraceptive efficacy of different preparations in KY Jelly base in female rabbits (Belgian strain). Treatments I) Vehical control (KY Jelly) II) Combination (DSE-37 +Saponins) and III) N-9. Number of animals treated in each group were 6 (n=6).

The results of the fertility trials are summarized in Table 5.1. Administration of DSE-37+Saponins at 20 mg dose resulted in a 100% fertility reduction with total
contraceptive effect in all the mated female rabbits. On the other hand, N-9 exhibited a 52.63% reduction in fertility with 33.33 % contraceptive efficacy at 20 mg. The vehicle KY Jelly did not affect fertility.

In vivo safety studies in Rabbit Model

In vivo safety of combination/compounds for topical use

Combination dose does not harm rabbit vaginal mucosa

![Histopathological changes in the rabbit vaginal mucosa after a 4-day exposure](image)

**Fig 5.1:** Histopathological changes in the rabbit vaginal mucosa after a 4-day exposure to (A) Control tissue treated with K-Y jelly alone,(B) tissues treated with DSE-37 + Saponins [5%] (C) and tissues treated with Nonoxynol-9 [2%]. (A, B, C), mid-vagina (A’, B’, C’), uro-vagina (A”, B”, C”) Note the intactness of vaginal epithelium and fewer leukocytes in the submucosa (green arrows) in DSE-37+Sap treated vagina. Conversely, N-9 treated rabbit vagina exhibited epithelial ulceration and leukocyte infiltration (red arrows), which are characteristic of inflammation. [Original magnification 200X].

- 108 -
In the 4-day intravaginal application of the new drug combination (DSE-37 + saponins; 5.0%) in KY jelly, no adverse signs of mucosal toxicity were evident. In contrast, N-9 application in rabbits for a similar duration induced marked epithelial ulceration and leukocyte infiltration. N-9 caused distinct erosion of the vaginal epithelial lining and exhibited conspicuous “washing-off” effects due to detergent action while the organs treated with combination of DSE-37 + saponin were largely comparable to those treated with KY Jelly control (Fig5.1). In animals that were administered N-9 (2%), leukocyte infiltration, erosion, hemorrhage, edema, exudates, congestion, decreases in epithelial height, and necrosis were increased compared to observations in vehicle treated animals. The histopathological architecture of rabbit cervico, mid and uro-vaginae suggested that the new drug combination at a higher concentration of 5% was much safer than lower concentration of N-9 (2%).

**In situ Apoptosis Detection in vaginal tissue**

Combination does not induce apoptosis in vaginal tissue

<table>
<thead>
<tr>
<th>Vehicle KY Jelly</th>
<th>DSE-37+Saponins</th>
<th>Nonoxynol-9</th>
</tr>
</thead>
</table>

![Image](image1.png)

**Fig 5.2:** The Fluorescence micrograph depicting lack of TUNEL staining of control and DSE-37 & Sap treated vaginal tissues. (A) Control tissue treated with K-Y jelly alone, (B) tissues treated with DSE-37 + Saponins [5%] (C) and tissues treated with Nonoxynol-9 [2%]. Blue fluorescence represents nuclei stained with DAPI. Green or yellow fluorescence represents apoptotic nuclei containing fragmented DNA (TUNEL positive). Red arrows indicate apoptotic nuclei. [Original magnification X 200].
The result of TUNEL assay performed on tissue sections of rabbit cervico-vagina treated with vehicle, combination of DSE-37 + saponins or Nonoxynol-9 indicated negligible TUNEL labeling of vaginal cells in vehicle and DSE-37+Sap treated vaginal tissues (Fig. 5.2, A & B). On the contrary, N-9 treatment caused distinct apoptotic changes as indicated by significant TUNEL labeling of rabbit cervico-vaginal cells (Fig 5.2, C).

**Determination of proinflammatory markers in vaginal tissue**

**Immunolocalization of Inflammatory marker NF-κB, E-selectin and VCAM-1 protein in rabbit vaginal tissue**

*Combination does not induce inflammatory biomarkers in vaginal tissue*

![Fig 5.3: Immunohistochemical localization and intensity of intraepithelial Nuclear Factor kappa B (NFkB) on the vaginal mucosa of animals treated with (A) Control tissue treated with K-Y jelly alone,(B) tissues treated with DSE-37 + Saponins [5%] (C) and tissues treated with Nonoxynol-9 [2%] . Upper panel NFkB expression, lower panel DAPI staining. [Instilled volume 1.0 ml; Magnification 200X]. Red arrows indicate instance staining of NFkB in N-9 treated group.](image)
**Fig. 5.4:** Representative photomicrographs comparing immunohistochemical staining for E-selectin in full-thickness paraffin-embedded vaginal tissue sections. Control tissue treated with K-Y jelly (A), experimental tissues treated with DSE-37 + Saponins [5%] (B) and reference control tissues treated with Nonoxynol-9 [2%] (C). [Instilled volume 1.0 ml; Magnification 200X]. Green arrows indicate intactness of vaginal epithelium in control and DSE-37+Saponins treated group, red arrow shows epithelial erosion and yellow arrow indicate instance E-selectin staining in N-9 treated group.

**Fig. 5.5:** Representative photomicrographs comparing immunohistochemical staining for vascular adhesion molecule (VCAM)-1 in full-thickness paraffin-embedded vaginal tissue sections. Control tissue treated with K-Y jelly (A), experimental tissues treated with DSE-37 + Saponins [5%] (B) and reference control tissues treated with Nonoxynol-9 [2%] (C). [Instilled volume 1.0 ml; Magnification 200X]. Green arrows indicate intactness of vaginal epithelium in control and DSE-37+Saponin treated group, red arrow shows epithelial erosion and yellow arrow indicate instance VCAM-1 staining in N-9 treated group.

Differential intensity and localization of NFκB staining in the vaginal tissues of nonoxynol-9 and [DSE-37+Saponin] treated animals was observed. Nonoxynol-treated vagina showed intense nuclear and cytoplasmic staining (especially in the apical region) (Fig 5.3, C). Vehicle controls and DSE-37+Saponin treated vagina conversely revealed less and diffuse staining (Fig 5.3 A, B). These differences in
staining pattern indicated significantly higher levels of neosynthesized NFκB and its active (nuclear) form in vaginal tissue of nonoxynol-9 treated as compared with DSE-37 + Saponin treated animal vaginae. Being a transcription factor capable of transactivating cytokine and chemokine genes, elevated levels of NFκB in the cervico-vaginal mucosa may be an early sign of the ensuing immune-inflammatory reaction.

The immunohistochemical and morphological observations of the stained vaginal tissue sections at the end of the study period revealed that the vaginal mucosa was intact in the DSE-37 + Saponin treated rabbits with leukocytes randomly spread throughout the tissue. The tissues from N-9 treated rabbits, however, showed various degrees of epithelial damage, vascular dilatation, leukocyte infiltration and immunocytochemical signs of cell activation (Figs 5.4 & 5.5). Although we could observe histopathologic changes after treatment with N-9, a more sensitive and quantifiable method to describe the alterations caused by N-9 would be preferable. With this goal in mind, we evaluated the expression of different inflammatory markers within the vaginal tissue, to verify our hypothesis that increased inflammation can be measured by increased level of NFκB, E-selectin and VCAM-1 production during the immune response.

E-selectin-positive staining was present in the lumen of all vaginal tissue samples, regardless of inflammatory condition or vascular distension (Fig 5.4). Excepting for some single positive cells in the lamina propria, no positive leukocyte infiltrates were found in the DSE-37 + Saponin treated rabbits, which were largely comparable with control group. E-selectin was expressed by infiltrating submucosal leukocytes in N-9 treated rabbits. The immunostaining for VCAM-1 in the rabbit vagina tissues showed distinct patterns among the different treatment groups (Fig 5.5). A faint positive endothelial cell staining and no positive leukocytes were observed in the tissues from animals in the vehicle and DSE-37 + saponin treated groups. In contrast, strong VCAM-1 positive leukocytes were abundant throughout the submucosa of all N9-treated animals, which also showed positive staining in the endothelium of dilated blood vessels.
Proposed intravaginal dose for dual protection in humans

The vaginal contraceptive dose of nonoxynol-9 for humans is 50 – 150 mg per application in different formulations with an in vitro spermicidal MEC of ~150µg/ml against human sperm. On the other hand, the new drug combination has an in vitro spermicidal MEC of 30 µg/ml against human sperm, which indicates that it has ~5-times higher spermicidal potential than N-9. Accordingly in vaginal contraceptive preparations for humans, the new drug combination could be effective at 5-times lower dose than N-9. Hence the calculated intravaginal dose of new drug combination would be ~10-30 mg per application in humans. Since the fertility rates in rabbits is much higher than that in humans (please see discussion below), hence the proposed human dose of about 10-30 mg may seem rational against the effective rabbit dose.

Reversibility of contraceptive effect on withdrawal of treatment

To become an acceptable contraceptive drug, a candidate agent is required to meet high efficacy standards with minimal to no toxicity. A second criterion is that the drugs have a high reversibility rate ideally, comparable to or better than those of existing female contraceptive methods. The third main criterion is the potential effects of the drug on pregnancies. The health of fetuses/infants/children from mothers who have taken the drug previously should not be affected; there must be no increase in the level of prenatal loss, birth defects, or infant mortality in the children of women who elect to stop using the drug and to have children again (Tash et al., 2008). The female rabbits which were treated with contraceptive vaginal dose of new drug combination at ≥ 20 mg per application and exhibited 100% contraceptive effects were mated again after a week without the application of drug. These animals showed totally normal reproductive performance and delivered healthy pups. Pups born to treated females were completely normal phenotypically in all general features. The pups were monitored for growth and general body development and were mated after reaching puberty to assess their reproductive performance. All such animals gave birth to normal number of pups with typically normal phenotypes. It was thus concluded that the new contraceptive drug combination (DSE-
37+Sap) may not apparently induce any birth defects in offsprings born to treated females, in case of contraceptive failure.

Discussion

Vaginal spermicides are available in several forms: spermicidal jellies, creams, foams, tablets, suppositories, sponges and films that are inserted into the vagina just before coitus to prevent pregnancy. Contraceptive jelly comes in tubes and is squeezed into its applicator, which is then inserted into the vagina. Normally jellies allow immediate protection, which lasts for about 1 hour and provides lubrication as well. Creams and gels are used the same way as spermicidal jelly and also provide lubrication. They are most effective when used not more than 30 minutes before having coitus and are preferred by users over other formulations (Grimes et al., 2005). Advantage 24 is a spermicide gel that steadily releases nonoxynol-9, providing 24 hour protection with one dose (Contracept Technol Update, 1995). Contraceptive foam comes in an aerosol can with an applicator and is of the consistency of shaving foams. The foam kills the sperm while also blocking the cervix (to prevent any surviving sperm from entering the uterus). Vaginal tablets and suppositories are solid forms of concentrated spermicide that melt into foam/gel. The suppository needs to be inserted in the vagina (and as close to the cervix as possible) and becomes fully active after 10-15 minutes and therefore somewhat less convenient than foam, cream or gel because it is hard to know if it has fully dissolved. Vaginal contraceptive film is generally a 2X2-inch thin sheet of film (similar to wax paper) and contains the spermicide, which is folded twice in half and then placed near the cervix with the help of the tip of the index finger. Spermicidal film melts into a thick gel consistency by absorbing vaginal secretions, hence also acts as a barrier to immobilize sperm. Currently all these formulations contain nonoxynol-9 as the active ingredient that has recently been contraindicated after a few clinical trials indicated its strong surfactant nature causing toxicity that increased vulnerability to STDs and HIV. We have proposed a new microbicidal contraceptive combination that has a non-surfactant spermicide (DSE-37) that is several times more potent than N-9 in killing human sperm, and natural saponins
from *Sapindus mukorossi* that exhibits potent microbicidal property at very weak, non-surfactant concentrations. A 1:1 combination of the two was found to be potently active against sperm and Trichomonas. In this chapter, we report the contraceptive efficacy and vaginal safety of a Jelly formulation that was developed by incorporating the new drug combination in sterile K-Y Jelly from Johnson and Johnson Ltd. K-Y Jelly is recommended for use in humans as a vaginal lubricator. The study reported in this chapter is a proof-of-concept for the suitability of the new drug combination for vaginal use as a prophylactic contraceptive. The new microbicidal sermicide was at least 3 times more efficacious in completely preventing pregnancy in rabbits than N-9.

The efficacy of a contraceptive microbicide depends on the balance between its specific activity (on-target effects) and its safety (minimal off-target {side} effects). The experience with nonoxynol-9 provides a good example of a compound with high *in vitro* activity and poor clinical performance because of underestimated local safety issues. It is of paramount importance to assess the local safety profile of a topical candidate as early as possible in the preclinical development. Given that HIV penetrates the cervicovaginal mucosa and infects immune cells, it is crucial to identify compounds that may induce epithelial toxicity and inflammation. The local toxic effects of test compounds are progressively characterized using human cervicovaginal cell lines, human cervical explants and animal models. We have described and validated *in vitro* and *in vivo* models that fit well with these goals. Microbicide candidates are assessed for their cytotoxicity and proinflammatory potential early in the discovery and/or preclinical phases, perhaps in combination with the characterization of specific activity. The proposed inflammatory mechanism of N-9 and other detergent based contraceptive microbicides in vaginal environment is as follows:
Fig. 5.6: Schematic summary of the key events of endothelial cell activation and leukocyte mucosal infiltration in response to compound-mediated cervicovaginal epithelial irritation.

The inflammatory cascade starts with release of interleukin (IL)-1 and other cytokines by damaged or stressed epithelial cells, followed by nuclear factor (NF)-κB and AP-1-mediated induction of endothelial vascular adhesion molecules and leukocyte trafficking and activation at the site of injury. The transendothelial migration involves a complex sequence of molecular and cellular events. The adhesion molecule E-selectin is involved in the early stages of leukocyte tethering and attachment to the endothelium, while VCAM-1 promotes late events such as firm adhesion and vascular diapedesis. The transmigrating leukocytes move up the chemokine gradient generated by the activated epithelium releasing more cytokines and shedding soluble(s) E-selectins, VCAM-1, CD 14 and/or myeloperoxidase (MPO) into the vaginal
secretions. TNF, tumour necrosis factor; MIP, macrophage inflammatory protein; IP, interferon-g-inducible protein; VCAM, vascular adhesion molecule (Fig 5.6). On the basis of the experiments reported in this chapter it can be concluded that a combination of DSE-37 + Saponins is not only a safer alternative of N-9 for prophylactic vaginal contraception but also more efficacious as a vaginal contraceptive. In the current study a 1:1 combination of DSE-37 + Saponins exhibited 100% in vivo contraceptive efficacy in rabbits at 20 mg vaginal dose at which N-9 was only partially active. It is relevant to note here that the fertility of rabbits far exceeds that of humans. Rabbits have a high conception rate of nearly 100% that is much higher than the average 30% conception rate reported in humans (Castle et al., 1998). On the other hand, the rabbits undergo hyperovulation and release 7-10 ova during mating, which is in contrast to humans where the number of ova is generally one. Moreover, the average inseminating dose of sperm in rabbit ejaculate is hundreds of fold in excess to the minimum fertilizing dose required while the same in humans is about 5 fold higher. Hence spermicides active in rabbits are expected to be active in human at much lower vaginal doses. In rabbit assay, the clinically used contraceptive agent nonoxynol-9 showed 33% efficacy at 20 mg dose. Since the fertility of rabbits far exceeds that of humans due to its ~100% conception rates (~30% in humans), extremely high inseminating doses of sperm and multiple ovulations after mating (Castle et al, 1998); even strong detergent based clinically-used vaginal products fail to show 100% contraceptive efficacy in rabbit assays. According to published studies a modest 30% reduction in fertility rate of rabbits has been reported with 25 mg N-9 (Homm et al., 1976). We treated rabbits with 20 mg N-9 in our study because DSE-37+Sap combination showed 100% effect at this dose, and our goal was to find-out the comparative contraceptive effect of N-9 at this dose. Additionally, the new drug combination was found to be safer and devoid of any adverse effect on morphology of cervicovaginal lining of vaginal tissues in comparison to N-9. Unlike N-9, the new contraceptive combination did not show any apoptotic and inflammatory effect on vaginal epithelia or trigger pro-inflammatory molecular markers like NF-κB, E-selectin and VCAM-1 in vaginal tissue. During inflammation, E-selectin plays an important part in recruiting leukocytes to the site of irritation/injury. The local release of cytokines IL-1 and
TNF by damaged cells induces the over-expression of E-selectin on endothelial cells of nearby blood vessels. Leukocytes in the blood bind with low affinity to E-selectin, causing the leukocytes to "roll" along the internal surface of the blood vessel as temporary interactions are made and broken. As the inflammatory response progresses, chemokines released by injured tissue enter the blood vessels which activate the rolling leukocytes, that tightly bind to the endothelial surface and begin making their way into the tissue (Cotran et al., 1999). VCAM-1 is involved in firm adhesion of leukocytes to the apical surface of endothelial cells through interactions with leukocyte CD49a/CD29. VCAM-1 clustering has been observed in the steps leading up to diapedesis. The inflammatory response is the body's stereotyped reaction to tissue damage of any kind. It involves rapidly and transiently delivering preformed soluble elements in the blood to the site of injury followed by a more prolonged delivery of leukocytes. Since leukocytes cannot swim, they are recruited locally at the site of inflammation in a series of adhesive steps that allow them to attach to the vessel wall, locomote along the wall to the endothelial borders, traverse the endothelium and the subendothelial basement membrane and migrate through the interstitial tissue (Ley et al., 2007). Transendothelial migration or diapedesis is arguably the point-of-no-return in the inflammatory response. The preceding steps of leukocyte rolling, activation, adhesion, and locomotion are all reversible, and most leukocytes that initiate contact with the postcapillary venule at the site of inflammation re-enter the circulation. However, once the leukocyte commits to diapedesis, it does not go back—at least not as the same cell type (Muller, 2009).

Leukocyte infiltration in vaginal tissues was a common observation in animals treated with N-9, however no such signs of inflammation were visible in animals treated with DSE-37 + Saponins. Thus the new contraceptive combination was apparently safer than N-9 towards exposed vaginal tissues. In fact higher concentration of combination (5%) was found to be safer than lower (2%) concentration of N-9, indicating the substantial margin of safety with the present drug combination.

NF-kB is considered a prototypical proinflammatory signaling molecule, largely based on the activation of NF-kB by proinflammatory cytokines such as
interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), and the role of NF-κB in the expression of other proinflammatory genes including cytokines, chemokines, and adhesion molecules. But inflammation is a complex physiological process and the role of NF-κB in the inflammatory response cannot be extrapolated from in vitro studies. Hence we probed NF-κB in situ, in vaginal tissues after in vivo exposure to spermicidal compounds. Once again the new drug combination was found to be safer than N-9 in inducing NF-κB signalling in vaginal tissues. Taking into consideration the reported adverse effects of N-9 in clinical situation and WHO/US-FDA issuing caution to people using N-9 based vaginal contraceptives; it is high time that we start searching for safer alternatives. DSE-37 + Saponins is a unique combination of a designed synthetic non-surfactant molecule and a natural, plant based (herbal) saponin fraction that has been found to be an effective Trichomonacidal vaginal contraceptive. Since Trichomoniasis is a major cause of HIV and other viral STD infections in humans, the new microbicidal contraceptive may also prevent new HIV/viral-STD infections.

It is proposed that the new drug combination could be effective at 5-times lower dose than N-9. The calculated intravaginal dose of new drug combination would be ~10-30 mg per application in humans.

The number of pups did not differ from controls in combination exposed group after the recovery period. Sex ratios were not altered by maternal exposure to drug combination. The mean weights of male and female fetuses were not affected in any treatment group, nor did exposure to combination induce an increase in the incidence of external malformations in the fetuses.

In summary, our present investigation reveals that the spermicidal effect of DSE-37 + Sap is not a non specific surfactant type action like N-9 on sperm; rather it is a cell specific phenomenon. Local toxicity studies on drug combination revealed that its intra vaginal administration at high doses neither results vaginal tissue inflammation nor it caused apoptosis to the vaginal tissues and the fertility potential of the treated animals were restored immediately after withdrawal of
the treatment and pups born after drug withdrawal or due to contraceptive failure were normal in physiological features.