Thesis Summary

“Novel microbicidal contraceptives for dual protection and their molecular mechanism of action”

Submitted to

Department of Biotechnology
Jamia Hamdard, New-Delhi

For the degree of

Doctor of Philosophy

By

Ashish Jain

Division of Endocrinology
CSIR-Central Drug Research Institute, Lucknow

(Prof. S. K. Jain) (Co-supervisor)
(Prof. S. K. Jain)
Professor,
Department of Biotechnology,
Jamia Hamdard, Hamdard University,
Hamdard Nagar, New Delhi- 110062.

(Dr. Gopal Gupta) (Supervisor)
(Dr. Gopal Gupta)
Principal Scientist,
Division of Endocrinology,
CSIR-Central Drug Research Institute,
Lucknow - 226 001.
Summary

In spite of a variety of contraceptive options, the world population has crossed the 7.0 billion mark and is still increasing at the rate of 1 billion every 10-11 years. There are over 1 million elective abortions every year in the United States and over 45 million in the whole world due to unintended pregnancies. This indicates that contraception still remains an unmet need for many. Also, one million new cases of sexually transmitted diseases (STDs) occur every day. Trichomoniasis is an extremely common, cosmopolitan, non-viral genitourinary STD in humans caused by the ancient protist *Trichomonas vaginalis*, which was described decades before *Chlamydia trachomatis* and the human papilloma virus (HPV) infections. Worldwide 248 million people are affected annually by trichomoniasis. Among this population, about 154 million people are in resource-limited settings, 8–10 million are in the United States and 11 million are in Europe. Since fertile sperm and STD pathogens are simultaneously transmitted during sexual intercourse, dually protective vaginal contraceptive preparations/devices are required to prevent both unwanted pregnancies as well as STDs. Condoms; the only dually-protective contraceptive barrier-device available, finds limited application due to personal preferences, cost and male dominance. Hence, there is an urgent need for a chemically active spermicidal microbicide that is safe to use, acceptable and effective. Nonoxynol-9 (N-9) is one such molecule that kills sperm, bacteria, virus, protozoa and epithelial cells *in vitro*, by its strong detergent (surfactant) action. Under *in vivo* conditions, N-9 induces a proinflammatory response in vagina resulting in increased (rather than decreased) incidence of STD and HIV infections. Presently almost all over-the-counter vaginal contraceptives incorporate this strong surfactant as the active ingredient for killing sperm by a general, non-specific cyto-toxic, surfactant action. However, the recent clinical trials’ revelation about N-9 increasing susceptibility to HIV and STDs by its detergent action on vaginal and cervical epithelium has made it unsuitable for vaginal use. The WHO and US-FDA have cautioned against the use of N-9 by people at a high risk of acquiring HIV. Therefore, an unanticipated void has been created as no other safe spermicide is
currently available to replace N-9 (or other detergents) in vaginal contraceptive preparations. Anticipating the need of spermicidal contraceptives for empowering women to have full control over their fertility and sexual health, the CSIR-Central Drug Research Institute designed several non-surfactant molecules that attacked sperm and STD pathogens by more specific, mechanism-based actions than N-9. In this study, an attempt has been made to combine the powerful spermicidal and microbicidal capabilities of two novel agents (a novel synthetic molecule and a plant product, respectively), to create a safe, dually-protective vaginal contraceptive.

We started our study with assessment of the most important aspect of vaginal contraceptives i.e. their spermicidal and microbicidal potential along with safety for vaginal application. This was established using live human sperm along with the most common non-viral sexually transmitted infection *T. vaginalis* and widely prevalent fungal reproductive tract infection of *Candida* spp, *in vitro*. Several natural products and chemically synthesized compounds of different structural series were evaluated for their spermicidal activity against human sperm and microbicidal activity against *T. vaginalis* and *Candida* spp *in vitro*. Two unique products were identified as under:

**I)** **DSE-37** [S,S”-{Disulfanediylbi (pyrrolidinopropane-2,1-diyl)] bis (piperidinothio-carbamate)] which displayed extremely potent spermicidal action and killed 100% human sperm almost instantaneously (in 20-30 s) *in vitro* at just ~15 g/ml concentration (~15 times more potent than N-9).

**II)** **Sapindus saponins** (A mixture of six natural sapindosides (sapindosides A, B, C, D and mukurozi saponins E1 and Y1), with sapindoside B as one of the major constituent, isolated by n-butanol extraction of the ethanolic extract of the fruit pericarp of *Sapindus mukorossi*), which exhibited potent anti-*Trichomonas* activity against Metronidazole susceptible and resistant strains of *T. vaginalis* at very dilute (~15 g/ml) concentration (~2.8 times more potent microbicide than N-9), also possessing moderate spermicidal activity.
A 1:1 (w/w) mixture of DSE-37 and Saponins (Combination) was capable of irreversibly inactivating 100% human sperms and completely eliminating 100% *T. vaginalis* at 30 μg/ml. N-9 demonstrated comparable dual activity at 2.5 - 5 times higher concentration. Both metronidazole-susceptible and resistant strains of *T. vaginalis* were vulnerable to this new drug combination. Dual activity was also discovered in some synthetic molecules that were newly designed to attack common targets (thiols) on sperm and *Trichomonas*. The most promising compound, *pyrrolidinium pyrrolidine-1-carbodithioate*, was more potent than N-9 in killing human sperm and more potent than metronidazole (the standard anti-Trichomonal drug) and N-9 in killing *T. vaginalis*. Moreover, the new synthetic compound eliminated both metronidazole-susceptible as well as metronidazole-resistant strains of *Trichomonas* with almost equal efficacy. However, due to superior spermicidal activity and presence of a natural component with proven safety for human use, the DSE-37+Saponins combination was selected for further study. In the in vivo contraceptive efficacy assay model (rodents), the new drug combination was found to be 100% efficacious in preventing pregnancy in rats at a vaginal dose of ≥400 µg. At corresponding dose, N-9 failed to inhibit pregnancy completely. Combination also exhibited considerable in vivo safety at much higher dose (20 mg) while N-9 failed as a safe compound in cytotoxicity assays.

These initial experiments showed that a 1:1 (w/w) combination of DSE-37 and Sapindus saponins was promising as spermicidal and microbicidal agent, as per the activities in vitro. The new drug combination was found to be both effective and safe in vivo in the rat model. It totally inhibited pregnancy at 400μg vaginal dose without causing any detectable damage to vaginal epithelium up to 20 mg vaginal dose in rats.

After primary evaluation of activity and safety of the new drug combination, experiments were performed to establish the compatibility of combination components towards human cervicovaginal cells and normal vaginal microflora in vitro. The precisely specific action of microbicidal spermicides in targeting *Trichomonas* and sperm exclusively and sparing the human cervical (HeLa) cells and normal vaginal microflora (*Lactobacillus*) at
effective contraceptive concentrations was studied in detail. The cytotoxicity of compounds toward human cervical (HeLa) cells was evaluated by MTT cell-viability assay while the membrane integrity of treated HeLa cells was evaluated by lactate dehydrogenase (LDH) release assay. The combination of DSE-37 + Saponins remained almost completely inert towards HeLa cells at spermicidal and microbicidal concentration (30µg/ml) for up to 24 hours in vitro. At similar concentration (30µg/ml), N-9 reduced the viability of HeLa by ~50% while its spermicidal concentration (150 µg/ml) made all the HeLa cells completely non-viable. Hence for all further experiments N-9 was used at a concentration of 30 µg/ml for comparisons with the new microbicidal spermicide formulation. Fluorescently labelling HeLa cells with FITC-conjugated phalloidin indicated negligible effect on cell morphology after treatment with DSE-37+Sap combination for 24 hours at 30µg/ml in vitro. Phalloidin binds actin molecules and makes the visualization of F-actin distribution in the cell easy through light microscopy. Cells treated with the new spermicide/microbicide combination displayed normal cell topography, comparable to controls. On the contrary, N-9 at 30 µg/ml caused lysis of ~40% cells while the remaining cells exhibited severe cell damage with formation of apoptotic bodies or necrotic pores. Similarly, fluorescent-DAPI stained nuclei of HeLa cells revealed normal nuclear integrity of HeLa treated with combination (DSE-37+Sap) and apoptotic nuclei with chromatin condensation in cells treated with N-9. Externalization of phosphatidylserine is an early sign of apoptosis and can be detected by fluorescent labeling of cells with FITC conjugated annexin-V. Likewise, depolarization of mitochondrial membrane potential is another event associated with cell apoptosis and can be detected with JC-1 labeling. Both these assays were run using a Flow Cytometer and it was clearly indicated that treatment of HeLa cells with new drug combination did not induce apoptosis in HeLa cells while N-9 induced apoptosis as well as necrosis of cells with significantly increased labeling for Annexin-V and propidium iodide, and acutely depolarized mitochondrial transmembrane potential. Toxic oxidative stress and ROS generation in Hela cells was also encountered but only with N-9 treatment while parallel treatment with new drug combination was apparently safe at 30µg/ml during 24 hours. Normal vaginal epithelial cell (Vk2/E6E7) and endocervical
(End1/E6E7) cell cultures were also used to re-confirm the results obtained with HeLa, and once again the new drug combination proved to be superior than N-9 for vaginal use.

The experiments in this section conclusively established that the new microbicidal spermicide combination (DSE-37+Sap) exerted a highly specific action on sperm and Trichomonas and remained almost completely inert (at effective spermicidal/microbicidal concentration) towards cervico-vaginal cells and Lactobacilli in vitro. This was in sharp contrast to N-9’s non-specific surfactant action that targeted cervico-vaginal cells and microflora more acutely than sperm.

After establishing in-vitro safety of promising agents next series of experiments were performed to evaluate the inflammatory potential of these agents/combinations towards cervico-vaginal and their efficacy in preventing inflammation caused by T.vaginalis infection. The action of DSE-37+Sap in inducing pro-inflammatory cytokines in HeLa cells was verified along with its efficacy to rescue HeLa cell from inflammation caused by T.vaginalis infection. Since vaginal inflammation significantly increases risk of acquiring HIV and other viral STDs, the pro-inflammatory response in HeLa cells to treatment with new vaginal contraceptive was evaluated in comparison with N-9 (reference control) at 30 µg/ml concentration for 12 hours. The gene (mRNA) expression of key inflammatory cytokines, IL-8, TNF-α, IL-1α, IL-6, and IL-1β was studied using Realtime-PCR. Results indicated marked proinflammatory response in HeLa cells treated with N-9 but not with DSE-37+Sap. The mRNA levels for the different pro-inflammatory cytokines remained at control levels in HeLa cells treated with DSE-37+Sap at its spermicidal/microbicidal concentration (30 µg/ml). However this was not the case with N-9 treated HeLa, where significant induction of all the five cytokines was evident. Subsequently, an in vitro model was developed to evaluate the potential of DSE-37+Sap to rescue Trichomonas infected HeLa cells from inflammation. HeLa cells were infected by T.vaginalis which significantly induced the proinflammatory response through augmented expression of cytokines. Following addition of DSE-37+Saponins at 30 µg/ml for 12 h, levels of all five cytokine mRNAs decreased to almost control
levels. On the contrary, parallel treatment of *Trichomonas* infected HeLa with N-9 at 30 g/ml for 12h did not rescue cells from inflammation. In fact, N-9 further contributed to increase in the levels of proinflammatory cytokine mRNAs.

*The experiments in this section not only proved the innocuous nature of new contraceptive drug combination towards cervical cells but also established its potential to rescue cells from toxicity associated with Trichomonas infection.*

**The next series of experiments dealt with molecular mechanism of Trichomonas infection and the anti-Trichomonas activity of the new drug combination.** *Trichomonas vaginalis* infection drastically reduced the viability of HeLa cells in vitro. In presence of N-9 (30 µg/ml) HeLa cells lost more viability as N-9 failed to offer any protection against *Trichomonas* but added further to its toxicity. On the other hand, *DSE-37+Saponins* successfully reduced the number of Trichomonads and rescued the HeLa cells from the cytotoxic effects of *Trichomonas*, maintaining ~80% viability of HeLa during 24 to 48 hours of infection. Cytoadherence of *Trichomonas* to HeLa, a crucial step for colonization and persistence of a pathogen during infection, was effectively reduced by *DSE-37+Saponins* indicating its potent anti-Trichomonal potential. Besides reducing cytoadherence, the new drug combination also caused several distinct changes in function and morphology of *T. vaginalis*, e.g. immotility, reduction in cell-size and disappearance of flagella. The hydrogenosomes in *Trichomonas* are equivalent to mitochondria of eukaryotic cells, and are sites of fermentative oxidation of pyruvate for production of ATP. Hydrogenosomal transmembrane potential of *T. vaginalis*, as determined by JC-1 labelling, was significantly depolarized by *DSE-37 + saponins* which led to apoptosis like cell-death of the parasites, an effect not seen in case of N-9. Cysteine Protease (CP) activity is essentially required for cytotoxicity, hemolytic potential and adherence of *T. vaginalis* to epithelial cells of the host. CP activity of trophozoites was reduced significantly by *DSE-37* and its combination with *Saponins*, which appeared to be a one of the major mechanisms for the anti-Trichomonal activity of the new drug combination.
The new drug combination (DSE-37 + Saponins) inhibited the growth of T. vaginalis by distinct mechanism(s), which included reduction of hydrogenosomal membrane potential, immobilization of trophozoits, weakening of cytoadherence with host cells and inhibition of crucial CP activity.

After promising in vitro results, drug combination was further evaluated for its in vivo efficacy and safety in animal model. We tried to establish the contraceptive efficacy and vaginal safety of the drug combination (DSE-37+Saponins) in rabbit model. The rabbit vagina is closest to human vagina in several respects and therefore, most suited for evaluation of contraceptive efficacy as well as irritation potential of vaginal contraceptive compositions. DSE-37 and Sapindus saponins in 1:1 (w/w) ratio were geometrically diluted in KY Jelly™ (a sterile lubricating jelly from Johnson & Johnson Ltd. intended for vaginal use in humans) for vaginal application in rabbits to achieve local contraception. Results revealed that a 20 mg dose of DSE-37+Sap (10 mg each) in 2.0 ml K-Y Jelly was able to prevent pregnancy in all the animals when applied intravaginally 10 min before coitus. At a similar dose (20 mg), N-9 was found to be only 33% effective in preventing pregnancy. The above study also established the dose dependent action of new contraceptive preparation. The histopathological evaluation of rabbit vaginae clearly demonstrated that the new drug combination is not damaging to vaginal mucosa of the rabbit. The combination did not cause leukocyte infiltration and any kind of erosion of cervico-vaginal, mid-vaginal and uro-vaginal epithelia, and did not show any apoptotic sign in cervico-vaginal tissues. No changes were also observed in expression of pro-inflammatory bio-markers i.e.NF-κB, VCAM-1 and E-selectin after treatment of rabbit vagina with this new drug combination. In all these experiments, N-9 exhibited significantly lower compatibility with rabbit vaginal tissues. Further studies on drug combination revealed that 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 (at subspermicidal dose) were normal in all phenotypic features.
Above results indicate that the drug combination (DSE-37+Saponins) in a suitable base (e.g. KY Jelly) appears to offer several benefits for vaginal contraception over N-9, including increased potential of contraceptive activity with significantly lower toxicity and inflammation risk. The contraceptive effect was totally reversible. Therefore, the drug preparation in a vaginally usable formulation like Vaginal Jelly shows unique potential in animals to justify its becoming a promising candidate for development as a clinically useful product capable of preventing the sexual transmission of STDs and unwanted pregnancies.

A new generation of dual-function spermicides that offers protection against STIs and pregnancy and whose action is mechanism-based are urgently needed by the society.

The present study has rationally designed and discovered a novel drug combination (DSE-37+Saponin) for dual protection. Experiments conducted on human cells (in vitro) and animals (in vivo) indicate that this new contraceptive/microbicidal drug combination (DSE-37+Saponin) is a promising candidate that fulfills the requirements of an ideal dually protective contraceptive, and thus warrants further study.