Comparative *in Vitro* Spermicidal Activity of Chelating Agents and Synergistic Effect with Nonoxynol-9 on Human Sperm Functionality

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Abstract □ Nonoxynol-9 (N-9), a nonionic surfactant, exerts both spermicidal and anti-viral activities and is the most widely used spermicide. Although N-9 has been regarded as an efficient spermicidal agent for barrier contraception, it has been reported to cause vaginal irritation and allergic vaginitis, and its spermicidal action in the vaginal mucus may be limited. To address these problems, the spermicidal activity of several chelating agents against human semen and their synergistic effect on the spermicidal activity of N-9 were evaluated using computer-assisted semen analysis and a cervical mucus penetration test. Carbopol 934P, chosen as a polymer base for dispersion of N-9 and chelating agent, was also evaluated for its potential spermicidal activity. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), ethylene bis(oxyethylenenitrilo)tetraacetic acid, and gramicidin, had spermicidal activity against human sperm at the tested concentration range and exerted spermicidal activity within mucus, impeding sperm penetration to the extended cervical space. A synergistic effect was shown between N-9 and EDTA on sperm motility. In dose–response curves, 0.1% EDTA significantly increased binding affinity constant and spermicidal potency of N-9 and reduced the concentration of N-9 at which 50% of the maximum response was observed from 144.5 to 66.4 (μg/mL). A synergistic effect was also shown between EDTA and carbopol 934P polymer on inhibition of sperm penetration through the cervical mucus. Therefore, EDTA can be used as a supplementary agent and potentiator for N-9. Development of a carbopol 934P-based drug delivery system for dual controlled release of N-9 in combination with chelating agents seems to be a promising approach for increasing the efficacy of fertility control.

Intravaginal controlled-release drug delivery systems have been used to achieve controlled delivery of locally active drugs, such as spermicides and antimicrobials. Nonoxynol-9 (N-9), a nonionic surfactant, exerts both spermicidal and antibacterial/antiviral activities against pathogens responsible for sexually transmitted disease (STD).[1−7] N-9, which has a long history of relatively safe use and efficacy, has been incorporated into several over-the-counter products[8] and is the most widely used spermicide for fertility control. Although N-9-releasing vaginal delivery systems offer the possibility of fertility control and protection against STD, they require the highest possible level of compliance by the users.[9] N-9 may cause vaginal irritation, allergic vaginitis, and genital irritation in male partners.[10] N-9 may have limited spermicidal activity within cervical mucus under conditions which should allow its unimpeded entry by diffusion, as previously reported.[11] To address these problems, various microbicidal agents can be screened for their spermicidal activities on human semen and for any synergistic spermicidal effect in combination with N-9.

In the search for a combination agent for fertility control, the role of calcium has been examined. Fertilization depends on the ability of spermatozoa to contact, bind to, and penetrate the oocyte, followed by sperm−egg fusion. These processes are primarily dependent on sperm motility, capacitation, and acrosome reaction. It has been reported that calcium is required for the mammalian sperm acrosome reaction and hyperactivation, a vigorous but nonprogressive type of movement.[12,13] Each step in the calcium-mediated regulatory process could serve as a potential target site for spermicidal action. To interrupt these steps using inhibitors or blocking agents could provide insight into the mechanisms of spermicidal action. Therefore, the possibility of using chelating agents for spermicides and their potential role in fertility control were investigated.

Ethylenediaminetetraacetic acid (EDTA) was found to have a relatively mild spermicidal effect on sperm motility.[14] EDTA appeared to decrease the *in vitro* percentage of motile sperm in both dose- and exposure time-dependent ways and exerted 100% spermicidal activity (EC_{100}) at concentrations above 5000 μg/mL. When EDTA was allowed to diffuse into lamb cervical mucus, restricted penetration and total loss of motility of sperm was observed at much lower concentrations of EDTA than that for spermicidal effect on sperm motility. After exposure to EDTA, the concentration of calcium ion in semen changed, showing a linear relationship between sperm motility and calcium ion concentration. The spermicidal activity of N-9 is much greater than that of EDTA. The total loss of sperm motility was achieved at a concentration of N-9 as low as 175 μg/mL. N-9 appeared to have a mild inhibitory effect on sperm penetration into mucus, even after N-9 was allowed to diffuse into capillaries for a prolonged contact period.[11,14] EDTA has a clear advantage over N-9 in that EDTA exerts the spermicidal activity in mucus and impedes sperm penetration to the extended cervical space. Therefore, the combination of EDTA and N-9 may enhance the efficacy of fertility control. This has heightened interest in development of a vaginal delivery system for dual controlled release of N-9 in combination with chelating agents for contraception and prophylaxis against STD.

Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) and gramicidin were also included to investigate the possible role of chelating agents in fertility control. EGTA, which has a property similar to EDTA, has been used as a fungal growth inhibitor.[15] Its action has been attributed to Ca^{2+} deprivation through the formation of Ca^{2+}−EGTA complexes. It was shown that nitrophenyl derivative of EGTA binds Ca^{2+} with higher affinity than the same derivative of EDTA.[16] In the presence of normal intracellular concentrations of Ca^{2+} and Mg^{+} ions, only Ca^{2+} was reportedly bound by the nitrophenyl derivative of EGTA. EGTA is also known to chelate essential divalent cations, such as Fe, Mn, Co, and Zn.[17]

Gramicidin is a linear peptide-type antibiotic, that has been reported to form specific channels across cell membranes and to enhance transport of cations. Its cation channel-forming property has been used in chelation. Since both amino and carboxy termini of the molecule are blocked, Gramicidin does not have any charged or hydrophilic chains and its aqueous solubility is low. It has been found to partition strongly into the hydrophobic region of phospholipid membranes.[18] Gramicidin is routinely used as a spermicide in Russia. Recently, its effectiveness against HIV was also reported.[19] The biologi-

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cal activity of gramicidin depends on the efficacy of its peptide-like interactions with the cell membrane and its chelating action, which induce pore formation across the cell membrane and subsequent deregulation of cation exchange.

The spermicidal activity of carbopol 934P, which was chosen as a polymer base for a mucoadhesive delivery system, was also evaluated. Carbopol 934P, which is a polyacrylic acid-based synthetic polymer, consists of particles that swell in water. Its degree of hydration is related to the expanded nature of its polymer network and charge density, which contribute significantly to the strength of mucoadhesion.[20] The most important consideration in the selection of carbopol 934P for this research is its high mucoadhessiveness even in the presence of mucus and its potential spermicidal activity exerted by interaction with cationic molecules in semen.[21]

In this report, the mechanism of spermicidal action of chelating agents was investigated, and the synergistic spermicidal effects of N-9 exerted by chelating agents were evaluated. The most potent and efficient combination was chosen to be developed into a mucoadhesive drug delivery system (AmDDS).

Experimental Section

Materials—All materials were obtained from commercial sources and used as received. Nonoxynol-9 (Igepal CO 630) from Rhone Poulenc Inc. (Cranbury, NJ), and EGTA, EDTA and gramicidin from Sigma (St. Louis, MO) were used as received. All buffering agents and solvents used were reagent grade. Distilled water purified by a Nanopure water purification system (Sybron-Barnstead, Boston, MA) was used in preparation of buffer solutions. In conducting *in vitro* assays, standard concentrations of all spermicidal agents were prepared in pH 7.4 phosphate buffer. Human semen which met the following criteria was used: Sperm count greater than 20×10^6 sperm/mL, sperm mobility greater than 50%, and sperm morphology greater than 30% of normal.

Computer-Assisted Semen Analysis (CASA)—The method previously described was used for evaluation of spermicidal potency of chelating agents on human sperm motility in semen.[14] A portion of the fresh semen was taken and the sperm concentration, percent motility, and normal morphology were determined using fully automated CASA (CellSoft, Cryo Resources Ltd., NY). Aliquots (0.2 mL) of various solutions of N-9 or agent were each pipetted into a glass test tube. Following the addition of semen (50 μL), the tube was vortexed for 10 s and left undisturbed for 2 min. An aliquot (10 μL) of solution was placed on a glass slide. After covering with a cover glass, the motility of the sperm was counted automatically by the CellSoft system.

Cervical Mucus Penetration Test—The extent of sperm penetration through cervical mucus can be objectively evaluated using the capillary tube method.[14,22,23] Fresh cervical mucus was obtained from lambs (6−8 months old) by aspiration and kept frozen until use. Precautions were taken during collection to avoid contamination. Glass capillary tubes (1.0 mm x 100 mm, Vitro Dynamics Rockaway, NJ) were filled with lamb cervical mucus and capped at one end. The open end of each tube was placed vertically into the test agent. After allowing agents to diffuse into the mucus for 30 min, the capillary tube was immersed vertically into pool of semen. After a 15 min. incubation, each capillary tube was examined under a phase contrast microscope at 400× magnification. The extent of sperm penetration was measured by counting the total number of sperm in sequential visual fields and noting the proportion of immotile sperm. Concentrations ranging from 100 to 5000 μg/mL for EDTA and EGTA, 100 to 500 μg/mL for N-9, and 1 to 10 μg/mL for gramicidin were examined. All tests were performed in triplicate at 25 °C. The results were analyzed by comparing equivalent visual fields along the capillaries containing control or test cervical mucus.

Construction of a Dose−Activity Relationship—After spermicidal activity measurement, *in vitro* spermicidal potencies of chelating agents (EC_{50} and EC_{100}) were quantitated and compared with N-9. Dose-response curves were constructed for sperm from at least three donors. A wide concentration range was tested to define the shape of the curve, which was analyzed by the NONLIN computer program using the following equation:[24]

$$R = \frac{R_m C^s}{\frac{1}{Q} + C^s}$$

$$\frac{R}{R_m} = \frac{C^2}{\frac{1}{Q} + C^s}$$

where R is the percentage of inhibition of the motility of sperm at the concentration of C, R_m is the maximum inhibition (100%), s is a parameter which determines the sigmoidicity of the response curve, and Q is the affinity constant showing R_{50} (0.5 R_m). The above equation can be derived into

$$\log(R/R_m - R) = s \log C + \log Q$$

The major advantage of the log dose−response plot is the approximate linearity of this relation over a relatively wide range in the vicinity of the half-maximal effect (R_{50}).

Synergism between Spermicidal Agents—To evaluate whether or not synergism existed between two spermicidal agents (N-9 and cation chelator), they were combined in various ratios and their spermicidal activities were evaluated using computer-assisted semen analysis. The result obtained can be expressed as the fractional spermicidal activity, dividing the spermicidal activity for the combination by the total spermicidal activity of each individual agent. Synergism exists if fractional spermicidal activity is more than 1.[25]

Table 1—Comparison of the Spermicidal Activity of EDTA and EGTA on Human Sperm Motility[a]

Concn (mg/mL)	Spermicidal Activity (% ± SD)[b,c]	
	EDTA	EGTA
0.10		
0.20	11.2(3.4)	10.4(3.6)
1.00	23.6(6.5)	23.5(3.4)
3.20	90.2(6.3)	85.9(6.2)
3.50	97.5(1.5)	92.2(4.0)
5.00	100	100

[a] Studies were conducted in whole semen and measured by computer-assisted semen analysis. [b] Expressed as percent of control. [c] The two values are not significantly different ($p > 0.01$).

Results and Discussion

Computer-Assisted Semen Analysis Test for Sperm Motility—The effects of EDTA and EGTA on sperm motility measured by CASA are described in Table 1. Upon exposuring to test agents, sperm was considered dead if it lacked any motion. The motionless sperm cannot fertilize eggs.[2] CASA revealed that exposure of semen to EGTA or EDTA for 2 min decreased the percentage of motile sperm in a concentration-dependent manner. The concentration−response curve formed a sigmoidal shape. EGTA inhibited human sperm motility in semen by 90% at concentrations as low as 3500 μg/mL, as shown in Figure 1. Total loss of sperm motility (EC_{100}) was observed at concentrations above 5500 μg/mL of EGTA. The mechanism of spermicidal action of EGTA seemed to be related to regulation of calcium ions, as was the case with EDTA. Even though EGTA was reported to be more specific and to have higher binding affinity to calcium than EDTA, there was no significant difference in concentrations of EGTA and EDTA required for total inhibition of sperm motility, as shown in Table 1. This result indicates that Ca^{2+} plays a key role in the fertilization process, but that some ions, such as Mn^{2+} and K^+, may work synergistically to support fusion.[26]

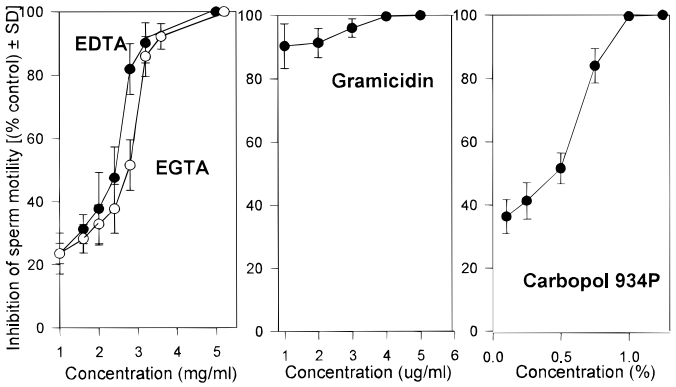

Figure 1—Dose-dependent spermicidal activity of EDTA, EGTA, gramicidin and carbopol 934P at 25 °C.

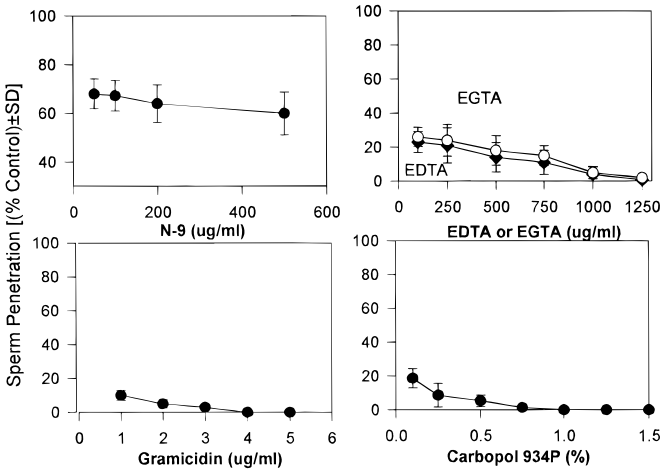

Figure 2—Distance travelled by sperm through lamb cervical mucus from whole semen after 15 min exposure to various concentrations of N-9, EDTA, EGTA, gramicidin, and carbopol 934P.

Gramicidin showed strong spermicidal activity. Total loss of sperm motility was observed at concentrations greater than 5 μg/mL, as shown in Figure 1. This high spermicidal potency may be due to its high partition to the hydrophobic region of the sperm membrane and a channel-forming property causing deregulation of cation exchange.[18] The spermicidal activity of gramicidin seemed to reflect the combined activities of N-9 and EDTA. Spermicidal activity of gramicidin may also be related to the activity of acrosin in sperm, causing reduced acrosin release and consequently preventing sperm mobility.[27] Gramicidin seems to be one of the most potent spermicidal agents, but its possible side effects, such as hemolytic action, and low aqueous solubility, may hinder its clinical application for fertility control.

Carbopol 934 P, chosen as a polymer base, showed 100% spermicidal activity at concentrations above 1.25% (w/w). CASA revealed that exposure of semen to solution- or gel-type carbopol for 2 min decreased the percentage of motile sperm in a concentration-dependent manner. Carbopol polymer immobilized human sperm in semen by 90% at concentrations as low as 1% (w/w), as shown in Figure 1. The anionic polymer, like carbopol 934P, clearly showed spermicidal activity, probably due to interaction with cationic molecules in semen.

Sperm Penetration of Mucus—EGTA, like EDTA, as reported earlier,[14] immobilizes sperm in mucus at concentrations even lower than that for 100% spermicidal effect on mobile sperm. There was a significant impedance of sperm penetration into mucus at concentrations within the tested range. The sperm never penetrated more than one-third of the distance traveled by sperm in the control, and all were immobile at the time of observation. As shown in Figure 2, EGTA decreased mucus penetration of sperm by as much as 99% of control after exposure to sperm for 15 min at a concentration 1000 μg/mL, which was only 24% effective in immobilization of sperm. This result indicated that EGTA diffused into mucus and potentiated spermicidal activity by various mechanisms, such as regulating mucus viscosity.[14] Total impedance of sperm penetration through cervical mucus was observed at concentrations of EGTA as low as 1300 μg/mL, which was much lower than that for 100% inhibition of sperm motility as estimated by the CASA test.

Gramicidin appeared to have a strong effect on impedance of sperm penetration into mucus, even at lower concentrations than those for total inhibition of sperm motility. Sperm penetrated less than 10% of the distance traveled by sperm in the control at the tested concentration range, as shown in Figure 2, and all were immobile at the time of observation. N-9, which diffused into the sperm plasma membrane, changed its semipermeable nature, and prevented its mobility, did not show spermicidal activity in cervical mucus. These results suggest that chelating action appears to be a major factor in impedance of sperm penetration in cervical mucus.

Table 2—Synergistic Effect of EDTA on the Spermicidal Activity of Nonoxynol-9 (N-9) on Human Sperm Motility[a]

	Spermicidal Activity (% ± SD)[b]	
N-9 Concn (μg/mL)	0% EDTA	0.1% EDTA
50	9.8(3.4)	21.4(7.3)
75	13.3(3.1)	52.4(10.2)
100	21.6(4.5)	99.3(0.9)
125	28.4(12.1)	100(0.0)
150	52.1(13.3)	100(0.0)
175	99.1(0.3)	100(0.0)

[a] Studies are conducted in whole semen and measured by computer-assisted seman analysis. [b] Expressed as percent of control.

Carbopol 934P in sperm-containing mucus also had an effect on impedance of sperm penetration into mucus. Sperm penetrated less than one-quarter of the distance traveled by the control at concentrations of carbopol 934P below 1% (w/w), as shown in Figure 2, and all were immobile at the time of observation. Its interaction with cationic molecules in mucus seems to be a major factor in impedance of sperm penetration by carbopol 934P in cervical mucus.

The role of mucus in fertilization process has not been fully elucidated yet, but mucus is known to act as the first barrier to sperm penetration to the cervix. The hostility of the cervical mucus to sperm penetration has been exploited as a mean of contraception, and low-dose oral contraceptive depends largely on this action for its effectiveness.[28] The mucus is a hydrogel with a nonuniform viscoelastic property. The major components are water (about 97%), glucose, and very small amounts of NaCl and NaOH. The highly viscous phase is composed of glycoproteins linked to peptide backbones.[29] Some components in mucus, such as sialic acid ($pK_a = 2.6$), are more negatively charged under physiological conditions than carbopol 934P ($pK_a = 4.75$), but the spermicidal activity of mucus in regard to ionic interaction has not been reported yet. The difference between mucus and carbopol 934P in binding affinity to calcium may play an important role in regulation of spermicidal action.

Synergism between Spermicidal Agents—The synergistic spermicidal activity of N-9 and EDTA is shown in Table 2. EDTA, at a concentration of 0.1%, showed mild spermicidal activity. N-9 at 150 μg/mL alone caused about 52% inhibition of sperm motility, while addition of 0.1% EDTA achieved 100%

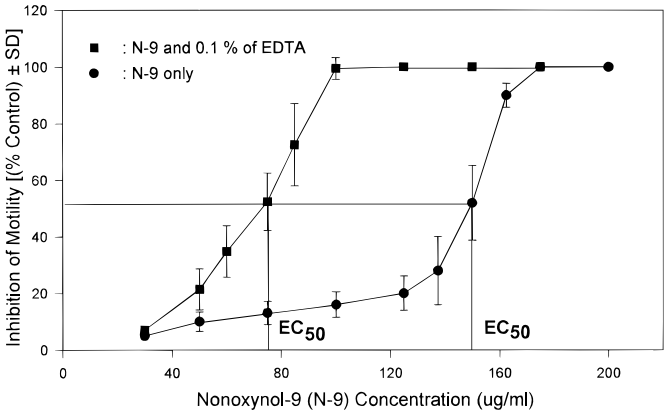

Figure 3—Dose–response relationship and the effect of 0.1% EDTA for inhibition of human sperm motility in semen by nonoxynol-9 (N-9).

Table 3—Statistical Values[a] of Sigmoidicity-Controlling Parameter (*s*), Intercept, Affinity Constants (*Q*), and Spermicidal Potency (C^s_{50}) of Nonoxynol-9 and Effect of EDTA (0.1%)

	s value (±SD)[b]	Intercept (±SD)	$1/Q$ (C^s_{50})
N-9	10.32(3.22)	–21.92(3.91)	144.5
N-9 and EDTA	8.38(1.39)	–15.28(2.58)	66.4

[a] Calculated from the equation $R/R_m = C^s/((1/Q)+C^s)$ using the SAS program. [b] The two values are not significantly different ($p < 0.05$).

inhibition. The EC_{100} of N-9 decreased to as low as 125 μg/mL as the added dose of EDTA increased up to 0.1%, indicating the presence of a synergistic effect between N-9 and EDTA on sperm motility. The mean effects of N-9 alone, and in combination with 0.1% EDTA, on motility of human sperm from three donors measured by CASA test are shown in Figure 3. Addition of 0.1% EDTA shifted the dose−response relationship curve further left and affected statistical values of the sigmoidicity coefficient (*S*), affinity constants ($1/Q$), and spermicide potency calculated from SAS, as shown in Table 3. Addition of 0.1% EDTA did not change the S of the dose−response curve of N-9, but significantly affected C^s_{50}, the concentration of N-9 at which 50% of maximum response was observed, reducing it from 144.5 to 66.4 (μg/mL). Because EGTA with N-9 showed a similar synergistic effect as EDTA with N-9, and because EDTA more clearly elucidated the effect of cationic ions on sperm motility, further combination studies were performed with EDTA and N-9.

Cell toxicity exerted by EDTA seems to be a major concern in its pharmaceutical application. EDTA was often used as a preservative potentiator in nasal drops or sprays at a concentration of 0.1%.[30] In those applications, EDTA was known to damage certain layers of the cell envelope and, in some instances, affect internal sites of the bacterial cell.[31] These activities can also damage the system application sites, such as nasal or vaginal mucosa. To avoid any possible side effects of EDTA, the synergistic effect of EDTA with carbopol 934P on inhibition of sperm penetration through cervical mucus was evaluated. Since both carbopol 934P and EDTA effectively inhibited sperm penetration through cervical mucus, the combination of two agents may further decrease the required dose of EDTA. The mean effects of EDTA alone, and in combination with 0.1% carbopol, on the penetration of human sperm from three donors measured by cervical mucus penetration test are shown in Figure 4. As described in Table 4, EDTA at a concentration of 0.01% caused about 77% inhibition of sperm penetration, while addition of 0.1% carbopol 934P achieved 100% inhibition. Therefore, carbopol 934P further reduced the EC_{100} of EDTA, the required dose for complete inhibition of sperm penetration through lamb

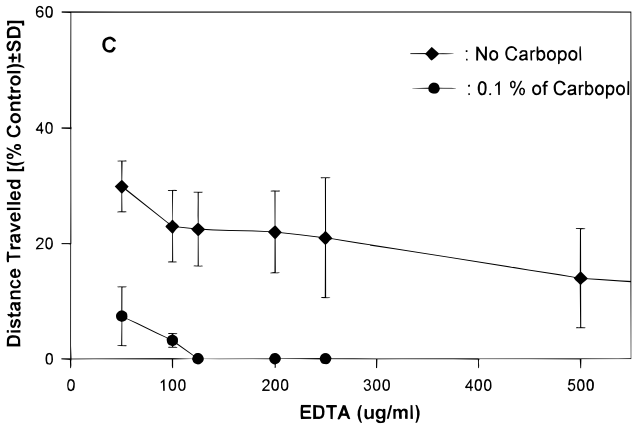

Figure 4—Distance travelled by sperm through lamb cervical mucus from whole semen after 15 min exposure to various concentations of EDTA and the effect of carbopol 934P (0.1%).

Table 4—Synergistic Effect of Carbopol 934P on the Inhibitory Action of EDTA on Cervical Mucus Penetration of Sperm[a]

	Spermicidal Activity (% ± SD)[b]	
EDTA Concn (mg/mL)	0% Carbopol	0.1% Carbopol
50	29.9(4.4)	7.4(5.1)
100	23.0(6.2)	3.2(1.2)
125	23.2(8.1)	0(0.0)
150	22.7(9.3)	0(0.0)
250	21.0(10.4)	0(0.0)

[a] Distance travelled by sperm was measured by the cervical mucus penetration test. [b] Expressed as percent of the control.

cervical mucus, down to 0.01%. The combination (100 μg/mL of N-9 and 0.01% EDTA in 0.1% carbopol 934P) showed 100% spermicidal action on human sperm motility. Consequently, the required loading dose of N-9 in a drug delivery system can be further reduced by controlling the carbopol 934P concentration.

Conclusion

In this report, the quantitative potency of the spermicidal activity of carbopol 934P and chelating agents, such as EDTA, EGTA and gramicidin, were evaluated using CASA and a cervical mucus penetration test. EDTA and EGTA show spermicidal activities at the tested concentration range, even though their potencies are much lower than that of N-9. Gramicidin has a strong spermicidal potency, completely immobilizing sperm at concentrations as low as 5 μg/mL. Chelating agents, like EDTA, have a clear advantage over N-9 in that EDTA exerts the spermicidal activity in mucus and impedes sperm penetration to the extended space in the cervix. There was a synergistic spermicidal effect between N-9 EDTA on sperm motility indicating that EDTA could reduce the loading dose of N-9 in the system. There also was a synergistic effect between EDTA and carbopol 934P on impedance of sperm penetration through cervical mucus. Therefore, EDTA not only decreases the loading dose of N-9 but also increases the efficacy of fertility control. The combination of EDTA and N-9 will be further evaluated for compatibility with a mucoadhesive polymer and tested for application of a mucoadhesive drug delivery system.

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