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In Vitro Testing for Potency of Various Spermicidal Agents*

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INTRODUCTION

The rapid progress and dramatic events in the field of fertility regulation since 1960 have prompted a reevaluation of the vaginal methods of contraception includes a consideration not only of the present status of the condom, diaphragm, and spermicides, but also of their potential for further development, increased effectiveness, and greater acceptance. Today's vaginal spermicides are available without a doctor's prescription like the condom and, when used in conjunction with the condom or diaphragm, are highly effective.

Spermicidal substances have been in use since pharaonic times, and form part of the folklore of most cultures. In more recent times, growing knowledge of cell biochemistry and of spermatozoal metabolism has provided information about substances that interfere with the functional activity of this cell. This basic approach has been paralleled by more or less empirical work aimed at finding substances with high spermicidal activity that are suitable for human use. In the latter context, the work of Baker (1935, 1937, 1939) in the 1930s, was outstanding both for its application of scientific experimentation to chemical contraception, and for its success in determining the relative in vitro activity of a large number of compounds. Baker's research led to the wide spread use of phenylmercuric acetate, benzoate, and nitrate as the active principles of contraceptive preparation (Baker, et al 1937).

Currently, a lot of research is undertaken for the development of more effective and harmless vaginal spermicides. For evaluation and approval of preparations for vaginal use vaginal spermicides are tested in the laboratory for their spermicidal activity in vitro. They are also tested in laboratory animals for toxicity and vaginal tolerance, and in humans for vaginal tolerance, aesthetic acceptability and clinical effectiveness.

This study was designed in order to evaluate the spermicidal activity in vitro of various compounds against human spermatozoa. Attempts were also made to study the influence of several factors on spermicidal activity, and to reappraise the methodology used for the in vitro testing of spermicidal agents.

MATERIALS AND METHOD

Materials

1) Semen: Semen was obtained from healthy fertile donors after two days of sexual abstinence. Specimens were collected manually into glass jars and were allowed to stand 30~60 minutes for complete liquefaction. All samples were used within 60 minutes after collection.

2) Heat inactivated serum: Serum was obtained from healthy fertile female during the late follicular phase of menstrual cycle. A pool of serum was heated at 56°C for 30 minutes to remove complement and prevent immobilization from complement-dependent immobilizing

* 本 論文의 要旨는 1981年 4月 3日 大韓産婦人科學會 第47次 春季 學術大會에서 發表하였음.

antibodies(Mishell and Davajan, 1979).

3) Diluent: Three types of solution were used.

1. Modified Baker's solution (MBS) buffer consisted of NaCl 2.0gm, Na_2HPO_4 1.4gm, KH_2PO_4 0.023gm and Glucose 3.0gm, which were diluted to a final volume of 100ml with distilled water and the pH adjusted to 7.8(Mishell and Davajan, 1979).

2. Ringer's Glucose Phosphate solution(RGP) buffer consisted of NaCl 0.9gm, KCl 0.046gm, KH_2PO_4 0.021gm, $MgSO_4$ 0.038gm, Na_2HPO_4 0.7gm, Glucose 254mg, which were diluted to a final volume of 127ml with distilled water and the pH adjusted to 7.4.

3. Isotonic 0.9% normal physiologic saline

4) Spermicidal agents: The following compounds were provided from World Health Organization for in vitro testing of spermicidal activity.

1. Compound 65/416, 2. Natural Sapindus 3. Synthetic Sapindus, 4. C-2,5. I-1, 6. GKP-3 (16/1010), 7. Nonoxynol-9, 8. GKP-15, 9. Emetine, 10. Quinine, 11. Sodium Deoxycholate(SDC), 12. N-Cetyl-N, N, N-Trimethylammonium bromide(NTB), 13. Cytochalasin A(Cyto-A), 14. Cytochalasin B(Cyto-B) 15. Typanalyse(Typ.), 16. 102/198-dilactate 17. N-Cetylpyridinium chloride(NCP), 18. Hexanal

Method

The following procedures were performed for spermicidal test in vitro:

1) Dilution of semen: After the semen analysed the semen was diluted with 1/54 heat-inactivated serum in buffer prewarmed at 37°C to achieve a final concentration 10 to 20×10^6 spermatozoa per ml. The final diluted semen, which had the same % motility and quality as before dilution, was used immediately in the testing of spermicidal activiy of compounds.

2) Serial dilution with agents: Spermicidal agents were dissolved in 0.9% physiologic saline to achieve a concentration of 500μg/ml, and diluted serially with 1/54 heat-inactivated serum in buffer prewarmed to 37°C. As a result of this serial dilution, each final concentration was obtained at 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, and 0.9μg/ml, respectively. Dissolved spermicidal agents were warmed in the 37°C bath.

3) Sperm motility assays: The experimental design is presented in Table 1. In the 37°C water bath 0.05ml of diluted semen was put into the control tube with buffer alone. After

Table 1. Experimental design

No.	Spermicidal agent (conc. of test solu.)	Volume of test solution	Volume of diluted whole semen	Spermicidal agent (final conc.)
1.	500.0 μg/ml (0.05 %)	500 μl	50 μl	454.5 μg/ml (0.045 %)
2.	250.0 μg/ml (0.025 %)	500 μl	50 μl	227.3 μg/ml (0.023 %)
3.	125.0 μg/ml (0.0125 %)	500 μl	50 μl	113.6 μg/ml (0.011 %)
4.	62.5 μg/ml (0.00625%)	500 μl	50 μl	56.8 μg/ml (0.0057 %)
5.	31.2 μg/ml (0.00312%)	500 μl	50 μl	28.4 μg/ml (0.0028 %)
6.	15.6 μg/ml (0.00156%)	500 μl	50 μl	14.2 μg/ml (0.0014 %)
7.	7.8 μg/ml (0.00078%)	500 μl	50 μl	7.1 μg/ml (0.00071%)
8.	3.9 μg/ml (0.00039%)	500 μl	50 μl	3.5 μg/ml (0.00035%)
9.	1.9 μg/ml (0.00019%)	500 μl	50 μl	1.7 μg/ml (0.00017%)
10.	0.9 μg/ml (0.00009%)	500 μl	50 μl	0.8 μg/ml (0.00008%)
Control	0	500μl of Buffer	50 μl	0

mixed quickly, a drop was placed on a slide glass and observed under the microscope to check for motility and quality of sperm. This procedure was done within 20 seconds. This procedure was repeated for other tubes starting from the tube containing the greatest dilution of the test material. At least 100 sperm were counted over multiple fields under a Zeiss microscope at 100×. The number of motile spermatozoa were estimated as a percentage of the total number of spermatozoa. The quality of motility was rated by the following system (Mishell and Davajan, 1979):

- 4^+: very rapid, forward motility
- 3^+: rapid, forward motility
- $2b^+$: sluggish, forward motility
- $2a^+$: rapid tailbeat without forward motility
- 1^+: sluggish tailbeat without forward motility
- 0: no motion

Table 2. The effect of pH on the spermicidal activity of Compound 65/416 and Quinine

Case 1:	pH 6.0		pH 6.5		pH 7.0		pH 7.6		pH 7.8		pH 8.0	
〈65/416〉	Motil.	Qual.	Motil.	Qual.	Motil.	Qual.	Motil.	Qual.	Motil.	Qual.	Motil.	Qual.
Control	40	2b	40	3	50	3	60	3	50	3	50	3
1.9 μg/ml	20	2a, 1	20	2b, 2a	30	2b, 2a	40	2b, 2a	40	2b, 2a	30	3
7.8	10	2a	10	2a	20	2a	30	2b, 2a	20	2a, 2b	20	2a
31.2	0	0	0	0	0	0	10	2a	0	0	0	0
62.5	0	0	0	0	0	0	0	0	0	0	0	0
250.0	0	0	0	0	0	0	0	0	0	0	0	0
〈Quinine〉												
Control	40	2b	40	3	50	3	60	3	50	3	50	3
1.9	30	2b, 2a	30	2b, 2a	40	2b, 2a	50	3, 2b	40	3	40	3
7.8	30	2a, 2b	20	2a, 2b	40	2b, 2a	40	2b2a	40	2b, 2a	40	2b, 3
31.2	20	2b, 1	20	2b, 2a	40	2b, 2a	40	2b, 2a	40	2b, 2a	40	2b, 2a
62.5	20	2a	20	2a, 2b	30	2a	40	2a, 2b	40	2a, 2b	40	2b, 2a
250.0	10	2a	10	2a, 1	20	2a	30	2a, 2b	20	2a	20	2a, 1
Case 2:												
〈65/416〉												
Control	40	2b, 2a	50	3	60	3, 2b	60	3, 2b	70	3	60	2b
1.9	20	2a, 2b	20	2a, 2b	40	2b, 2a	40	2b, 2a	40	2b, 2a	40	2a, 2b
7.8	5	2a, 1	10	2a	30	2b, 2a	30	2b, 2a	30	2b, 2a	20	2a
31.2	0	0	0	0	0	0	10	1	5	2a	0	0
62.5	0	0	0	0	0	0	0	0	0	0	0	0
250.0	0	0	0	0	0	0	0	0	0	0	0	0
〈Quinine〉												
Control	40	2b, 2a	50	3	60	3, 2b	60	3, 2b	70	3	60	2b
1.9	30	2b, 2a	40	2b, 2a	60	3, 2b	60	2b, 2a	60	3, 2b	60	3, 2b
7.8	30	2a, 2b	30	2a, 2b	40	2b, 2a	50	3, 2b	50	3, 2b	50	2b, 2a
31.2	20	2a, 2b	30	2a, 2b	40	2a, 2b	50	2b, 2a	50	3, 2b	40	2a, 2b
62.5	10	2a, 2b	20	2a, 2b	30	2a	40	2a	40	2a, 2b	40	2a, 2b
250.0	10	2a	10	2a	20	2a	20	2a, 1	30	2a, 1	30	2a

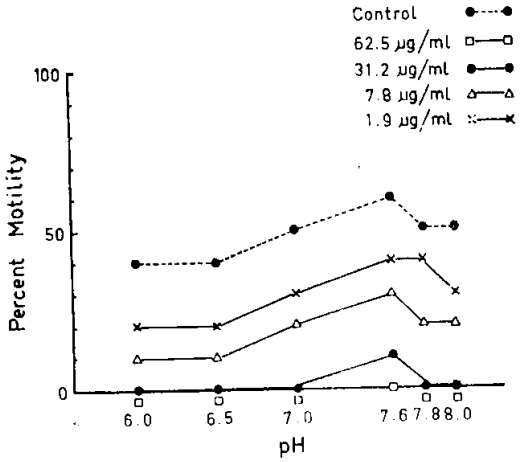

Fig. 1. Effect of pH of test preparations on the spermicidal activity of Compound 65/416.

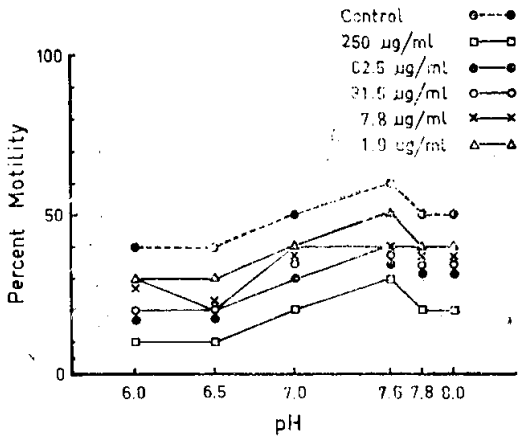

Fig. 2. Effect of pH of test preparations on the spermicidal activity of quinine.

RESULTS

First of all, various factors were studied for their influence on spermicidal activity. Tests were carried out under the various pH of solution to determine the effects of pH on the motility of sperm. The results are shown in Table 2, Fig. 1 and Fig. 2, which indicated that immobilizing activity was stronger in

Table 3. The effect of various diluents used in in vitro test on the spermicidal activity of Nonoxynol-9 and Compound 65/416

	Saline		MBS		RGP	
	Motil.	Qual.	Motil.	Qual.	Motil.	Qual.
〈Nonoxynol-9〉						
Control	60	3	60	3	60	3
0.9 μg/ml	40	3	50	3	50	3
3.9	40	3, 2b	50	3	50	3, 2b
7.8	0	0	10	2a	0	0
15.6	0	0	0	0	0	0
62.5	0	0	0	0	0	0
125.0						
500.0	0	0	0	0	0	0
〈65/416〉						
Control	60	3	60	3	60	3
0.9	50	3, 2b	50	3	60	3
3.9	50	3, 2a	50	3, 2a	60	2a, 2b
7.8						
15.6	30	2a, 1	40	3, 2a	50	3, 2b
62.5	0	0	30	2a, 1	40	2b, 2a
125.0			0	0	20	2a
500.0	0	0	0	0	0	0

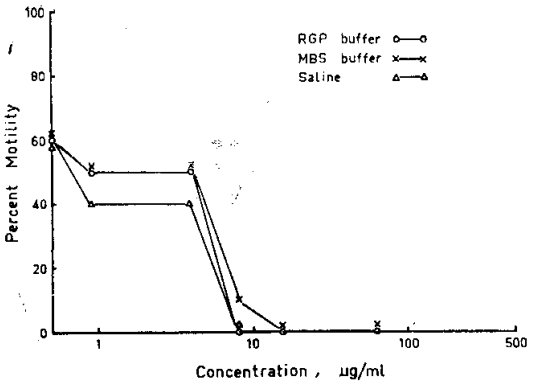

Fig. 3. Effect of various diluents used in the test preparations on the spermicidal activity of Nonoxynol-9.

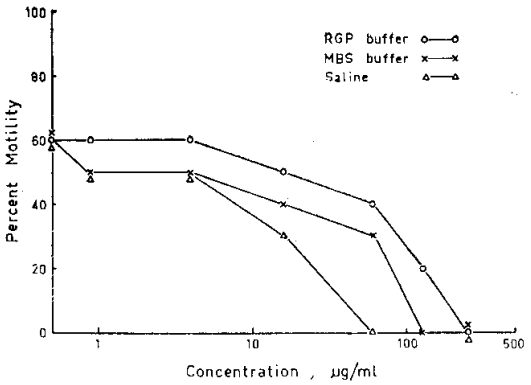

Fig. 4. Effect of various diluents used in the test preparations on the spermicidal activity of Compound 65/416.

acidic (pH 7.0) than in alkaline (pH 7.0～8.0) solution at the same concentrated test solution. However, spermicidal activity was effective even at an optimal pH for sperm migration in the cervix in the case of high concentrated test solution.

The other tests were carried out to determine the effect of various diluents, MBS, RGP and saline, on the activity of spermicidal agent. The results are presented in Table 3, Fig. 3 and Fig. 4. Although total immobilization of sperm was observed at the lower concentrated test solution when tests were conducted using saline as a diluent for serial dilution of spermicidal agent than when tests were conducted using MBS or RGP, the difference was not great.

In the next experiment, in vitro spermicidal activities of various compounds were determined according to a modified method of Sander and Cramer (1941). The result of the assays is summarized in Table 4. A representative experiment on each compound is also presented in Fig. 5, 6, 7, 8 and 9.

Nonoxynol-9 and Compound 65/416 resulted in total immobilization of sperm in 20 seconds at 62.5 μg/ml and 125 μg/ml, respectively. Therefore, both of them indicated most potent spermicidal activity among all the compounds tested. Natural Sapindus, synthetic Sapindus, C-2, E-1, GKP-3, GKP-15, NTB, NCP and Hexanal belonged to the next potent spermicidal group. On the other hand, quinine, emetin, cyto-A, cyto-B, SDC, Typ. and 102/198 belonged to the group of least effective spermicidal activity, which did not show zero motility of sperm even at 250 μg/ml.

In order to evaluate the result of Modified Sander-Cramer Test, another in vitro spermicidal test, the so-called "comparative survival test" (Suter, 1978), was performed on quinine, Nonoxynol-9 and 65/416.

The procedure of this test is described as follows: Human semen was diluted with 1/54 heat-inactivated serum in MBS buffer prewarmed at 37°C to achieve a final concentration 10 to 20×10^6 spermatozoa/ml. To 0.5 ml of each test compound in same buffer or the buffer alone was added 0.5 ml of diluted whole semen. The sample was incubated in a stoppered test tube in a slowly shaking water-bath at 37°C for 4 to 6 hours. At 60, 120, 240 and 360-minute intervals during incubation, 0.02 ml aliquots were placed on microscope slides and

Table 4. Antimotility effect of various compounds on human sperm tested by Modified Sander-Cramer Test

Agent	Conc.(μg/ml)	Case (Ⅰ)		Case (Ⅱ)		Case (Ⅲ)	
		Mot.(%)	Qual.	Mot.(%)	Qual.	Mot.(%)	Qual.
65/416	Control	60	3	60	4, 3,	60	3
	0.9	50	3	60	3, 2b	50	3
	3.9	40	3	40	3, 2b	40	3, 2a
	15.6	30	2b, 2a	40	3, 2b	40	3, 2b
	31.2			20	2a	30	2b, 2a
	62.5	5	1	10	2a	10	2a, 1
	125.0			0	0	0	0
	250.0	0	0	0	0	0	0
Natural	Control	60	3	60	4, 3	60	3
Sapindus	0.9	50	3, 2a	50	3	60	3
	3.9	40	3	40	3, 2a	50	3
	15.6	40	3, 2b	40	3, 2a	40	3, 2a
	31.2						
	62.5	40	3, 2a	30	3, 2a	30	2b, 2a
	125.0			20	2a, 1	20	2b, 2a
	250.0	0	0	0	0	20	2b, 2a
Synthetic	Control	60	3	60	4, 3	60	3
Sapindus	0.9	50	3	50	3, 2b	50	3, 2a
	3.9	50	3, 2b	40	3, 2b	40	3, 2b
	15.6	40	3, 2b	40	2b, 2a	30	3, 2b
	31.2						
	62.5	30	3, 2b	30	2b, 2a	20	2b, 2a
	125.0			20	2a, 1	20	2b, 2a
	250.0	0	0	0	0	0	0
C-2	Control	50	3	50	3, 2b	50	3
	0.9					40	3, 2b
	3.9	40	2b, 2a	40	2b, 2a	40	3, 2b
	7.8			40	2b, 2a	40	2b, 2a
	15.6					40	2b, 2a
	31.2	40	2b, 2a				
	62.5			40	2b, 2a	30	2b, 2a
	125.0	30	2b, 2a	30	2b, 2a	30	2b, 2a
	250.0	0	0	0	0	0	0
I-1	Control	45	2b, 2a	50	3, 2b	50	3
	0.9					50	3, 2b
	3.9	45	2b, 2a	40	2b, 2a	40	3, 2b
	7.8			40	2b, 2a		
	15.6					40	2b, 2a
	31.2	30	2b, 2a				

Agent	Conc.(μg/ml)	Case (Ⅰ)		Case (Ⅱ)		Case (Ⅲ)	
		Mot.(%)	Qual.	Mot.(%)	Qual.	Mot.(%)	Qual.
	62.5			20	2b, 2a	30	2b, 2a
	125.0	30	2b, 2a			20	2b, 2a
	250.0	0	0	0	0	0	0
GKR-3	Control	50	3	45	3, 2b	50	3
	0.9					40	3, 2b
	3.9	45	3			40	3, 2b
	7.8			30	2b, 2a		
	15.6					40	2b, 2a
	31.2	10	2b				
	62.5			30	2b, 2a	20	2b, 2a
	125.0	0	0			10	2b, 2a
	250.0	0	0	0	0	0	0
Nonoxynol-9	Control	50	3	60	3	50	3, 2b
	0.9	40	3, 2b	50	3	50	3, 2b
	3.9	30	2b, 2a	50	2b, 2a	40	3, 2b
	7.8			40	2b, 2a		
	15.6	10	2b, 2a	20	2b, 2a	0	0
	62.5	0	0	0	0	0	0
	250.0	0	0	0	0	0	0
Emetine	Control	50	3	60	3	50	3
	0.9			50	2b, 2a	50	3
	3.9	40	2b, 2a	50	2b, 2a	50	2b
	7.8			40	2b, 2a		
	15.6	30	2b, 2a	30	2b, 2a	30	2b, 2a
	62.5	20	2b, 2a	20	2b, 2a	20	2a, 1
	250.0	10	2b, 2a	20	2b, 2a	15	2a, 1
GKR-15	Control	50	3	60	3, 2b	60	3
	0.9			50	3, 2b	50	3, 2b
	3.9	40	2b, 2a	50	3, 2b	50	3, 2b
	7.8						
	15.6	20	2b, 2a	50	3, 2b	50	3, 2b
	62.5	10	2a, 1	50	3, 2b	50	2b, 1
	125.0			40	3, 2b	40	2b, 1
	250.0	0	0	0	0	0	0
Quinine	Control	50	3	60	3	50	3, 2b
	0.9			50	3, 2b	50	3, 2b
	3.9	50	2b, 2a	50	2b, 2a	50	3, 2b
	7.8			40	2b, 2a		
	15.6	40	2b, 2a	50	2b, 2a	50	3, 2b
	62.5	20	2b, 2a	30	2b, 2a	40	2b, 2a
	250.0	10	2b, 2a	20	2a, 1	30	2b, 2a

Agent	Conc.(μg/ml)	Case (Ⅰ)		Case (Ⅱ)		Case (Ⅲ)	
		Mot.(%)	Qual.	Mot.(%)	Qual.	Mot.(%)	Qual.
Cyto-A	Control	60	3	60	3	50	3, 2b
	0.9			60	3, 2b	50	3, 2b
	3.9	50	3, 2b	60	3, 2b	50	3, 2b
	15.6	50	3, 2b	60	3, 2b	40	2b, 2a
	62.5	50	3, 2a	60	3, 2b	30	2b, 2a
	250.0	40	3, 2a	60	3, 2b	30	2a, 1
Cyto-B	Control	60	3	60	3	50	3, 2b
	0.9			60	3, 2b	40	3, 2b
	3.9	40	3, 2b	60	3, 2b	40	3, 2b
	15.6	40	2b, 2a	50	3, 2b	40	2b, 2a
	62.5	40	2b, 2a	50	3, 2b	30	2b, 2a
	250.0	40	2b, 2a	50	3, 2b	30	2b, 2a
SDC	Control	60	3	60	3	50	3, 2b
	0.9			55	3, 2b	50	3, 2b
	3.9	40	2b, 2a	50	3, 2b	50	3, 2b
	15.6	40	2b, 2a	50	3, 2b	50	3, 2b
	62.5	40	2b, 2a	50	3, 2b	50	3, 2b
	250.0	30	2b, 2a	50	3, 2b	20	2a, 1
NTB	Control	60	3	60	3, 2b	50	3, 2b
	0.9			60	3, 2b	50	3, 2b
	3.9	50	3, 2b	60	3, 2b	50	3, 2b
	15.6	40	3, 2b	60	3, 2b	50	3, 2b
	31.2			60	3		
	62.5	40	3, 2b	0	0	50	3, 2b
	125.0					50	3, 2b
	250.0	0	0	0	0	0	0
Typ.	Control	50	3	60	3	50	3
	0.9			40	3, 2b	40	3, 2a
	3.9	40	3, 2b	30	2b, 2a	40	2b, 2a
	15.6	40	3, 2a	30	2b, 2a	40	2b, 2a
	62.5	30	2b, 2a	30	2b, 2a	40	2b, 2a
	125.0						
	250.0	30	2b, 2a	20	2b, 2a	30	2b, 2a
NCP	Control	50	3	60	3	50	3
	0.9	50	3, 2b	30	3, 2b	50	3, 2b
	3.9	40	2b, 2a	30	3, 2b	40	3, 2b
	15.6	30	2b, 2a	20	2b, 2a	40	3, 2b
	62.5	30	2b, 2a	10	2b, 2a	20	2b, 2a
	125.0	5	2a			10	2b, 2a
	250.0	0	0	0	0	0	0
102/198	Control	50	3	60	3	50	3, 2b

Agent	Conc. (μg/ml)	Case (Ⅰ)		Case (Ⅱ)		Case (Ⅲ)	
		Mot. (%)	Qual.	Mot. (%)	Qual.	Mot, (%)	Qual,
	0.9	40	2b, 2a	40	3, 2b	40	3, 2b
	3.9	30	2a	30	2b, 2a	40	2b, 2a
	15.6	10	2a	10	2a, 1	30	2b, 2a
	62.5	10	2a, 1	10	2a, 1	20	2b, 2a
	250.0	10	2a	10	2a, 1	20	2b, 2a
Hexanal	Control	50	3	60	3	50	3
	0.9	40	3, 2a	40	3, 2b	30	2b, 2a
	3.9	30	2b, 2a	20	2b, 2a	20	2b, 2a
	15.6	10	2a	5	2a	10	2a
	62.5	0	0	5	1	0	0
	250.0	0	0	0	0	0	0

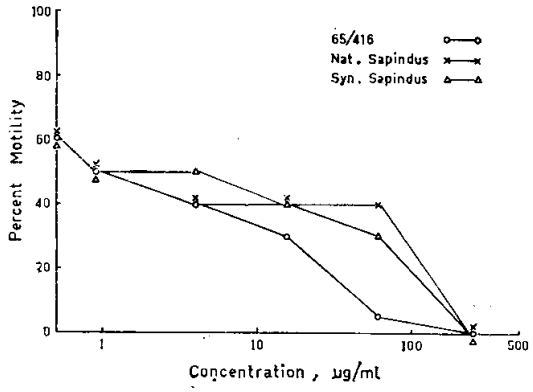

Fig. 5. Antimotility effect of Compound 65/416, Natural Sapindus and Synthetic Sapindus on human sperm.

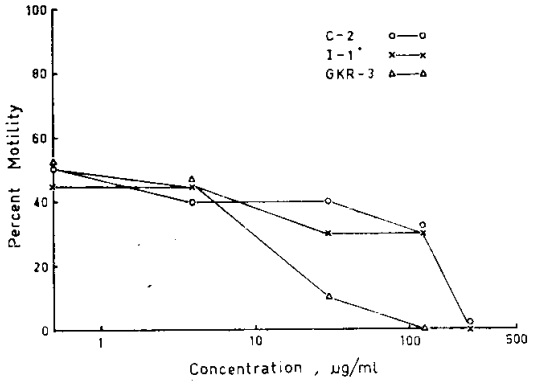

Fig. 6. Antimotility effect of C-2, I-1 and GKR-3 on human sperm.

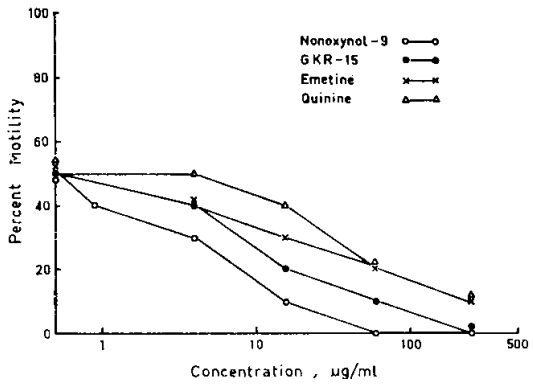

Fig. 7. Antimotility effect of Nonoxynol-9, GKR-15, emetine and quinine on human sperm.

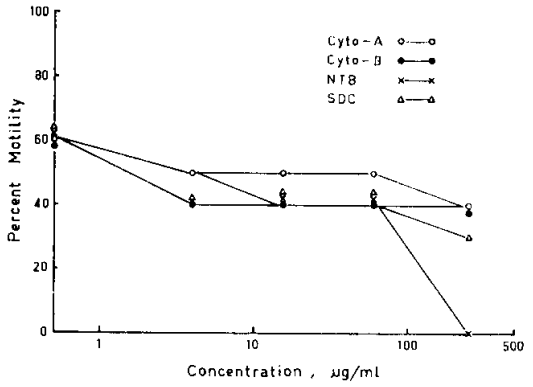

Fig. 8. Antimotility effect of cytochalasin-A, cytochalasin-B, N-Cetyl-N, N, N-Trimetlyammonium bromide and sodium deoxycholate on human sperm.

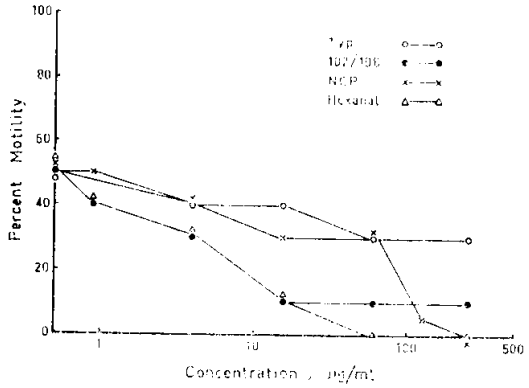

Fig. 9. Antimotility effect of Typanalyse, Compound 102/198-dilactate, N-Cetylpyridinium chloride and Hexanal on human sperm

examined under a microscope for assessment of motility. The result of this test, as shown in Table 5, revealed that either Nonoxynol-9 or 65/416 had more potent sperm immobilizing activity than quinine.

DISCUSSION

All of spermicidal products presently on the market have two components: a relatively inert vehicle or carrier and active spermicidal agent. The effectiveness of vaginal spermicides depends on a number of interacting factor, including: 1) the spermicidal potency of the active ingredient, 2) the release of the active ingredient from the dosage forms, and 3) the physical properties of the formulation as a barrier to sperm penetration through the cervix.

In reviewing the literatures(Baker, 1935;

Table 5. Comparative survival test showing the antimotility effect of Quinine, 65/415 and Nonoxynol-9 on human sperm

Agent	Conc. (μg/ml)	Case I (Initial Motil./Qual. 70/4, 3) Incubation time (min.)				Case II (Initial Motil./Qual. 80/4) Incubation time(min.)		
		60′	120′	240′	360′	60′	120′	240′
Quinine	Control	40/3	40/3, 2b	30/3, 2b	10/2a, 1	50/3	30/2a	20/2b
	1.9	40/4, 3	40/2b, 2a	20/2a	0/0	40/2b, 2a	20/2b	20/2b, 2a
	7.8	40/2b, 2a	30/2b, 2a	30/2b, 2a	0/0	30/2b, 2a	20/2b	10/2a, 1
	31.2	30/2a	30/2b, 2a	30/2b, 2a	0/0	20/2b, 2a	10/2b	0/0
	125.0	20/2a	30/2a	10/2b, 2a	0/0	20/2b	10/2b	0/0
	500.0	10/1	0/0	0/0	0/0	10/1	5/2b	0/0
65/416	Control	50/3	40/2b	40/3	20/2a, 1	30/2b	40/3	30/2b, 2a
	1.9	50/3	40/2b, 2a	30/3, 2b	10/1	20/2b, 2a	20/2b, 2a	20/2b, 2a
	7.8	40/3, 2b	30/2b, 2a	20/2b, 2a	0/0	20/2b, 2a	10/2a	5/1
	31.2	30/2a	10/2a, 1	20/2a, 1	0/0	10/2b	10/2a	0/0
	125.1	10/1	10/1	0/0	0/0	0/0	0/0	0/0
	500.0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Nonoxynol-9	Control	50/3	40/2b	30/3	10/2a, 1	50/2a	30/2a	30/2b, 2a
	1.9	40/3, 2b	30/2b, 2a	20/2b, 2b	10/2a, 1	40/2b, 2a	20/2b, 2a	20/2b, 2a
	7.8	30/2b, 2a	30/2b, 2a	30/2a	0/0	40/2b, 2a	10/2b, 2b	20/2a, 1
	31.2	20/2a	20/2a	20/1	0/0	30/2b	10/2b, 2a	0/0
	125.0	0/0	0/0	0/0	0/0	10/2a	0/0	0/0
	500.0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

Table 6. Methodological differences among the Sander-Cramer test, modified Sander-Cramer test and IPPF Agreed test.

Variable	Sander-Cramer Test	Modified Sander-Cramer Test	IPPF Agreed Test
Semen quality	Examine volume, sperm count and motility	≥50M/ml, 40~50% rapid forward motion	≥50M/ml, 40~50% rapid forward motion
Semen age	≤1 hour	≤1 hour	≤4 hours
Semen donors	Not specified	Minimum of 3	Minimum of 3, maximum of 6
Temperature	Room temperature (22±1°C)	37°C	35~37°
Testing solution	Serial duilutions	Serial dilutions	1gm/11ml
Mix time	5 seconds	5 seconds	10 seconds
Elapsed time	20 seconds	20 seconds	40 seconds
Slide preparation	Hanging drop	Slide plus coverglass	Slide plus coverglass
Endpoint	Greatest dilution that immobilizes sperm in<20 seconds	Greatest dilution that immobilizes sperm in<20 sec.	Must immobilize sperm from 3 or 5 of 6 donors
Confirmation	Retest endpoint dilution	Average value of donors	Unable to revive motility with buffered glucose solution

Baker et al. 1937, 1939; Brown and Gamble, 1940, 1941, 1943, 1943; Millman, 1952; Davidson, 1953; Carruthers, 1958) dealing with in vitro spermicidal tests, it is apparent that new methodology has not been published since the work of Harris in 1962 and the tests most commonly used at present are the Sander-Cramer(1941) and the IPPF Agreed(Kleinman, 1964) Tests. The Sander-Cramer test, which was developed in the laboratories of Ortho Pharmaceutical Corporation in 1940, involves the addition of 1.0 ml of diluted test compound or formulation to 0.2 ml of human semen at room temperature. The endpoint is the greatest dilution of the test material which immobilizes all sperm within 20 seconds. In the IPPF Agreed test, all spermicides are diluted with 0.9% saline to a constant concentration of 1 g/11 ml. For a product to "pass" the IPPF test, 1 ml of this solution at 35°-37°C must immobilize the sperm in 0.2 ml of semen from each of three different donors or from five of six donors within 40 seconds. Major variables between these two tests, as shown in Table 6, include semen age, temperature, number of different semen donors, and test solution preparation.

It must be noted that while there are several procedural differences between these two tests, the principal difference is in the endpoint of the tests. The Sander-Cramer test can provide quantitative potency data, whereas the IPPF Agreed test only provides qualitative data with respect to the activity: a product either "passes" or "fails" the IPPF test.

It has been the general consensus that there is a large difference in criteria for spermicidal testing and various factors have been known to affect the result (Millman, 1952; Harris, 1962). Among the factors which could have an influence on the results of spermicidal activities are the pH of test solutions, diluent for serial dilution of test compound, semen age, temperature, and semen donor variations.

Sperm are susceptible to changes in the pH of cervical mucus. Acid mucus immobilizes sperm, whereas alkaline mucus enhances their motility (Moghissi, 1973). The pH of semen obtained from the donors for all the tests was 7.4-8.0. The optimal pH for sperm migration and survival in cervical mucus is between 7.0 and 8.5. This is also the pH range of normal midcycle cervical mucus (Moghissi, 1973).

Our result indicated that sperm immobilizing activity was stronger in acidic (pH 7.0) than in alkaline (pH 7.0-8.0) solution at the same concentrated test solution. However, spermicidal activity was effective even at an optimal pH for sperm migration in the cervix in the case of high concentrated test solution. Therefore, in order to evaluate accurately spermicidal potency of tested compound it seems to be reasonable to perform in vitro spermicidal activity testing at the PH 7.0-8.0, which is optimal pH for sperm survival and migration as well as physiologic range of normal midcycle cervical mucus.

The other tests were carried out to determine the effect of various diluents, MBS, RGP and saline, on the activity of spermicidal agents. Although the difference was not great among the results of three of them, it was suggested that MBS (pH 7.8) is desirable as a diluent for serial dilution of spermicidal agents in in vitro testing. Hahn et al (Hahn et al, 1979) performed comparative studies of the effects of age of semen, room temperature (22 ±1°C), and 37°C on six different contraceptive formulations. Statistical analysis of the data indicated that spermicidal potency was not affected by altering temperature when 30-min, ejaculates were used. When 2-hr-aged semen was used, there was also no significant difference in the spermicidal potencies obtained for all formulations, with the exception of Ortho-Forms, a melting suppository tested at 37°C. They felt that it is not necessary to evaluate spermicidal potency at 37°C because evaluations conducted at room temperature using fresh ejaclates yield similar results and are more convenient.

However, because sperm are subjected in vivo to 37°C temperature, it may seem logical to subject them in the in vitro tests to spermicides at 37°C. Our criteria in research practice was that fresh semen should be used, ideally within 1 hour ejaculation, to assure consistent spermicidal results, and that in vitro testing should be assayed at 37°C to prevent the influence of any unphysiologic factor on spermicidal potency.

The problem of semen variation among different donors has been pointed out by many investigators in the past (Baker, 1939; Brown and Gamble, 1940; Millman, 1952; Harris, 1962) and there appears to be no single solution. It has become our research practice to test each compound with three different donors' semen in order to minimize variations due to "susceptible" or "resistant" sperm (Table 6).

The large majority of spermicides fall into one of five categories with some considerable overlap(Sobrero, 1979); electrolytes; sulphydryl-binding substances; bactericides; surfactant agents; and enzyme inhibitors.

1) Electrolytes: Changes in spermatozoal activity and metabolism associated with change in tonicity has been reviewed by Blackshaw and Emmens (Blackshaw and Emmons, 1951).

2) Sulphydryl-binding compounds; These compounds exert their inhibitory action on cellular functions by several mechanisms, thus allowing their subclassification into oxidizing substances, mercaptide-foaming compounds, and alkylating compounds.

3) Bactericides: Substances that were highly active microbicidals would, like all disinfectants, act effectively against spermatozoa and also

protect against unwanted pregnancies and venereal diseases.

4) Surfactant agents: There are a large number of long-chain detergents and soaps possessing spermicidal activity. Their effect is the irreversible loss of motility and permanent disruption of the cell membrane. All surface-active agents exert a potent spermicidal effect in spite of their different membrane affinities, depending on their electric charge. Different surface-active agents—almost exculsively nonionic—are the principal ingredients of most currently available spermicidal products, worldwide. The most frequently found is Nonoxyno 1-9. Many of compounds tested in author's in vitro spermicidal test belong to this group.

5) Enzyme inhibitors; Many enzyme inhibitors are spermiostatic rather than spermicidal. Some do not have any effect on spermatozoal motility in vitro, but have proved effective in vivo. More recently, Joice, Freund and Peterson (Joyce et al, 1979) reported a series of experiment exploring the contraceptive activity of two acrosin inhibitors: TLCK (N-a-p-tosyl-L-lysine chlormethylketone HCL) and NPGB (p-nitro-phenyl-p-guanidinobenzoate) and two hyaluronidase inhibitors, compounds 53 D/K and phosphorylated hesperidin.

In author's experiment, in vitro spermicidal activities of 18 compounds were determined according to a modified method of Sander-Cramer. Nonoxynol-9 and compound 65/416 resulted in total immobilization of sperm in 20 seconds at 62.5 μg/ml and 125 μg/ml, respectively, while Quinine did not resulted in zero motility of sperm even at 250 μg/ml. Among all the compounds tested, Nonoxynol-9 and compound 65/416 was revealed to possess the most potent spermicidal activity.

This result was confirmed with another in vitro spermicidal test, the so-called comparative survival test(Suter, 1978). The modified Sander-Cramer Test is regarded as a simple effective in vitro testing procedure for the screening and evaluation of spermicidal potency of the active ingredient and the vaginal contraceptive formulation as compared to the so-called comparative survival test requiring long incubation.

Kamboj (1978) evaluated in vitro spermicidal activity of 12 compounds including 65/416, Nonoxynol-9 and Quinine. He used the Spot test in which the results were scored positive if 100% of the spermatozoa became immobile instantaneously, within 10 seconds. The minimum effective dose was considered as that concentration which killed or immobilized all the spermatozoa instantaneously. His result indicated that compound 65/416 showed spermicidal property upto a concentration of 0.003% in saline and 0.015% in buffer (Blackshaw and Emmens, 1951) (Sorenson's isotonic phosphate buffer, pH 7.1) in the Spot test. Nonoxynol-9 was spermicidal at a concentration of 0.05% in saline and 0.25% in buffer. None of another compounds including Quinine immobilized sperm at a concentration of less than 0.1% in physiologic saline in the Spot test. Therefore, his results were almost same as ours.

As briefly mentioned before, one of the important aspect in the development of new vaginal spermicides is that in combination with an in vitro screening technique, we must use an in vivo animal model in the selection of potential new active ingredients and dosage forms for future clinical evaluation. In the safety evaluation, in addition to assessing topical effects, we need to study the potential for absorption from vagina and the potential systemic effects of a product.

To date, the most fundamental question pertaining to preclinical efficacy studies remain unanswered. Is there a direct correlation between preclinical efficacy as judged by various in vitro and in vivo test procedures and clinical

efficacy as judged by valid clinical trials? It is reasonable to assume that any product which can demonstrate high standards of in vitro and in vivo preclinical efficacy may also show similar effects in clinical trials.

SUMMARY

This study was performed in an attempt to evaluate the spermicidal activity in vitro of various compounds against human spermatozoa.

First of all, several factors were studied for their influence of spermicidal activity in vitro. The methodology used for the in vitro testing of spermicidal agents was also reappraised.

Tests were carried out under the various pH of solution to determine the effect of pH on the motility of sperm. Our result indicated that sperm immobilizing activity was stronger in acidic (pH<7.0) than in alkaline(pH 7.0~8.5) solution at the same concentated test solution. However, spermicidal activity was effective even at an optimal pH for sperm migration in the cervix. It was felt to be logical to subject test preparations in the in vitro test to spermicides at the pH 7.0~8.0. The other tests were carried out to determine the effect of various diluents, MBS(Modified Baker's solution), RGP (Ringer's Glucose Phosphate) and Saline, on the activity of spermicidal agents. Modified Baker's Solution Buffer(pH 7.8) was suggested to be desirable as a diluent for serial dilution of spermicidal agents. Although the original Sander-Cramer Test does not indicate the number of semen samples that should be used for each evaluation, it appears that variability is likely to occur among semen donors. It has become the author's research practice to test each spermicidal agent with semen obtained from three different donors in order to minimize variations.

In the next experiment, in vitro spermicidal activities of 18 compounds provided by W.H.O. were determined according to a modified method of Sander and Cramer, which had been established on the basis of above mentioned preliminary experiments. The results of in vitro spermicidal test showed that of all the compounds tested, Nonoxynol-9 and Compound 65/416 possessed maxium spermicidal activity and they were considerably potent. Nonoxynol -9 and Compound 65/416 resulted in total immobilization of sperm in 20 seconds at 62.5 μg/ml, respectively. On the other hand, Quinine, Emetin, Cyto-A, Cyto-B, SDC, Typ., and Compound 102/198 belonged to the group of least effective spermicidal activity, which did not show zero motility of sperm even at 250 μg/ml.

Meanwhile, in order to evaluate the result of Modified Sander-Cramer Test, another in vitro spermicidal test, the so-called "Comparative Survival Test" was performed on Quinine, Compound 65/416 and Nonoxynol-9. From the result of this test, it was also revealed that either Nonoxynol-9 or Compound 65/416 exerted more potent spermicidal effect than Quinine.

=國 文 抄 錄=

殺精蟲劑의 作用力에 關한 in vitro 實驗

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이 硏究는 사람 精蟲에 대한 殺精蟲劑의 殺精蟲作用力(in vitro)을 評價하기 위하여 試行되었다.

우선 in vitro 殺精蟲 作用力에 影響을 미치는 여러 因子에 대하여 調査하였으며, 또한 殺精蟲劑의 in vitro test에 利用되는 方法들을 評價하였다.

精蟲運動性에 미치는 pH의 效果를 調査하기 위하여 여러가지 pH의 溶液下에서 試驗을 實施하였다. 그 結果 同濃度의 test 溶液에서는 알칼리性(pH 7.0~8.5) 溶液보다 酸性(pH<7.0)溶液에서 精蟲不動化 作用力이 더 强함을 認知하였다. 그러나 子宮頸管內 精蟲移動에

適切한 pH에 있어서도 殺精蟲作用이 効果的이었음으로 미루어 殺精蟲劑의 in vitro test에 있어서 試驗準備液을 pH 7.0~8.0下에서 實施하는 것이 妥當하다고 생각되었다. 다음 實施된 test는 各種 稀釋液인 modified Baker's solution(MBS), Ringer's glucose phosphate (RGP), saline등이 殺精蟲劑의 作用力에 미치는 効果를 觀察하는 것이었다. 그 結果 modified Baker's solution buffer (pH 7.8)가 殺精蟲劑의 連續的 稀釋을 위한 稀釋劑로서 바람직함을 알았다. 原來 Sander-Cramer test는 各 評價에 使用되어야하는 精液샘플의 數를 指摘하지는 않았으나 精液提供者間에 變異性이 있기 쉬우므로 著者의 研究施行에서는 이 變異性을 極小化하기 위하여 3名의 서로 다른 提供者로부터 採取한 精液을 사용해서 各殺精蟲劑를 test하였다.

다음 實驗으로서 上記한 豫備實驗을 基礎로 하여 確立된 Sander-Cramer變法을 利用하여 WHO에 의하여 提供된 18種의 殺精蟲劑의 in vitro 作用力을 測定하였다. in vitro 殺精蟲test의 結果를 보면 test된 製劑中 Nonoxynol-9과 Compound 65/416이 最高의 殺精蟲 作用力을 가졌으며 상당히 强力하였다. 即 Nonoxynol-9과 Compound 65/416은 62.5 μg/ml과 125 μg/ml에서 各各 20秒內 精蟲의 全不動化를 나타내었다. 한편 quinine, emetin, cyto-A, cyto-B, SDC, Typ., Compound 102/198은 250 μg/ml에서도 精蟲의 運動性 零을 나타내지 않아 殺精蟲作用이 가장 적은 群에 속하였다.

한편 modified Sander-Cramer test의 結果를 評價하기 위하여 또 다른 in vitro 殺精蟲 test인 所謂 "比較 生存力 test"를 quinine, Compound 65/416, Nonoxynol-9에 대하여 實施하였다. 이 test의 結果도 역시 Nonoxynol-9이나 Compound 65/416이 quinine보다 殺精蟲効果가 더 强力함을 나타내었다.

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