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SPERMICIDAL ACTIVITY OF NONOXYNOL-9 AND ANALOGS: QUANTITATIVE ASSESSMENTS BY FLOW CYTOMETRY

K. Yu[1], R. E. Bagdon and Y. W. Chien[2]
Controlled Drug-Delivery Research Center, College of Pharmacy, Rutgers University, 41-D Gordon Road, Piscataway, N.J. 08854.

E. J. Yurkow
Environmental and Occupational Health Sciences Institute, College of Pharmacy, Rutgers University, Piscataway, N.J. 08855

ABSTRACT

A flow cytometer-based method was developed for the quantitative assessment of spermicidal action of nonoxynol-9 (N-9) against human sperm. Two fluorescent dyes were chosen: carboxyfluorescein diacetate (CFDA) was employed as an indicator for viable sperm, while propidium iodide (PI) identified the sperm membrane integrity disrupted by spermicidal agents. Living, motile sperm were identified by the green fluorescence of CFDA, while the red fluorescence of PI reflected the ability of the spermicide to kill sperm. Both living and dead sperm were effectively resolved from the acellular components of seminal fluid. N-9 was used as a model spermicidal agent to study the feasibility as well as establish the

1. Recipient of Parke-Davis Graduate Fellowship (Current address: Cibus Pharmaceutical, Inc., 200 D Twin Dolphin Drive, Redwood City, CA 94065
2. To whom correspondence should be addressed.

various technical aspects of this method. Further studies with a series of structurally-related nonoxynol analogs, using the method established, demonstrated that N-9 is the most effective spermicidal agent. The spermicidal activity of N-9 and analogs was also determined by a a computer-assisted semen analysis (CASA) method for comparison. This conventional CASA method is designed to detect sperm motility, in contrast to the flow cytometry method which is capable of identifying the loss of membrane integrity. A discrepancy in the concentration needed for comparable spermicidal action between these assay methods was observed, which could imply that there is a concentration dependency on the disruption of membrane integrity and the complete loss of motility.

INTRODUCTION

One of the first tests developed to study the spermicidal activity of various agents was the Sander-Cramer method [1]. Today, variations of the Sander-Cramer method, utilizing advanced instrumentation introduced over the years, have been commonly employed as standard tests to examine the effects of pharmacological agents on sperm motility [2]. An example of such a method is the computer-assisted semen analysis method. Using contrast phase optics and image capture, sperm motility is normally determined for evaluation of fertility. A new approach to the assessment of sperm activity is the application of flow cytometry. The versatility of the data from this instrumentation is determined by the indicator dyes chosen. Flow cytometry has been extensively employed throughout reproduction research for the determination of semen quality and fertility, testicular neoplasia, as well as for gender preselection [3]. Flow cytometry analyzes large sperm populations on a cell-by-cell basis, consequently providing robust statistical data.

A flow cytometer is capable of measuring light scatter and fluorescence from each cell. Briefly, samples are delivered into a flow cell apparatus by a built-in syringe. In the apparatus, the individual cells pass through, single file, into a laminar flowing sheath fluid which acts to contain the sample core flow into a sensing area (Figure 1).

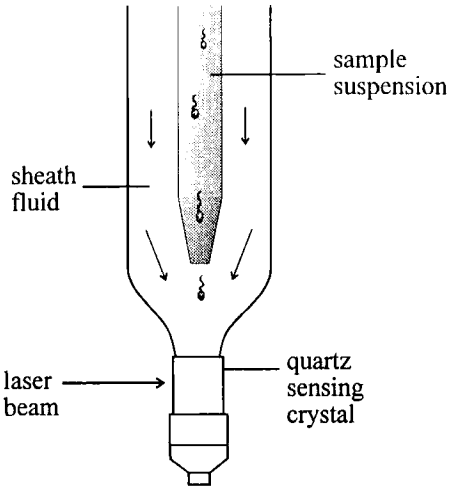


FIGURE 1

The flow cell apparatus of a flow cytometer. Cells flow single file past an argon laser that excites the fluorescent dyes labeling each cell.

There, the fluorescent-labeled cells are excited by an argon laser source. The resulting light scatter and fluorescence are filtered and collected at respective detectors (Figure 2). The detectors convert the light first to an electric current and then to a voltage. By analog-to-digital conversion, the light from each cell is assigned to a particular channel. Over a range of channels, a count for each of the channels is recorded, resulting in a histogram for each run. When two fluorescent materials are used, a dual-parameter histogram resembling a topographical map is created. The counts for each of the fluorescent signals are produced, allowing for the identification and quantitation of the individual cell characteristics.

Nonoxynol-9 exists as a homologous series of oligomers containing a 9-carbon alkyl chain and varying units of ethylene oxide. The purpose of this study was to establish flow cytometry as a sensitive and quantitative spermicidal assay method which can be applied to the evaluation of nonoxynol-9 and its structurally-related analogs, to gain more insight into the structure-activity relationship of spermicidal action.

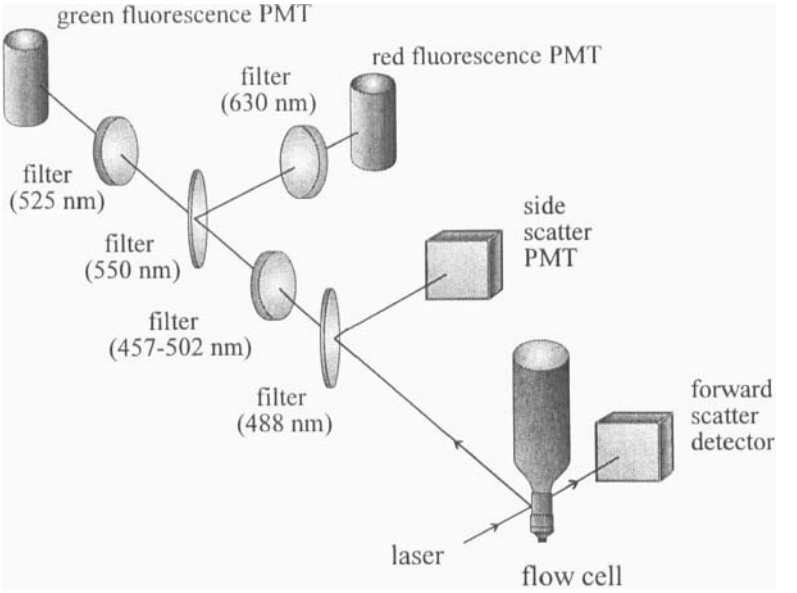


FIGURE 2

The fluorescence and light scatter path of a flow cytometer. The light scatter and fluorescence are directed to their respective detectors by appropriate filters.

MATERIALS AND METHODS

Chemicals

The pharmaceutical grade of nonoxynol-9 (CO-630SP) and its analogs (CO-610, CO-660, CO-720, CO-730, CO-850, CA-630G and DM-530) were obtained as samples from Rhône-Poulenc (Cranbury, NJ). All other HPLC grade solvents and chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Semen Specimen Preparation

Semen specimens were prepared using a Percoll density gradient separation technique (Pharmacia / Piscataway, NJ). This preparatory method has been found to improve the isolation of motile human spermatozoa, making the specimens free of the major contaminating constituents of seminal fluid [4]. For sperm isolation, Percoll was

diluted with modified human tubal fluid (Irvine Scientific / Santa Ana, CA) and a discontinuous gradient was generated in a sterile polystyrene centrifuge tube (15 ml) by carefully pipetting 1.0 ml of the 90, 70, and 50% Percoll solutions, respectively. Approximately 2 ml of the semen sample was layered over the gradient and the preparation was centrifuged at 300 x g and room temperature for 30 min. After centrifugation, the bottom half of the 90% layer, which contained the sperm, was isolated. To remove residual Percoll, the isolated sperm were resuspended in 2 ml of Ham's F-10 media (Gibco / Grand Island, NY) and centrifuged at 300 x g and room temperature for 5 minutes. The supernatant was removed and the resulting sperm pellet was resuspended in Ham's F-10 media. The specimen was analyzed, using a computer-assisted semen analyzer (Cryo Resources / New York, NY), to determine cell count. A portion of the specimen was then diluted to approximately 5 x 10^6 sperm/ml with Ham's F10 medium for analysis by flow cytometry. The time between acquisition of a semen sample and the analytical run averaged approximately 2 hours.

Fluorescent Staining

The esterase-specific dye, 6-carboxyfluorescein diacetate [(CFDA), Sigma Chemical Co. / St. Louis, MO], was used to label viable sperm. The functional groups of CFDA were modified by cellular esterases to produce a molecule with intense green fluorescence when excited with a 488nm light. In a typical experiment, CFDA was added to a sperm sample to a final concentration of 5 μM and incubated at 35 °C for 15 min. Aliquots (100 μl each) of the labeled sperm were then pipetted into polypropylene culture tubes (Fisher Scientific / Fair Lawn, NJ) each containing 400 μl of potassium phosphate buffer (pH 7.4) and either nonoxynol-9, a structurally related analog, or a nonoxynol-9 oligomer component. The sperm samples were exposed to the spermicide solutions for a period of 15 minutes prior to analysis. The phosphate buffer was made isotonic with NaCl and the isotonicity (300 mOsm) was confirmed using an osmometer (μOsmette, Precision Systems, Inc./ Natick, MA). Immediately before analysis, propidium iodide [(PI), Sigma Chemical Co. / St. Louis, MO] was added to each tube to a final concentration of 10 μM.

Flow Cytometry

Stained samples of sperm were analyzed using an EPICS Profile flow cytometer (Coulter Electronics, Inc. / Hialeah, FL) equipped with an Omnichrome 25 mW argon laser, which emits at 488 nm with 15 mW of power. After collection through a series of filters at 457-502 and 550 nm, green fluorescence signals were transmitted through a 525 nm band pass filter and processed through a linear amplifier. At the same time, red fluorescence signals were collected through a 630 nm long pass filter and processed through a four-decade logarithmic amplifier. Acellular debris was excluded from fluorescence analysis using forward angle and log 90° light scatter gates. Electronic color compensation was used to correct the signal overlap, which was observed in samples containing both red (PI) and green (CFDA) fluorescence. Using the filters and laser setting described above, a signal subtraction of 3% and 25% was used to correct the green and red fluorescence, respectively. A minimum of 10,000 gated events was collected for each sample. The histograms generated in these experiments were analyzed using EPICS CytoLogic Software (Version 2.0, Coulter Electronics, Inc. / Hialeah, FL).

Computer-Assisted Semen Analysis

For each analysis, a sample (40 μl) was pipetted from each spermicide solution into a glass tube containing 10 μl of sperm suspension. The sperm suspension was prepared by diluting a fresh semen sample from a donor with Ham's F10 media (Gibco / Grand Island, NY) to a sperm count of approximately 40-60 million sperm/ml. The spermicide-treated sperm suspensions were vortexed for 10 seconds and left undisturbed for another 30 seconds. A 10 μl sample was placed on a Makler chamber and analyzed by a computer-assisted semen analyzer (Cryo Resources / New York, NY), using 5 fields, for measurement of sperm count and sperm motility.

RESULTS AND DISCUSSION

Establishing Cytometry Parameters

Some preliminary experiments were conducted to examine feasibility and the results indicated that sperm samples contain

extraneous tissue and debris which obscures flow-cytometric analysis. In addition, the dead sperm which are present in normal semen samples may confound data interpretation, resulting in the overestimation of spermicidal activity. To address these concerns, a Percoll gradient was included as a preparatory step in the study protocol. Following the density gradient isolation procedure, the viable sperm emerged as a distinct population which permitted accurate analysis and provided a highly motile population ideal for the screening of spermicides.

For these experiments, the two fluorescent dyes were chosen for their ability to distinguish between viable and dead/dying sperm. CFDA readily enters living, intact cells. Once inside the cells, resident esterases cleave the diacetate functional groups, leaving a green fluorophore (carboxyfluorescein). On the other hand, PI cannot permeate into the viable cells, but can enter the cells to intercalate with DNA when the cellular membrane integrity has been compromised. Since membrane integrity is disrupted by the action of a surface-active spermicidal agent [5,6], the consequence of spermicidal treatment can be detected by the increase in the red fluorescing population under the appropriate instrumental settings.

The data generated are produced in the form of a dual parameter histogram. The green fluorescence is shown on a linear scale along the ordinate while the red fluorescence is displayed logarithmically along the abscissa. To obtain counts of the different cell populations, the histograms were separated into quadrants (Figure 3). It was established through optimization and validation experimentation that Quadrant 1 contained the highly green fluorescing sperm population, quantitating the viable sperm. Previous work evaluating bovine spermatozoa demonstrated that CFDA-stained sperm are viable and motile as well [7]. On the other hand, the events in Quadrant 2 comprised those cells that were stained with both green and red, having a significantly greater amount of red fluorescence. By fluorescence microscopic examination, these dual-labeled sperm were determined to be non-motile, while the sperm which stained exclusively with CFDA (Quadrant 1) were observed to be highly motile. Since PI is known to specifically stain the nuclei of dead cells, the increase in red

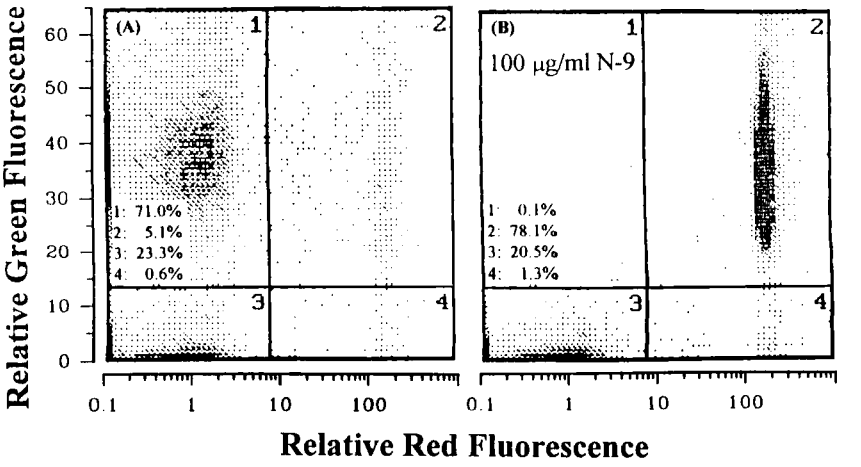


FIGURE 3

Dual-parameter histograms showing a non-treated sperm sample (A) and one treated with nonoxynol-9 at a concentration of 100 µg/ml (B). Quadrant 1 gives a count of the green fluorescing viable sperm while Quadrant 2 represents the red fluorescing dead/dying sperm. Quadrant 3 and 4 were found to be extraneous data consisting of acellular debris and non-viable sperm (prior to staining), respectively.

fluorescence reflected the ability of N-9 to kill the sperm. Figure 3 (B) demonstrates the change in the dual-parameter histogram resulting from the treatment of nonoxynol-9 (100 µg/ml). The shift in fluorescing character of the cells from green to primarily red is observed. The remaining Quadrants 3 and 4 were found to be acellular debris and non-viable sperm (prior to staining), respectively.

With the populations of each quadrant identified, the activity of various spermicides could be assessed using data collected from Quadrants 1 and 2. Since the events in Quadrants 3 and 4 were considered irrelevant to the assessment of spermicidal activity, they were excluded from data calculation. To account for the Quadrant 2 sperm that die without spermicide treatment, samples of sperm in buffer alone (blank) were analyzed in a minimum of triplicate. The mean value was then subtracted from each analytical run. The

spermicidal activity was calculated as :

$$\text{Percent Sperm Killed} = \frac{\left(N_{quad_2} - \bar{N}_{blank}\right)}{\left(N_{quad_1} + \left(N_{quad_2} - \bar{N}_{blank}\right)\right)} \times 100$$

where N is the number of sperm in the designated quadrant and $\bar{N}_{blank}$ is the average number of dead sperm found in Quadrant 2 using the blank buffer.

Assessment of Nonoxynol-9 Spermicidal Activity

The effect of increasing the concentration of nonoxynol-9 on the viability of sperm was determined using samples from three different donors (Table I). Nonoxynol-9 concentrations below 40 μg/ml were found to have no effect on sperm viability under these experimental conditions. At 60 μg/ml, 10.6% of the sperm sample was observed to be killed. Interestingly, a dramatic increase in spermicidal activity to 90% was noted between a narrow range of 60-65 μg/ml. At concentrations greater than 70 μg/ml, essentially all of the sperm from all three donors became non-viable (>98% sperm kill) following a 15-min. exposure. Concentrations of ≥80 μg/ml were found to kill 100% of the sperm within 1 minute. Furthermore, the results were found to be highly reproducible with minimal variation among the donors.

Screening Potential of Spermicidal Agents

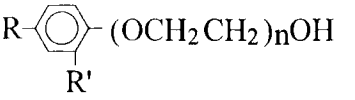
The screening potential of this cytometer-based technique was investigated by measuring the spermicidal activity of nonoxynol-9 and a series of structurally-related analogs which vary in either carbon chain length or ethylene oxide unit. The structure of these compounds tested are given in Figure 4. Nonoxynol-9 and its analogs all exist as a mixture of oligomers differing in the chain length of ethylene oxide units. The distribution of these oligomers follow a Poisson pattern in which the number of ethylene oxide units giving the highest concentration yields the product nomenclature (*i.e.*, nonoxynol-9 has 9 units of ethylene oxide). For these experiments, samples were

Table I

The Dose-Dependent Spermicidal Activity of Nonoxynol-9

Concentration	Percent Sperm Killed (%)[1]			
(μg/ml)	Donor 1	Donor 2	Donor 3	Mean (± s.d.)[2]
20	0.0	0.0	0.0	0.0 (0.0)
40	0.0	0.0	0.0	0.0 (0.0)
60	12.0	9.8	9.7	10.6 (1.4)
65	99.4	86.7	84.8	90.3 (7.9)
70	100.0	99.5	96.8	98.8 (1.7)
75	100.0	99.8	97.9	99.2 (1.2)
80	100.0	99.9	99.9	99.9 (0.1)
100	100.0	99.9	99.9	99.9 (0.1)

1) Assayed by the flow cytometric analysis.
2) Mean (± standard deviation) of triplicate determination from 3 donors.



Analog	R	R'	n
CO-610	C_9H_{19}		7-8
CO-630SP	C_9H_{19}		9
CO-660	C_9H_{19}		10
CO-720	C_9H_{19}		12
CO-730	C_9H_{19}		15
CO-850	C_9H_{19}		20
CA-630G	C_8H_{17}		9
DM-530	C_9H_{19}	C_9H_{19}	9

FIGURE 4

The general chemical structure of nonoxynol-9 analogs evaluated. n gives the mean number of ethylene oxide units.

performed in triplicate with a semen sample from a single donor (Donor 1). The results on the percent sperm killed attained by each analog at the same concentration range (60 and 100 μg/ml) are compared in Table II. The analogs CO-730, CO-850, CA-630G and DM-530 exhibited significantly lower spermicidal activity with 0.5 to 65.5% sperm killed at 100 μg/ml, compared to virtually 100% spermicidal activity with nonoxynol analogs with 7-12 units of ethylene oxide. At lower concentrations (60 μg/ml), a structure-activity relationship was observed for these nonoxynol analogs (Table II) in which the greatest spermicidal potency is achieved by the nonoxynol analog having 9 units of ethylene oxide (CO-630SP, nonoxynol-9). The spermicidal activity of the nonoxynol analogs then declines as the ethylene oxide chain increases or decreases in its length.

Comparison to a Computer-Assisted Semen Analyzer

Computer-assisted semen analyzers (CASA) have been commonly used in fertility assessment, with motility as the primary parameter. This apparatus uses image capture and phase contrast optics for analysis. The spermicidal activities of nonoxynol-9 and other spermicidal agents have been evaluated using similar instrumentation [8]. In contrast to this, the flow cytometry method was developed to assess the spermicidal activity by the loss of membrane integrity. A comparative study was conducted and the are shown in Table III. In this study, the concentrations of nonoxynol-9 (CO-630SP) that produced 100% spermicidal activity in each method was used for all the nonoxynol analogs: 250 μg/ml for the CASA analysis and 100 μg/ml for the flow cytometry analysis. The spermicidal activities obtained by the two methods were found to be comparable even though the concentrations differed by 2.5 times. Both of these methods used similar sperm counts and concentration per sample, but differed in the time of spermicide exposure prior to analysis (CASA: 30 seconds, flow cytometry: 15 min.). It should be pointed out that complete spermicidal activity has been found for nonoxynol-9 concentrations ≥ 80 μg/ml within 1 minute when analyzed by flow cytometry. The difference in spermicidally-effective concentrations determined by

Table II

Comparative Spermicidal Activities of Nonoxynol-9 and Its Analogs

	Percent Sperm Killed (% ± s.d.)	
ANALOG	60 μg/ml	100 μg/ml
CO-610	5.5*	99.7 (0.3)
CO-630SP	21.6 (8.0)	99.9 (0.0)
CO-660	15.4*	99.9 (0.0)
CO-720	2.6 (1.3)	99.9 (0.0)
CO-730	1.5 (1.6)	65.5 (12.4)
CO-850	—	2.3 (2.2)
CA-630G	—	60.6 (8.7)
DM-530	—	0.5 (0.6)

*Single data points obtained in separate experiments.

Table III

Comparison of Spermicidal Assay Data Between Flow Cytometry and Computer Assisted Semen Analysis (CASA)

	Percent Sperm Killed (% ± s.d.)*	
ANALOG	CASA	Flow Cytometry
CO-610	94	99.7 (0.3)
CO-630SP	100	99.9 (0.0)
CO-660	99	99.9 (0.0)
CO-720	96	99.9 (0.0)
CO-730	64	65.5 (12.4)
CO-850	60	2.3 (2.2)

* data determined from two replicate samples (250 μg/ml) for CASA and triplicate (100 μg/ml) for flow cytometry.

these two types of instrumentation may be an indication that the mechanism of spermicidal action is concentration dependent for these nonoxynol-type agents. The data suggests that the sperm may first undergo the disruption of membrane integrity produced by nonoxynol analogs at lower concentrations (100 μg/ml), while total cessation of sperm motility is achieved when high enough concentrations (250 μg/ml) are reached. Nonoxynol-9 has been demonstrated by ultrastructural studies to significantly disrupt sperm plasma membrane at a concentration of 500 μg/ml [5,6]. While flow cytometry offers the capability of analyzing a large population of sperm on a cell-by-cell basis, the data from the two instruments complement each other in the overall evaluation of spermicidal activity.

Overall, this investigation demonstrated that flow cytometry provides a powerful analytical system for the quantitative assessment of new and old spermicidally-active agents.

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