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Assessment of Spermatozoal Function Using Dual Fluorescent Staining and Flow Cytometric Analyses[1]

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ABSTRACT

Spermatozoa from bulls, boars, dogs, horses, mice, and men were examined using a fluorogenic stain consisting of the membrane-permeant substrate carboxyfluorescin diacetate (CFDA) and the relatively membrane-impermeant nuclear stain propidium iodide (PI). Three distinct populations of spermatozoa were discernible in samples from each species upon microscopic examination. Individual spermatozoa, presumed to be viable because of their motility, retained products of the fluorescein chromophore throughout the cell. A second population of spermatozoa in which the nuclei stained red with PI retained the green fluorescein fluorophore mainly in the acrosome. A third population, presumed to be degenerate spermatozoa, possessed only red fluorescent nuclei. These populations were quantified using dual parameter flow cytometry in 14 samples of cryopreserved bovine spermatozoa for which fertility and seminal quality data were available. Flow cytometric analyses were highly correlated with other seminal quality measurements. Sequential flow cytometric analyses provided the ability to rapidly quantitate changes in specific fluorescently stained populations. The ability to make rapid quantitative measurements should allow development of new and presumably more reliable information on the functional aspects of spermatozoa.

INTRODUCTION

Rapid and accurate assessment of spermatozoan viability is important in determining seminal quality. Methods used to evaluate the viability of spermatozoa have been reviewed by Pace et al. (1981). Most of the currently used procedures are very time-consuming and evaluate relatively few spermatozoa. Supravital stains, which are known also as "live-dead stains," generally consist of eosin Y or an eosin derivative alone or in combination with either nigrosin or analine blue (Elliot, 1978). The discriminatory ability of these supravital stains is dependent on the ability of functionally intact membranes to exclude the eosin chromophores from the nuclear compartment of the spermatozoa. An additional problem with classical supravital stains is that they are not appropriate for assessing viability in cryopreserved spermatozoa because glycerol, a common and necessary ingredient of most cryopreservation media, interferes with the stain (Mixner and Saroff, 1954).

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Assessment of spermatozoan viability is difficult when the cells are suspended in opaque media. This problem can be overcome somewhat by using fluorescent stains (VanDemark et al., 1959). More recently, fluorescent stains have been used either singularly (Babcock, 1980, 1983) or in combination (Evenson et al., 1982) to assess some cellular functions of spermatozoa. Specific combinations of fluorophores could provide quantitative data provided that the relative membrane permeabilities and staining specificites of the fluorophores were discriminatory between functional and nonfunctional spermatozoa. A combination of 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) could provide quantifiable information on functional aspects of spermatozoa due to their molecular characteristics. The CFDA fluorophore, which readily permeates membranes, is nonfluorescent. However, hydrolysis of the ester bonds results in formation of a highly fluorescent, membrane-impermeant green fluorophore. Intracellular esterases readily hydrolyze CFDA and the resultant product, 6-carboxyfluorescein, is trapped within cellular structures possessing intact membranes. The counterstain, PI, is a bright red, nucleic acid-specific fluorophore. This fluorophore is relatively impermeant to membranes and thus does not readily stain nuclei of viable spermatozoa. Our preliminary evidence indicated that the combination of CFDA and PI could be used for semen from several mammalian orders (Garner and Johnson, 1984; Garner et al., 1984; Johnson and Garner, 1984). This particular differential stain is similar to the fluorescein diacetate and propidium iodide combination used by Resli et al. (1983) to qualitatively assess the functionality of bovine spermatozoa.

Flow cytometry offers a means for rapid and systematic quantification of the spermatozoal populations differentially stained with the CFDA and PI combination (Melamed et al., 1979). Thus, a combination of dual fluorescent staining and flow cytometric analysis may make it possible to quantitatively assess spermatozoan viability and potential fertility. The specific objectives of this study were: 1) to assess the staining characteristics of spermatozoa representing several mammalian orders using a combination of CFDA and PI, and 2) to determine the quantitative relationships among subpopulations of bovine spermatozoa identified by fluorogenic staining and flow cytometry to those characterics measured by classical semen evaluation methods.

MATERIALS AND METHODS

Materials

The CFDA (6-carboxyfluorescein diacetate), PI [2, 7-diamino-9-phenyl-10-(diethylaminopropyl)-phenanthridinium iodide methiodide], and Miracloth were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Digitonin and BAEE (*N*α-benzoyl-L-arginine ethyl ester hydrochloride) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade. The stock solution of 6 mM CFDA was prepared by dissolving 4 mg in 1 ml of dimethylsulfoxide (DMSO). A 0.58 mM solution of PI was prepared in 0.1 M potassium phosphate buffer (pH 7.1).

Procurement of Spermatozoa

Samples of ejaculated spermatozoa were obtained from bulls, boars, dogs, horses, and men. Samples of bovine and equine semen were collected using artificial vaginas, whereas the canine semen was collected by manual manipulation. The human ejaculates were donated by healthy young volunteers and were collected by masturbation. The samples of boar semen were collected by the gloved-hand technique from mature boars of proven fertility and were cryopreserved as described by Johnson et al. (1981). Epididymal mouse spermatozoa were obtained from freshly removed epididymides. The isolated epididymides were minced in 1 ml of 0.11 mM sodium citrate to facilitate sperm recovery. Residual tissue debris was removed from the spermatozoa by passing the minced sample through Miracloth.

Sample Preparation and Quality Evaluations

Cryopreserved samples of bovine spermatozoa from the same semen samples of the 14 bulls that were used by Pace et al. (1981) to compare several viability assays to fertility were obtained for the present study. Data on the 14 samples, including fertility as determined by 90-day nonreturn to estrus rates, were obtained from the 1981 study. The seminal quality measurements included progressive motility as determined by the photographic method of Elliott et al. (1973) and acrosomal morphology as described by Saacke and Marshall (1968) and modified by Pace and Sullivan (1978), and proacrosin content was estimated from acrosin (EC 3.4.21.10) activity expressed as International Units [1 IU equals the hydrolysis of 1 μmol of BAEE/(min·10^9 sperma-

tozoa)]. These quality evaluations were made at post-thaw examination and again after 1.5 h for motility and after 3 h incubation at 37°C for the remaining parameters.

Additional cryopreserved samples of bovine spermatozoa were used to determine the relationship of the fluorogenic staining properties to the functional aspects of spermatozoa. Membrane disruption of stained spermatozoa was carried out using a stock solution of digitonin (2.6 mM in ethanol) (Babcock, 1983). The stock solution was added to stained spermatozoal suspensions in 0.11 mM sodium citrate to yield a final concentration of 0.1 mM digitonin. Suspensions containing an increased proportion of motile spermatozoa were obtained from aliquots of these samples by using the swim-up technique of Blazak et al. (1982).

Fluorogenic Staining of Spermatozoa for Microscopic Examination

Seminal samples were diluted with about 2 vol of 0.11 mM sodium citrate (22 ± 2°C) before sequentially staining the spermatozoa with CFDA and PI. Ten microliters of CFDA stock solution (4 mg/ml of DMSO) were added to each diluted sample and uniformly distributed within the sample by careful repipetting. After 15 min, 10 μl of PI stock solution (0.27 mg/ml in phosphate buffer) were added, the sample was mixed, and the sperm cells were collected by centrifugation at 500 × *g* for 5 min. Each supernatant was aspirated and the resultant pellets were resuspended in 1 ml of isotonic sodium citrate to which 10 μl of PI stock solution had been added. In some cases samples were processed using only half of these volumes. Each sample was prepared for microscopic examination by pipetting 5 μl of the stained sperm suspension onto a clean slide and covering it with a 24 × 50-mm coverglass to form a very thin film. The prepared slides of stained spermatozoa were examined using an HBO 50 mercury lamp illuminated, epi-fluorescence Zeiss Universal microscope equipped with a fluorescein isothiocyanate (FITC) filter set. This set consisted of a BP 485/20 excitation filter, an FT 510 dichromatic beam splitter, and an LP 520 barrier filter. Photomicrographs of the stained sperm were taken using Kodak Ektrachrome 400 slide film.

Combined Fluorogenic Staining and Flow Cytometric Analyses

Only the cryopreserved bovine semen samples were analyzed by dual parameter flow cytometry. The 14 samples of bovine semen for which the fertilizing capacity had been measured previously (Pace et al., 1981) were thawed by immersion of the sealed straws in warm water (37°C) for 30 s. Each sample was extruded into 2 vol of isotonic sodium citrate (22 ± 2°C). For each sample a portion containing one-half of the thawed spermatozoa was removed, pipetted into a 1.5 ml polypropylene microcentrifuge tube, sealed, and placed in an oven at 37°C to incubate for 3 h before fluorogenic staining. The remaining thawed spermatozoa were stained immediately with CFDA and PI as previously described for microscopy. The stained samples were allowed to reach equilibrium by standing (22 ± 2°C) for a minimum of 15 min before flow cytometric analysis. Approximately 100,000 sperm cells per sample were measured at a rate of about 500 cells/s. Two fluorescence parameters were measured simultaneously as each sperm cell passed through the flow cytometer (Fig. 1). The stained cells were excited at 488 nm and both green CFDA (560 nm short pass and Corning 3–69 filters) and red PI (Corning 2–73 filter) fluorescence were measured (Melamed et al., 1979). Each fluorescence intensity was digitized to a resolution of 64 intensity levels. The number of cells in a sample with a particular combination of red and green fluorescence intensity was stored in a two-dimensional array. Each analysis was completed within a 20-min period of staining.

Quantification of Fluorescent Spermatozoal Populations

Spermatozoa in each sample could be grouped into characteristic subpopulations based on the fluorescence measurements. These subpopulations were quantified by computer analyses using a DEC PDP 11/44 computer (Digital Equipment Corp., Maynard MA) and the SUMSAV software program (Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, CA). The background fluorescence from cellular debris was excluded from the spermatozoal populations by including only those fluorescence combinations having greater than 50 events per 100,000 measurements. Comparisons among subpopulations were made using the information from the initial post-thaw cytometric evaluations and those obtained from aliquots of the same samples after incubation for 3 h. Indices were developed to take into account incubative increases in fluorescently stained populations of presumably degenerative sper-

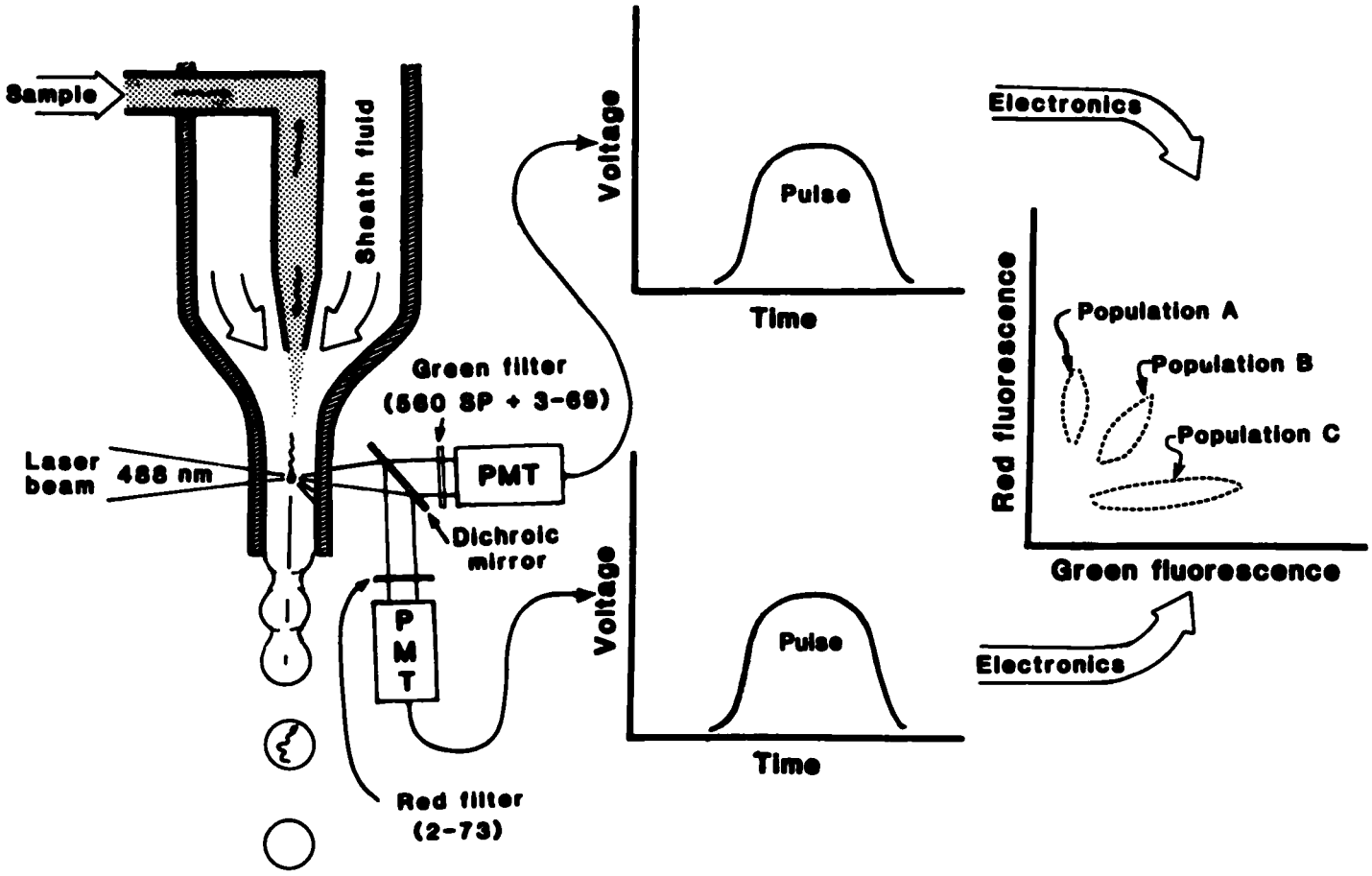

FIG. 1. Schematic illustration of a dual-parameter flow cytometer showing laser beam illumination (488 nm) of the flow chamber and the dual fluorescent light detectors (photomultiplier, *PMT*). The emitted light is split by a dichroic mirror so that green and red fluorescence can be measured individually. The spermatozoa are carried in a fast-moving flow of fluid past the optical detection system where the emitted fluorescent light is measured by the PMTs. The red and green fluorescence from each sperm cell is quantified by pulse height analyzers. The green versus red fluorescence is digitized across 256 channels and stored on magnetic tape for quantitative comparisons.

matozoa and decreases in the apparently functional spermatozoa.

Statistical Methods

Simple correlations were used to establish relationships between the classical spermatozoal quality traits (Daniel and Wood, 1971) and the flow cytometric measurements (Steel and Torrie, 1960). Correlation coefficients between the seminal quality measurements and fertility were calculated according to the Statistical Analysis System (Barr et al., 1982).

RESULTS

Dual Fluorescent Staining of Mammalian Spermatozoa

Microscopic evaluations of the fluorescently stained spermatozoa showed similar patterns for all five mammalian orders (Fig. 2). Motile spermatozoa retained the carboxyfluorescein fluorophore throughout the cytoplasm of the cell and did not stain with PI. Some spermatozoa, presumably moribund cells, also retained the green fluorophore throughout the cytoplasm but were immotile. The percentage of spermatozoa that retained the carboxyfluorescein increased when samples were enriched by the swim-up technique (Blazak et al., 1982). A second subpopulation of spermatozoa retained the green fluorophore within the acrosome, but lacked notable staining within the cytoplasm of the cell. None of the spermatozoa in this subpopulation were motile. Although a portion of the cells within this subpopulation retained some green fluorophore within their mitochondria, the nuclei of all cells within this subpopulation stained red with PI. A third subpopulation of spermatozoa, which was presumed to be degenerative because prolonged digitonin treatment shifted all cells to this population, possessed

TABLE 1. Spermatozoal quality evaluations of cryopreserved bovine semen from 14 bulls as determined from initial post-thaw assessments and after 1.5 or 3 h incubation at 37.5°C.

Bull sample	Progressive motility (%)		Intact acrosomes (%)		Normal acrosomes (%)		Proacrosin content (IU*)	
	Initial	1.5 h	Initial	3 h	Initial	3 h	Initial	3 h
A	55	35	90	73	77	59	13.9	13.0
B	58	36	84	64	60	53	11.6	12.5
C	62	39	89	57	70	32	13.1	11.3
D	36	21	70	45	42	26	12.0	10.6
E	59	28	84	64	70	50	12.8	14.3
F	44	16	82	43	49	34	11.9	9.8
G	59	37	88	60	56	48	10.8	11.3
H	39	19	80	45	58	36	11 8	11.3
I	44	25	80	45	38	29	10.3	9.2
J	53	17	83	40	56	28	11.5	9.0
K	42	26	74	51	45	42	13.9	12.4
L	49	22	84	44	67	33	10.3	9.2
M	67	49	89	66	60	53	14.5	13.2
N	55	36	84	55	51	39	13.4	12.0

*BAEE hydrolysis expressed as International Units(min·10^9 spermatozoa).

only brightly stained red nuclei. The brightness of the spermatozoal nuclei that stained red with PI is not evident in the micrographs shown in Fig. 2.

Classical Seminal Quality Evaluations

The results of analyses of the cryopreserved samples from 14 bulls are given in Table 1. The percentage of progressively motile spermatozoa at post-thaw evaluation averaged 51 ± 9% and ranged from 36% to 67%. Evaluation of aliquots of the samples following incubation of the samples at 37.5°C for 1.5 h showed that the average dropped to 29 ± 10% with a range of 16% to 49%. Initially, the percentage of spermatozoa with intact acrosomes was 83 ± 6% and the range was 70% to 90%. Following incubation for 3 h the average was 54 ± 10% and the range was 40% to 73%. The subcategory of percentage of spermatozoa with normal acrosomes (intact apical ridges) averaged 57 ± 11% and ranged from 38% to 77% initially. After incubation the percentage of spermatozoa with normal acrosomes dropped to 40 ± 11% with a range of 26% to 59%. The initial proacrosin content of spermatozoa averaged 12.2 ± 1.4 IU/(min·10^9 cells) and ranged from 10.3 to 14.5 IU/(min·10^9 spermatozoa). The average dropped to 11.4 ± 1.7 IU/(min·10^9 spermatozoa) with a range from 9.0 to 14.3 IU/(min·10^9 spermatozoa) following 3 h at 37.5°C.

Initial Dual Parameter Flow Cytometric Analyses

Cryopreserved bovine semen samples were evaluated by flow cytometric analyses following staining with CFDA and PI. Three rapidly discernible populations of spermatozoa, as had been noted using fluorescence microscopy, were detected also using flow cytometric analyses. These three populations were termed spermatozoal populations A, B, and C. The A population consisted of spermatozoa with brightly stained red nuclei. The B population contained spermatozoa in which the nuclei had stained red with PI, but some green fluorophore was retained within the acrosome and/or mitochondria. The C population consisted of those spermatozoa that had retained the carboxyfluorescein throughout the cytoplasm and excluded PI. These three populations, which were quantified by computer analyses of each flow histogram, are shown in Fig. 3. This example shows that, with the threshold set at 50 counts, the total spermatozoa analyzed for this sample was 86,871. Population A accounted for 9300 cells, population B some 12,900 cells, and population C the majority with 61,700 spermatozoa. The lines on Fig. 3 that encompass population C in the shape of a trapezoid are computer traces that were used to define the area quantified as population C. The other two spermatozoal populations were quantified similarly.

The shift in spermatozoal populations that had been seen by microscopic examination as a result of

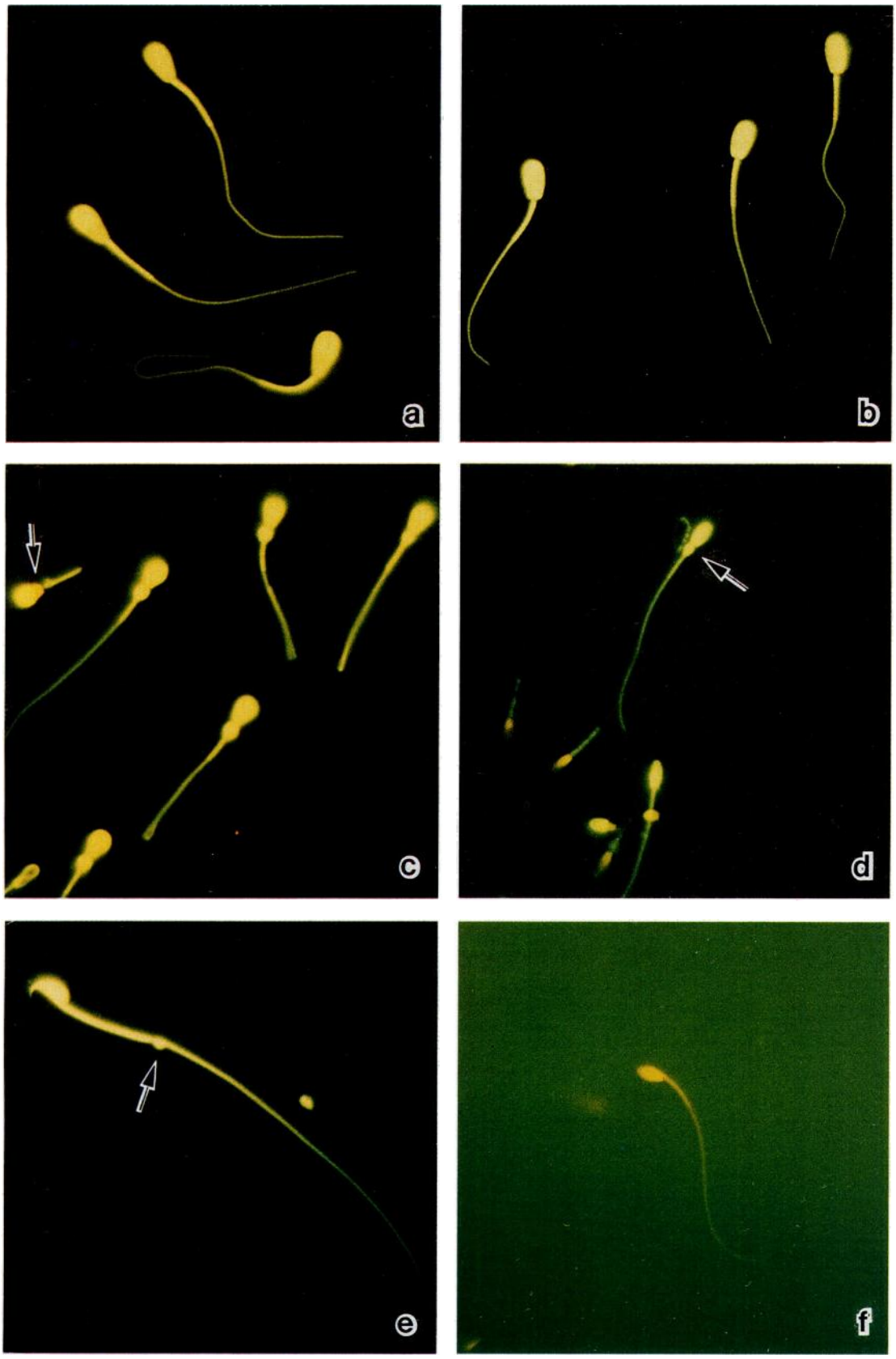

FIG. 2. Micrographs of bovine (*a*), porcine (*b*), canine (*c*), equine (*d*), murine (*e*), and human (*f*) spermatozoa that had been stained sequentially with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) and viewed with an epifluorescence microscope (×800). Spermatozoa with intact membranes retained the CFDA hydrolysis product carboxyfluorescein and fluoresced bright green. Although not evident in these micrographs, the nuclei of spermatozoa that have lost membrane integrity stained bright red with PI. Both bovine (*a*) and porcine (*b*) spermatozoa were from cryopreserved samples that were thawed just prior to staining. Only spermatozoa that had retained the green fluorophore are shown. The ejaculated canine spermatozoa that were examined (*c*) had prominent cytoplasmic droplets. One canine spermatozoon (*c, arrow*) retained the carboxyfluorescein within the acrosome and mitochondria but had lost the fluorophore from the cytoplasm. The nucleus had stained with PI. Proximal cytoplasmic droplets were noted also on some of the ejaculated equine spermatozoa (*d, arrow*). Epididymal murine spermatozoa possessed distal protoplasmic droplets (*e, arrow*). Human spermatozoa also stained with CFDA-PI, but notable background staining occurred that was apparently due to residual seminal plasma esterases.

the swim-up enrichment or the digitonin treatment was confirmed by flow cytometry. Enrichment by swim-up increased the proportion of cells in population C by only 7%, but the membrane disruption resulting from exposure to digitonin reduced this population to 0%. Digitonin treatment increased the proportion of spermatozoa in subpopulation B by 28% and that in A by 30%. The rate of redistribution and the final sample composition was dependent on digitonin concentration and exposure time (data not shown).

Incubative Changes as Quantified by Dual Parameter Flow Cytometric Analyses

The flow cytometric quantification of the fluorescently stained spermatozoal populations from the 14 samples of known fertility were carried out immediately upon thawing and again after aliquots of the samples had been diluted with isotonic sodium citrate and incubated at 37°C for 3 h before staining. The percentage change from the initial values obtained for spermatozoal populations A, B, and C for the 14 samples are shown in Fig. 4. The bulls were ranked according to their field-tested fertility with Bull A having the highest and Bull N having the lowest percentage of nonreturn to estrus after 90 days. A decrease in population C and an increase in the proportion of cells in the combined populations A and B was noted for samples from all but one bull. Only the sample from Bull H showed an opposing trend during incubation. Differences in the magnitude of incubationally induced changes were noted among the samples from other bulls. These changes tended to be less with samples from the higher fertility bulls.

Relationship among Seminal Quality Evaluations and Flow Cytometric Analyses

Simple correlation coefficients between the percentage of spermatozoa falling into each seminal quality category are given in Table 2. Companion data in which the measurements were expressed as the number of spermatozoa contained in the cryopreserved sample (0.5-ml straw) possessing a given characteristic are given in Table 3. Both percentage ($r = 0.85$, $p<0.01$) and number ($r = 0.65$, $p<0.05$) of progressively motile spermatozoa were correlated with the values obtained for intact acrosomes. Likewise, the percentage of spermatozoa with intact acrosomes ($r = 0.84$, $p<0.01$) and normal acrosomes ($r = 0.81$, $p<0.01$) was correlated with proacrosin content.

Comparisons made between the flow cytometric analyses of the CFDA- and PI-stained spermatozoa and the other seminal parameters revealed important relationships. The percentage of spermatozoa in population A was negatively correlated with percent-

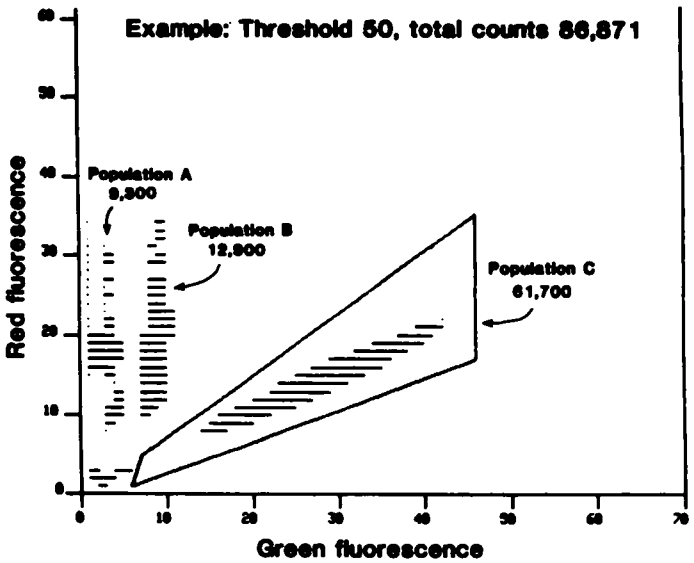

FIG. 3. Dual parameter analysis of a sample of cryopreserved bovine spermatozoa that had been stained with carboxyfluorescein diacetate and propidium iodide. Of the approximately 100,000 spermatozoa analyzed, 86,871 were above the background 50 counts per channel threshold, 9300 were in population A, 12,900 were in population B, and 61,700 were in population C. The lines that encompass population C in the shape of a trapezoid are a computer trace to define the area to be quantified as population C.

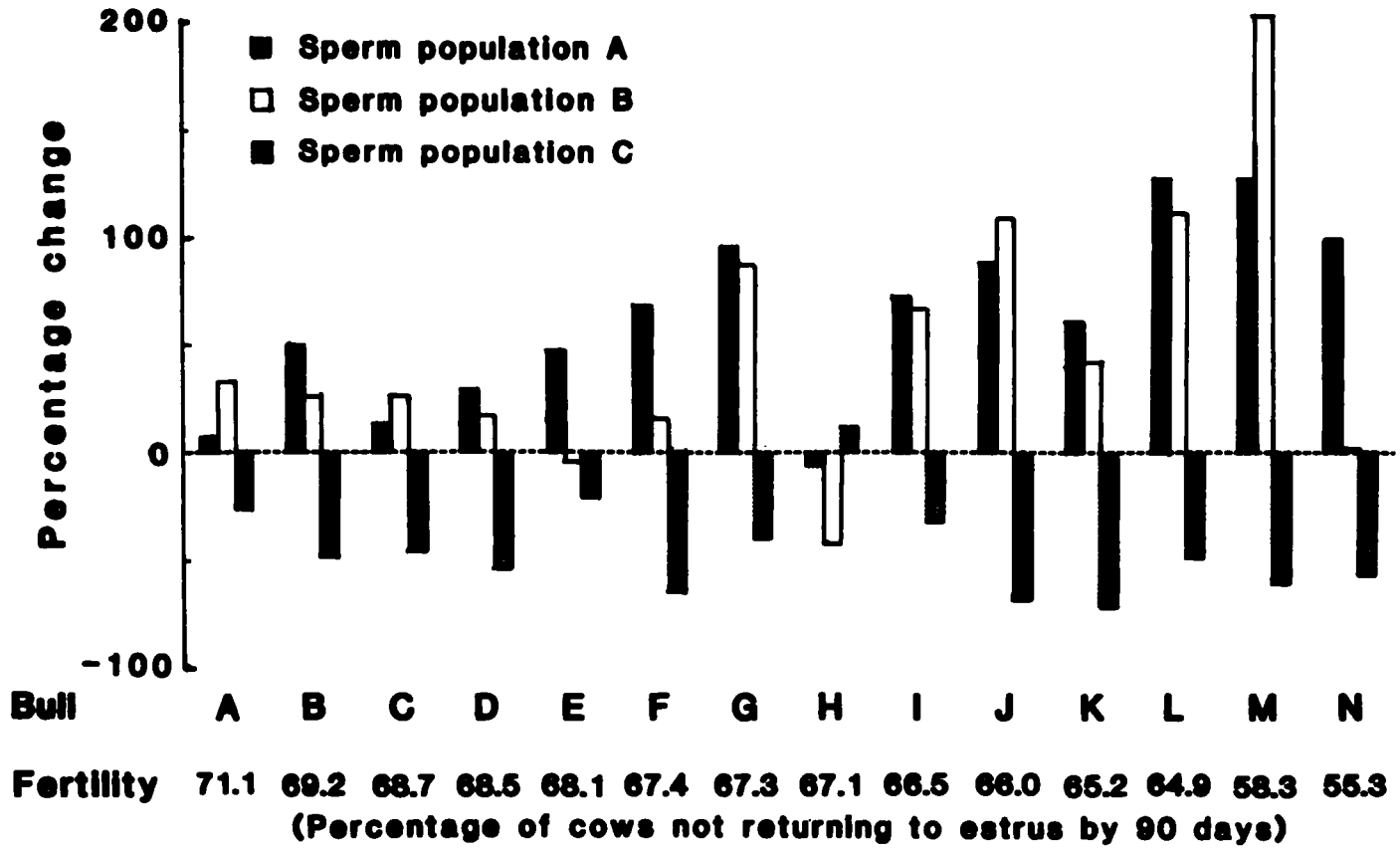

FIG. 4. Spermatozoal population shifts occurring during incubation of thawed, cryopreserved bovine spermatozoa of known fertilizing capacity as determined from differences in flow cytometric analyses of populations A (*solid columns*), B (*open columns*), and C (*striped columns*) conducted immediately after thawing and again after 3 h at 37°C. Aliquots of spermatozoa were stained with carboxyfluorescein diacetate and propidium iodide and analyzed by flow cytometry. Data are shown as the percentage of change from the quantitative values obtained immediately after thawing to those obtained from aliquots incubated for 3 h prior to staining and analysis. The samples were from 14 Holstein bulls (A through N) in which fertility data (90-day nonreturn to estrus rates, percentage) were available from 16,474 units of semen.

age of progressively motile cells ($r = -0.61$, $p<0.05$) and with the percentage of spermatozoa with intact acrosomes ($r = -0.77$, $p<0.01$) (Table 2). The number of spermatozoa in population B was correlated only with initial proacrosin content ($r = 0.54$, $p<0.05$) (Table 3). However, the percentage of spermatozoa in population C was positively correlated with the percentage of spermatozoa possessing progressive motility ($r = 0.55$, $p<0.05$) and having intact acrosomes ($r = 0.60$, $p<0.05$), but negatively correlated with populations A ($r = 0.84$, $p<0.01$) and B ($r = -0.76$, $p<0.01$) (Table 2).

Other interrelationships became evident when the evaluations were carried out on aliquots that had been incubated for 3 h. Examination of the relationships among the populations of spermatozoa quantified by flow cytometry revealed that the percentage of spermatozoa in population A was negatively correlated ($r = -0.71$, $p<0.01$) with the percentage of spermatozoa in population C.

Fluorescent Staining of Spermatozoa

Several methods for measuring sperm cell function using specific fluorescent probes have been developed recently (Evenson et al., 1980; Blazak et al., 1982; Babcock, 1983). Furthermore, combinations of fluorogenic probes such as fluorescein diacetate and PI can be useful in ascertaining sperm cell functionality (Resli et al., 1983). Although the noncarboxylated fluorescein derivatives such as fluorescein diacetate permit assessment of cell viability, the carboxylated derivatives have greater utility because they are retained inside the cell much longer (Lizak and Grumet, 1980; Tangelder et al., 1982). Our work followed that of Babcock (1980, 1983) in using CFDA to measure functional aspects of spermatozoal organelles and membranes. Our flow cytometric analyses using the combined fluorophores indicated that digitonin treatment resulted both in the loss of carboxyfluorescein from the spermatozoal cytoplasm and in staining of the nuclei with the membrane-impermeant probe PI.

TABLE 2. Correlation coefficients between the percentage of spermatozoa with a specific characteristic and the fluorescent spermatozoal populations (A, B, and C) as determined by flow cytometric analyses of CFDA- and PI-stained bovine spermatozoa.

Parameter	Progressive motility	Intact acrosomes	Normal acrosomes	Proacrosin content	Fluorescent population A	Fluorescent population B	Fluorescent population C	Difference A	Difference B
Initial									
Intact acrosomes	0.85**								
Normal acrosomes	0.61*	0.73**							
Proacrosin	0.35	0.19	0.28						
Population A	−0.61*	−0.77**	−0.29	0.05					
Population B	−0.25	−0.12	−0.015	0.23	0.31				
Population C	0.55*	0.60*	0.26	−0.11	−0.84**	−0.76**			
3 hours									
Intact acrosomes	0.82**								
Normal acrosomes	0.64*	0.91**							
Proacrosin	0.61*	0.84**	0.81**						
Population A	−0.53	−0.58*	−0.58*	−0.21					
Population B	0.44	0.19	0.13	0.09	−0.24				
Population C	0.28	0.45	0.39	0.14	−0.71**	−0.38			
Indices									
A Difference 3 h	0.02	−0.25	−0.34	−0.24	0.01	−0.42	0.24		
B Difference 3 h	0.55*	0.38	0.09	0.09	−0.50	−0.70**	0.74**	0.39	
C Difference 3 h	0.39	0.21	−0.10	0.08	−0.47	−0.56*	0.63*	0.72**	0.85**

*Significantly different ($p<0.05$) from zero.

**Significantly different ($p<0.01$) from zero.

The population shifts that we quantified by flow cytometry following treatment of fluorescently stained spermatozoa with digitonin support the conclusions of Babcock (1983) that the addition of digitonin to suspensions of viable spermatozoa results in disruption of the plasma membrane.

It is also noteworthy that the fluorescent staining was readily carried out on cryopreserved samples

TABLE 3. Correlation coefficients between the number of spermatozoa with a specific characteristic and the fluorescent populations (A, B, and C) as determined by flow cytometric analyses of CFDA- and PI-stained bovine spermatozoa.

Parameter	Progressive motility	Intact acrosomes	Normal acrosomes	Proacrosin content	Fluorescent population A	Fluorescent population B	Fluorescent population C	Difference A	Difference B
Initial									
Intact acrosomes	0.65*								
Normal acrosomes	0.61*	0.69**							
Proacrosin	0.42	0.80**	0.56*						
Population A	−0.29	0.20	0.01	0.38					
Population B	−0.19	0.44	0.28	0.54*	0.50				
Population C	0.72**	0 64**	0.38	0.33	−0.40	- 0.34			
3 Hours									
Intact acrosomes	0.61*								
Normal acrosomes	0.49	0.91**							
Proacrosin	0.39	0.27	0.42						
Population A	−0.69**	−0.48	−0.49	−0.56*					
Population B	0.17	0.29	0.21	−0.38	0.19				
Population C	0.34	0.65**	0.46	−0.15	−0.36	−0.05			
Indices									
A Difference 3 h	0.20	0.15	−0.15	0.11	0.11	−0.27	0.35		
B Difference 3 h	0.55	0.11	−0.002	0.14	−0.49	−0.66*	−0.66**	0.52	
C Difference 3 h	0.48	0.29	0.05	0.19	−0.31	−0.40	0.66**	0.88**	0.86**

*Significantly different ($p<0.05$) from zero.

**Significantly different ($p<0.01$) from zero.

containing glycerol. Combinations of CFDA and PI can be readily used to evaluate the apparent viability of spermatozoa in cryopreserved samples. This is a major advantage since classical supravital staining techniques are not useful on samples containing glycerol (Mixner and Saroff, 1954).

Seminal Quality Evaluations

Considerable variability was noted in the percentage of progressively motile spermatozoa at post-thaw evaluation among the bovine samples evaluated in the present study, and no definitive relationship of this characteristic to fertility was evident. Even the percentage of progressively motile spermatozoa remaining after incubation was not significantly related to the percent fertility ($r = -0.34$, $p=0.23$). The ability of spermatozoa to maintain progressive motility during incubation has been considered as a potentially important discriminatory parameter in the assessment of seminal quality (Saacke et al., 1980; Pace et al., 1981). However, Pace et al. (1981) found that it was the number, not the percentage, of progressively motile spermatozoa in a sample that was important in predicting potential fertility.

Relationship of Seminal Analyses to Fertility

Information on the fertility of these 14 samples of cryopreserved bovine semen has been published (Pace et al., 1981), but data on individual bulls were not reported. The potential fertility of these semen samples was estimated using 90-day nonreturn rates and involved 16,464 units of semen. The average nonreturn rate for the semen from these bulls was 66.0 ± 4.2% and the range was 55.3% to 71.1% (Fig. 4). Although some seminal parameters measured by flow cytometry approached significance, none of the simple correlation coefficients between percentage of 90-day nonreturns and seminal parameters was significant. More complex comparisons, such as an index that summed the increase in populations A and B and the decrease in population C, tended to be correlated with fertility but not at a significant level ($r = -0.57$, $p = 0.06$).

DISCUSSION

Although our method of identifying the apparently viable, moribund, and dead populations of spermatozoa by differential fluorogenic staining was not definitive, it does present a new quantitative approach for assessment of seminal quality. The reliability of the classical laboratory tests for seminal quality is questionable because such tests rely mainly on physical and biochemical measurements made on a relatively small, presumably representative population of spermatozoa. The resultant data are averages and do not take into account variations within cell populations. This type of variability can be overcome somewhat by microscopic evaluations in which individual spermatozoa are evaluated. Microscopic evaluations using differential interference contrast have yielded relatively reliable data on the potential fertilizing capacity of spermatozoa (Saacke and White, 1972; Saacke et al., 1980). The problem with such technique is that they are inherently time-consuming and the resultant data are based on evaluations of only a few hundred cells. Flow cytometry offers even greater versatility for evaluating functional aspects of spermatozoa not only because data can be acquired on a large number of cells, but because more than one characteristic can be measured simultaneously on the same cell (Melamed et al., 1979; Loken, 1980).

The significant correlations between the percentage of spermatozoa with normal and intact acrosomes and the percentage of progressively motile spermatozoa were not unexpected since loss of the acrosomal contents under the incubation conditions used in this study would not likely be indicative of cell death.

The relatively little change noted in proacrosin content in this study could be related to difficulties in obtaining accurate measurements of acrosin activity. First, the quantity of the inactive precursor must be estimated from assay of the active enzyme. Uniformity in both the extraction and activation processes can be a problem even if carefully controlled (Brown and Harrison, 1978). Second, spermatozoa contain a natural inhibitor that interferes with the assay (Brown and Hartree, 1975). Third, acrosin tends to degrade itself through autoproteolysis (Zaneveld et al., 1973). Nonetheless, the retention of proacrosin by spermatozoa should be an important parameter in predicting fertility because of its purported role in fertilization.

Although combinations of seminal quality assessments might have a greater predictive value, Pace et al. (1981) reported that no combination of physiologic characteristics that they used in a multiple regression analysis explained any more of the variation in fertility among the 14 bulls used in the present study than did the most predictive single characteristic.

Classification of spermatozoal population A as dead cells is supported by the correlative data. Population A was negatively correlated with the percentage of progressively motile cells ($r = -0.61$, $p<0.05$) and the percentage of spermatozoa with intact acrosomes ($r = -0.77$, $p<0.01$). Although the significance of these relationships decreased with incubation, the staining of spermatozoal nuclei with PI was consistent with other criteria that appear to measure cell death.

Population B was considered to be moribund spermatozoa for two major reasons. First, the nuclei of these cells stained with PI but other cellular organelles, mainly the acrosome and mitochondria, retained, at least temporarily, the carboxyfluorescein. Second, the spermatozoa were apparently damaged to the extent that centrifugation resulted in loss of the carboxyfluorescein from the cytoplasm. Spermatozoal population B was correlated ($r = 0.54$, $p<0.05$) only with the initial proacrosin content of the spermatozoa. The intact acrosomal membranes thus appeared to retain both proacrosin and carboxyfluorescein.

Spermatozoal population C was considered to be viable because: 1) motile spermatozoa retained the carboxyfluorescein, 2) the percentage of spermatozoa in population C was highly correlated with the percentage of spermatozoa with intact acrosomes and with normal acrosomes, and 3) the percentage of cells in C was correlated with the percentage of progressively motile cells at post-thaw evaluation.

The population shifts that occurred during incubation also supported classification of population A as dead cells, population B as moribund cells, and population C as viable spermatozoa. The only exception to this trend was Bull H, in which an opposing trend was noted. Elimination of the data contributed by Bull H resulted in a significant correlation ($r = -0.57$, $p=0.04$) between fertility and the percentage of increase in population A occurring between the initial flow cytometric analysis and the analysis following incubation for 3 h. Removal of Bull H also resulted in significant correlations between fertility and the decrease in the percentage of spermatozoa in population C occurring during incubation ($r = -0.56$, $p<0.05$) and the percentage of spermatozoa remaining in population C following incubation ($r = 0.56$, $p<0.05$).

Although the results presented in this report are encouraging and certainly supportive of the concept that flow cytometric measurements of spermatozoan viability can be meaningful, the goal of accurately predicting fertilizing capacity from laboratory assessment of seminal quality remains to be achieved.

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