



Viability Assessment of Mammalian Sperm Using SYBR-14 and Propidium Iodide¹

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ABSTRACT

The proportion of living sperm in semen from six representative mammals was assessed by means of a dual staining technique using the stains SYBR-14 and propidium iodide (PI). SYBR-14, a newly developed fluorescent nucleic acid stain, maximally absorbs at 488 nm and emits at 518 nm when bound to DNA. Microscopic examination revealed that SYBR-14 stained the nuclei of living sperm bright green as determined by simultaneous examination of fluorescence and motility. Conversely, PI stained only nonmotile sperm that had lost their membrane integrity. Sperm from bulls, boars, rams, rabbits, mice, and men were stained and examined through use of fluorescence microscopy. The proportions of living and dead sperm were determined by first staining with SYBR-14 and PI and then assessing stain uptake by flow cytometry. Similar staining patterns were observed in all six mammalian species tested. Three populations of sperm were identified: living—SYBR-14 stained, dead—PI stained, and moribund—doubly stained. The SYBR-14 staining was replaced by PI staining as sperm progressed from living to moribund. The transition from green (SYBR-14) to red (PI) fluorescence started at the posterior region of the sperm head and proceeded anteriorly. The proportions of living and dead sperm in mammalian semen were readily identified through use of dual staining with SYBR-14 and PI and quantified through use of flow cytometry.

INTRODUCTION

Recent advances in staining technology have provided new means for assessing the functional capacity of sperm. Much of the emphasis has been directed toward using combinations of stains to determine sperm viability. One of the first attempts to assess sperm viability utilized rhodamine 123 (R123) to assess mitochondrial membrane potential and ethidium bromide to determine membrane integrity using flow cytometry [1]. Other combinations that have been used to examine the functional capacity of sperm are carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) [2–4]; carboxydimethylfluorescein diacetate (CMFDA), R123, and PI [5, 6]; and PI, pisum sativum agglutinin (PSA), and R123 [7]. Most of these stain combinations suffer from time-dependency problems because they are based on enzyme substrate conversion to a fluorescent product. In addition, the latency period from the time of cell death until loss of the fluorescent label adds variability to the staining outcome. A more logical approach would be to use two stains that react with the same cellular constituent—one stain that identifies only living sperm and a second that stains only dead sperm. The cellular target is also important. Sperm DNA is very stable and easily quantified by staining. Among the more commonly used stains for assessing sperm DNA content and viability have been the bisbenzimidazole stains,

Hoechst 33342 [8] and 33258 [9]. These stains must be excited with ultraviolet (UV) light to emit blue fluorescence. UV-generated fluorescence, however, may be detrimental to cellular function and DNA integrity in some cell types. Excitation with visible light (488 nm) is always preferable to the use of UV light where viability is an issue. To this end, a new membrane-permeant nuclear stain that brightly stains the nuclei of living cells, SYBR-14, has been developed by Molecular Probes, Inc. (Eugene, OR). In earlier studies, we found that SYBR-14, in combination with propidium iodide (PI), was useful in estimating the proportions of living and dead sperm in both fresh and cryopreserved bovine semen samples [10].

The present study sought to determine whether the combination of SYBR-14 and PI could be used to differentiate between living and dead sperm from a variety of mammals as had been previously demonstrated only for the bull [10]. The overall objective was to determine whether the staining characteristics of sperm from four males from each of six mammalian groups (bulls, boars, rams, mice, rabbits, and men) were similar when the combination of SYBR-14 and PI was used. These eutherian mammalian groups represent four different mammalian orders: artiodactyla, lagomorpha, rodentia, and primates. Two specific objectives were pursued: 1) to microscopically examine the staining characteristics of living and dead sperm from six representative mammals using SYBR-14 in combination with PI and 2) to quantify the proportions of SYBR-14- and PI-stained sperm in semen samples from four males from each of the six mammals through use of flow cytometry.

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MATERIALS AND METHODS

Semen Samples

Semen was obtained from four males from each of six mammals; the mammals tested were bovine, porcine, ovine, lagomorphs, murine, and human males.

Bull ejaculates. Bovine ejaculates were collected from four mature bulls by means of an artificial vagina. The sperm were diluted 1:40 in HEPES-buffered saline containing 0.1% BSA (HEPES-0.1% BSA). The HEPES-0.1% BSA, which contained 0.760 g NaCl, 0.030 g KCl, 0.252 g fructose, 0.238 g HEPES, 0.015 g CaCl₂, 0.010 g MgCl₂, and 0.10 g BSA/100 ml, was made up in tissue culture-grade water and titrated to pH 7.4 with 0.1 N NaOH before use [10]. The sperm were then counted with a hemocytometer, and the samples were adjusted to approximately 30×10^6 sperm/ml. One-half-milliliter aliquots of the diluted samples were pipetted into warmed (36°C) 1.5-ml Eppendorf centrifuge tubes for staining.

Boar ejaculates. Semen was collected from four mature boars by the gloved hand technique and filtered to remove the gel. The samples were diluted with Beltsville TS (BTS) [11]; as used for liquid semen extension [11], to a sperm concentration of 10×10^6 sperm/ml. Portions (500 μ l) of the diluted samples containing 5×10^6 sperm were pipetted into warmed (36°C) 1.5-ml Eppendorf centrifuge tubes.

Ram ejaculates. Ovine semen was collected from four mature rams through use of an artificial vagina. The semen was diluted 1:3 with HEPES-0.1% BSA and counted. Sperm numbers were adjusted so that each sample contained approximately 30×10^6 sperm/ml. For each sample, 500- μ l aliquots of the sperm suspension, which contained 15×10^6 sperm, were aliquoted for staining as described for the bovine samples.

Rabbit ejaculates. Rabbit semen was collected from four mature males via an artificial vagina. The semen was diluted 1:3 with HEPES-0.1% BSA and counted. Sperm numbers were adjusted so that each sample contained approximately 30×10^6 sperm/ml. For each sample, 500- μ l aliquots of the sperm suspension containing 15×10^6 sperm were placed in 1.5-ml tubes for staining as described for the bovine samples.

Mouse epididymal sperm. Four mice were killed by cervical dislocation, and each pair of epididymides was removed. The excised epididymides were dissected free of excess tissue. Each pair was placed in 300 μ l medium, HEPES-0.1% BSA, under mineral oil held at 36°C in 60-mm polystyrene tissue culture dishes. The epididymides were carefully minced while under oil to prevent atmospheric damage to the sensitive mouse sperm. Approximately 5 min was allowed so that the sperm could swim out of the minced epididymides. Tissue debris was removed, and 300

μ l of the sperm suspension was placed under 100 μ l mineral oil in warmed (36°C) 1.5-ml Eppendorf tubes for staining. Actual sperm concentration was not determined.

Human cryopreserved sperm. The four samples of human sperm were collected and processed according to World Health Organization criteria [12]. These samples contained approximately 10×10^6 sperm/ml. The samples (0.5 ml) were thawed at 37°C and diluted 1:3 with HEPES-buffered medium containing 0.1% BSA, which had been warmed to 36°C. The sample was then divided into three 500- μ l aliquots and pipetted into warmed (36°C) Eppendorf tubes for staining.

Fluorescent Staining

The newly developed living cell nucleic acid stain, SYBR-14, was used in combination with propidium iodide (PI). This stain combination is now marketed as *FertLight* (Molecular Probes, Eugene, OR). The SYBR-14 was prepared in anhydrous methyl sulfoxide (DMSO) from Aldrich Chemical Company (Milwaukee, WI) at a concentration of 1 mg/ml. A working solution of SYBR-14 diluted 1:10 with DMSO was used for staining the sperm of all of the mammals tested. The PI was dissolved in Tyrode's salt solution at 2 mg/ml (Sigma Chemical Company, St. Louis, MO).

Aliquots (500 μ l) of diluted or cryopreserved semen were stained at 36°C with 0.27 μ l of the working solution of SYBR-14 and 2 μ l of the PI stock solution. An exception was the mouse samples, where the stain volumes had to be adjusted to 0.16 μ l (for the SYBR-14) and 1.2 μ l (for the PI) for the 300- μ l samples. The small stain volumes were added through use of P-2 Pipetman (Rainin Instrument Co., Emeryville, CA) and FluoroPel pipette tips (No. T30510F; Ulster Scientific, Inc., New Paltz, NY). The samples were incubated for 15 min at 36°C before examination. When this stain combination was excited at 488 nm, the nucleus of the SYBR-14-stained cells fluoresced bright green while the dead sperm nuclei exhibited red fluorescence (PI). The fluorescent staining of sperm was monitored and photographed with a Zeiss Axiophot epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a fluorescein isothiocyanate filter set (Zeiss #487909). Ektachrome 400 (Eastman Kodak, Rochester, NY) slide film was used to photograph sperm (30 sec).

Flow Cytometry

Quantitative data on the fluorescently stained sperm populations were collected through use of an EPICS PROFILE II (Coulter Corporation, Miami, FL). The Profile II uses an air-cooled argon ion laser emitting at 488 nm and was equipped with the PowerPak option that provided for 3-color fluorescence detection in addition to the side and forward light scatter parameters. Gates were set for the side

and forward light scatter parameters so that only those cells possessing the light scatter characteristics of sperm were analyzed for fluorescence intensity. The green fluorescence that passed through a 525-nm band pass filter was collected as the log of green fluorescence 1 (LFL1). The red fluorescence parameters, i.e., fluorescence 2 (LFL2) and fluorescence 3 (LFL3), were collected through 575-nm band and 635-nm band-pass filters, respectively, as a log function. Compensation (25%) was used to minimize spillover of green fluorescence into the 635-nm red channel (LFL3). The adjustment does not change the number of sperm that reside within any given population, but rather shifts the relative position of the populations so that quantification is easier and more precise. A total of 10 000 sperm per sample were analyzed for the log of their fluorescence for each sample. The generated data were then analyzed for the relative fluorescence of the LFL1 and LFL3 using the Coulter Histogram Analysis program (Coulter Corporation).

Statistical Analyses

Differences among the sperm populations both within and among the mammalian groups were examined by ANOVA using the least squares procedure and general linear models procedure of Statistical Analysis System [13]. The data, which were acquired as percentages, were arcsine transformed before analysis. The interrelationships among the sperm populations were evaluated through use of Pearson correlation coefficients generated by SAS.

RESULTS

Fluorescent Staining

The living sperm in samples representing each of the six mammals exhibited bright green fluorescence in the nucleus when stained with SYBR-14 and excited at 488 nm. Nonmotile sperm, apparently dead, fluoresced when stained with SYBR-14 alone, but the staining intensity was much less than that of living motile sperm. With the addition of PI, the dead sperm nuclei stained bright red. Some sperm, apparently moribund, stained with both dyes. The change from green to red began at the posterior portion of the sperm head and proceeded anteriorly. This change could be observed microscopically as sperm became nonmotile and then died, as occurs along the edge of a coverslip. The change from green to red took about one min. The three major populations—living sperm that stained with SYBR-14, dead sperm that stained with PI, and moribund sperm that fluoresced both green and red—were evident for all mammals tested (Fig. 1). The staining of sperm with the combination of SYBR-14 and PI was observed microscopically almost immediately, but a slight gain in fluorescence intensity was observed with time. Although staining of sperm began immediately upon addition of the SYBR-14 to the sample and the intensity reached an apparent equilibrium within a few minutes, a

staining time of 15 ± 2 min at 36°C was utilized to assure an equilibrium between the stain and the sperm nucleic acids. Exact timing was not critical for staining, but experimental control was established by standardizing this among the various samples.

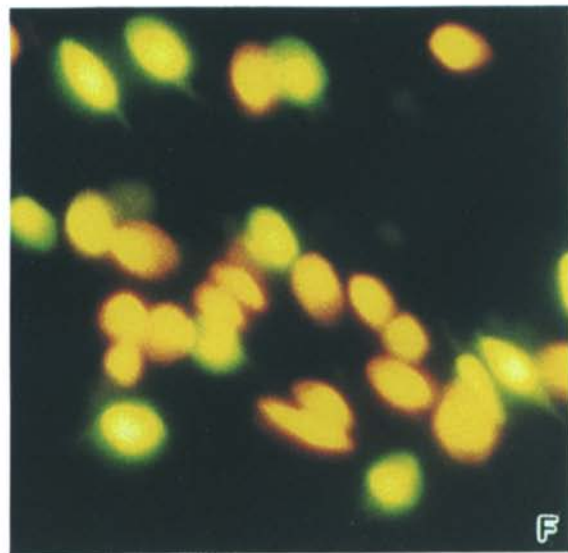
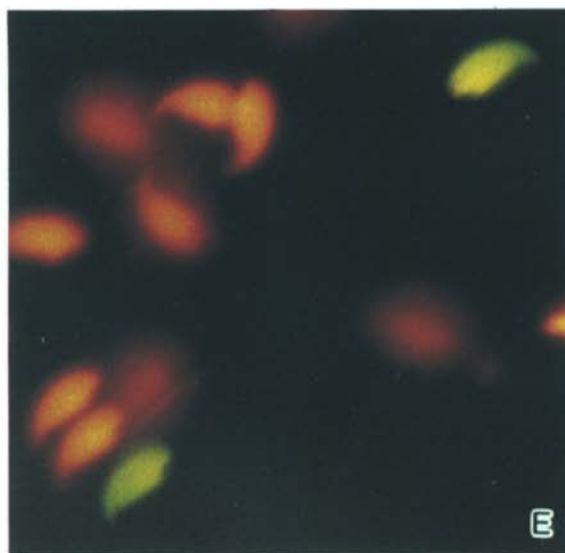
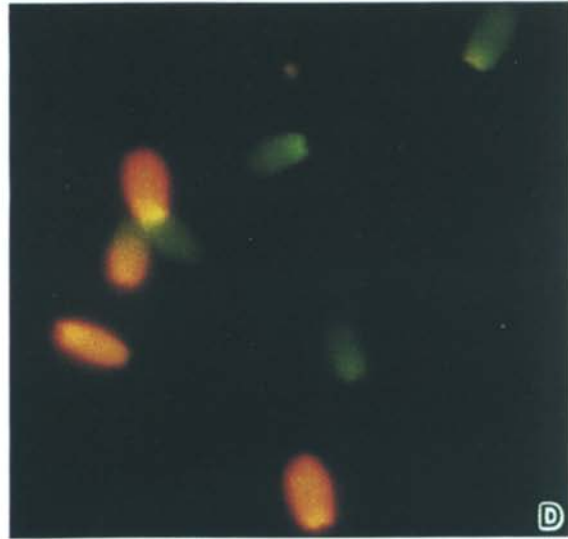
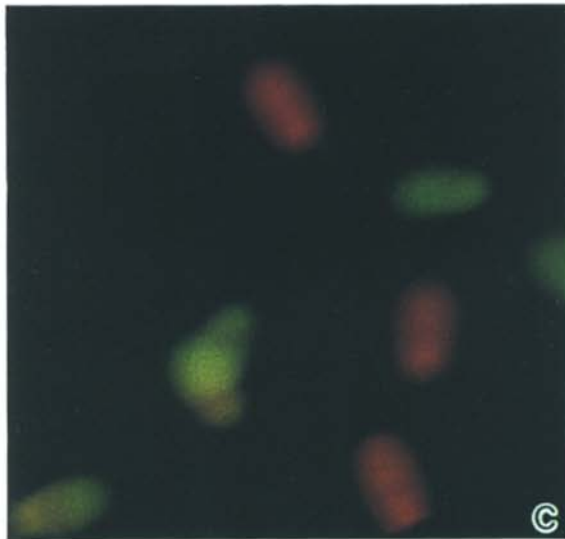
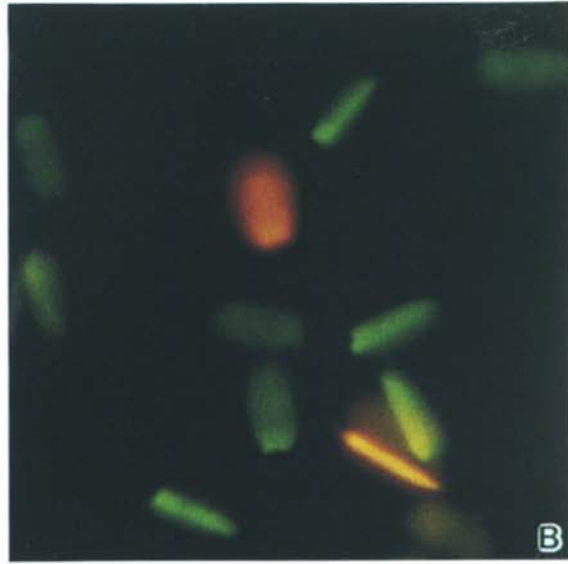
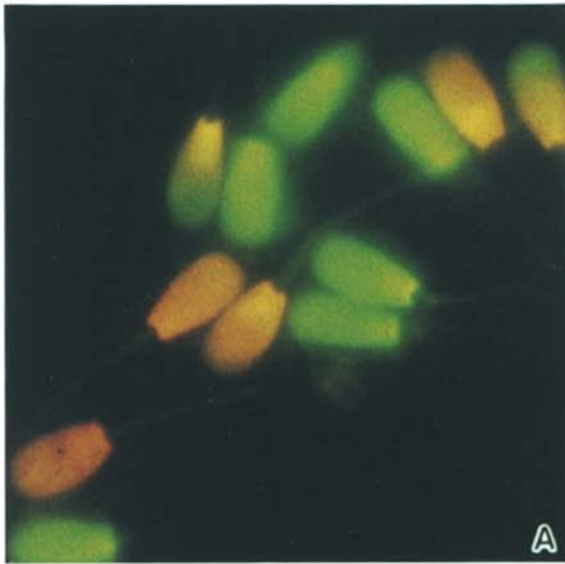
Flow Cytometry

Dot-plot scattergrams from each sperm sample analysis (10 000 sperm) were generated for each replicate ($n = 3$) for the four individuals representing each mammalian group. Control samples, which were unstained or were stained with SYBR-14 alone or PI alone (data not shown), were also examined for each species to adjust the range of fluorescence to be examined by the flow cytometer. Scattergram dot plots showing typical flow cytometric analyses for sperm that were stained with both SYBR-14 and PI, representing each of six mammals, are given in Figure 2.

The sperm were stained with SYBR-14 and PI to assess the differences in sperm viability among four males for each of six mammals. Examples of the dot plots of the logs of green and red fluorescence for sperm representing each mammal are provided along with the window analyses showing the mean proportion of PI (population 1), dual (population 2), and SYBR-14 (population 3) staining for an individual bull (A), boar (B), ram (C), rabbit (D), mouse (E), and human (F). Cytoplasmic droplets and other debris, which also includes diluent constituents such as egg yolk granules and milk fat droplets in some cases, were noted in some samples (identified as population 4 in Figure 2, B and E). These debris particles, however, were relatively much less fluorescent than were sperm. Although not specifically examined in the present studies, we have found that the amount of debris tends to be related to the initial sperm concentration and the type of medium in which the sperm are suspended.

The relative proportions of sperm staining with PI, SYBR-14, or both PI and SYBR-14 (dual stained) for each of the four individual bulls, boars, rams, rabbits, mice, and men are provided as mean values of three replicates (Table 1). Differences in the proportion of sperm that stained with PI were found among the individual bulls, rams, rabbits, and men ($p < 0.05$) but not among the boars and mice. Differences ($p < 0.05$) were found for the proportion of living sperm (SYBR-14 staining) among the four males for each of the mammals examined with the exception of the mice (Fig.

FIG. 1. Photomicrographs of fluorescently stained mammalian sperm. Sperm were fluorescently stained through use of a combination of SYBR-14 and PI (*FertLight*). Examples of stained sperm from bulls (A), boars (B), rams (C), rabbits (D), mice (E), and men (F) are shown. Three major populations of sperm were evident for all mammals tested. The living sperm stained bright green (SYBR-14); the membrane-damaged sperm stained red (PI); and the moribund sperm stained with both. A transition from green to red, beginning at the posterior region of the nuclei and proceeding anteriorly, occurred in sperm from all mammals.



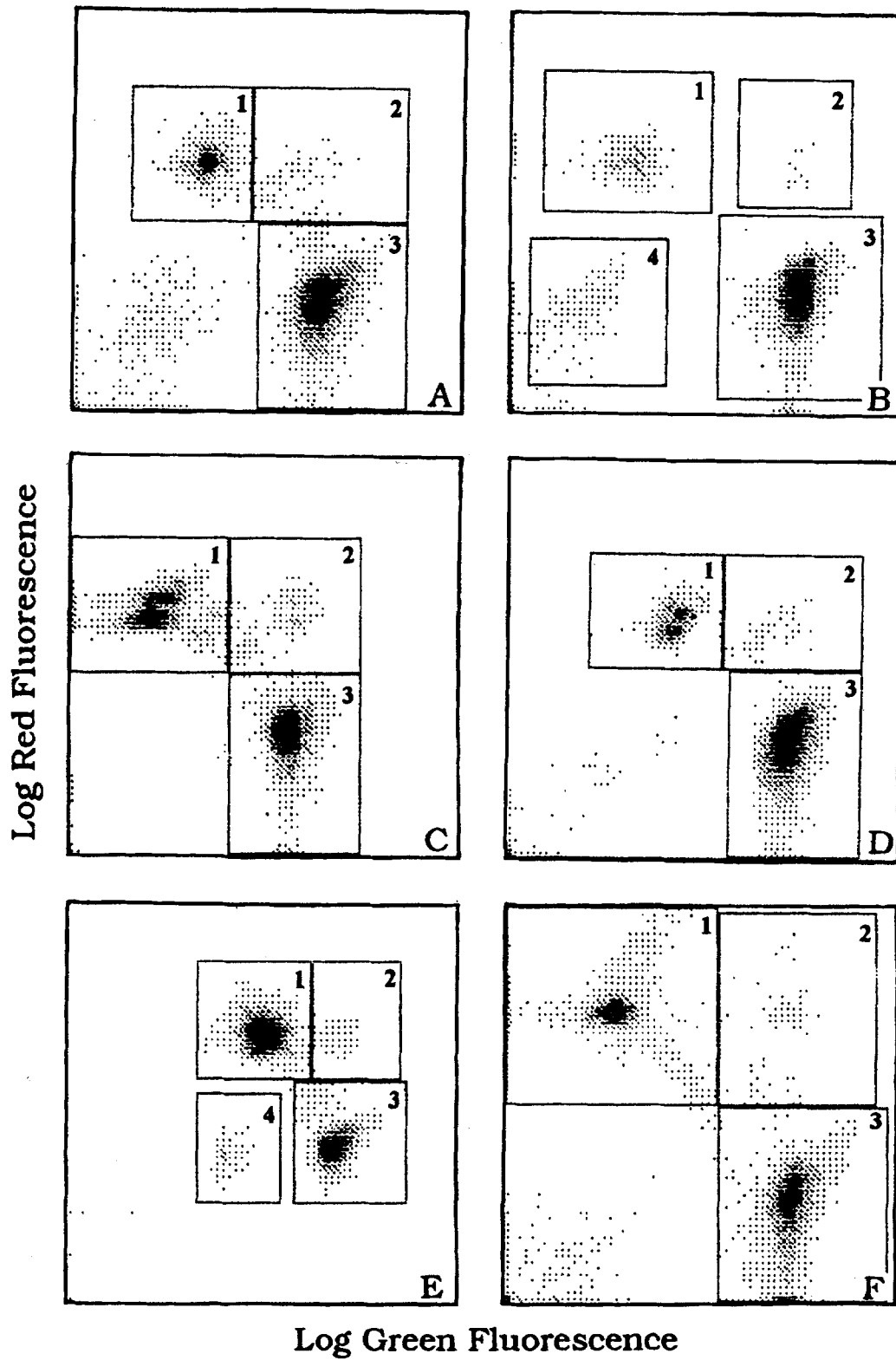


FIG. 2. Three sperm populations were quantified for one of the four representatives of each mammal group: bulls (A), boars (B), rams (C), rabbits (D), mice (E), and men (F). The samples were quantified for the proportions of PI- (1), dual- (2), and SYBR-14 (3)-stained sperm. Debris is identified in (4) in B and E. Dot plots (A to F) show flow cytometric analyses of a sperm sample from one of the three replicates run for that particular animal.

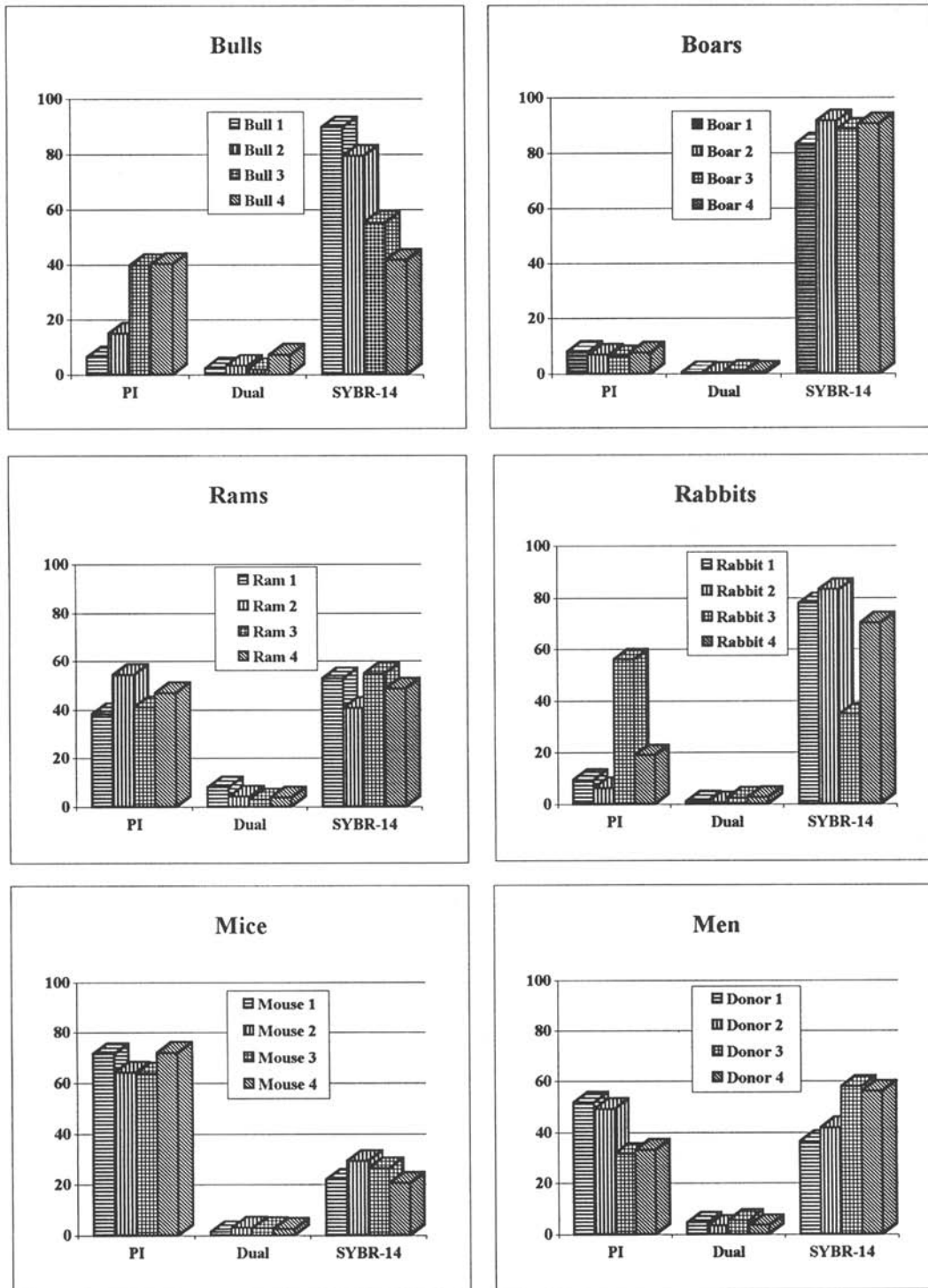


FIG. 3. Histograms showing the three major populations of sperm that were identified by dual DNA staining with SYBR-14 and PI. The relative proportions of PI-, dual-, and SYBR-14-stained sperm are means ($n = 3$) for each of the four individual males tested for bulls, boars, rams, rabbits, mice, and men.

TABLE 1. Mean values (\pm SE for individual means) for the three major populations of sperm that were identified by dual DNA staining with SYBR-14 and PI for semen from bulls, boars, rams, rabbits, mice, and men ($n = 4$).

Stain	Bulls	Boars	Rams	Rabbits	Mice	Men
PI	25.4 \pm 4.5 ^a	7.3 \pm 0.2 ^b	45.2 \pm 2.0 ^{*c}	22.6 \pm 5.7 ^{*a}	67.9 \pm 2.1 ^d	41.5 \pm 2.8 ^{*c}
Dual	3.7 \pm 0.7 ^{*a}	0.6 \pm 0.1 ^b	4.8 \pm 0.8 ^{b,c}	1.6 \pm 0.3 ^{*b}	2.5 \pm 0.3 ^{a,b}	4.2 \pm 0.4 ^{a,c}
SYBR-14	66.6 \pm 5.7 ^{*a}	88.6 \pm 1.0 ^{*b}	49.2 \pm 1.7 ^{*c}	66.6 \pm 5.7 ^{*a}	24.5 \pm 2.2 ^d	48.1 \pm 2.8 ^{*c}

* Difference among individual males within a mammalian group for that particular sperm staining parameter ($p < 0.05$).

^{a,b,c,d} Mammalian groups with the same letter do not differ for that particular sperm staining parameter ($p < 0.05$).

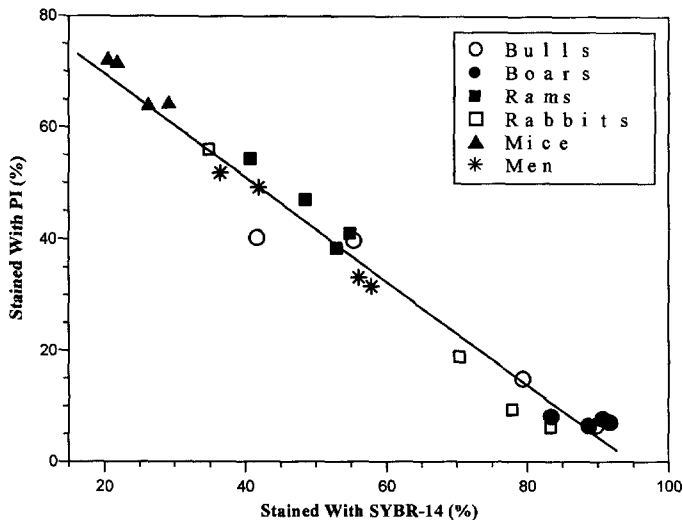


FIG. 4. Correlation between living (SYBR-14) and dead (PI) sperm for all mammalian males tested.

3). Only the rabbits exhibited differences ($p < 0.05$) in dual staining among the four males examined. The means for the proportion of sperm that stained with PI or SYBR-14 for the four individual males were not different ($p > 0.05$) between bulls and rabbits (Table 1) or between rams and men.

The negative correlations between living and dead sperm populations for each of the mammalian groups were significant ($p < 0.05$) with the exception of those for the boar sperm. These correlations were -0.97 , -0.47 , -0.93 , -0.99 , -0.98 , -0.99 for bulls, boars, rams, rabbits, mice, and men, respectively. The proportions of sperm that stained with PI for boars 1, 2, 3, and 4 were 8.1, 7.0, 6.4, and 7.7% and the proportions for SYBR-14 were 83.4, 91.6, 88.7, and 90.7%, respectively. The overall correlation between living and dead sperm for all males was -0.99 ($p < 0.05$) (Fig. 4).

DISCUSSION

The combination of SYBR-14 and PI effectively identified the living and dead sperm populations in semen from bulls, boars, rams, rabbits, mice, and men. The validity of this staining combination had been previously demonstrated

with bull semen through the use of a predetermined mixture of living and dead sperm [10]. In that initial study [10] on SYBR-14 and PI staining, living sperm were prepared by filtration of diluted semen through glass wool and Sephadex to remove dead and damaged sperm. A portion of this filtered sperm sample was then killed by freezing-thawing before being recombined with the living sperm in particular ratios to establish the viability of the SYBR-14 and PI combination for determining the proportion of living and dead sperm in a sample. When this staining technology was applied to other animals as it was in the present study, it was demonstrated that the flow cytometric patterns of the SYBR-14- and PI-stained sperm were similar for the various groups. Furthermore, the change in SYBR-14 staining in relation to PI staining as sperm died was evident in semen from all of the mammalian groups. A possible explanation for this phenomenon is that as sperm die, they lose their ability to resist the influx of the membrane-impermeant dye, PI, which upon entering the sperm apparently replaces or quenches the SYBR-14 staining. The PI likely enters the nuclear compartment through pores in the nuclear membrane that are located in the diverticulum or membrane folds at the posterior aspect of the head near the implantation fossa [14]. We have also seen a similar effect when PI was used in combination with another nucleic acid stain, Hoechst 33342. The change in Hoechst 33342 staining intensity, which began at the posterior aspect of the sperm head, was similar to that seen with SYBR-14 and PI [15]. The mechanism by which SYBR-14 stains living sperm more brightly than dead sperm is unknown. Although no data are currently available, it is possible that some biophysical phenomena, e.g., membrane potential, play a role in enhancing the fluorescence of SYBR-14 in living sperm.

There are two major advantages of using SYBR-14 over enzyme-based stains: staining time is not as critical, and background staining is virtually nonexistent. The esterase-based stains for assessing membrane integrity, such as CFDA [2] and CDMFDA [5], require very careful timing because cellular fluorescence continues to increase over time. In contrast, SYBR-14 rapidly reaches an equilibrium (in less than 15 min) with the nucleic acid and is thereafter relatively stable. Sperm samples that have been fluorescently stained with CMFDA and the mitochondrial stain R123 tend to ex-

hibit heavy background staining due to extracellular esterase. The background in SYBR-14-stained samples is, however, nonexistent. Thus, staining artifacts are minimal with the combination of SYBR-14 and PI.

Differences among the individual bulls, rams, rabbits, and men in the proportion of sperm staining with SYBR-14 and PI, representing the living and dead sperm populations, were readily quantified. In addition, a difference among boars was found in the proportion of sperm that stained with SYBR-14. These differences among males likely reflect true animal differences because no selection of males for seminal quality within a mammalian group was done prior to the studies reported.

Populations of individual sperm exhibiting both red and green fluorescence were found in all individual males. These doubly stained sperm populations represent moribund or slightly damaged sperm that have lost their ability to exclude PI. One must consider that at least a portion of the doubly stained sperm observed in the scattergrams may be due to coincidence in which two sperm, one green and one red, were simultaneously measured as they passed through the flow cell. An effort was made to minimize this problem by gating with volume and light scatter to eliminate particles that were larger than sperm. In addition, doubly stained sperm were also identified microscopically in samples from all of the mammals tested.

The negative correlations between the SYBR-14- and PI-stained sperm populations, which ranged between $r = -0.47$ for the boars and $r = -0.99$ for the rabbits and men, are indicative of the interaction between SYBR-14 and PI. These values represent assay characteristics in which the selected fluorescent sperm were subpopulations of the 10 000 sperm sample. It is, however, notable that this significant relationship held up for all of the mammalian groups tested with the exception of the boars ($r = 0.47$, $p = 0.12$). The boars differed from the other mammals in having a very small percentage of PI-stained sperm. The range of these values (6.4% to 8.1%) was so narrow that it negated the potential relationship between PI- and SYBR-14-stained sperm for these four samples. This was the only group of males from which semen had been routinely collected for artificial insemination purposes. The semen quality was, therefore, more uniform in the boars. It should be noted that the values obtained for the four males for each of the six groups were representative of the males used in these experiments and may not be representative of males of that mammalian group. The values that we obtained might have been affected by the conditions of semen collection and processing. Semen from the bulls, boars, rams, and rabbits was freshly collected while the mouse samples were epididymal sperm and the human samples were cryopreserved. Considering these different collection conditions, the fluorescent patterns of the flow cytometric anal-

yses were markedly similar in their staining patterns. The variation found in the four human samples was not as great as that noted for cryopreserved human samples that had been stained with CMFDA, PI, and R123 [16]. This was not unexpected in that the combination of CMFDA, PI, and R123 is more variable among replicates because of its critical dependency on staining time.

In the use of data derived from the staining of sperm with the combination of SYBR-14 and PI, the doubly stained sperm should be considered part of the dead sperm population because they are, in fact, dead on the basis of their functional capacity. The staining pattern indicates that they are in a transitional phase and beginning to show signs of losing their membrane integrity.

We have stained sperm from other mammals, including cats, tigers, and chinchillas, using SYBR-14 and PI (Garner and Johnson, unpublished). The staining combination of SYBR-14 and PI has also been used on turkey sperm [17].

Summary

Dual DNA staining with SYBR-14 and PI (*FertiLight*) effectively identified the living and dead sperm populations in semen from bulls, boars, rams, rabbits, mice, and men. The living sperm, which stained green with SYBR-14, and the dead sperm, which stained red with PI, were readily quantified by flow cytometry in samples from four males from each of the six mammal types. Flow cytometry detected differences among the individual bulls, rams, rabbits, and men in the proportions of sperm that stained with SYBR-14 and PI. In addition, a difference among boars was found in the proportion of sperm that stained with SYBR-14.

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