A Competitive *In Vitro* Assay of Human Sperm Fertilizing Ability Utilizing Contrasting Fluorescent Sperm Markers

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Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) were evaluated for use as contrasting fluorescent labels on living human spermatozoa. Unlabeled (control), FITC-labeled (green fluorescence), TRITC-labeled (red fluorescence), and mixed FITC-TRITC-labeled sperm suspensions were incubated with non-fertilizable human oocytes for assessing sperm penetration of the zona pellucida and with zona-free hamster eggs for assessing sperm incorporation into the ooplasm. Fluorochrome-labeled spermatozoa were as efficient as unlabeled cells from the same donors in penetrating the human zona pellucida and in entering the hamster ooplasm. The middle and principal pieces of spermatozoa undergoing nuclear decondensation within the hamster ooplasm retained their fluorescent label, allowing visual differentiation between FITC- and TRITC-labeled spermatozoa. Videomicrographic analyses of the movement characteristics (percentage of motile cells and mean swimming speeds) of labeled and unlabeled spermatozoa before and after incubation with ova revealed no detrimental effect on the motility of labeled cells. We conclude that the fluorescent dyes FITC and TRITC do not impair the function of human spermatozoa as assessed by motility characteristics and by their ability to penetrate ova *in vitro*. The contrasting colors of the two fluorochromes make them particularly useful in the competitive assessment of the *in vitro* fertilizing potential of human spermatozoa from different donors or after different experimental treatments.

Key words: Spermatozoa, fertilization, sperm motility.

Techniques for labeling living spermatozoa have become valuable tools in the study of mammalian sperm transport and fertilization. Mellish and Baker (1970) were the first to explore the use of fluorescein isothiocyanate (FITC) for marking and subsequently for recognizing sperm populations. Their studies showed no detrimental effect of FITC on the transport or fertilizing capacity of boar spermatozoa. Overstreet and colleagues used FITC for labeling rabbit spermatozoa and concluded that the fluorescent label had no deleterious effect on sperm transport, capacitation, or fertilizing ability (Overstreet and Adams, 1971; Overstreet and Bedford, 1974).

Overstreet and Hembree (1976) first applied fluorochrome labeling techniques to human spermatozoa *in vitro*. They reported that FITC-labeled spermatozoa from fertile donors were able to penetrate the human zona pellucida as frequently as unlabeled spermatozoa from the same donors. In some experiments, mixtures of FITC-labeled spermatozoa from a fertile donor and unlabeled spermatozoa from an infertile patient were incubated with non-fertilizable human oocytes. Observation of the fluorescence of spermatozoa in the zona pellucida and perivitelline space allowed identification of the source of the penetrating

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spermatozoa. Thus, the donor spermatozoa served as an internal control for technical variables in the *in vitro* system.

To interpret experiments with mixtures of labeled and unlabeled spermatozoa *in vitro*, it must be assumed that no unbound fluorochrome is present (to contaminate the unlabeled sperm) and that the fluorochrome label remains associated with the cell throughout the experiment (i.e., the labeled sperm do not lose their fluorescence). A better design for such experiments would include two contrasting fluorescent labels to identify the two competing sperm populations. The objective of the present study was to evaluate the feasibility of utilizing two fluorescent dyes, fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC), as contrasting fluorescent markers of living human spermatozoa. In this paper, we report experiments that show that human sperm motility is not affected by these compounds. We also conclude that neither sperm interaction with the human zona pellucida nor the zona-free hamster egg is affected by labeling the sperm cells with these compounds.

Materials and Methods

Experimental Design

A protocol was designed to assess the effect of fluorescein isothiocyanate (FITC, Sigma) and tetramethyl rhodamine isothiocyanate (TRITC, Research Organics) on human sperm motility and fertilizing capacity *in vitro*. Eight replicates of the experiment were conducted. A single semen specimen was studied in each replicate, but with a different semen donor utilized. Sperm motility in the semen (percentage of motile sperm and mean swimming speeds) was analyzed by videomicrography (Katz and Overstreet, 1981), and the specimen was divided into three aliquots. An aliquot was labeled with each of the two fluorochromes and one remained unlabeled as a control. Following the labeling procedure, the sperm motility in the three suspensions was assessed a second time and a group of non-fertilizable human oocytes and zona-free hamster eggs was added to each. A fourth mixed suspension (FITC-labeled sperm plus TRITC-labeled sperm) was also incubated with human oocytes and hamster eggs. Following these incubations, sperm motility in each suspension was assessed a final time and sperm penetration into the human zona pellucida and hamster vitellus was evaluated.

Preparation of Fluorochrome-Labeled Sperm Suspensions

Preliminary dose-response studies were carried out to determine the optimum concentration of each fluorescent dye in the labeling solution, i.e., strong fluorescence of the sperm cells without an adverse effect on flagellar activity. Since there is significant lot-to-lot variation in the interaction of these dyes with spermatozoa, such preliminary studies are essential whenever a new dye lot is utilized. Each dye was dissolved initially in 0.05 ml of 0.1 M KOH and this concentrated solution was then diluted with the modified culture medium of Biggers, Whitten, and Whittingham (Biggers et al, 1971 as modified by Yanagimachi et al, 1979) to a final volume of 5 ml. This medium, supplemented with 3 mg/ml of bovine serum albumin (BSA, Sigma, Fraction V), is hereafter referred to as BWW medium. The final concentration of FITC in the BWW medium was 0.3 mg/ml and that of TRITC was 0.2 mg/ml. The two semen aliquots that were labeled with FITC and TRITC were each diluted 1:2 with respective dye solutions; the control aliquot was similarly diluted with BWW medium alone. After incubation for 10 minutes at 37 C, 0.75 ml of each diluted sperm suspension was layered beneath 5 ml of BWW medium in 15 ml glass centrifuge tubes. Sealed tubes were inclined at a 30 angle and incubated for 1 hour at 37 C to allow spermatozoa to swim upward out of the seminal plasma and into the medium. Spermatozoa were recovered after incubation by removing the BWW over-layer with a Pasteur pipette. The cells were washed twice by centrifugation at 600 × g for 6 minutes and resuspension with 5 ml of BWW medium. Washed sperm were diluted to a concentration of 10×10^6 cells/ml with a capacitating medium (BWW medium containing 35 mg/ml human serum albumin, Sigma, Fraction V). Fluorochrome-labeled spermatozoa were protected at all times from fluorescent light to prevent light induced free-radical formation and cross-linking of membrane proteins that are spermicidal (Lepock et al, 1978).

Assessment of Sperm Motility

Spermatozoan motility was recorded at 37 C by videomicrography three times during the experiments (see experimental design). Tungsten illumination was used for motility assessment and the sperm suspensions were never exposed to ultraviolet illumination until the end of the experiment. The videotapes were analyzed as described by Katz and Overstreet (1981). A total of 50 spermatozoa in 7 to 12 independent microscopic fields were analyzed in each suspension to determine the percentage of motile cells, and swimming speeds of 25 individual cells per suspension were measured.

Preparation of Gametes For Sperm-Egg Interaction

Aliquots of FITC, TRITC, and control sperm suspensions (0.15 to 0.20 ml) were placed in 35 mm × 10mm plastic petri dishes (Falcon) and covered with paraffin oil (Fisher, SV 125/135). Mixtures of FITC and TRITC-labeled spermatozoa in 0.15 ml to 0.20 ml droplets were similarly prepared. Sperm suspensions were held at 37°C in air until oocytes were added (30–45 minutes).

Human oocytes were recovered from ovarian tissue, stored at −80 C and prepared for incubation as described by Overstreet et al (1980). Mature golden ham-

ster oocytes were recovered from superovulated adult females and stored with zonae intact at −80 C. The methods for superovulation as well as oocyte recovery, freezing, and thawing were the same as those of Fleming et al (1979). The zonae pellucidae were removed after thawing by exposure to 0.1% bovine pancreatic trypsin (2 × cryst., 10^4 BAEE units/ml, Sigma) in BWW medium for 1 to 2 minutes. Hamster eggs with signs of freezing damage (Fleming et al, 1979) were excluded from the experiments. One or two human oocytes and eight to 15 zona-free hamster eggs were transferred in approximately 10 μl of the capacitating medium to each sperm suspension. The dishes were incubated in air for 4 hours at 37 C, at which time ova were analyzed for evidence of sperm penetration; and aliquots of unlabeled, FITC-labeled, and TRITC-labeled spermatozoa were videotaped for motility assessment.

Examination of Spermatozoa and Oocytes With Phase Contrast Fluorescence Microscopy

An Olympus Vanox universal microscope equipped with phase contrast optics and a 200W mercury-fluorescence vertical illuminator were used. For simultaneous observation of FITC- and TRITC-labeled spermatozoa, the FITC exciter filter and Y495 barrier filter (blue excitation) were used. A second, green excitation filter combination (exciter filter IF545 plus BG36) allowed observation of TRITC-labeled sperm but did not excite fluorescence of the FITC label. Observations were made of sperm suspensions (7 μl) on plain glass slides covered by #1½ (22 mm × 22 mm) coverglasses. Human oocytes were mounted between a glass slide and a coverslip and were repeatedly "rolled" to identify spermatozoa within the zona pellucida and perivitelline space (PVS). Zona-free hamster eggs were examined for the presence of swollen sperm heads (developing male pronuclei) and associated sperm tails, as described byYanagimachi et al (1976). The fluorescence of the sperm in all preparations was assessed with blue and green excitation.

Statistical Analysis

The mean swimming speeds of spermatozoa were analyzed by a three-way analysis of variance with donors, fluorochrome dyes (ie, unlabeled, FITC- and TRITC-labeled) and incubation as the main effects. A mixed model analysis was employed, with fluorochrome dyes and incubation regarded as fixed effects and donors as random effects. All possible interaction terms between the main effects were fitted into the statistical model. Differences in the percentages of motile spermatozoa and the proportions of penetrated zona-free hamster ova were analyzed by the Student's *t*-test after the angular transformation or by chi-square tests.

Results

The Fluorescence of Human Spermatozoa Labeled With FITC and TRITC

With blue excitation, FITC-labeled sperm were easily recognized by a bright green fluorescence in the region of the middle piece. A majority of the dead cells also had a bright green fluorescence in the head region. TRITC-labeled cells could be distinguished from FITC-labeled cells under blue excitation, the former displaying an orange-yellow fluorescence of the middle piece and post-acrosomal region of the head. The identity of the two labels could be confirmed with green excitation that resulted in a red fluorescence of the TRITC-label, whereas the FITC label became invisible. When viewed under a microscope equipped with an ultraviolet light source, FITC- and TRITC-labeled human spermatozoa become immotile shortly after exposure to the ultraviolet illumination.

The Effect of FITC and TRITC on Sperm Motility

Labeling with FITC or TRITC did not impair the motility of human spermatozoa, provided they were protected from ultraviolet light. A high percentage of motile spermatozoa was initially isolated from each semen specimen and the percentage of motile spermatozoa remained greater than or equal to 90% after 4 hours of incubation with ova. The pooled results of the eight replicates are given in Table 1. The movement characteristics of fluorochrome-labeled and unlabeled spermatozoa were not significantly different (Table 1, Fig. 1). Significant differences were noted among suspensions from the different donors (Fig. 1). The overall mean swimming speeds were significantly higher after 4 hours of incubation (Table 1). This increase after incubation, however, was observed in sperm suspensions from some donors and not others as evidenced by the significant donor × incubation interaction term in the analysis of variance. The unlabeled control spermatozoa from Donor F (Fig. 1) had a significantly lower swimming speed than the FITC- or TRITC-labeled spermatozoa, both before and after incubation, and this resulted in a significant donor × dye interaction term in the analysis of variance. The cause of this result was not determined, but the collective results of the other seven replicates argue against a true stimulative effect of either dye on sperm motility.

The Effect of FITC and TRITC on Sperm-Egg Interaction

Spermatozoa from each fertile man were not equally efficient in penetrating zona-free hamster

TABLE 1. Movement Characteristics of Fluorochrome-Labeled and Unlabeled Human Spermatozoa Before and After Incubation with Ova*

	Unlabeled		FITC		TRITC	
	Percentage Motile ± SEM†	Mean ± SEM Swimming Speed (μm/sec)‡	Percentage Motile ± SEM	Mean ± SEM Swimming Speed (μm/sec)	Percentage Motile ± SEM	Mean ± SEM Swimming Speed (μm/sec)
Pre-incubation	92 ± 3	61 ± 3	95 ± 1	63 ± 3	91 ± 3	62 ± 4
Post-incubation	91 ± 2	67 ± 3	92 ± 2	70 ± 5	90 ± 3	71 ± 4

* Pooled results from eight replicates with eight different semen donors.
† Overall percentages based on 400 cells (50 cells per donor).
‡ Overall means based on 200 cells (25 cells per donor).

ova (Fig. 2; $X^2 7df = 35.4$, $P < 0.005$). The overall percentages of penetrated ova ranged from 24% (8/33, Donor D) to 85% (35/41, Donor C) for spermatozoa from each man. To investigate whether swimming speed was associated with these differences, correlation coefficients were calculated between the proportion of penetrated ova and swimming speeds before and after incubation with the ova. All correlations were low and statistically insignificant. Fluorochrome-labeled and unlabeled spermatozoa were equally capable of penetrating zona-free hamster ova (Fig. 2, Table 2). The FITC and TRITC labels remained associated with the sperm middle piece and principal piece which could be easily identified with the decondensing sperm head (Fig. 3). By alternate use of blue and green excitation, it was possible to unequivocally differentiate the two labeled sperm populations within the same hamster egg. In the experiments with mixed FITC/TRITC-labeled sperm suspensions, the proportions of FITC- and TRITC-labeled sperm within the hamster ooplasm were similar to their proportions in the mixed sperm suspensions (Table 3, $P > 0.40$).

Observations on the penetration of fluorochrome-labeled sperm into the human zona pellucida are also presented in Table 2. Statistical analysis of the variation between donors was not feasible due to the small number of human oocytes used in the experiments. The pooled results, however, indicate that the fluorochrome labels had no effect on the ability of human spermatozoa to attach to and penetrate the human zona pellucida (Table 2, $P > 0.50$). The distribution and intensity of fluorescence on the labeled sperm was unchanged from that observed on cells prior to incubation with ova. The distribution of FITC- and TRITC-labeled spermatozoa within the zona pellucida or perivitelline space of oocytes incubated in mixed sperm suspensions was equivalent to the relative proportions of each population in the mixed suspension (Table 3).

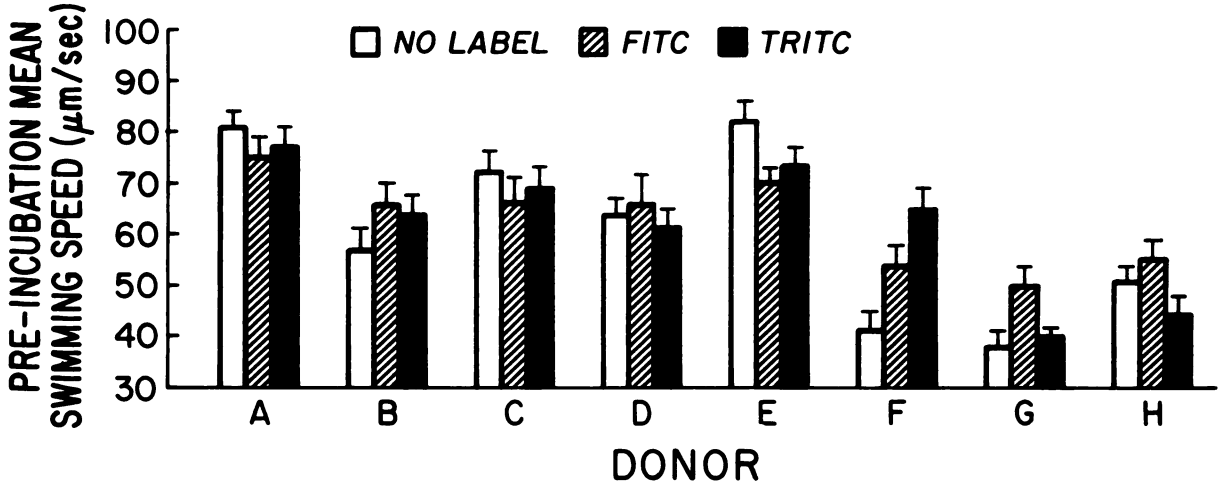

Fig. 1. Mean pre-incubation swimming speeds of fluorochrome-labeled and unlabeled spermatozoa from eight fertile donors. Each mean represents measurements made on 25 spermatozoa. Bars represent one standard error of the mean.

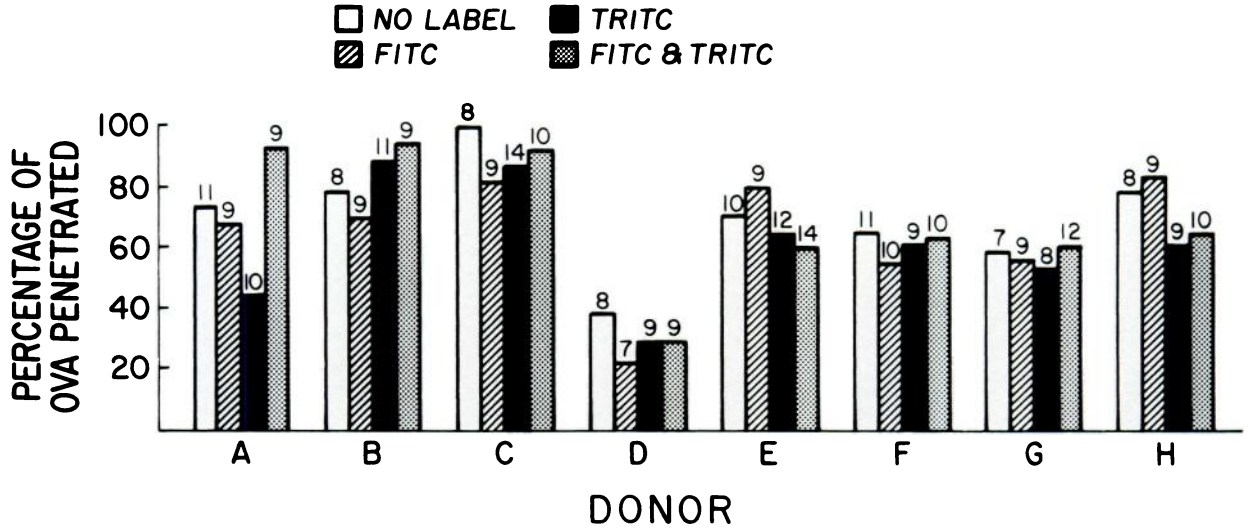

Fig. 2. Penetration of zona-free hamster eggs by fluorochrome-labeled and unlabeled sperm from eight fertile donors. (Number above each bar represents the number of eggs evaluated).

Discussion

There are both clinical and basic scientific applications for laboratory methods that assess physiologic functions of human spermatozoa, such as capacitation and the acrosome reaction. Two principal types of assay have been suggested for measuring the fertilizing capacity of human spermatozoa. One of these systems uses nonfertilizable human oocytes to assess the ability of spermatozoa to penetrate the acellular zona pellucida, an event that requires both capacitation and an acrosome reaction (Overstreet and Hembree, 1976). The second system utilizes mature, living hamster ova from which the zona pellucida has been enzymatically removed (Yanagimachi et al, 1976; Barros et al, 1979; Rogers et al, 1979). This eliminates one of the blocks to interspecies fertilization and allows the human sperm to enter the hamster ooplasm. The assay is also believed to measure the ability of spermatozoa to undergo *in vitro* capacitation and an acrosome reaction (Yanagimachi et al, 1976). The two systems have also been combined in a mixed gamete assay in which sperm penetration of the human zona pellucida and the hamster vitellus were simultaneously assessed (Overstreet et al, 1980).

A major technical problem with *in vitro* gamete interaction systems is the identification of false negative results, ie the apparent poor performance

TABLE 2. Interaction of Fluorochrome-Labeled and Unlabeled Human Spermatozoa with Zona-Free Hamster Ova and the Human Zone Pellucida*

	Sperm Suspension			
	No Label	FITC	TRITC	FITC and TRITC
Number of zona-free hamster ova	71	71	82	83
Percentage ± SEM of hamster ova penetrated	75 ± 6	70 ± 6	68 ± 6	75 ± 7
Number of human ova	9	11	13	15
Mean number ± SEM of sperm in the zona pellucida	27 ± 4	24 ± 4	27 ± 3	31 ± 3
Percentage of ova with sperm in the perivitelline space	67	73	62	73

* Pooled results from eight replicates with eight different semen donors.

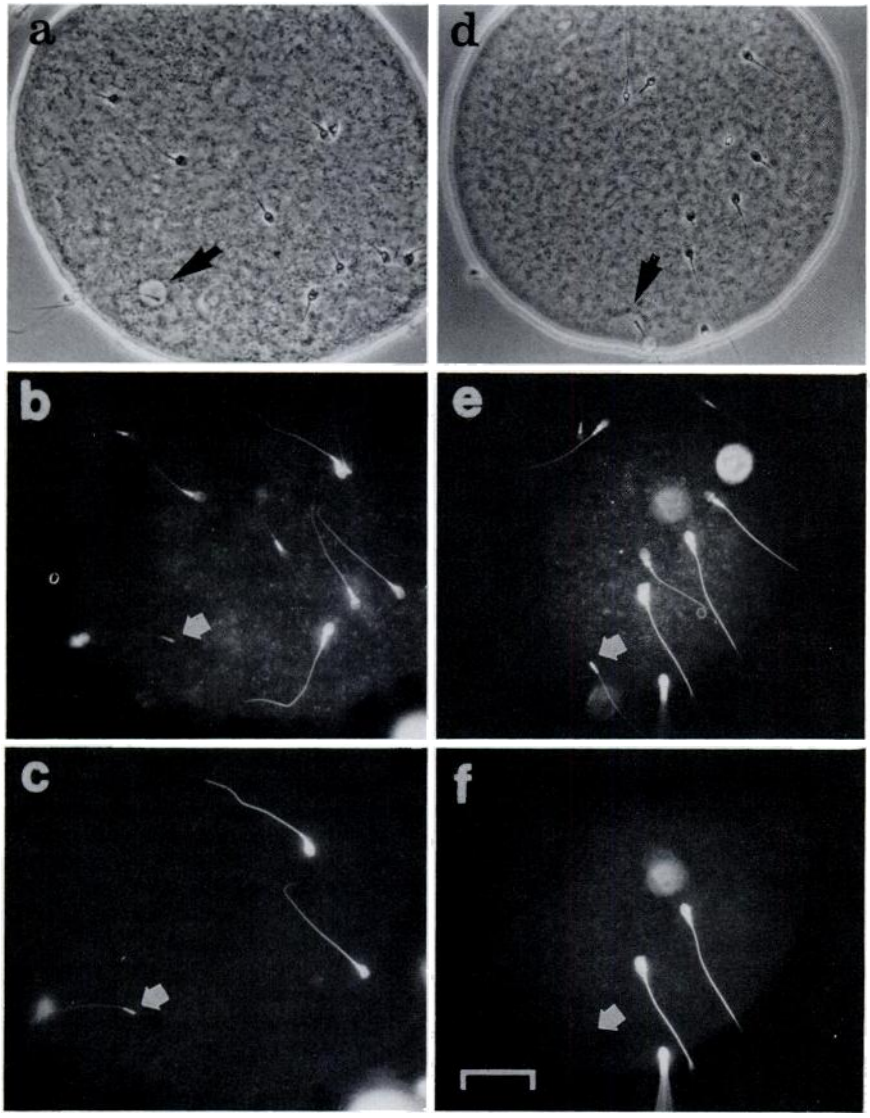

Fig. 3. Zona-free hamster eggs incubated in a mixed FITC/TRITC sperm suspension and penetrated by a TRITC-labeled sperm (A–C) and an FITC-labeled sperm (D–F). Bar represents 30 μm. The phase contrast micrographs (A, D) show a single decondensing sperm nucleus (arrow) with associated tail in each egg. Same eggs viewed under blue excitation (B, E) show the fluorescence on the midpiece and tail of the sperm undergoing nuclear decondensation (arrow). Same eggs viewed under green excitation (C, F) show that FITC-labeled-sperm are no longer visible whereas those bearing the TRITC label remain fluorescent.

of a sperm suspension in a given experiment because of deficiencies in the oocytes, fertilization medium, or technical procedures. The FITC- and TRITC-labeling techniques demonstrated in this study allow experimental designs in which an internal control (eg known fertile sperm population) can be included in the same culture dish with the sperm population being tested. Since zona-free hamster ova exhibit no apparent block to polyspermic fertilization by human spermatozoa

TABLE 3. Distribution of FITC- and TRITC-Labeled Human Spermatozoa in Ova Incubated with Mixed Sperm Populations*

Sperm Label	Percentage ± SEM of Total Sperm in Suspension	Percentage ± SEM of Total Sperm in Hamster Ova	Percentage ± SEM of Total Sperm in Human Zona
FITC	61 ± 4	54 ± 7	58 ± 5
TRITC	39 ± 4	46 ± 7	42 ± 5

* Pooled results from eight replicates with eight different semen donors.

(Binor et al, 1980), the number of "fertilizing" sperm per egg as well as the proportion of eggs penetrated by spermatozoa from each of the labeled sperm populations could be used to compare the fertilizing potential of the two sperm populations. The assay is equally applicable to the clinical assessment of semen quality and to the assessment of the effects of different experimental manipulations.

A similar competitive assay of sperm fertilizing ability *in vitro* could be usefully applied for economically important livestock species. After capacitation, spermatozoa from the boar (Imai et al, 1977) and bull (Lorton and First, 1979) become capable of penetrating zona-free hamster ova. At the present time, the precise relationship between results of *in vitro* fertilization assays and male fertility remains unknown. If a significant correlation between the two exists, competitive *in vitro* fertilization systems may become powerful, inexpensive laboratory methods for evaluating mammalian male fertility potential.

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