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Use of Hoechst 33342 Stain to Evaluate Live Fresh and Frozen Bull Sperm by Computer-Assisted Analysis

ANGELA L. TARDIF, PAUL B. FARRELL, VICTORIA TROUERN-TREND, MICHAEL E. SIMKIN, AND ROBERT H. FOOTE

From the Department of Animal Science, Cornell University, Ithaca, New York.

ABSTRACT: The objective of this research was to investigate possible procedures for evaluating living bull sperm stained with Hoechst 33342 while in a simple medium and in commonly used complex egg yolk–glycerol-Tris (EYGT) and whole milk–glycerol (WMG) extenders. The two semen extenders provide good cryoprotection, but the latter one virtually obscures the sperm. To evaluate sperm motion characteristics when static nonsperm particles are present, a new Hamilton Thorne epifluorescent optical system (UV) with a strobe light was developed for potential use with DNA-stained sperm. This system permitted examination for the first time of sperm motion characteristics in milk. In Experiment 1 (four bull semen replicates with five dye concentrations and three incubation times), 2.5 μg/ml of Hoechst 33342 stained live and dead sperm sufficiently in a modified Tyrode's solution to measure all sperm characteristics without depressing motility, which was validated by using phase-contrast to analyze stained and unstained controls. In Experiments 2a and 2b, each using semen from four bulls with a 5 × 5 factorial arrangement, it was determined that 40 to 60 μg/ml of dye in EYGT or WMG, with UV illumination for 20 minutes, was optimal. There was no detrimental effect on sperm motility. In Experiment 3, analyses of two ejaculates, from each of eight bulls, confirmed that motion characteristics of sperm in EYGT and WMG were not depressed when the sperm were stained with Hoechst 33342. These experiments demonstrate that the dye concentrations and exposure times developed for use with the new epifluorescent optics facilitate evaluating bull sperm frozen in particle-filled whole milk and should be useful for sperm evaluation of a variety of species when nonsperm particulate matter may otherwise interfere.

Key words: Sperm motility, DNA, ultraviolet illumination.

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Evaluation of semen quality is of prime importance in the operation of any andrology laboratory or major artificial insemination organization, and many tests of bull semen quality have been reported (Bratton et al, 1956; Pace, 1980; O'Connor et al, 1981; Amann and Hammerstedt, 1993). With the development of computer-assisted sperm analysis (CASA), new criteria for sperm evaluation were available for many species (Amann and Hammerstedt, 1980; O'Connor et al, 1981; Budworth et al, 1988; Anzar et al, 1991; Schrader et al, 1992; Tuli et al, 1992; Cran et al, 1993; Davis and Katz, 1993; Barisic et al, 1994; Farrell et al, 1996a; Seed et al, 1996). These studies depended on the identification of sperm with phase-contrast optics. However, semen from some species and several semen buffers and extenders, such as egg yolk or whole milk, contain particulate matter, which can prevent analysis by CASA or can lead to erroneous results. The use of DNA-specific dyes to stain sperm heads and instrumentation equipped with fluorescence illumination of appropriate wavelengths (hereafter referred to as UV) can overcome this problem, providing that the exposure time is minimal. For example, DNA staining has been combined with flow cytometry to examine a few characteristics of sperm (Evenson et al, 1982; Garner et al, 1988; Graham et al, 1990; Kramer et al, 1993), but many sperm characteristics that are considered to be important for a thorough semen examination cannot be evaluated by flow cytometry.

Recently, Hamilton Thorne Research (Beverly, Massachusetts) produced an integrated visual optical system (IVOS) equipped with strobe light UV illumination to be used with the DNA-specific Hoechst 33342 stain for CASA (Farrell et al, 1996b). Preliminary observations indicated that this equipment had the potential to evaluate characteristics of sperm cells that were frozen in media containing many nonsperm particles.

The current study was designed such that the same ejaculates of bull semen were frozen in egg yolk–glycerol-Tris (EYGT) and in whole milk–glycerol (WMG), media widely used for sperm cryopreservation and artificial insemination (Almquist, 1962; Foote, 1970; Foote et al, 1993). Sperm in EYGT could be examined with both phase-contrast and UV optics, whereas sperm in WMG could be examined with UV illumination only. For comparison, fresh sperm were diluted with a Tyrode's solution containing albumin, lactate, and pyruvate (TALP), which is compatible with the sperm of several species (Tuli et al, 1992; Farrell et al, 1996a). Thus, experiments

Correspondence to: R. H. Foote, Department of Animal Science, Cornell University, Ithaca, New York 14853-4801.

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were designed to test the hypothesis that bull sperm could be stained with sufficient Hoechst 33342 to detect all motile and static sperm in optically clear and in particle-filled media without altering sperm cell motion characteristics. This determination was possible because of the brief exposure of sperm to UV illumination with the strobe light.

Materials and Methods

Bull Semen

Semen was obtained from fertile bulls on a regular semen collection schedule at Genex Cooperative, Inc. (Ithaca, New York). Standard procedures (in use at this large artificial breeding center) were followed for semen collection and for initial semen processing (Foote et al, 1993).

Semen Extenders and Semen Processing

Following the initial determinations of the percentage of motile sperm and the sperm concentration in fresh semen by personnel at this center, aliquots of semen were mixed at 35°C with TALP, EYGT, and WMG. The optically clear TALP buffer was tested only with fresh semen, as it does not protect sperm during cooling or freezing. The composition of TALP was described recently (Farrell et al, 1996a): it is isotonic with bull sperm, and it has a pH of 6.8. The Hoechst 33342 stain (catalog no. B2261; Sigma Chemical Co., St. Louis, Missouri) was added directly to this medium, which was free from possible interfering particulate material Semen was diluted with TALP to produce a final concentration of 20×10^6 sperm/ml for CASA. Details of the design for each experiment are given subsequently.

The EYGT, which is isotonic with sperm and has a pH of 6.8, was prepared as described by Foote (1970). The heated WMG, which is also isotonic with sperm and has a pH of 6.7, was prepared according to procedures described by Foote et al (1993). Semen in EYGT and WMG was surrounded by a water jacket and was cooled from 35°C to 5°C over a period of 3 hours. After cooling, the semen was extended to 40×10^6 total sperm/ml. The final concentration of glycerol in both EYGT and WMG was 7% (v/v).

Extended semen was packaged in 0.5-ml coded straws and was tightly sealed. Within 4 hours after the glycerol was added, the packaged semen was ready for freezing. Semen was frozen in straws cooled at approximately −15°C/minute from 5°C to −115°C. Straws were then transferred to liquid nitrogen at −196°C. A sufficient number of straws were processed so that multiple replicates and experiments could be conducted over time using the same supply of semen for the different treatments, thus adding precision to the experiments.

The frozen semen was stored in liquid nitrogen at −196°C until used. Straws were thawed at 35°C for 30 seconds in a water bath. Duplicate straws for each treatment were thawed and pooled for CASA.

CASA

For all experiments, the IVOS (version 10 model, Hamilton Thorne Research) equipped with UV illumination was used. This unit has a strobed xenon light source with a double band-pass filter. For fluorescence, the 50% excitation band-pass is 327 to 395 nm, and the 50% emission band-pass is 423 to 487 nm. The peak emission of Hoechst 33342 stain in solution was increased approximately 45 nm from the emission peak of stained DNA, so fluorescence of unattached stain in solution essentially was excluded by the emission filter (Douglas-Hamilton, personal communication).

For all experiments, both 20-μm microcell chambers (Conception Technologies, La Jolla, California) per slide were filled with 7 μl of the same sperm suspension containing 20×10^6 sperm/ml. To reduce the frozen-thawed preparations to 20×10^6 sperm/ml, Tris buffer without egg yolk was added to EYGT in an equal volume, and WMG was added similarly to sperm frozen in WMG. Microcell chambers were maintained at 37°C. The same fields of sperm were examined with both phase-contrast and UV systems. The internal optics of the IVOS system were used to record six fields for 10 seconds each with a Super Video Home Systems (JVC, Tokyo, Japan) video recorder. Although six fields per chamber were evaluated, two fields at each end of the chamber were omitted. Unpublished observations indicated that these fields tended to provide divergent results. Therefore, each subclass of data was based upon evaluating sperm in 12 fields.

Individual fields were examined visually to establish settings for the IVOS unit that would result in the proper identification of motile and nonmotile fresh and frozen-thawed sperm in different media, while ignoring static particles. It was especially important to determine that unusual swimming patterns, such as the starlike patterns (Mortimer and Mortimer, 1990) of hyperactive sperm, were accurately identified by CASA. Instrument settings were similar to those previously reported (Farrell et al, 1996a,b).

Experiment 1

Experiment 1 was a randomized block design with four bulls and a 5×3 factorial arrangement to establish the effects of Hoechst 33342 dye on sperm motion characteristics in a simple TALP diluent. Semen from each of the four bulls was diluted to 20×10^6 sperm/ml with TALP containing 0, 2.5, 5, 10, and 20 μg/ml of Hoechst 33342. Phase-contrast was used for CASA after 0 and 30 minutes of incubation at 37°C. Ultraviolet illumination was used to evaluate the stained preparations after 30 minutes, as there was only minimal fluorescence at 0 minutes. The CASA was performed as described previously.

Experiments 2a and 2b

Experiment 2a was a randomized block design with four bulls and a 5×5 factorial arrangement of treatments to establish the effects of Hoechst 33342 dye on sperm motion characteristics in EYGT, a medium containing substantial amounts of organic particles. Semen from two ejaculates from each of four bulls was frozen in EYGT, and, upon thawing, the extended semen from two straws per dye treatment was mixed 1:1 (v/v) with Tris buffer (no egg yolk) to yield 20×10^6 sperm/ml. Hoechst 33342 dye was added from a concentrated stock solution (1 mg/ml) to a series of tubes of semen to produce 0, 10, 20, 40, and 60 μg/ml

Table 1. *Experiment 1. Means for nine variables estimated by CASA for sperm diluted with TALP solution containing different concentrations of Hoechst 33342 dye**

Stain (μg/ml)	Time (minutes)	Illumination	VAP	VSL	VCL	ALH	BCF	STR	LIN	MOT	PRG
0	0	Phase	140	124	233	7.0	46	87	54	80	61
0	30	Phase	128	116	205	6.2	46	88	57	71	55
Mean			134	120	219	6.6	46	88	56	76	58
2.5	0	Phase	135	116	228	6.9	46	85	53	85	60
2.5	30	Phase	123	110	200	6.2	46	87	55	73	53
2.5	30	UV	131	119	207	6.1	45	91	60	70	60
Mean			130	115	212	6.4	46	87	56	76	58
5.0	0	Phase	132	113	227	7.2	45	84	51	86	59
5.0	30	Phase	114	101	193	6.3	44	87	54	68	48
5.0	30	UV	121	108	200	6.6	42	89	56	67	55
Mean			123	107	207	6.7	44	87	54	74	54
10.0	0	Phase	133	109	235	7.4	44	82	48	84	52
10.0	30	Phase	111	95	191	6.4	41	85	52	76	50
10.0	30	UV	113	101	191	6.3	41	89	55	66	53
Mean			119	102	205	6.7	42	85	52	75	52
20.0	0	Phase	128	104	230	7.5	43	80	47	83	50
20.0	30	Phase	58	41	126	6.6	32	66	32	57	18
20.0	30	UV	76	60	155	6.6	35	81	42	36	17
Mean			87	68	170	6.9	37	76	40	58	28

CASA, computer-assisted sperm analysis; TALP, Tyrode's albumin-lactate-pyruvate; VAP, mean path velocity (μm/second); VSL, straight line velocity or progressive velocity (μm/second); VCL, critical velocity or track speed (μm/second); ALH, amplitude of lateral head displacement (μm); BCF, beat cross frequency (Hz); STR, straightness (percent, VSL/VAP); LIN, linearity (percent, VSL/VCL); MOT, percentage of motile sperm; PRG, percentage of progressive motile sperm with >80% threshold straightness and with medium VAP cutoff of >60 μm/second for frozen semen and >75 μm/second for fresh semen.

* Significant differences are not indicated in the table because, after reanalyzing the data and excluding the 20 μg/ml concentration of dye, which was toxic, most differences were not significant (see text).

of the dye. The semen was incubated at 37°C for 0, 10, 20, 30, and 45 minutes before performing CASA.

Experiment 2b was identical in design to Experiment 2a, but the semen extender was WMG, in which bull sperm cannot be measured with visible light. Therefore, sperm could be analyzed only using the UV mode.

Experiment 3

Two ejaculates of semen from each of four bulls (in addition to the bulls used in Experiments 1 and 2) were collected and were frozen in EYGT and WMG extenders, providing a total of 16 ejaculates from eight bulls, to compare CASA procedures and extenders. Based on the results of Experiment 2b, a subset of straws, with sperm frozen in EYGT, was processed as before but was stained with 40 and 60 μg/ml of Hoechst 33342 dye and was incubated at 37°C for 20 minutes. Sperm that were frozen and thawed in WMG were stained with 50 μg/ml of Hoechst 33342 dye and were incubated at 37°C for 20 minutes. Sperm in EYGT and WMG were evaluated by CASA using UV illumination, and sperm in EYGT without stain were analyzed with phase-contrast illumination.

Statistical Analysis

Nearly 250,000 sperm were analyzed to validate the procedures. A general linear mixed model was used for the analysis of variance. The analysis for each of the variables determined by CASA consisted of a random bull effect and fixed effects of media or extender, dye concentration, and dye exposure time. The bull effect represented the combined effect of two semen collections per bull in Experiments 2a and 2b. Chambers and interactions contributed little to the variance, so these variables were included in the error term. Differences among means were considered to be statistically significant at $P \leq 0.01$, when tested by Tukey's honest significant difference. With 12 fields per treatment, factorially arranged treatments, and multiple replications, the number of degrees of freedom for error was large, and many small differences were significant at $P \leq 0.01$.

Results

Experiment 1

Table 1 presents the means for the variables measured in fresh semen diluted with TALP containing various concentrations of Hoechst 33342 dye. Details are provided as these are the first published data comparing UV and phase-contrast illumination of bull sperm. With the large number of degrees of freedom, the standard errors were small and were not particularly informative (1–3% of the means). For simplicity, these standard errors are not included in Table 1. None of the dye concentrations at 0 minutes stained sperm sufficiently to evaluate the sperm using UV illumination, so only the results with phase-contrast illumination were obtained at 0 minutes. By 30 minutes, both static and motile sperm fluoresced brightly.

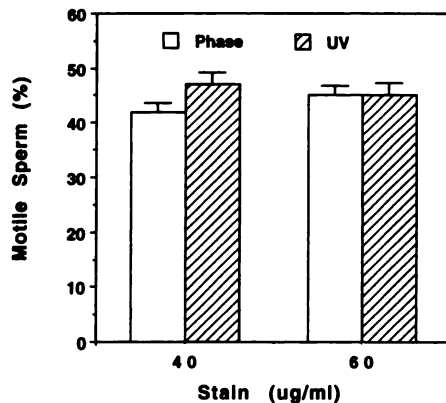

FIG. 1. Experiment 2a. The percentage of motile sperm in egg yolk-glycerol-Tris (EYGT) measured with phase-contrast and UV illumination after exposure of the sperm for 20 minutes to 40 or 60 μg/ml of Hoechst 33342 stain.

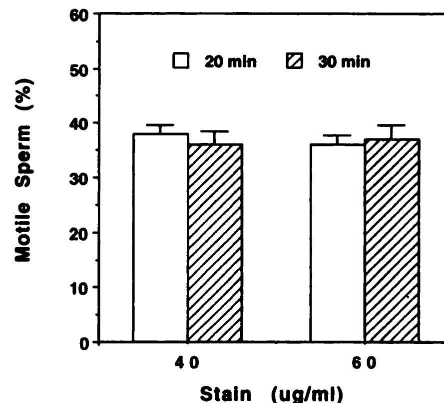

FIG. 2. Experiment 2b. The percentage of motile sperm in whole milk-glycerol (WMG) measured with UV illumination after exposure of sperm for 20 or 30 minutes to 40 or 60 μg/ml of Hoechst 33342 stain.

All of the main sources of variation (bulls, stain concentration, and time and wavelength of illumination), as well as most of the first order interactions, were significant ($P \leq 0.01$) in the initial analysis. The data were reanalyzed after excluding the 20 μg/ml dye treatment, which was somewhat toxic. Then most characteristics analyzed with phase-contrast and UV illumination no longer were different ($P > 0.05$).

Experiment 2a

In this experiment, Hoechst 33342 dye concentrations of 0, 10, 20, 40, and 60 μg/ml in EYGT and incubation times of 0, 10, 20, 30, and 45 minutes were compared. A dye concentration of 20 μg/ml or greater with an exposure time of 20 minutes was required to provide uniform staining of the sperm heads. No increase in intensity of the stain was detected visually after 20 or 30 minutes. Analysis of variance of semen exposed to the dye concentrations of 20 μg/ml or higher for 20 minutes or longer resulted in no significant differences ($P > 0.01$). Because of the lack of differences, only example means for the percentage of motile sperm, considered to be one of the most important sperm characteristics, are shown in Figure 1. The bull effect was significant in this and in all experiments ($P < 0.01$), signifying that differences in semen quality among bulls were detected by CASA.

Experiment 2b

This experiment was conducted with semen in WMG from the same bulls that were used in Experiment 2a, with the same five dye concentrations and the same five exposure times. However, sperm in WMG could be observed only with UV illumination. As in EYGT, Hoechst 33342 dye concentrations of 20, 40, or 60 μg/ml for 20 and 30 minutes produced brightly stained sperm that could be analyzed. There were no differences among the various treatment effects ($P > 0.01$); however, there were small but significant effects on track speed and beat cross frequency. For comparison with Experiment 2a, the results measured with UV light after exposure of sperm for 20 or 30 minutes in WMG are shown in Figure 2. Note the similarity of sperm motility results between the two dye concentrations and the two exposure times.

Experiment 3

To provide further replication of the most useful treatments in the previous experiments, sperm from eight bulls were frozen in EYGT, were stained with 40 and 60 μg/ml of Hoechst 33342 dye, were incubated for 20 minutes, and were examined with UV illumination. There were no differences caused by dye concentration for any of the variables ($P > 0.01$). Therefore, only the overall means for the two dye concentrations for EYGT are compared in Table 2 with sperm frozen in WMG, stained with 50 μg/ml of dye, and examined with fluorescence after 20

Table 2. *Experiment 3. Comparison of CASA characteristics obtained with UV illumination of frozen-thawed sperm in EYGT and WMG stained for 20 minutes*

Characteristics*	EYGT	WMG
VAP	82†	88†
VSL	73†	81‡
VCL	130†	127†
ALH	5.3†	4.5‡
BCF	36†	36†
STR	89†	92†
LIN	58†	66‡
MOT	47†	46†
PRG	32†	35†
HYP	2.5†	0.6†

CASA, computer-assisted sperm analysis; EYGT, egg yolk-glycerol-Tris; WMG, whole milk-glycerol; HYP, percentage of hyperactivated sperm.

* For definitions of abbreviations, see Table 1.

†,‡ Means within a row with different superscripts differ ($P < 0.01$).

minutes of incubation. The percentage of motile sperm in the two semen extenders was similar. Unstained sperm in EYGT, estimated with phase-contrast optics, averaged 45% motile, which was similar to the 47% estimated for stained sperm in EYGT and the 46% in WMG.

Fully hyperactivated sperm averaged 2.5% in EYGT and 0.6% in WMG. These low values were expected because sperm were not incubated for capacitation and because partially capacitated sperm probably were not detected.

Discussion

The development of CASA equipment introduced a new dimension in the evaluation of semen (Amann and Hammerstedt, 1980, 1993; O'Connor et al, 1981; Budworth et al, 1988; Anzar et al, 1991; Schrader et al, 1992; Tuli et al, 1992; Davis and Katz, 1993; Iqbal and Hunter, 1995; Farrell et al, 1996a). However, sperm in semen with many nonsperm particles, such as bull semen processed with WMG extender (Foote et al, 1993), cannot be detected with phase-contrast optics. An alternative to overcome this problem was to develop equipment that could detect the high density DNA in sperm heads and that would provide minimal exposure of sperm to UV light.

The DNA-specific Hoechst 33342 stain has been useful in flow cytometric studies of sperm evaluation (Evenson et al, 1982; Garner et al, 1988; Graham et al, 1990; Kramer et al, 1993). The stain does not render sperm cells infertile since semen stained with Hoechst 33342 has produced normal calves (Cran et al, 1993), and fertility of sperm used for artificial insemination following staining with Hoechst 33342 was not affected (Morrell and Dresser, 1989). Initial studies with human sperm (Farrell et al, 1996b) indicated that this relatively inexpensive stain could be useful.

The development of CASA instrumentation with UV illumination described here makes the potential evaluation of multiple characteristics of sperm motion obscured by particulate matter when using visible light microscopy possible. After establishing a procedure for distinguishing sperm from other opaque material, such as WMG, it was important to establish that the dye itself did not alter sperm motion characteristics of fresh and frozen-thawed semen during the evaluation procedure at 37°C. Consequently, experiments were designed to test the effects of the dye in a simple medium with no macromolecule protection as well as in a complex, more viscous media used for freezing sperm. Both stained and unstained sperm preparations were examined with phase-contrast optics and with UV illumination.

As a result of these experiments, concentrations of dye and convenient operational exposure times were established that did not appreciably alter the proportion of motile sperm or other motion characteristics. It should be noted that 2.5 μg/ml of Hoechst 33342 in TALP stained live and dead sperm sufficiently for CASA (Table 1). However, when a substantial number of macromolecules were present, such as in EYGT and in WMG, considerably higher concentrations of the Hoechst 33342 were required for proper staining. These higher concentrations of dye caused no detectable toxicity (Figs. 1, 2; Table 2). The reasons for the difference are unknown, but presumably the complex media interact with the sperm membrane and alter staining. The EYGT and WMG also slightly altered the type of motion of the sperm cells (Table 2). The greater lateral head displacement in EYGT could contribute to the lower progressive velocity, which, along with track speed, is used to calculate linearity.

Although continuous exposure of fluorescently stained cells to UV illumination is often harmful, the illumination in the Hamilton Thorne IVOS unit is not continuous. The exposure to the strobe light is about 10 to 15 microseconds, with a frequency of 60 cycles/second, so sperm are exposed very briefly. Furthermore, only a small subsample is used for analysis, and the sperm used for biological purposes are not exposed to the DNA stain.

The need to sample semen carefully and to control all operating procedures in order to obtain precise and accurate estimates of sperm motion has been discussed (Budworth et al, 1988). In the present study, analysis of sperm in two chambers, with 12 fields, usually resulted in the analysis of more than 200 sperm per subclass, which was helpful in reducing sampling variation (Schrader et al, 1992).

From a clinical standpoint, of particular interest is the fact that the majority of the variance for most traits in these experiments was associated with semen from different bulls. For example, in Experiment 3 the motility for unstained sperm in EYGT, stained sperm in EYGT, and stained sperm in WMG for the eight bulls ranged from 30 to 55%, 37 to 54%, and 32 to 66%, respectively, and more than 50% of the total variance was due to the bulls. This large bull variation provides a basis for anticipating a relationship to fertility. Whereas O'Connor et al (1981) and Budworth et al (1988) reported low correlations between CASA measurements and fertility estimates, O'Connor et al (1981) pointed out that fertility was not measured very precisely. When a competitive fertility index was compared with multiple measurements of sperm motion, Budworth et al (1988) reported high correlations (≥0.87).

The Hamilton Thorne equipment with UV optics provides an opportunity to investigate sperm characteristics under many conditions where sperm movement may be obscured or inaccurately monitored. An increase in precision by accurately discriminating between sperm and

debris can facilitate studying the relationship between sperm characteristics and their potential fertilizing ability.

In conclusion, bull sperm can be stained with Hoechst 33342 dye at concentrations that permit the Hamilton Thorne unit, equipped with appropriate UV optics, to discriminate accurately between sperm and nonsperm particles. Different protocols were established for CASA with stained living sperm suspended in various media in which sperm motion characteristics were not altered appreciably by the DNA-specific Hoechst 33342 dye. Thus, the capability of distinguishing between sperm and nonsperm interfering particulate material expands the opportunity to study sperm function under a variety of conditions and to relate these conditions to the fertilizing potential of the sperm.

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