Effect of Cryopreservation on Bovine Sperm Organelle Function and Viability As Determined by Flow Cytometry[1]

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ABSTRACT

Flow cytometry was used to compare the functional status of fluorescently stained sperm organelles from 12 Holstein bulls after storage for 24 h at 5°C and after cryopreservation. The organelle-specific stains, SYBR-14 and LysoTracker Green DND-26, identified spermatozoa with intact plasmalemma and those with intact acrosomes, respectively. The mitochondria-specific stain, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), identified two populations of spermatozoa. One population stained red-orange because the JC-1 accumulated in the mitochondria as aggregates (characteristic of cells exhibiting a high membrane potential); a second population stained green because of JC-1 monomers within the mitochondria (characteristic of cells exhibiting a lower membrane potential). Analysis of variance revealed that within bulls, the properties of sperm viability, intact acrosomes, and mitochondrial status differed in spermatozoa stored for 24 h ($p < 0.001$) but not in cryopreserved spermatozoa ($p > 0.11$). Linear regression analyses resulted in significant models in which the proportions of stained spermatozoa stored for 24 h were indicative of those proportions observed in the cryopreserved fractions. These findings suggest that the plasmalemma, the acrosome, and the mitochondria of unfrozen spermatozoa varied as to their functional status. The cryopreservation process, however, resulted in a more uniform status of sperm organelles.

INTRODUCTION

It is generally known that the process of cryopreservation causes approximately a 50% decrease in sperm viability due to temperature and osmotic effects, and that morphological changes occur in the organization, fluidity, permeability, and lipid composition of the sperm membranes. A review of new research on the cryopreservation of mammalian spermatozoa [1] enumerates studies demonstrating that the injuries are caused during cooling but manifested during thawing. In his review, Watson [1] stated that although the assumption has been that the post-thaw motile spermatozoon is similar to its pre-freeze counterpart, it is becoming more clear that the survivors are different, appearing to be in a capacitated-like state [1, 2].

Ultrastructural observations of post-thaw ram spermatozoa that were cryopreserved without benefit of any cryoprotectant demonstrated differences in membrane stability between major cellular compartments [3]. The damage to sperm mitochondria [4–6] caused a decrease in ATP production and thus in sperm motility. Cryoprotective agents such as glycerol helped to increase post-thaw motility but did not protect the mitochondria from freeze-thaw injury [6]. In fact, glycerol, the very agent that helps stabilize sperm membranes during cryopreservation, had detrimental

effects on compartmental membranes to varying degrees [7], but these effects differed among species [1].

Some commercial bull stud organizations assess the quality of fresh and post-thaw ejaculates primarily by using three parameters: 1) the number of sperm present or concentration of the ejaculate; 2) the percentage of progressively motile spermatozoa; and 3) the percentage of intact acrosomes present. Sperm counts are usually made using spectrophotometry. Motilities of incubated and nonincubated sperm are routinely estimated visually, while acrosomal integrities are determined using differential counts of 100–200 spermatozoa. These methods indirectly assess metabolic activity and viability, are labor-intensive and time-consuming, and have inherent variability. In contrast, various combinations of organelle-specific fluorescent stains and flow cytometric quantification allow a rapid and precise assessment of the organelle status of thousands of individual spermatozoa in each semen sample [8].

The fluorometric staining combination, SYBR-14 and propidium iodide (PI), has been shown to be a rapid and reliable means for determining the proportions of living and dead spermatozoa in semen [9–19]. These nucleic acid-specific probes stain living spermatozoa green while dead, membrane-damaged spermatozoa stain red. Upon cell death, the PI rapidly overwhelms the fluorescence exhibited by the SYBR-14.

The acrosomal probe, LysoTracker, is an acidotropic probe that identifies living spermatozoa with intact acrosomes and stains the acrosome green. It has been demonstrated that the percentage of spermatozoa staining with LysoTracker was correlated with the microscopic determination of the percentage of spermatozoa displaying normal acrosomal ridges ($r = 0.97$; $p < 0.001$) [11].

The mitochondrial probe, JC-1, exists as a monomer with excitation and emission peaks in the green wavelengths (510–520 nm). However, it also exhibits a second peak in the red-orange range (590 nm) depending on dye concentration, pH, ionic strength, and temperature [20]. The phenomenon producing the red-orange fluorescence has been referred to as J-aggregate formation [20–23]. It was reported that in isolated rat mitochondria, the aggregates were correlated with membrane potential ($r = 0.97$), but the monomers were not ($r = 0.20$) [24]. To date, data on JC-1 staining have been reported primarily for isolated mitochondria in humans and rats. In samples of cryopreserved bovine spermatozoa stained with JC-1, the combined proportions of monomers and aggregates were correlated with visual estimates of progressive forward motility ($r = 0.97$) [10].

In this experiment, we assessed the effects of cryopreservation on different sperm compartments using three different organelle-specific probes: SYBR-14, LysoTracker, and JC-1. To assess the sperm plasmalemma, acrosomal status, and mitochondrial function, the statuses of these organelles were compared with each other before and after

cryopreservation, using visual estimates of motility and differential counts of acrosomal integrity. The JC-1-stained samples were evaluated for four characteristics to determine which were most sensitive to cryopreservation effects. These characteristics were the proportions of aggregates, the proportions of monomers, the ratio of aggregates:monomers, and the sum total of JC-1-stained spermatozoa.

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: from Molecular Probes, Inc. (Eugene, OR)—SYBR-14 (available as Component A of the LIVE/DEAD Sperm Viability Kit, L-7011), LysoTracker Green DND-26 (LYSO; L-7526), 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; T-3168), and PI (P-1304); from Sigma Chemical Co. (St. Louis, MO)—Tyrode's salt solution (T-2397), Hepes (H-3375), and BSA Fraction V (A-4503); and from Aldrich Chemical Co.—anhydrous dimethyl sulfoxide (DMSO; 27,685-5).

Semen Samples

Ejaculated semen from 12 Holstein bulls was diluted, processed, and cryopreserved in a 20% egg yolk, 2.9% sodium citrate medium containing 7% glycerol, and packaged at a concentration of 70×10^6 spermatozoa/ml in 0.5-ml French straws (Select Sires, Inc., Plain City, OH). The 12 bulls used in this study were selected to provide a range of high to low semen quality based on historical individual records. Samples having greater than 80% viable or normal spermatozoa were considered to be high-quality semen while those exhibiting less than 50% were considered to be low-quality semen. At the Select Sires facility, straws were thawed or warmed standing vertically or incubated for 3 h in a 37°C water bath, and analyzed for the classical seminal quality parameters of initial motility (MOT), post-thaw motility (MOT0), post-thaw motility after 3 h (MOT3), and intact acrosomes (IA). Aliquots of the samples were shipped to Reno, Nevada, at 5°C; ice packs were shielded from direct contact with the straws. Frozen samples were cryopreserved in a static vapor freezer and shipped to Reno, Nevada, in liquid nitrogen (−196°C).

Flow Cytometry

Information on 10 000 spermatozoa examined fluorometrically was collected in list mode on a FACS Analyzer flow cytometer (Becton-Dickinson, Sunnyvale, CA). The generated data were examined using a Hewlett Packard (Palo Alto, CA) Consort 30, a 200-series computer. SuperCyt Analyst 3 software (Sierra Cytometry, Reno, NV) was used to partition and quantify dot plot sperm populations. The flow cytometer was equipped with 1) a standard fluorescein isothiocyanate and phycoerythrin dichroic filter (DF) set; 2) 400-nm long pass (LP) and DF 485/22 nm band pass excitation filters; 3) a DM-560 dichroic mirror to separate fluorescent signals; 4) a photomultiplier tube 1 (FL1) collecting 515–545-nm light through a DF 530/30 bandpass filter; and 5) an FL2 collecting light through an LP 570 filter. Electronic compensation was used to minimize spill-over of green fluorescence into the red channels.

Microscopic Examination

At Select Sires, the percentages of progressively motile spermatozoa were classified using the methods of Saacke

and Marshall [25]. Sperm motility estimates were made before freezing (MOT), immediately after thawing (MOT0), and again after 3-h incubation (MOT3). Motility percentage values were obtained using a thermostatically warmed stage (37°C) and light microscopy at ×100 magnification. Estimates of intact acrosomes (IA) were determined by differentially counting the number of spermatozoa with and without intact acrosomal ridges using differential interference contrast microscopy at ×1000 magnification.

Fluorescent Examinations of Organelle Function

Three stock solutions were prepared in Hepes saline medium (130 mM NaCl, 4 mM KCl, 14 mM fructose, 10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% BSA). Each contained 12 μl of 2.99 mM PI in Tyrode's salt solution/ml Hepes/BSA, plus either 1.4 μl of 0.1 mM SYBR-14 in DMSO/ml Hepes/BSA, 5.0 μl of 0.1 mM LYSO in DMSO/ml Hepes/BSA, or 2.0 μl of 1.53 mM JC-1 in DMSO/ml Hepes/BSA. One milliliter from each stock solution was dispensed into 36 Eppendorf microcentrifuge tubes and warmed in heater blocks at 37°C. Three 0.5-ml straws from each bull were warmed or thawed at 37°C in a CITO Warm Water Thaw (CITO Products, Inc., Watertown, WI) approximately 9 min apart to accommodate running time on the flow cytometer. Each straw was dispensed into a 1-ml prewarmed stock solution and was incubated at 37°C for 30 min before flow cytometric analyses. Final stain concentrations present in sperm samples were, therefore, 23.9 μM PI, 93.3 nM SYBR-14, 333.3 nM LysoTracker, and 2.0 μM JC-1.

Statistical Analyses

Data were analyzed using ANOVA, regression analysis, and correlations. Orthogonal contrast analysis compared stained populations within bulls, one against another. Regression analyses used Statistical Analysis Systems (SAS) macros for regression analysis (Fernandez GCJ. Simple linear regression: a SAS macro for doing linear regression and checking assumptions. In: APST705 Linear and Nonlinear Regression Class Notes; 1996; University of Nevada, Reno, 89557); these macros produced both numeric test results and visual plots to check the underlying assumptions necessary for valid regression models. The relationships among variables were evaluated using correlation coefficients generated by SAS. The experimental design was a 12 × 3 split-plot in randomized complete block design using the general linear model of SAS. Three straws from each bull provided blocked replications; the main plot (A) included bulls 1 through 12; and the split plot (B) included the populations of spermatozoa stained with SYBR-14, LysoTracker, and JC-1.

RESULTS

Microscopic Examinations

The mean percentages of spermatozoa displaying progressive forward motility immediately after ejaculation (Table 1), immediately after thawing, and post-thaw after 3-h incubation at 37°C (Table 2) ranged from 75% to 88%, 60% to 75%, and 25% to 35%, respectively. The mean percentages of spermatozoa displaying intact acrosomes in post-thaw samples after 3-h incubation at 37°C ranged from 39% to 88%.

Microscopic examination of spermatozoa that had been fluorescently stained with SYBR-14 and PI appeared as

TABLE 1. Percentages of motile spermatozoa observed immediately after ejaculation and fluorometric percentages of stained spermatozoa in semen that was stored 24 h at 5°C (means ± SD).

Bull no.	Motility[a]	SYBR-14[b]	LYSO[c]	Monomers[d]	Aggregates[e]	Total JC-1[f]
1	87.5 ± 3.5	76.4 ± 6.9	71.7 ± 5.4	6.2 ± 1.0	73.8 ± 0.4	79.9 ± 1.3
2	77.5 ± 3.5	68.7 ± 2.9	72.5 ± 1.4	30.6 ± 2.5	42.3 ± 4.7	72.9 ± 2.4
3	80.0 ± 7.1	71.6 ± 3.7	77.5 ± 0.7	24.5 ± 1.6	56.9 ± 2.1	81.5 ± 0.8
4	75.0 ± 0.0	68.1 ± 3.1	70.1 ± 2.2	64.2 ± 2.9	9.6 ± 0.8	73.9 ± 2.1
5	75.0 ± 0.0	70.5 ± 3.6	73.6 ± 2.0	52.3 ± 1.2	27.1 ± 1.0	79.4 ± 1.3
6	85 ± *	38.3 ± 3.6	40.9 ± 1.1	34.0 ± 2.4	12.9 ± 1.8	46.9 ± 1.0
7	82.5 ± 3.5	54.2 ± 3.3	60.2 ± 4.1	35.5 ± 4.1	30.9 ± 4.7	66.5 ± 3.2
8	82.5 ± 3.5	73.1 ± 3.2	76.8 ± 3.9	24.8 ± 0.7	58.6 ± 0.3	83.3 ± 0.9
9	75.0 ± 0.0	58.2 ± 1.3	57.0 ± 1.8	44.7 ± 1.4	19.2 ± 2.0	63.9 ± 1.1
10	75 ± *	49.2 ± 0.9	47.0 ± 2.6	42.7 ± 3.2	24.3 ± 1.3	67.0 ± 1.9
11	82.5 ± 3.5	68.6 ± 4.5	70.1 ± 0.3	28.4 ± 1.2	44.2 ± 2.1	72.7 ± 1.1
12	82.5 ± 3.5	69.1 ± 5.3	76.8 ± 1.2	31.3 ± 5.6	49.0 ± 5.7	80.3 ± 0.7
Mean ± SEM	80.0 ± 1.3	63.8 ± 3.3	66.2 ± 3.5	34.9 ± 4.3	37.4 ± 5.8	72.3 ± 3.0

[a] Visual estimate of sperm motility (%; n = 2).
[b] Percentage of 10 000 spermatozoa that stained with SYBR-14 (n = 3).
[c] Percentage of 10 000 spermatozoa that stained with LysoTracker (n = 3).
[d] Percentage of 10 000 spermatozoa that stained with JC-1 and emitted green fluorescence, termed monomers (n = 3).
[e] Percentage of 10 000 spermatozoa that stained with JC-1 and emitted red-orange fluorescence, termed aggregates (n = 3).
[f] Percentage of 10 000 spermatozoa that stained with JC-1 (monomers plus aggregates (n = 3).
* Only one motility value was determined.

motile green cells or as red immotile cells, as shown previously in micrographs at ×1000 [11]. LysoTracker/PI-stained spermatozoa were seen as either a dull green acrosomal cap covering an unstained cell or, in moribund spermatozoa, as a dull green cap overlying a red sperm head [11]. Sperm samples to which JC-1 and PI had been applied displayed three fluorescent types: those with red heads and faint green mitochondria, those with unstained heads and bright green mitochondria, and those with unstained heads and bright orange clusters amid green mitochondria. Color micrographs of the latter have been previously published [10].

Fluorometric Evaluations

Dot plots of SYBR-14/PI-stained spermatozoa showed three populations [9], as did JC-1/PI dot plots (Fig. 1). The upper right population of the SYBR-14-treated samples doubly stained red and green. The upper right population of the JC-1-treated samples exhibited red-orange aggregates in the mitochondria. Dot plots of LysoTracker-treated samples (Fig. 2) showed only two populations, the upper being the red PI-stained dead spermatozoa and the lower being those with intact green-stained acrosomes.

Dot plots (Figs. 1 and 2) display samples from bulls 6, 7, and 8. These bulls represented individuals known to be low-, average-, and high-quality semen producers, respectively. Bull 8 had a high proportion of sperm mitochondria with JC-1 aggregates in the 24-h stored samples. This bull also showed relatively few monomers and few dead, PI-stained spermatozoa. In contrast, bull 6 had few aggregates, many monomers, and many dead spermatozoa. After cryopreservation, the semen of all three bulls had almost no aggregates. However, the semen of bull 8 had a significantly greater proportion of monomers and lesser proportion of dead spermatozoa than that observed in samples from bull 6.

TABLE 2. Percentages of motile spermatozoa observed immediately after thawing (MOT0) or after 3 h incubations at 37°C (MOT3); percentages of intact acrosomes (IA) determined 3 h post-thaw; and fluorometric percentages of stained spermatozoa in semen that was cryopreserved and thawed at 37°C (means ± SD[a]).

Bull no.	MOT0[b]	MOT3[c]	IA[d]	SYBR-14[e]	LYSO[f]	JC-1[g]
1	70	35	76	51.1 ± 4.5	53.8 ± 2.5	60.2 ± 7.3
2	75	25	63	39.7 ± 2.3	44.3 ± 2.1	46.0 ± 3.9
3	75	35	80	53.9 ± 3.2	58.2 ± 2.0	54.3 ± 2.5
4	70	30	70	45.6 ± 2.8	42.0 ± 5.2	42.9 ± 1.7
5	70	25	63	57.7 ± 0.7	54.6 ± 1.6	54.7 ± 0.4
6	60	20	39	22.5 ± 0.1	26.7 ± 1.4	26.3 ± 0.5
7	75	30	67	44.6 ± 3.2	44.9 ± 2.6	40.6 ± 3.2
8	75	35	88	61.5 ± 0.5	67.4 ± 0.8	63.4 ± 3.9
9	70	30	73	43.5 ± 4.9	40.5 ± 2.5	37.7 ± 1.6
10	70	30	67	39.4 ± 1.4	40.1 ± 2.0	39.6 ± 1.1
11	75	35	74	49.3 ± 2.3	49.8 ± 2.6	45.7 ± 1.3
12	75	30	80	61.6 ± 1.1	61.7 ± 4.7	63.7 ± 2.4
Mean ± SE				47.5 ± 3.2	48.7 ± 3.2	47.9 ± 3.3

[a] Standard deviations not available for MOT0, MOT3, and IA because only one assessment was made for post-thaw motility and intact acrosomes.
[b] Visual estimate of sperm motility (%) immediately after thawing cryopreserved straws (n = 1).
[c] Visual estimate of sperm motility (%) after thawed, cryopreserved straws were incubated 3 h at 37°C (n = 1).
[d] Percentage of 100–200 spermatozoa that exhibited intact acrosomal ridges from thawed, cryopreserved straws (n = 1).
[e] Percentage of 10 000 spermatozoa that stained with SYBR-14.
[f] Percentage of 10 000 spermatozoa that stained with LysoTracker.
[g] Percentage of 10 000 spermatozoa that stained with JC-1 (monomers plus aggregates).
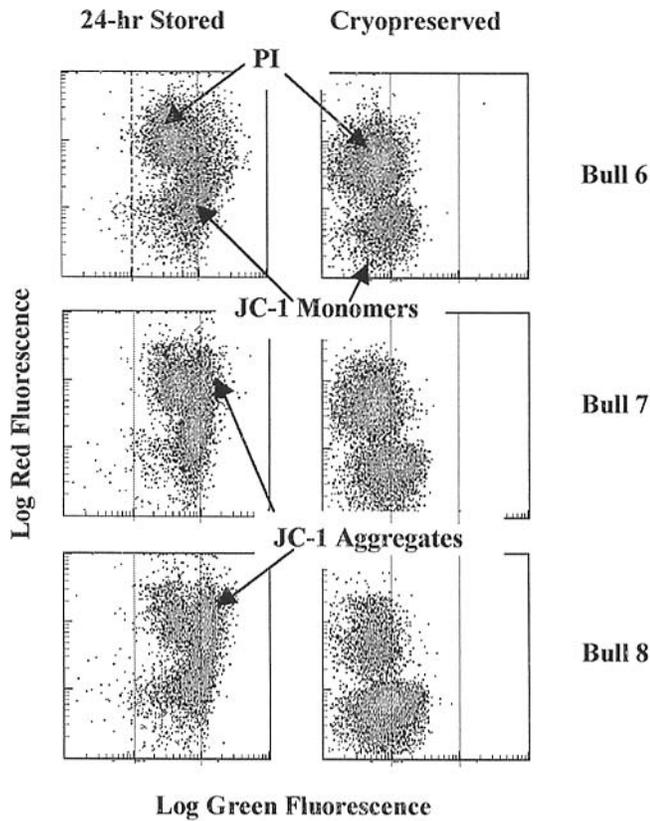
FIG. 1.   Density (dot) plots showing the effects of cryopreservation on spermatozoa stained with JC-1 and PI, displayed on 3-decade logarithmic scales. Bulls 6, 7, and 8 represent bulls possessing, respectively, low, medium, and high proportions of JC-1 aggregates and viable spermatozoa. The mean percentages of JC-1 aggregates in 24-h stored spermatozoa for bulls 6, 7, and 8 were 12.9%, 30.9%, and 58.6%, respectively. After cryopreservation, these values were reduced to 0.5%, 1.3%, and 0.9%, respectively.
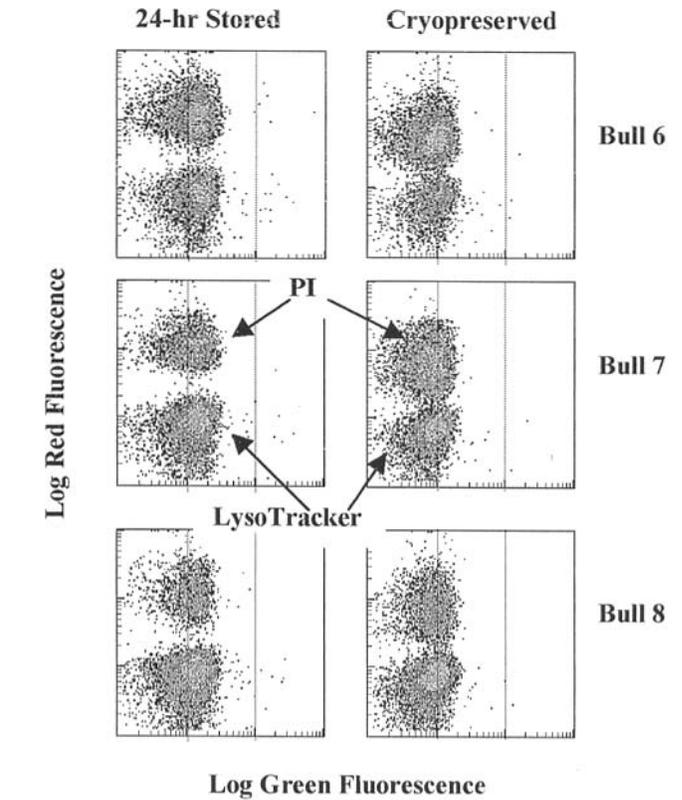

FIG. 2.   Density (dot) plots showing the effects of cryopreservation on spermatozoa stained with LysoTracker and PI, displayed on 3-decade logarithmic scales. Bulls 6, 7, and 8 represent bulls possessing, respectively, low, medium, and high proportions of LysoTracker-stained spermatozoa. The mean percentages of LysoTracker in 24-h stored spermatozoa for bulls 6, 7, and 8 were 40.9%, 60.2%, and 76.8%, respectively. After cryopreservation, these values were reduced to 26.7%, 44.9%, and 67.4%, respectively.

Within all bulls, the proportion of 24-h stored spermatozoa that stained with JC-1 was greater than the proportion of spermatozoa that stained with LysoTracker or SYBR-14 (Table 1). However, after cryopreservation, the same ejaculates for each bull showed approximately the same proportions of spermatozoa that stained with SYBR-14, LysoTracker, and JC-1. Of the total JC-1-stained spermatozoa, 37.4% were red-orange aggregates, and 34.9% were green monomers (Fig. 3). The percentage of JC-1 aggre-

gates decreased after cryopreservation to 0.9%, and the percentage of monomers increased to 47.1%.

ANOVA

Within each staining method, ANOVA revealed differences among bulls (factor A; $p < 0.001$), both before (Table 3) and after cryopreservation (Table 4). Contrast analysis showed that the pre-freeze populations of spermatozoa treated with the three stains differed from one another (Ta-
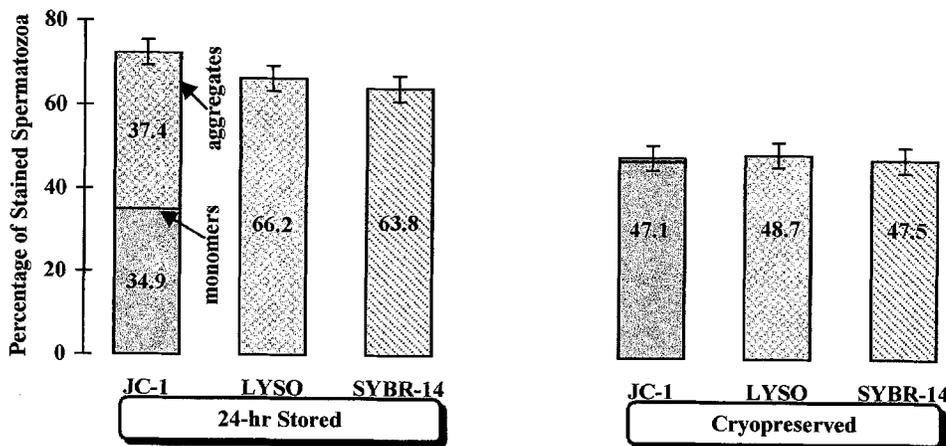

FIG. 3.   Histogram showing the mean percentages of stained spermatozoa. The two columns for JC-1-stained spermatozoa—24-h stored and cryopreserved—are partitioned to show the proportions of aggregates (red-orange-stained mitochondria) and monomers (green-stained mitochondria). The percentage of aggregates decreased from 37.4% before freezing to 0.88% post-thaw.

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TABLE 3. ANOVA and contrast analysis[a] for 24-h stored spermatozoa.

Analysis	df	Type III SS	MS	F-value	Probability
ANOVA					
Main plot					
Straws (block)	2	15.270	7.635	0.87	0.4315
Bulls (Factor A)	11	11,983.430	1,089.403	124.63	0.0001
Error Term-A	22	192.310	8.741		
Split plot					
Staining treatment (Factor B)	2	1,355.442	677.721	84.63	0.0001
A*B	22	581.687	26.440	3.30	0.0003
Error Term B	47	376.379	8.008		
Total	106	14,478.786			
Contrast					
Staining treatments (Factor B)					
JC-1[b] vs LysoTracker[c]	1	681.174	681.174	85.06	0.0001
JC-1 vs SYBR-14[d]	1	1,256.808	1,256.808	156.54	0.0001
LysoTracker vs SYBR-14	1	95.076	95.076	11.87	0.0012

[a] Analyses were performed using the general linear model (GLM) of SAS; A split plot randomized complete block design was used separately for 24 h stored and cryopreserved sperm data, with bulls in the main plot and staining treatments in the split plot.
[b] Percentage of 10 000 spermatozoa that stained with JC-1 (monomers plus aggregates).
[c] Percentage of 10 000 spermatozoa that stained with LysoTracker.
[d] Percentage of 10 000 spermatozoa that stained with SYBR-14.

ble 3; $p < 0.002$), but the post-thaw spermatozoa did not (Table 4; $p > 0.11$).

Regression Analysis

For regression analyses, the conditions of normal distribution, independent residuals with equal variance were examined and were satisfied. The predictor variable, the proportion of spermatozoa in 24-h stored samples that stained with SYBR-14, had a significant linear relationship with the response variable, the proportion of cryopreserved spermatozoa that stained with SYBR-14. The estimated regression model is as follows: Y-hat $= -3.211 + 0.795\times$ ($r^2 = 0.68$, n = 12) (Fig. 4a).

No significant outliers or influential observations were present in these data; the studentized residuals all lay within 2.5 standard deviations, and none of the Cook's D-values were significant. The plotted residuals (not shown) did not appear to have any particular shape, suggesting that the residuals had equal variance. A normal probability plot showed data close to the reference line, indicating that the

residuals were normally distributed. Probability values for skewness and kurtosis were less than 0.05.

A 95% confidence interval (Fig. 3a) suggested that for each 1% increase in any bull ejaculate stored 24 h and stained with SYBR-14, an increase between 0.41% and 1.18% in SYBR-14-stained spermatozoa after cryopreservation could be expected. The confidence interval plots for LysoTracker- and JC-1-stained spermatozoa, for which cryopreserved samples were regressed on 24-h stored samples, are shown in Figure 4, b and c, respectively. The percentages used for JC-1 analyses consisted of the sum total of aggregates plus monomers for each sample. A 1% increase in a 24-h stored sample stained with LysoTracker reflected an increase after cryopreservation of somewhere between 0.45% and 1.12%. Likewise for JC-1-stained samples, the increase would be somewhere between 0.76% and 1.33%.

The estimated regression models (underlying assumptions were met as described for the SYBR-14 analyses) are as follows: LysoTracker—Y-hat $= -3.278 + 0.785\times$ (r^2

TABLE 4. ANOVA and contrast analysis[a] for cryopreserved spermatozoa.

Analysis	df	Type III SS	MS	F-value	Probability
ANOVA					
Main plot					
Straws (block)	2	15.391	7.696	1.15	0.3348
Bulls (Factor A)	11	12,034.058	1,094.005	163.57	0.0001
Error Term-A	22	147.142	6.688		
Split plot					
Staining treatment (Factor B)	2	23.878	11.939	1.34	0.2706
A*B	22	457.771	20.808	2.34	0.0070
Error Term B	48	426.546			
Total	107	13,104.787			
Contrast					
Staining treatments (Factor B)					
JC-1[b] vs LysoTracker[c]	1	9.469	9.469	1.07	0.3071
JC-1 vs SYBR-14[d]	1	3.059	3.059	0.34	0.5602
LysoTracker vs SYBR-14	1	23.290	23.290	2.62	0.1120

[a] Analyses were performed using the general linear model (GLM) of SAS. A split plot randomized complete block design was used separately for 24 h stored and cryopreserved sperm data, with bulls in the main plot and staining treatments in the split plot.
[b] Percentage of 10 000 spermatozoa that stained with JC-1 (monomers plus aggregates).
[c] Percentage of 10 000 spermatozoa that stained with LysoTracker.
[d] Percentage of 10 000 spermatozoa that stained with SYBR-14.
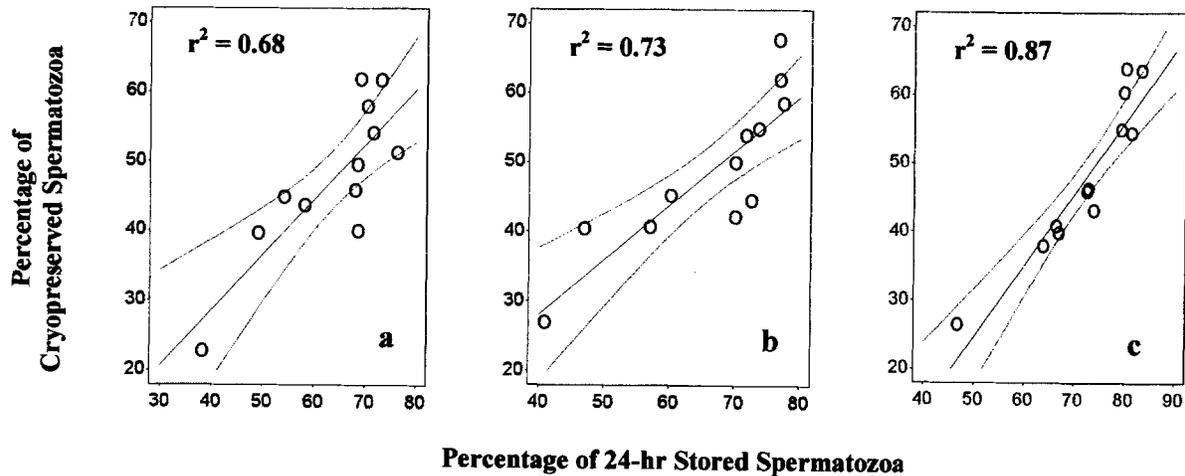
Percentage of 24-hr Stored Spermatozoa

FIG. 4. Scatter plots showing the regression lines and confidence intervals for the mean percentages of cryopreserved, stained spermatozoa for each bull versus the mean percentages of stained spermatozoa in 24-h stored semen from the same ejaculate (n = 3). The regression models and 95% confidence intervals (CI) are: a) SYBR-14-stained spermatozoa: Y-hat = $-3.211 + 0.795\times$; upper CI = 1.181; lower CI = 0.409; b) LysoTracker-stained spermatozoa: Y-hat = $-3.278 + 0.785\times$; upper CI = 1.118; lower CI = 0.451; and c) JC-1 stained spermatozoa: Y-hat = $-27.540 + 1.043\times$; upper CI = 1.331; lower CI = 0.756.

= 0.73, n = 12) and JC-1—Y-hat = $-27.540 + 1.043\times$ ($r^2 = 0.87$, n = 12) (Fig. 4, b and c, respectively).

Correlation Analysis

When JC-1-stained spermatozoa were analyzed for correlations with microscopic determinations of motility, pre-freeze motility (MOT) was negatively related to the proportion of sperm mitochondria that fluoresced green ($r = -0.81$) and positively related to those displaying red-orange fluorescence ($r = 0.60$) (Table 5). The microscopic parameter, percentage of post-thaw intact acrosomes (IA), was positively correlated with the percentage of Lyso-Tracker-stained spermatozoa, both before freezing ($r = 0.72$) and after cryopreservation ($r = 0.85$). It was also correlated with post-thaw motility immediately after thawing ($r = 0.78$) and after 3-h incubation ($r = 0.88$). All 24-

h stained samples were highly correlated with one another ($r > 0.92$; $p < 0.001$, Table 5).

DISCUSSION

Cryopreservation resulted in not only a shift in population ratios, as demonstrated by the monochrome density dot plots but also in a decrease in the fluorescence intensity of PI-stained sperm. For example, the PI red fluorescence intensity for bull 6 within the JC-1/PI dual staining system was slightly higher before cryopreservation (mean channel 174) than after cryopreservation (mean channel 143). In the LysoTracker/PI dual-staining systems, the mean channels for PI red fluorescence were 165 and 151 for 24-h stored and cryopreserved samples, respectively. The JC-1 and LysoTracker populations also shifted to lower green fluorescence intensity values, appearing as movement to the left

TABLE 5. Correlations among fluorometric assessments of sperm function and microscopic measurements of sperm motility and acrosomal status.

	SYBR-14[a] (r)	LYSO[b] (r)	Monomers[c] (r)	Aggregates[d] (r)	Total JC-1[e] (r)	MOT[f] (r)	Ia[g] (r)	MOTO[h] (r)	MOT3[i] (r)
Total JC-1[j]	0.86***	0.86***	−0.42	0.79**	0.93***	0.25	0.78**	0.62*	0.58*
MOT3[i]	0.62*	0.53	−0.43	0.66*	0.66*	0.23	0.88***	0.64*	—
MOTO[h]	0.66*	0.73**	−0.22	0.55	0.74**	−0.02	0.78**	—	—
Ia[g]	0.75**	0.72**	−0.29	0.64*	0.78**	0.05	—	—	—
MOT[f]	0.07	0.08	−0.81***	0.60*	−0.00	—	—	—	—
Total JC-1[e]	0.93***	0.92***	−0.23	0.69**	—	—	—	—	—
Aggregates[d]	0.69*	0.64*	−0.87***	—	—	—	—	—	—
Monomers[c]	−0.28	−0.22	—	—	—	—	—	—	—
LYSO[b]	0.96***	—	—	—	—	—	—	—	—

[a] Percentage of pre-freeze SYBR-14-stained spermatozoa in 24-h stored samples that exhibited green fluorescent DNA.
[b] Percentage of pre-freeze LysoTracker Green DND-26-stained spermatozoa in 24-h stored samples that exhibited green fluorescent acrosomes.
[c] Percentage of pre-freeze JC-1-stained spermatozoa in 24-h stored samples that exhibited green fluorescent mitochondria ($\lambda = 510–520$ nm).
[d] Percentage of pre-freeze JC-1-stained spermatozoa in 24-h stored samples that exhibited red-orange fluorescent mitochondria ($\lambda = 590$ nm).
[e] Sum of the percentages of pre-freeze monomers and aggregates in 24-h stored samples of spermatozoa that were stained with JC-1.
[f] Percentage of pre-freeze spermatozoa that exhibited progressive forward motility before cryopreservation; estimates were obtained using a thermostatically warmed state (37°) and light microscopy at ×100 magnification.
[g] Percentage of spermatozoa that exhibited intact acrosomal ridges after 3-h incubation post-thaw (microscopic examination).
[h] Percentage of spermatozoa that exhibited progressive forward motility immediately after thawing (microscopic examination).
[i] Percentage of spermatozoa that exhibited progressive forward motility after 3-h incubation post-thaw (microscopic examination).
[j] Sum of the percentages of post-thaw monomers and aggregates in cryopreserved samples of spermatozoa that were stained with JC-1.
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

on dot plots. This was apparent microscopically as well; post-thaw LysoTracker-stained sperm acrosomes were difficult to see, even at ×1000 magnification. It is unclear what caused these decreases in the fluorescence intensity of the spermatozoa. These shifts in the mean fluorescence intensities, however, did not alter the actual number of sperm in that particular population.

The disappearance of high mitochondrial membrane potential after cryopreservation was obvious (Figs. 1 and 3). However, we encountered difficulty trying to segregate the JC-1 aggregate and PI populations because they tended to merge together (Fig. 1). Since our software is limited to drawing rectangles around the population of interest, it was sometimes difficult to accurately separate the two populations. In some samples, it was also difficult to determine where the boundary between them occurred because they appeared on the dot plot as almost a single population. Color density plots (Sierra Cytometry) greatly helped to discriminate between the two, but further experimentation will be necessary to develop a staining approach that results in more discrete populations.

The difference between the proportion of spermatozoa with intact plasmalemma, intact acrosomes, and functional mitochondria in 24-h stored samples appeared to be small (Fig. 3). These differences were statistically significant, nevertheless. The ANOVA contrast F-values (Table 3) indicated a significance level of $p < 0.001$ where mitochondrial function > acrosomal integrity > plasma membrane integrity. Although it has been shown that differences existed in the effect of cryopreservation on the membranes that enclose the various sperm organelles [2–7], it is not known whether the differences in the present analyses were partly due to staining artifacts, or if they accurately reflected the hierarchy of sperm compartment functionality.

It was expected that fluorometric assessments of mitochondrial function and motility estimates in both frozen and unfrozen samples would be correlated. On a scatter plot, however, of total JC-1 from 24-h stored samples (n = 3) vs. pre-freeze motility (n = 2; plot not shown), no discernible relationship was evident. When JC-1 was plotted against motility separately as either monomers or aggregates, the percentage of JC-1 monomers displayed a negatively linear pattern ($r = -0.81$, $p = 0.001$). Likewise, when motility was plotted against the percentage of aggregates, a positive, somewhat linear pattern appeared ($r = 0.60$; $p = 0.04$). This effectively canceled out any relationship that motility would have with the total percentage of JC-1. It has been demonstrated that an increase in the monomer:aggregate ratio (green:red-orange) reflected a decrease in membrane potential [24, 26, 27] and decreased membrane potential reflected or caused decreased motility [6]. The fact that pre-freeze motility had a highly significant negative correlation with JC-1 monomers is interesting in light of the extremely low correlation coefficients motility had with respect to SYBR-14 ($r = 0.07$) and LysoTracker ($r = 0.08$). The possibility exists that more precise measurements of motility with computer-aided semen analysis (CASA), or some other automated means of motility assessment, would disclose an even greater relationship between JC-1 staining and motility.

After cryopreservation, the proportion of JC-1 aggregates was less than 1.0%, indicating a low to moderate membrane potential in all of the spermatozoa. Thus, there was no "cancellation" effect between monomers and aggregates in cryopreserved samples. In a previous study using only cryopreserved samples [10], the effects of three

different mitochondrial probes on bovine spermatozoa were examined. In that study, total JC-1 was correlated with post-thaw motility to a greater degree than in the present study ($r = 0.97$ vs. $r = 0.62$). Although both studies used twelve bulls, the mean percentages of JC-1-stained sperm ± SEM and post-thaw motility ± SEM in the present study were 47.9 ± 3.3 and 71.7 ± 1.3, respectively; the same values in the previous study were 59.0 ± 7.4 and 65.8 ± 5.8, respectively. The reason for the difference was obvious when one compared the two scatter plots (not shown). The previous study [10] used bull semen that exhibited a much wider range of bulls with respect to semen quality (27–83% MOT0), and each motility value was the mean of three observations. Although the historical individual records of the 12 bulls used in the present study exhibited a wide range of quality, the ejaculates here were more uniform (60–75% MOT0), and a single motility assessment was performed for each bull. Thus, 50% of the bulls were evaluated as equal in percentages of motile spermatozoa. Total JC-1 ranged previously from 5% to 79% [10], whereas the range of total JC-1-stained spermatozoa in this study was 26–64% (Table 2). Another point of difference was that the previous data showed the presence of a population of post-thaw JC-1 aggregates in 8 out of the 12 bulls. The post-thaw samples described herein exhibited almost no aggregates. Since none of the bulls were common to both studies, it is possible that this set of bulls, by chance, were those that had moderate to low cryopreservation tolerance. Alternatively, some unknown physical variable may have interfered with the cryopreservation process, such as the time of year that the ejaculates were collected [28]. It seems unlikely that the difference was due to a staining artifact because the unfrozen fractions of the samples from all 12 bulls displayed significant proportions of aggregates.

Assessment of intact acrosomes observed in thawed, cryopreserved samples showed the greatest relationship with sperm motility after 3 h of incubation ($r = 0.88$; $p = 0.0001$). This relationship may have existed because both parameters were measured at 3 h post-thaw and therefore reflected an overall weakened state of the spermatozoa that occurs over time. Karabinus et al. [29]) found a significant decrease of approximately 20% in mitochondrial function of thawed bovine spermatozoa extended in egg yolk citrate between 0 min and 3 h, as determine by rhodamine 123 staining; it was not stated, however, whether the change was linear or quadratic. Contrast analysis F-values in Tables 3 and 4 signified that the plasmalemma, acrosome, and mitochondria differed in their functional status before freezing but were similar after cryopreservation and thawing. These data clearly illustrate that cryopreservation and thawing reduced the range of sperm mitochondrial membrane potentials to a rather homogenous level. Thawed spermatozoa exhibited relatively low membrane potentials when compared to those seen in the 24-h stored samples. The proportions of sperm exhibiting JC-1 aggregates decreased markedly relative to concomitant increase in JC-1 monomeric staining.

Regression analyses revealed a closer association of pre-freeze and post-thaw mitochondrial function than for pre-freeze and post-thaw assessments of the plasmalemma or acrosomes (Fig. 4). The coefficient of determination (r^2) for the JC-1-model was 0.87, while the values for the SYBR-14 and LysoTracker models were 0.68 and 0.73, respectively. Since the underlying assumptions were thoroughly checked by means of visual plots and quantitative statistics, it can be concluded from this study that JC-1 was

a more reliable indicator of the freezing tolerance of bull spermatozoa.

In conclusion, this study demonstrated that the functional status of the sperm plasmalemma, acrosomes, and mitochondria differed before cryopreservation but not in post-thaw samples. Although among-bull variability existed both before and after cryopreservation, there were no differences among straws. The greatest differences were observed in the degree of membrane potential: less than 1% of the sperm exhibited aggregates after cryopreservation and thawing. A linear relationship existed between the percentage of stained spermatozoa in the 24-h stored samples and the percentage of stained spermatozoa in the cryopreserved fractions. The mitochondrial probe JC-1 appeared to be the most reliable indicator of the ability of sperm to survive the cryopreservation process.

REFERENCES

1. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995; 7:871–891.
2. Maxwell WMC, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Mol Reprod Dev 1997; 46:408–418.
3. Holt WV, North RD. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol Reprod 1994; 51: 414–424.
4. Jones RC, Stewart DL. The effects of cooling to 5°C and freezing and thawing on the ultrastructure of bull spermatozoa. J Reprod Fertil 1979; 56:233–238.
5. Lindemann CB, Fisher M, Lipton M. A comparative study of the effects of freezing and frozen storage on intact and demembranated bull spermatozoa. Cryobiology 1982; 19:20–28.
6. Windsor DP. Mitochondrial function and ram sperm fertility. Reprod Fertil Dev 1997; 9:279–284.
7. Hammerstedt RH, Graham JK. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology 1992; 29:26–38.
8. Evenson DP, Darzynkiewicz Z, Melamed MR. Simultaneous measurement by flow cytometry of sperm viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 1982; 30:279–280.
9. Garner DL, Thomas CA, Allen CH. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. J Androl 1997; 18:324–331.
10. Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997; 57:1401–1406.
11. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997; 56:991–998.
12. Garner DL, Dobrinsky JR, Welsh GR, Johnson LA. Porcine sperm

13. Garner DL, Johnson LA, Allen CH, Palencia DD, Chambers CS. Comparison of seminal quality in Holstein bulls as yearlings and as mature sires. Theriogenology 1996; 45:923–934.
14. Maxwell WM, Welch GR, Johnson LA. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 1996; 8:1165–1178.
15. Medrano A, Holt WV. Protective effects of glycerol during cold shock in boar spermatozoa. A cryomicroscope study using propidium iodide and SYBR-14. Reprod Domest Anim 1996; 31:281–282.
16. Donoghue AM, Garner DL, Donoghue DJ, Johnson LA. Viability assessment of turkey sperm using fluorescent staining and flow cytometry. Poult Sci 1995; 74:1191–1200.
17. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995; 53:276–284.
18. Garner DL, Johnson LA, Yue ST, Roth BL. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 1994; 15:620–629.
19. Maxwell WMC, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Mol Reprod Dev 1997; 46:408–418.
20. Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991; 30:4480–4486.
21. Cossariza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorometric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993; 197:40–45.
22. Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD Jr, Chen LB. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Natl Acad Sci 1991; 88:3671–3675.
23. Sims PJ, Waggoner AS, Wang H-H, Hoffmann JF. Studies on the mechanisms by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry 1974; 13:3315–3330.
24. Cossarizza A, Ceccarelli D, Masini A. Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorometric analysis at the single organelle level. Exp Cell Res 1996; 222:84–94.
25. Saacke RG, Marshall CE. Observations on the acrosomal cap of fixed and unfixed bovine spermatozoa. J Reprod Fertil 1968; 16:511–514.
26. Kuhnel JM, Perrot JY, Faussat AM, Marie JP, Schwaller MA. Functional assay of multidrug resistant cells using JC-1, a carbocyanine fluorescent probe. Leukemia 1997; 11:1147–1155.
27. Troyan MB, Gilman VR, Gay CV. Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp Cell Res 1997; 233:274–280.
28. Vogler CJ, Saacke RG, Bame JH, DeJarnette JM, McGilliard ML. Effects of scrotal insulation on viability characteristics of cryopreserved bovine semen. J Dairy Sci 1991; 74:3827–3835.
29. Karabinus DS, Evenson DP, Kaproth MT. Effects of egg yolk-citrate and milk extenders on chromatin structure and viability of cryopreserved bull sperm. J Dairy Sci 1991; 74:3836–3848.