Effects of Egg Yolk-Citrate and Milk Extenders on Chromatin Structure and Viability of Cryopreserved Bull Sperm

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ABSTRACT

Semen from four Holstein bulls was evaluated to compare effects of four extender treatments on postthaw semen quality. Extender fractions A and B, either heated whole milk or 20% egg yolk-citrate, were combined to yield the extender treatments 1) milk and milk, 2) milk and egg yolk-citrate, 3) egg yolk-citrate and milk, and 4) egg yolk-citrate and egg yolk-citrate. Semen was evaluated at thawing and after 30, 60, 120, and 180 min of incubation at 38.5°C. Flow cytometry showed that acridine orange-stained sperm were most susceptible to in situ DNA denaturation when fraction A was milk. For sperm stained with rhodamine 123, flow cytometry showed that the proportion with intact mitochondrial membrane potential was lowest of all treatments at thawing but greatest at 180-min incubation with milk and milk extender. Flow cytometry of propidium iodine-stained sperm showed greatest proportion of cell membrane intact sperm when fraction A was egg yolk-citrate. Light microscopy showed the lowest proportion of cell membrane intact sperm with milk and milk extender after eosin-aniline blue vital staining. Postthaw motility scores tended to be reduced when both extender fractions were egg yolk-citrate. Results demonstrate differential extender effects on postthaw semen quality and indicate that altering extender composition or sequence of adding extender components may improve postthaw quality of cryopreserved sperm.
(Key words: bull, extender, semen quality, flow cytometry)

Abbreviation key: α_t = the ratio of red fluorescence to total fluorescence, %COMPα_t = percentage of cells outside the main population of α_t, E-E = extender fractions A and B egg yolk-citrate, E-M = extender fraction A egg yolk-citrate; extender fraction B heated whole milk, FC = flow cytometric, %Gf = percentage of sperm exhibiting green mitochondrial fluorescence, M-M = extender fractions A and B heated whole milk, M-E = extender fraction A heated whole milk; extender fraction B egg yolk-citrate, %Rf = percentage of sperm exhibiting red nuclear fluorescence, %RG = percentage of sperm exhibiting red nuclear fluorescence and green mitochondrial fluorescence, SCSA = sperm chromatin structure assay, SDα_t = standard deviation of α_t, Xα_t = mean of α_t.

INTRODUCTION

Prior to processing and freezing semen for artificial insemination, sperm morphology and viability are evaluated to cull low quality samples. Postthaw viability is evaluated to assess resistance to cryopreservation damage. Although sperm morphology and viability traits are related to fertility (25, 26, 27), little is known about how the quality of chromatin contained in the sperm nucleus affects fertility. During spermiogenesis, chromatin condensation results in sperm nuclear morphology char-

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acteristic of the species. Sperm head morphology is, on a gross organizational level, a reflection of chromatin structure that may be important to fertility. Misshapen bovine sperm heads from subfertile bulls have been reported to contain structurally abnormal chromatin (16, 22), probably a result of abnormal chromatin condensation (16, 17). The susceptibility of sperm nuclear DNA to denaturation is inversely related to chromatin organization, heterospermic fertility ranking, normal sperm morphology, and sperm viability (1, 2, 21). It is likely that the quality of the genetic material contained in the sperm affects its ability to support normal embryonic development should fertilization occur.

Semen extenders function to protect the fertility of sperm and to increase the total volume of a sperm dose to a usable and practical size (12). Many semen extenders contain milk or egg yolk as primary ingredients because components in each protect sperm from cold shock (13, 23). Results of fertility trials have shown little difference between milk and egg yolk-citrate extenders when used separately or in combination for unfrozen semen (12) or for frozen-thawed semen (29).

Differences have been noted between milk and egg yolk-citrate extended bull sperm in their ability to resist in situ denaturation of nuclear DNA (Karabinus and Evenson, unpublished) as measured by flow cytometry by the sperm chromatin structure assay [SCSA; (1, 2, 21)]. These observations involved different bulls' semen, cryopreserved in one or the other extender. In light of the high negative relationship between measures of bull fertility and flow cytometric (FC) measures of sperm nuclear DNA resistance to denaturation (1, 2, 21), the potential for differences between the extenders' effects on sperm chromatin quality appeared to warrant further investigation. The study described in this paper was designed and conducted as an initial investigation into post-thaw viability and chromatin structure differences for bovine sperm cryopreserved in milk and egg yolk-citrate extenders.

MATERIALS AND METHODS

Semen Processing

Semen from one collection from each of four different bulls, owned by Eastern Artificial Insemination Cooperative, Inc., Ithaca, NY, was used in this split ejaculate study. A 2 × 2 factorial design with two extender components and two extender fractions yielded four extender treatments. After initial evaluation by Eastern Artificial Insemination Cooperative personnel, semen from each bull was divided into four aliquots. Each aliquot was processed in one of the four extender treatments, frozen in .5-ml polyvinyl chloride straws, and stored in liquid nitrogen. Sperm concentration of all extended semen was 40×10^6 sperm/ml.

Extender Treatments

Extender fraction A consisted of either whole milk or 20% (vol/vol) egg yolk in 2.9% (wt/vol) sodium citrate dihydrate (egg yolk-citrate). Milk was heated to 95°C for 10 min, then cooled to 20°C, filtered to remove skin, and held overnight at 5°C. Egg yolk-citrate was clarified by allowing it to stand overnight at 5°C in a 300-ml graduated cylinder and then decanting for use the upper 250 ml of the 300-ml total volume. Antibiotics were added to extender fraction A as per the Certified Semen Services requirements (6). Extender fraction A was rewarmed to 35°C prior to adding semen and then cooled at 5°C for approximately 2.5 h. Extender fraction B, either whole milk or egg yolk-citrate prepared as described, contained glycerol (14% vol/vol) and was cooled to 5°C. Fraction B was added 1:1 (vol/vol) in a dropwise fashion to semen diluted in extender fraction A. The two extender fractions factored with the two extender components yielded the following four extender treatments: 1) M-M = fractions A and B milk; 2) M-E = fraction A milk, fraction B egg yolk-citrate; 3) E-M = fraction A egg yolk-citrate, fraction B milk; and 4) E-E = fractions A and B egg yolk-citrate. Extended semen was packaged in straws and frozen after 4 h of equilibration at 5°C.

Semen Handling

Straws of semen were thawed 30 s in 37°C water immediately upon removal from liquid nitrogen and emptied into polyethylene microcentrifuge tubes. For analysis of sperm chromatin structure, three straws of thawed semen were pooled and then incubated in wa-

ter at 38.5°C in closed 1.5-ml polyethylene microcentrifuge tubes. At time of thawing and at 30, 60, 120, and 180 min thereafter, 50 μl of semen were pipetted into .25-ml polypropylene microcentrifuge tubes precooled on ice, placed in a –20°C freezer for approximately 4 h, and then stored at –95°C until FC evaluation.

For FC and light microscopic evaluation of sperm viability, three straws of semen were thawed, pooled, and incubated as described. At thawing and at 180 min of incubation, semen was sampled, and the viability assays were immediately performed.

Flow Cytometry

All FC measurements were performed using an Ortho Diagnostics System Cytofluorograf II equipped with a Lexel 100-mW argon ion laser and interfaced with an Ortho Diagnostics Systems model 2150 computer system (Becton Dickinson Immunocytometry Systems, San Jose, CA). Green (515 to 530 nm) and red (>600 nm) fluorescence emitted by each sperm after laser excitation (35 mW, 488 nm) were processed through photomultiplier tubes and quantified. The 2150 computer utilized software programs to calculate values for sperm traits measured by flow cytometry.

Sperm Chromatin Structure. Sperm chromatin was evaluated by flow cytometry using the SCSA (5, 8, 9). Upon removal from the –95°C freezer, each sample was thawed for 30 s in 37°C water and pipetted into a 16- × 125-mm polystyrene tube (Falcon 3033, Becton Dickinson Labware, Lincoln Park, NJ) containing 1.0 ml of ice-cold buffer (.15 *M* NaCl, .01 *M* Tris·HCl, 1.0 m*M* EDTA, pH 7.4), making the final concentration approximately 1×10^6 sperm/ml. The semen suspension was then sonicated (Biosonik IV, VWR Scientific, San Francisco, CA; 30 s, 50% power, low setting, 1.27-cm probe). Preliminary work indicated that, in addition to separating sperm heads from tails, sonication removed an apparent artifact present in E-E extended semen, possibly debris not removed by clarification of egg yolk-citrate extender.

A 200-μl sample of sonicated semen was admixed with 400 μl of ice-cold .1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in .08*N* HCl and .15 *M* NaCl, pH 1.2, to potentially denature sperm nuclear DNA in situ. The DNA in sperm having normal chromatin structure is resistant to this level of physical stress; varying degrees of susceptibility to potentially denaturing conditions are a manifestation of abnormal chromatin structure (9, 11). After 30 s, 1.2 ml of ice-cold acridine orange staining solution [.2 *M* Na_2HPO_4, 1.0 m*M* Na_2-EDTA, .15 *M* NaCl, .1 *M* citric acid monohydrate, 6.0 μg/ml of chromatographically purified acridine orange (Polysciences, Warrington, PA), pH 6.0] were added (5), and the SCSA was performed 3 min later. Because of the metachromatic nature of acridine orange, the dye fluoresces green when intercalated into native, double-stranded (undenatured) DNA but fluoresces red when associated with single-stranded (denatured) DNA. In each of two replicates, two consecutive FC analyses of each coded semen sample quantified the red and green peak mode fluorescence emitted by each of 5000 sperm.

The ratio of red fluorescence to total (red plus green) fluorescence [α_t; (5)] was calculated for each sperm analyzed in the semen sample. The SCSA results, expressed as the mean and standard deviation of α_t ($X\alpha_t$ and $SD\alpha_t$, respectively) and the percentage of cells lying outside the main population of α_t ($\%COMP\alpha_t$) for sperm analyzed in each semen sample, were computed. The $X\alpha_t$ describes the average α_t value for the sperm analyzed in a semen sample. The $\%COMP\alpha_t$ indicates the percentage of cells having increased α_t (increased susceptibility to DNA denaturation; abnormal chromatin structure) relative to the main population of cells. The $SD\alpha_t$ is a measure of cell to cell variation for α_t and describes the extent of chromatin abnormality in the sample of cells. A high negative relationship exists between $SD\alpha_t$ and measures of bull fertility, namely, fertility rating ($r = -.58$, $P < .01$; 2), nonreturn rate [$r = -.77$, $P < .001$; (21)], and competitive index based upon heterospermic performance [$r = -.94$, $P < .01$; (1)]. A typical cytogram (Figure 1A) and α_t frequency histogram (Figure 1B) generated from an FC analysis of 5000 sperm illustrate the population from which values for the SCSA variables ($X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$) were derived for each semen sample.

Sperm Viability. Evaluation of sperm viability by FC employed rhodamine 123 (laser grade, Eastman Kodak Co., Rochester, NY), a

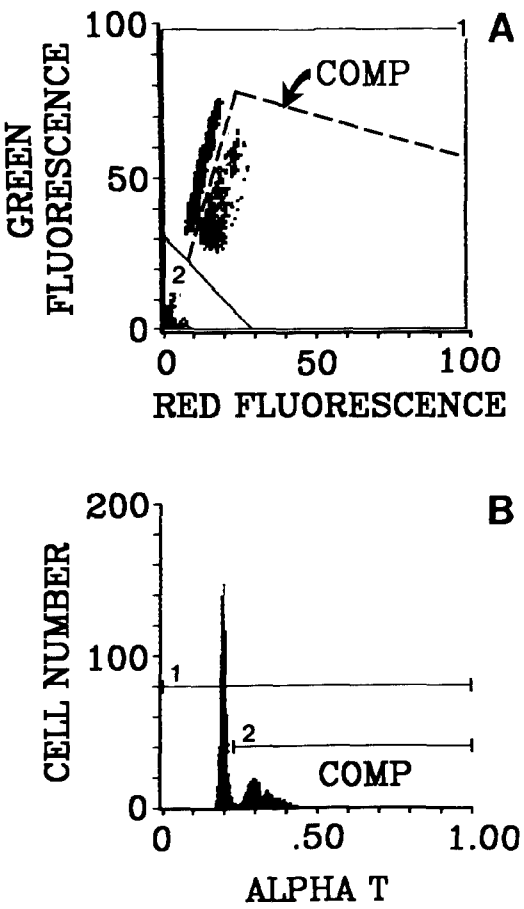

Figure 1. Example of cytogram (A) and frequency histogram (B) generated from flow cytometric evaluation of chromatin structure in 5000 sperm. A) debris (region 2) excluded from sperm population to be analyzed. Cells outside the main population of α_t (COMPα_t) separated from main population by broken line; α_t is the ratio of red fluorescence to total fluorescence. B) region 1 indicates entire sperm population. Region 2 indicates COMPα_t.

mitochondria-specific stain that emits green fluorescence (4, 9, 20), with propidium iodine (A grade, Calbiochem, San Diego, CA), a DNA-specific stain that emits red fluorescence (15), as a counterstain. This stain combination will be referred to as rhodamine 123-propidium iodide. Semen samples were pipetted into Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++} (Gibco, Grand Island, NY) to a final concentration of 1 to 2 $\times$ 10^6 sperm/ml. Aqueous stock rhodamine 123 solution (1 mg/ml) was pipetted into the diluted semen to a final concentration of 10 μg of rhodamine 123/ml and then incubated 10 min at room temperature. After 8 min of centrifugation at 300 $\times$ *g*, the supernatant was discarded, and the pellet was resuspended with buffer. Aqueous stock propidium iodide solution (1 mg/ml) was pipetted into this sperm suspension to a final concentration of 10 μg of propidium iodide/ml and incubated 10 min at room temperature; FC analysis was performed 3 min later. In each of two replicates, two consecutive FC analyses were performed on each coded semen sample, quantifying the red and green peak mode fluorescence emitted by each of 10,000 sperm.

As shown in the example cytogram (Figure 2A), the total number of sperm evaluated in a sample was equal to those in region 1 but excluded region 4 (indicated by arrow), which enclosed debris. Figure 2B is a computer enlarged view of the origin in Figure 2A, illustrating exclusion of debris (region 4) from the sperm population to be analyzed. Results of rhodamine 123-propidium iodide staining are expressed as %Gf, %Rf, and %RG. The %Gf (Figure 2A, region 2) are those sperm that exhibited green mitochondrial fluorescence, indicating an intact electrical potential across the mitochondrial membrane and, presumably, uncompromised mitochondrial function (3, 7, 10, 18, 20). The %Rf (Figure 2A, region 3) are those sperm that exhibited red nuclear fluorescence, which indicated compromised cell membranes, and were presumed nonviable (15). The %RG [100% – Σ (%Gf + %Rf)] are those sperm that exhibited both red and green fluorescence and were presumably in transition from the green (viable) population to the red (nonviable) population (7, 15).

Light Microscopy

Eosin-aniline blue vital-stained smears were prepared from each semen sample using the procedure of Shaffer and Almquist (31) as described by Saacke (26). In two replicates, 200 sperm were randomly observed at 400× magnification on each of a pair of coded slides prepared from a semen sample. The number of stained and unstained sperm were recorded.

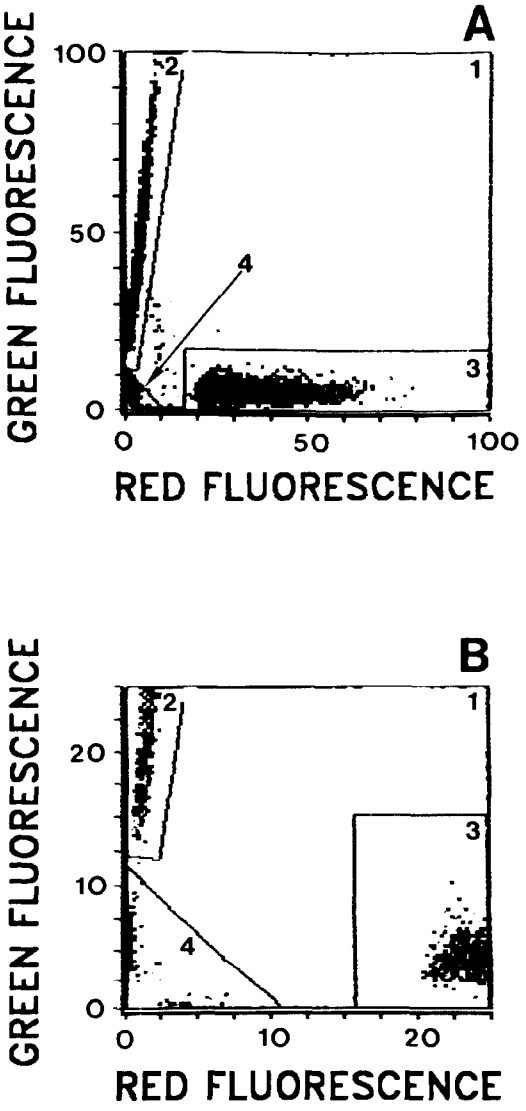

Figure 2. Examples of cytograms generated from a flow cytometric viability evaluation of 10,000 sperm stained with rhodamine 123 and propidium iodide. A) Entire sperm population enclosed by region 1. Regions 2 and 3 enclose subpopulations exhibiting green fluorescence from rhodamine 123 staining of mitochondria and red fluorescence from propidium iodide staining of nuclear DNA, respectively. B) Computer-enlarged view of cytogram origin in A illustrating exclusion of debris (diagonal line, region 4) from sperm population.

Unstained cells were considered to have intact cell membranes and were presumed viable. Cells exhibiting red staining were considered to have compromised cell membranes and were presumed nonviable.

Postthaw motility was estimated using a heated stage (35°C) phase contrast microscope equipped with a Dage 650 video camera and a Javelin BWM-15 monitor (Spectrum Services, Inc., Rochester, NY). In each of two replicates, motility was evaluated within 30 min of thawing one straw of semen in 5 to 10°C water for 5 min. The percentage of progressively motile sperm was estimated by examining five random fields of view on the video display (750× final magnification). A motility rating was then assigned to each sample: 5 = exceptional, 4 = superior, 3 = normal, 2 = conditional, and 1 = discard.

Statistical Analysis

Data were analyzed by least squares procedures using the general linear models procedure of SAS (28). Main effects were bull, extender, time, and replicate. The extender effect, the time effect, and the replicate effect were tested using the interactions of bull with extender, bull with time, and bull with replicate as error terms. The residual was used to test all other effects. Means comparisons were performed using the general linear models "contrast" specification. Pearson product-moment correlations were calculated using the CORR procedure of SAS. Results are presented as least squares means (± SEM) unless otherwise stated.

RESULTS

Sperm Chromatin Structure

The results of analysis of variance for sperm evaluated by the SCSA are presented in Table 1. The SCSA results for this study are summarized in Figure 3. Elevated values for SCSA variables ($X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$) indicate increased susceptibility of nuclear DNA to denaturation. This corresponds to increased heterogeneity of sperm chromatin structure and is associated with reduced fertility (1, 2, 21).

At all sampling times, sperm in M-M extender had greater $X\alpha_t$ ($P < .001$) than sperm in the other extenders (Figure 3A). The $X\alpha_t$ for

sperm extended in E-M versus E-E did not differ significantly at each sampling time. For sperm in M-E extender, $X\alpha_t$ values were not significantly different from those for E-M and E-E extenders at thawing and at 30 min of incubation. At 60 and 120 min of incubation, $X\alpha_t$ values for sperm in M-E extender exceeded those for E-M and E-E ($P < .05$). At 180 min of incubation, $X\alpha_t$ for sperm extended in M-E remained greater than for E-M ($P < .05$) but did not differ from E-E.

The $SD\alpha_t$ for sperm in M-M extender did not differ significantly from M-E extender at thawing; otherwise, it exceeded ($P < .001$) that for sperm in the other extenders at each sampling time (Figure 3B). Sperm extended in E-M versus E-E did not differ significantly for $SD\alpha_t$ at each sampling time. At 30 min of incubation, sperm in M-E extender did not differ from those in E-E for $SD\alpha_t$; otherwise, sperm in M-E exhibited greater $SD\alpha_t$ values than E-M and E-E extenders to 120 min incubation ($P < .01$). At 180 min of incubation, sperm extended in M-E, E-M, and E-E did not differ significantly for $SD\alpha_t$.

Sperm extended in M-M had greater ($P < .001$) $\%COMP\alpha_t$ than those in the other extenders at each sampling time (Figure 3C). The $\%COMP\alpha_t$ for sperm in M-E extender were greater ($P < .001$) than those for E-M and E-E extender at each sampling time. Sperm in E-M versus E-E extender did not differ significantly for $\%COMP\alpha_t$ at any sampling time.

Rhodamine 123-Propidium Iodide Vital Staining

The results of analysis of variance for sperm evaluated by rhodamine 123-propidium iodide vital staining are presented in Table 2. Results of rhodamine 123-propidium iodide vital staining of sperm at thawing and at 180 min of incubation are summarized in Table 3. The %GF indicates those that had intact electrical potential across the mitochondrial membrane and, presumably, uncompromised mitochondrial function (4, 7, 10, 18, 20). The %Rf indicates those sperm that exhibited red nuclear fluorescence, indicating compromised cell membranes, and were presumed nonviable (15). The %RG were presumably in transition from the green fluorescent (viable) sperm population to the red fluorescent (nonviable) sperm population (7, 15).

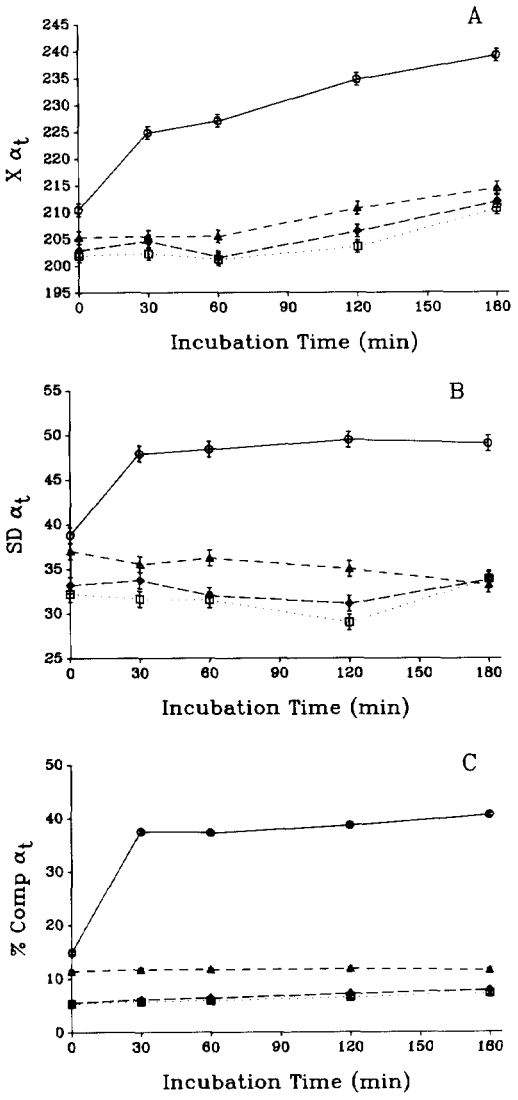

Figure 3. Comparison of extender composition effects on susceptibility of sperm nuclear DNA to denaturation after 0, 30, 60, 120, and 180 min of incubation at 38.5°C. O——O = Both extender fractions milk; Δ– – –Δ = fraction A milk, fraction B egg yolk-citrate; □· · ·□ = fraction A egg yolk-citrate, fraction B milk; ◊– – –◊ = both extender fractions egg yolk-citrate. A) Mean of α_t ($X\alpha_t$) where α_t = ratio of ref fluorescence to total fluorescence, SEM = 1.2, n = 16; B) standard $SD\alpha_t$ ($SD\alpha_t$), SEM = .9, n = 16; and C) percentage of cells outside the main population of α_t ($\%COMP\alpha_t$), SEM = .4, n = 16. Elevated values for $X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$ indicate increased susceptibility of sperm nuclear DNA to denaturation and heterogeneity of sperm chromatin structure.

TABLE 1. Mean squares and tests of significance for sperm traits measured by flow cytometry using the sperm chromatin structure assay.

Source	df	$X\alpha_t$[1]	$SD\alpha_t$[2]	$\%COMP\alpha_t$[3]
Bull (B)	3	366.85***	555.27***	462.30***
Extender (E)	3	9299.26***	3777.47***	13,525.91***
Time (T)	4	1854.42***	50.82	589.71***
Replicate (R)	1	3995.64*	1118.77	16.87
B × E	9	101.83***	28.57*	124.11***
B × T	12	99.35***	26.82*	8.53***
B × R	3	218.72***	146.54***	27.22***
E × T	12	294.59***	125.81***	420.19***
Error	270	23.00	13.20	2.42

[1]Mean of α_t, where α_t = the ratio of red fluorescence to total fluorescence.

[2]Standard $SD\alpha_t$.

[3]Percentage of cells outside the main population of α_t.

*$P < .05$.

***$P < .001$.

At thawing, %Gf was greatest for E-E and E-M extenders and lowest for M-M extender ($P < .05$; Table 3). Conversely, %Rf at thawing was least for E-M and E-E extenders, intermediate for M-E extender, and greatest for M-M extender ($P < .05$). At 180 min of incubation, %Gf was greatest for M-M and least for M-E ($P < .05$) with similar, intermediate values for E-M and E-E extenders. The %Rf was lowest for E-E extender and greatest for M-E extender ($P < .05$) at 180 min of incubation. For sperm in M-M and E-M extenders, %Rf was similar and intermediate between those for E-E and M-E.

Percentage RG was greater at thawing for E-M and E-E extenders than for M-M or M-E extenders ($P < .05$; Table 3). At 180 min of incubation, %RG was greatest for E-E extender, least for M-M extender ($P < .05$), and intermediate for M-E and E-M extenders.

Eosin-Aniline Blue Vital Staining

Analysis of variance (Table 4) showed that the interaction of extender treatment with incubation time was not a significant source of variation. When data were pooled across incubation times, the proportion of unstained

TABLE 2. Mean squares and tests of significance for sperm traits evaluated by flow cytometry using rhodamine 123-propidium iodide vital staining.

Source	df	%Gf[1]	%Rf[2]	%RG[3]
Bull (B)	3	776.91***	1674.80***	172.71***
Extender (E)	3	108.81*	505.98***	173.58*
Time (T)	1	8007.29*	12,723.89**	543.67*
Replicate (R)	1	292.16	38.52	118.49
B × E	9	19.63*	30.83***	27.18***
B × T	3	244.51***	188.29***	19.69**
B × R	3	30.14*	19.07*	26.22***
E × T	3	313.78***	326.85***	31.54***
Error	101	23.00	13.20	2.42

[1]Percentage of sperm exhibiting green mitochondrial fluorescence.

[2]Percentage of sperm exhibiting red nuclear fluorescence.

[3]Percentage of sperm exhibiting red nuclear fluorescence and green mitochondrial fluorescence.

*$P < .05$.

**$P < .01$.

***$P < .001$.

TABLE 3. Least squares means for percentages of total sperm in fluorescent subpopulations after rhodamine 123-propidium iodide staining of sperm incubated in extender composed of combinations of milk and egg yolk-citrate as extender fractions A and B.

Fluorescent criterion	Extender treatment[1]	Incubation time		SEM
		0 min (n = 16)	180 min (n = 16)	
%Gf[2] (High green, low red)	M-M	40.5[a]	34.2[a]	.8
	M-E	46.1[b]	27.2[b]	.8
	E-M	49.8[c]	30.7[c]	.8
	E-E	49.5[c]	30.6[c]	.8
%Rf[3] (High red, low green)	M-M	51.0[a]	61.7[a]	.7
	M-E	43.7[b]	66.6[b]	.7
	E-M	37.0[c]	62.5[a]	.7
	E-E	38.0[c]	58.6[c]	.7
%RG[4] (Transitional)	M-M	8.5[a]	4.1[a]	.5
	M-E	10.2[b]	6.2[b]	.5
	E-M	13.2[c]	6.7[b]	.5
	E-E	12.5[c]	10.9[c]	.5

[a,b,c]Means within fluorescent criterion within a column having unlike superscripts differ ($P < .05$).

[1]M = Milk, E = egg yolk-citrate; for each character pair, left character indicates fraction A component, right character indicates fraction B component.

[2]Percentage of sperm with green mitochondrial fluorescence, indicating intact mitochondrial membrane potential.

[3]Percentage of sperm with red nuclear fluorescence, indicating compromised cell membrane integrity.

[4]Percentage of sperm with green mitochondrial fluorescence and red nuclear fluorescence, indicating declining or transitional viability.

sperm (i.e., those sperm that had intact cell membranes and were presumed viable) in E-E, E-M, and M-E extenders exceeded ($P < .01$) the proportion in M-M extender (Table 5). The percentage of unstained sperm progressively increased with increased extender content of egg yolk-citrate.

Postthaw Motility

Results of analysis of variance for postthaw motility evaluation are shown in Table 5. Postthaw motility scores among the M-M, M-E, and E-M extender treatments did not differ (Table 6). The motility score for sperm in the E-E extender treatment differed only from that of sperm in the E-M treatment ($P < .05$).

Correlations among the sperm viability traits are shown in Table 7. For M-E, E-M, and E-E treatments, the significant correlations of %Rf and %Gf with percentage of unstained cells at thawing were mostly unchanged by 180 min of incubation. However, those relationships for the M-M treatment became nonsignificant after 180 min of incubation. Results of motility evaluation were mostly unrelated to viability traits measured by rhodamine 123-propidium iodide and eosin-aniline blue staining.

DISCUSSION

The SCSA results show that extender containing milk as fraction A (M-M and M-E) had greater values for the SCSA variables than the other extenders. The $X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$ for M-M extender treatment were greater than for the other treatments at almost all sampling times (Figure 3). The M-E treatment yielded SCSA results that were lower than M-M on all occasions except one but which tended to exceed those for E-E and E-M. This suggests that extenders having milk as fraction A or as both fractions stimulated detrimental changes in sperm chromatin structure relative to extenders similarly composed of egg yolk-citrate. The SCSA results for M-M extender treatment are consistent with other SCSA results for sperm incubated in milk extender (21). Taken together, the results of the two studies appear to indicate that such changes may be characteristic of the behavior of the chromatin of milk extended bull sperm upon in vitro incubation at temperatures com-

TABLE 4. Mean squares and tests of significance for sperm traits evaluated by light microscopy.

Source	df[1]	% Unstained sperm[2]	Motility score[3]
Bull (B)	3 (3)	1350.95***	1.46*
Extender (E)	3 (3)	441.76**	.71*
Time (T)	1	2856.15*	
Replicate (R)	1 (1)	1.74	.50***
B × E	9 (9)	34.99	.15
B × T	3	103.60**	
B × R	3 (3)	8.09	.00
E × T	3	7.94	
Error	101 (12)	19.18	.29

[1]Degrees of freedom in parentheses are for motility score model effects.

[2]Percentage of sperm having intact cell membrane, as indicated by eosin-aniline blue vital staining.

[3]5 = Exceptional, 4 = superior, 3 = normal, 2 = conditional, 1 = discard.

*$P < .05$.

**$P < .01$.

***$P < .001$.

parable with those of the female genital tract.

Elevated values for the SCSA variables indicate increased susceptibility of sperm nuclear DNA to in situ acid denaturation. Because measures of bull fertility are inversely related to the SCSA variables, particularly SDα_t, (1, 2, 21), the present results, if taken alone, would suggest that fertility of sperm frozen in M-M or M-E extenders relative to E-E and E-M may potentially be compromised. The elevated SDα_t for M-M and M-E treatments relative to E-M and E-E indicate the greater cell to cell variation for α_t in the former pair of treatments. Increased SDα_t describes a greater range of susceptibility of sperm nuclear DNA to denaturation and indicates greater heterogeneity of sperm nuclear chromatin. Other studies (2, 3, 11, 21) have shown that increased heterogeneity of sperm nuclear chromatin is associated with spermatogenic disturbances, morphologically abnormal sperm, and reduced fertility.

It appeared that extender-related effects on sperm chromatin may have occurred upon ini-

TABLE 5. Least squares means for percentages of unstained[1] sperm in semen incubated in extender composed of combinations of milk and egg yolk-citrate as extender fractions A and B and stained with eosin-aniline blue. Pooled across incubation times.

Extender treatment[2]	% Unstained sperm (n = 32)	SEM
M-M	57.8[a]	.8
M-E	63.0[b]	.8
E-M	64.6[bc]	.8
E-E	66.5[c]	.8

[a,b,c]Means within a column having unlike superscripts differ ($P < .01$).

[1]Indicates intact cell membrane.

[2]M = Milk, E = egg yolk-citrate; for each character pair, left character indicates fraction A component, right character indicates fraction B component.

TABLE 6. Least squares means for postthaw motility scores[1] of sperm in extender composed of combinations of milk and egg yolk-citrate as extender fractions A and B.

Extender treatment[2]	Motility score (n = 8)	SEM
M-M	3.1[ab]	.2
M-E	2.8[ab]	.2
E-M	3.3[a]	.2
E-E	2.6[b]	.2

[a,b,c]Means within a column having unlike superscripts differ ($P < .05$).

[1]5 = Exceptional, 4 = superior, 3 = normal, 2 = conditional, 1 = discard.

[2]M = Milk, E = egg yolk-citrate; for each character pair, left character indicates fraction A component, right character indicates fraction B component.

tial dilution of semen with extender fraction A. For the most part, treatments with milk as fraction A had greater immediate postthaw values for the SCSA variables than did treatments having egg yolk-citrate as fraction A. The lack of differences between E-M and E-E for values of each SCSA variable demonstrates that the effects of milk on sperm chromatin structure were not evident when milk was used only as extender fraction B. The intermediate nature of values for the SCSA variables for M-E treatment (between those of M-M and those of E-M and E-E) suggests that fraction A effects may have been modulated by fraction B. This offers an alternative explanation for differences in extender effects on sperm chromatin quality. The E-E and E-M extender treatments may have exerted a protective or inhibitory effect on the normal course of changes in denaturability of sperm nuclear DNA as opposed to M-M and M-E extender treatments having had an actively detrimental effect on the resistance of sperm nuclear DNA to denaturation.

For semen extended in M-M, it is notable that a large proportion of the total increase in $X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$ values occurring during in vitro incubation took place during the first 30 min (51.4, 88.8, and 87.3%, respectively). In another study (21), changes of a similar nature also occurred within the same time period when milk-extended sperm were incubated in vitro. In both cases, the changes occurred at temperatures comparable with those of the cow's genital tract and within the time period corresponding to the postinsemination interval during which sperm transport begins (19) and sperm enter the secretions of the female reproductive tract. In that context, the effects of extender on the chromatin structure of sperm in frozen-thawed semen may be particularly pertinent to the timing of postthaw

TABLE 7. Correlations among sperm viability traits measured at thawing (T = 0) and after 180 min of incubation (T = 180) for cryopreserved semen extended in combinations[1] of heated whole milk and egg yolk-citrate as extender fractions A and B.

		T = 0		T = 180
Extender treatment[1]	Viability trait	% Unstained cells (n = 16)[2]	Motility score (n = 8)[3]	% Unstained cells (n = 16)[2]
M-M		−.83***	.45	−.39
M-E	%Rf[4]	−.79***	.05	−.82***
E-M	(n = 16)	−.68**	−.31	−.83***
E-E		−.90***	−.66	−.81***
M-M		.72**	.55	.35
M-E	%Gf[5]	.52*	−.46	.80***
E-M	(n = 16)	.77***	.35	.82***
E-E		.64**	.50	.81***
M-M		.64		
M-E	Motility	−.16		
E-M	score[3]	.54		
E-E	(n = 8)	.73*		

[1]M = Milk, E = egg yolk-citrate; for each character pair, left character indicates fraction A component, right character indicates fraction B component.

[2]Percentage of sperm having intact cell membrane, as indicated by eosin-aniline blue vital staining.

[3]5 = Exceptional, 4 = superior, 3 = normal, 2 = conditional, 1 = discard.

[4]Percentage of sperm having disrupted cell membrane, as indicated by propidium iodide staining.

[5]Percentage of sperm having intact electrical potential across mitochondrial membrane, indicating intact mitochondrial function, as determined by rhodamine 123 staining.

*$P < .05$.

**$P < .01$.

***$P < .001$.

semen deposition in artificial insemination. These results may also have relevance to the composition of semen extenders, the formulation or physical characteristics of which are intended to increase sperm retention in the female genital tract. During the early postthaw period in vitro, the longer the sperm remained in the M-M extender, the greater the values for the SCSA variables became. This indicates that the heterogeneity of the already apparently compromised sperm chromatin structure became more pronounced. As suggested earlier, the increases in values for the SCSA variables during incubation in vitro, particularly during the first 30 min, may reflect characteristic chromatin changes for sperm extended in milk. However, it is not clear whether those changes resulted from events that would normally have occurred, persistent detrimental extender effects, effects of incubation on compromised chromatin, or a combination of those effects.

For freshly thawed semen, results of rhodamine 123-propidium iodide staining show the disadvantage to sperm cell membrane integrity and potential for mitochondrial function with M-M. Best immediate postthaw viability occurred when at least one extender fraction, particularly fraction A, was egg yolk-citrate. Similarly, the eosin-aniline blue vital staining results show the advantage to cell membrane integrity of using egg yolk-citrate as at least one extender fraction. These results agree with the reports of others (14, 29, 30) that have shown sperm cryopreserved in egg yolk-citrate to have an immediate postthaw viability advantage over those frozen in whole milk immediately postthaw. Conversely, the results of postthaw motility evaluation in the present study illustrate the similarities among effects of the extender treatments but indicate that sperm cryopreserved in extenders containing some milk may have had a motility advantage.

After 180 min of incubation, rhodamine 123-propidium iodide vital staining results showed that the cell membrane integrity advantage remained with extenders having egg yolk-citrate as one fraction, particularly fraction A. This is in agreement with reports of others (14, 30) that have shown egg yolk-citrate to be superior to milk for maintenance of viability during postthaw incubation. The benefit to cell membrane integrity of using egg yolk-citrate as extender fraction A suggests that this viability advantage may have stemmed from some protection imparted during initial dilution and corresponds to similar observations for SCSA results. Although SCSA variables and sperm viability traits have been shown to be inversely related (1, 2, 21), no significant correlations resulted in the present study. It is notable, however, that at 180 min of incubation, sperm in M-M showed the greatest potential for sperm mitochondrial function (Table 3).

The correlations between results of cell membrane integrity evaluation (%Rf and percentage of unstained cells, Table 7) demonstrate that, although absolute values for the results of two tests may not have been in complete agreement, the inverse relationship between them was strong. The lack of significant correlation between motility score and mitochondrial fluorescence (%Gf) may have been due to low variation among the motility scores.

The results of the viability evaluations indicate that milk was a more hospitable incubation medium than egg yolk-citrate for maintaining mitochondrial function postthaw, and cell membrane integrity was best preserved during incubation by egg yolk-citrate. Because optimal maintenance of cell membrane integrity and mitochondrial function of the sperm are presumably desirable, these results suggest that an extender combining the attributes of milk and egg yolk-citrate would best serve both purposes. However, the incubation of semen in extender treatments other than M-M may have induced the manifestation of latent mitochondrial damage, i.e., that which was not apparent immediately postthaw. From this point of view, milk extender would be desirable if use of the other extenders actually resulted in greater latent damage to mitochondrial function, and thus motility potential, than was apparent by immediate postthaw evaluation of motility or %Gf. This remains to be determined.

CONCLUSIONS

Optimal protection of sperm cell membrane integrity, mitochondrial function, and chromatin quality should be primary considerations when selecting an extender for cryopreservation of sperm. The results of this initial study

show that sperm chromatin structure and cell membrane integrity evaluation were consistent in demonstrating the potential benefit of extender containing egg yolk-citrate to these semen quality traits. The results of postthaw motility evaluation and rhodamine 123-propidium iodide staining after 180 min of incubation demonstrated the likely benefit of potential for sperm motility imparted by extenders containing milk. It is not clear why sperm cryopreserved in milk versus egg yolk-citrate differed for chromatin structure, cell membrane integrity, and maintenance of mitochondrial function. Differences in composition between the extender components may have been a possible cause. Milk and egg yolk differ in levels of minerals, vitamins, lipid, and amino acids (24). Differences may also have stemmed from the sequence in which the different components were combined with raw semen. Because the extender treatments in the present study appeared to have beneficial effects on different aspects of postthaw semen quality, perhaps the general lack of published differences in fertility between milk and egg yolk-citrate extenders (12, 29) may have been due to the beneficial effects of each extender being canceled by their individual detrimental effects on some other aspect or aspects of semen quality. The results of this preliminary study indicate that further, larger scale investigations of extender effects on semen quality traits should be conducted in conjunction with fertility trials. Better understanding of extender effects on semen quality may result in enhanced postthaw semen quality through modified semen processing and cryopreservation techniques.

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