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Cryopreservation of Ram Semen Facilitates Sperm DNA Damage: Relationship Between Sperm Andrological Parameters and the Sperm Chromatin Structure Assay

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ABSTRACT: We hypothesized that cryopreservation and incubation in conditions that mimic the female genital tract following insemination increases the susceptibility of ram sperm DNA to denaturation. Ram sperm samples (n = 12) underwent the sperm chromatin structure assay (SCSA) and semen quality tests, including motility parameters, viability, and chlortetracycline fluorescence (CTC) patterns. We also assessed correlations between SCSA variables and semen quality parameters. Analyses were performed for both fresh and cryopreserved samples at 0, 3, and 20 hours of incubation in synthetic oviductal fluid (SOF; 39°C, 5% CO_2). The SCSA variables, mean alpha t ($X\alpha_t$) and standard deviation of alpha t ($SD\alpha_t$), were higher because of cryopreservation ($P < .05$, $P < .001$, respectively) after 20 hours in SOF. For both fresh and frozen spermatozoa, SCSA values ($X\alpha_t$, $SD\alpha_t$, and the percentage of cells outside the main population of α_t [$\%COMP\alpha_t$]) increased during incubation in SOF. Motility was negatively correlated with both $SD\alpha_t$ and $\%COMP\alpha_t$, ranging from −0.39 ($P < .01$) to −0.59 ($P < .001$) for both fresh and cryopreserved semen; viability also was negatively correlated with $X\alpha_t$, $SD\alpha_t$, or $\%COMP\alpha_t$ (−0.36; $P < .05$, −.40 and -.46; $P < .01$, respectively) in fresh semen. The $\%COMP\alpha_t$ was positively correlated to the percentage of CTC pattern AR ($P < .001$) and negatively correlated to the percentages of patterns F and B (−0.33 to −0.60, $P < .05$ to $P < .001$). Variation among ejaculates within ram was observed ($P < .01$). Cryopreservation clearly facilitates DNA damage in physiological conditions. The low to moderate correlations between SCSA variables and classical semen quality parameters indicate that the SCSA provides additional information to standard tests for evaluating ram sperm quality.

Key words: Artificial insemination, semen quality, motility, chlortetracycline, viability, SCSA.

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Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection programs (Maxwell, 1984). However, the biggest obstacle to exploiting cryopreserved semen of many species is that cooling, freezing, and thawing generally damage sperm membrane structures, leading to fewer viable and motile cells postthaw (Nath, 1972; Hammerstedt et al, 1990). Consequently, fertility following AI is poorer than with fresh semen in most species (Salamon and Maxwell, 1995; Watson, 1995).

Semen quality and its relationship to fertility are of major concern in animal production. Quality tests are routinely used to determine acceptability of processed semen for breeding purposes. Thus accurate measurement is of major importance. Conventionally, the principal laboratory tests for standard semen analysis at most AI centers use light microscopy to estimate sperm survival and the percentage of motile (and progressively motile) spermatozoa (Rowe et al, 1993). Although useful, these tests are not completely reliable or repeatable because of the small numbers of sperm evaluated, lack of objectivity, and human bias (Graham et al, 1980). More objectivity and repeatability in assessing sperm motility can be achieved by computer-assisted sperm analysis (CASA) (Davis and Siemers, 1995). In addition to CASA, flow cytometry (Shapiro, 1988) has emerged as a powerful technique for providing rapid, multiparameter, objective, and nonbiased measurements of parameters on very high numbers of sperm per ejaculate, which might be necessary for predicting fertility potential (Evenson et al, 1994). Flow cytometric measurements of sperm parameters have included mitochondrial function (Evenson et al, 1985b; Graham et al, 1990), viability (Garner et al, 1986), DNA content for sex determination (Johnson et al, 1987, 1989), acrosome integrity (Graham et al, 1990), sperm calcium level (Collin et al, 2000), and chromatin structure integrity, which is defined as the susceptibility of DNA to acid- or

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heat-induced denaturation in situ (Evenson et al, 1980; Ballachey et al, 1987).

The sperm chromatin structure assay (SCSA) is a technique based on the assumption that structurally abnormal sperm chromatin is more susceptible to denaturation (Evenson et al, 1980). This assay uses the metachromatic properties of acridine orange, which fluoresces green when combined with the intact double DNA helix and red when combined with RNA and denatured DNA (ie, single-stranded helices) (Darzynkiewicz, 1990). Mild acid treatment of spermatozoa denatures structurally abnormal chromatin, thereby intensifying red band fluorescence (Darzynkiewicz, 1990).

The SCSA can determine the importance of DNA structure in assisted reproductive techniques outcomes such as AI. The SCSA is reported to be an unbiased, quantitative assessment of sperm chromatin integrity, and its variables are apparently consistent within individuals over time if they are not exposed to reproductive stressors (Evenson et al, 1991). Furthermore, SCSA parameters are correlated with DNA strand breaks (Sailer et al, 1995b) and fertility in vivo (Evenson et al, 1980, 1999). However, SCSA parameters are only weakly to moderately correlated with classical criteria of sperm quality, including concentration, total number, motility and morphology (Evenson et al, 1991), and viability (Januskauskas et al, 2001). Therefore, SCSA parameters are considered independent descriptors of semen quality and might complement the information derived from the classical andrological assessment. The SCSA has not been used extensively on ram sperm, and associations between traditional andrological endpoints and sperm chromatin structure in this species have not been established.

This study was therefore performed to investigate the effect of commercial semen cryopreservation on DNA structure of ram spermatozoa by evaluating the level of sperm nuclear DNA damage during incubation in medium resembling the natural environment of the female genital tract. Our hypothesis is that the freeze-thaw process reduces sperm fertilizing competence by destabilizing the chromatin, as reflected by increased DNA susceptibility to denaturation in situ. A secondary objective was to determine the relationship between the parameters of SCSA and the classical criteria of ram sperm quality.

Materials and Methods

Chemicals and Animal Care

All experimental protocols were approved by the university animal protection committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care (1993). Chemicals used in this study were obtained from Sigma Chemical Co (St. Louis, Mo), except acridine orange, which was purchased from Polysciences, Inc (Warrington, Penn).

Semen Handling and Cryopreservation

Semen was obtained from 5 mature Dorset rams (aged 2 to 4 years) maintained at the Center d'Expertise en Production Ovine du Québec (CEPOQ, La Pocatière, Canada) under uniform feeding, housing, and light conditions. Semen from these rams was collected with the use of an artificial vagina. Semen volume, concentration, and subjective motility (using phase contrast microscopy, X400) were measured. Sperm concentration was assessed with a spectrophotometer previously calibrated by hemocytometry, and samples with total motility higher than 65% were used in this study.

Immediately after collection, the semen was diluted to a final concentration of 4×10^8 spermatozoa mL^{-1} with a commercial egg yolk–based extender (Triladyl®; Minitube Canada, Woodstock, Canada) then slowly cooled to 5°C over 3 hours in a transport box (CEPOQ) as reported earlier (Morrier et al, 2002). On arrival at the laboratory, diluted semen was divided into 2 portions: 1 remained fresh, and the other was cryopreserved. Aliquots (0.25 mL) of the fresh samples were diluted to about 50×10^6 spermatozoa mL^{-1} in synthetic oviductal fluid (SOF: Morrier et al, 2002). The SOF consisted of 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM glucose, 3.3 mM sodium lactate, 0.33 mM sodium pyruvate, 1 mM glutamine, 20 μM penicillamine, 10 μM hypotaurine, and 50 mg L^{-1} gentamycin; 20% heat-inactivated estrus sheep serum was added to this solution and adjusted to pH 7.36.

Classical semen quality tests (motility, viability, and chlortetracycline fluorescence [CTC]) and SCSA were assessed immediately following dilution in SOF (time 0). The samples diluted in SOF were incubated in a humidified atmosphere of 5% CO_2 in air at 39°C. After 3 and 20 hours of incubation, the classical tests and SCSA were reassessed.

The remaining semen was frozen according to industry standards (Morrier et al, 2002) in 250-μL straws (IMV, l'Aigle, France) sealed with polyvinyl acid. The straws were frozen by plunging directly into liquid nitrogen at −196°C. Straws were stored in liquid nitrogen for at least 2 weeks before analysis. Semen was thawed by plunging straws into a 37°C water bath for 60 seconds. As described above for fresh semen, a straw was thawed and then diluted in SOF (50×10^6 spermatozoa mL^{-1}). Again, classical sperm quality tests (motility, viability, CTC) and SCSA were evaluated immediately following dilution in SOF (time 0), then after 3 and 20 hours of incubation (time 3 and 20 hours). Figure 1 shows an outline of the protocol.

Sperm Motility Analysis

Sperm motility parameters were analyzed with a Hamilton-Thorn motility analyzer (Ceros Analyzer with software version 12.0 F; Hamilton Thorn Research, Beverly, Mass). Samples of spermatozoa in SOF (50×10^6 cells mL^{-1}) were placed in a prewarmed (37°C) MicroCell counting chamber (20 μM depth; Conception Technologies, San Diego, Calif) and loaded into the analyzer, and at least 200 motile sperm were analyzed (phase contrast, 100×) for each sample. The motility parameters mea-

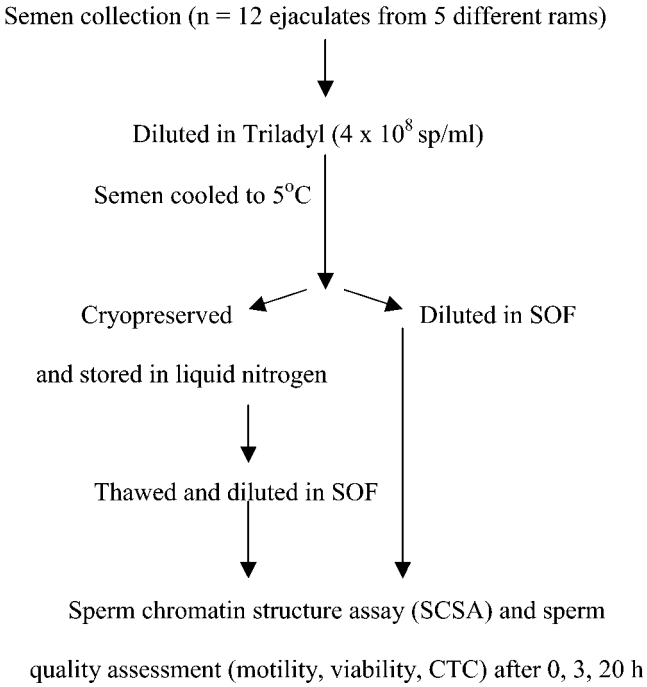

Figure 1. Schematic for testing the effect of freezing and incubation time on ram semen and measuring by the sperm chromatin structure assay.

sured were as follows: percentage of motile spermatozoa, percentage of rapidly progressively motile spermatozoa, straight-line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and straightness (STR). The settings of the sperm analyzer used in this study (Morrier et al, 2002) and the description of the parameters of sperm motility are essentially as previously described (Bilodeau et al, 2001)

Sperm Viability Assay

Sperm viability was assessed by using eosin-negrosin staining as described by (Barth and Oko, 1989; Morrier et al, 2002). At least 200 spermatozoa were recorded per slide by light microscopy (400×), and the percentage of dead (colored pink) and live (unstained) cells were evaluated.

Chlortetracycline Fluorescence Assay

The chlortetracycline (CTC) fluorescence assay was used previously and described by Morrier et al (2002). All slides were prepared in duplicate. At least 200 spermatozoa per slide were classified according to 1 of the 3 CTC staining patterns, as described by Gillan et al (1997): F, or noncapacitated spermatozoa for those with uniform head fluorescence; B, or capacitated spermatozoa for those fluorescent only along the acrosome; and AR, for acrosome-reacted spermatozoa with pale fluorescence over the head.

Flow Cytometry

All flow cytometry measurements were performed with the use of an EPICS XL (Beckman-Coulter, Miami, Fla). Green (525 band-pass filter) and red (620 band-pass filter) fluorescence emitted by each sperm after laser excitation (35 mW, 488 nm wavelength) were processed through photomultiplier tubes and quantified. The flow cytometer is capable of distinguishing up to 1024 channels (fluorescence intensities) of both red and green fluorescence on each cell. Data on at least 5000 spermatozoa per sample were collected and analyzed. The flow cytometer detected the green and red fluorescence of each cell as it passed through the focal point in the quartz flow cell at rates of about 300 cells/s.

Sperm Staining With Acridine Orange

The procedure of sperm staining with acridine orange was as previously described (Evenson et al, 1989, 1994). Briefly, fresh or frozen-thawed semen was diluted in TNE buffer (0.15 M NaCl, 0.001 M EDTA, and 0.01 M Tris, pH 7.4) to a final concentration of 1 to 2 million cells/mL. Then, 0.2 mL of semen was added to 0.40 mL of a detergent/acid solution consisting of 0.1% Triton X-100 in 0.08 N HCl and 0.15 M NaCl (pH 1.4). After 30 seconds, 1.2 mL of staining solution containing 6 μg/mL electrophoretically purified acridine orange in staining buffer (prepared by mixing 370 mL of 0.1 M citric acid monohydrate and 630 mL of 0.2 M Na_2HPO_4 [dibasic] and adding 0.372 g disodium EDTA and 8.77 g NaCl [pH 6.0]) was added to the sample. All steps were conducted at 4°C, and flow cytometry was begun 3 minutes after staining.

Sperm Chromatin Structure Assay (SCSA)

The SCSA is an acridine orange staining technique used to study sperm chromatin structure (Evenson et al, 1980, 1983, 1985a; Ballachey et al, 1987) and measure the susceptibility of DNA in sperm chromatin to acid-induced denaturation in situ. It is quantified by flow cytometric measurement of the metachromatic shift from green (double-stranded DNA [dsDNA]) to red (denatured, single-stranded DNA [ssDNA]) of acridine orange fluorescence. This shift is expressed as the function alpha t (α_t) which is the ratio of red to total (red + green) fluorescence intensity (Darzynkiewicz et al, 1975), representing the amount of denatured ssDNA over the total cellular DNA. In the SCSA, α_t was calculated for each spermatozoon in a sample, and the results were expressed as the mean ($X\alpha_t$) for all 5000 sperm assessed, the standard deviation ($SD\alpha_t$) of the α_t distribution, and the percentage of cells with high α_t values (cells outside the main population, $\%COMP\alpha_t$), representing spermatozoa with an excess of ssDNA. Sperm populations with normal chromatin structure have small $X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$ (ie, percentage of cells with denatured DNA). The α_t values were expressed within a range of 0 and 1024 channels of fluorescence.

Statistical Analyses

Data were normalized by square root transformation, and the least squares means were analyzed by the General Linear Model (GLM) ANOVA procedure of Statistical Analysis System, version 8.2 (SAS Institute Inc, Cary, NC). The statistical model used to analyze SCSA parameters ($X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$) and sperm quality tests (motility, viability, CTC) include the effect of ram, ejaculate within ram, type of semen (fresh or cryopreserved), and incubation time (0, 3, 20 hours). When main

Table 1. *Mean squares and tests of significance for sperm chromatin structure assay (SCSA) measured by flow cytometry*

Source†	df	$X\alpha_t$‡	$SD\alpha_t$§	% $COMP\alpha_t$‖
R	4	188.6	78.0	0.4460
E(R)	7	208.1**	304.7**	2.1**
F	1	175.8*	400.5**	2.4***
R × F	4	18.5	6.6	0.027
E × F(R)	7	28.5	35.2*	0.288*
E × T(R)	14	21.8	10.60	0.090
R × F × T	8	14.8	61.8**	0.059
T	2	1884**	616 033**	10.8***
R × T	8	16.7	66.0	0.053
F × T	2	35.5	875 151	0.005

Values are significant at * $P < .05$; ** $P < .01$; *** $P < .001$.

† R indicates ram; E(R), ejaculates within ram; F, treatment (fresh vs cryopreserved); T, time of incubation in synthetic oviductal fluid (0, 3, 20 hours).

‡ Mean of α_t, where α_t is the ratio of red fluorescence to total fluorescence (red + green).

§ Mean of $SD\alpha_t$.

‖ Percentage of cells outside the main population of α_t.

effects were significant ($P < .05$), multiple comparisons using Tukey's test were made to determine the significance between the fresh and cryopreserved semen and between the different incubation times. Pearson correlation coefficients were used to calculate the relationships between SCSA parameters and sperm quality tests. The results are presented as nontransformed means ± SEM and were considered statistically significant at $P < .05$.

Results

Sperm Chromatin Structure Assay Variables

Analysis of variance revealed that rams were not a significant source of variation for all SCSA variables ($X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$), although the differences between the ejaculates within the ram were statistically significant ($P < .01$; Table 1). Figure 2 shows values of SCSA variables. Differences between fresh and cryopreserved semen for SCSA variables were not significant at most incubation times, except that $X\alpha_t$ and $SD\alpha_t$ were higher as a result of cryopreservation ($P < .05$ and .001, respectively) at 20 hours of incubation in SOF. There was a significant effect of incubation time in SOF (Figure 2) on DNA denaturability, as measured by the SCSA. These values ($X\alpha_t$, $SD\alpha_t$, and $\%COMP\alpha_t$) increased with the duration of incubation in SOF ($P < .01$ and .001, respectively), and the rate of increased denaturability for cryopreserved sperm was greater than for fresh sperm.

Classical Semen Quality Parameters

Table 2 shows the analysis of variance results for the classical andrological parameters. Ram and ejaculate within ram had a minor effect on semen parameters (CTC patterns and motility parameters), whereas the main effects of treatment (fresh vs cryopreserved) and incubation time

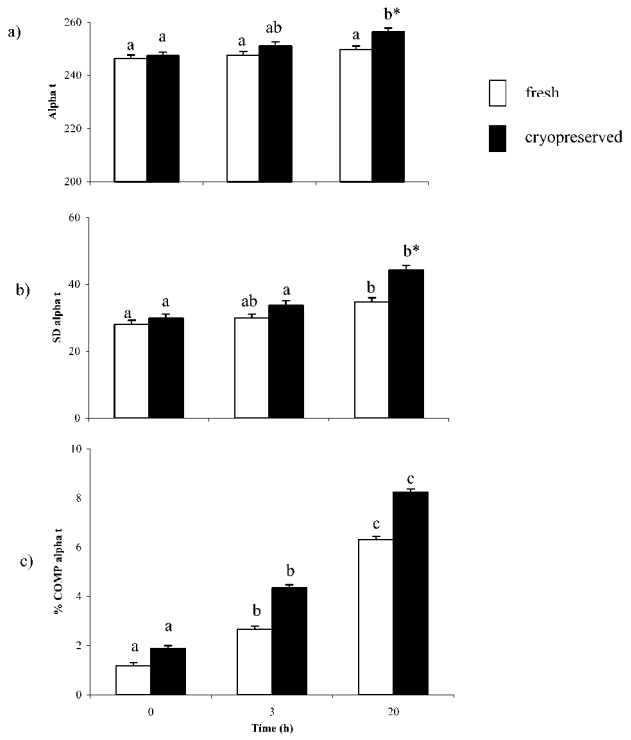

Figure 2. Effect of cryopreservation and subsequent incubation of sperm in synthetic oviductal fluid (SOF) for 0, 3, and 20 hours (39°C, 5% CO_2) on sperm chromatin structure assay (SCSA) parameters. (**A, B, C**) Mean alpha t ($X\alpha_t$), standard deviation of alpha t ($SD\alpha_t$), and percent cells outside the main population of alpha t ($\%COMP\alpha_t$), respectively. Within treatment (fresh, cryopreserved), different letters (a, b) indicate significant differences due to duration of incubation in SOF ($P < .05$). Within each time in SOF (0, 3, 20 hours), an asterisk indicates a significant difference as a result of cryopreservation ($P < .05$). Values are means ± SEM (n = 12 ejaculates from 5 different rams).

in SOF had a more widespread effect on most parameters (motility, viability, and CTC).

As shown in Figure 3, cryopreservation decreased ($P < .01$) the percentages of total and progressively motile sperm at 0 and 3 hours of incubation, but was not significant at 20 hours. No significant differences were found between fresh and cryopreserved semen for any of the other motility parameters (VAP, VSL, VCL, ALH, BCF, STR, and LIN) over all incubation times (data not shown).

Results of analysis of variance indicate that the differences in the percentage of viable sperm between fresh and cryopreserved semen were significant ($P < .01$; Figure 3) only at time 0 of incubation in SOF, but not at 3 and 20 hours.

With respect to CTC fluorescence, both cryopreservation and duration of incubation in SOF affected ($P < .001$) the distribution of sperm displaying patterns F, B, and AR (Figure 4). For both fresh and cryopreserved semen, the percentage of sperm showing pattern F (noncapacitated) decreased ($P < .05$) with increasing duration of incubation in SOF, corresponding to an increase in the

Table 2. *Mean squares and tests of significance for semen quality parameters*

		Motility Parameters‡								Chlortetracycline Patterns		
Source†	df	% Motile	% Prog Mot	VAP	VSL	VCL	ALH	BCF	STR	LIN	% Viable	% F	% B	% AR
R	4	254.1	56.8	447.8	305.2	1119	3.4	59.3	45.1	2.2	391.4	17.4***	28.6***	33.3***
E(R)	7	501.1**	93.5	254	174.8***	905	3.4	62.5	235.7	60.5	450	5.2	158.3	151.5
F	1	14 168**	1699.9**	1343	570.9	4994.6	0.37	41.7	221.9	18.2	2585.8**	9080.2***	600.5***	14 200***
R × F	4	509.2*	47.7***	273.9	124.6***	944	6.2	10.7	78.7	55.4	185.8	23.6	10.7	28.7
E × F(R)	7	46.7	18	166.7	78.2	545	4.4	106.5	81.9	61.4	292***	71.1	215.2**	413.9**
E × T(R)	14	107.7*	33.9	236.2	136.4	890	6.2	63.9	227.8*	89.5	24.9	14.7	53.2	76.6
E × F × T	8	63.9	12.6	74.1	42.8	248	2.8	7.9	67.8	50.0	29.2	45.2	14.9	46.6
T	2	10 454***	204.3***	20 405***	14 696***	45 680***	54.9	609.5**	2086.9***	1420***	2036.7***	6750.1***	1883.5***	15 163***
R × T	8	16.7	16.7	198.7***	103.9***	739.5	5.5	64.6***	66.7	24.4	41.6	25.7	40.0	76.4
F × T	2	409.8***	409.8***	44	23.8	7.7	0.16	137.9**	329.9*	109.4	296.4	1975.6***	45.4	1857.3***

Values are significant at * $P < .05$; ** $P < .01$; *** $P < .001$.
† R indicates ram; E(R), ejaculate within ram; F, cryopreservation treatment (fresh vs cryopreserved); T, time of incubation in synthetic oviductal fluid (0, 3, 20 hours).
‡ % Motile indicates percentage of motile spermatozoa; % Prog Mot, percentage of rapidly progressively motile spermatozoa; VSL, straight-line velocity; VAP, average path velocity; VCL, curvilinear velocity; LIN, linearity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; % Viable, percentage of viable spermatozoa; % F, % B, % AR, percentages of spermatozoa displaying chlortetracycline fluorescent patterns F, B, and AR, respectively.

Figure 3. Effect of cryopreservation and subsequent incubation in synthetic oviductal fluid (SOF) for 0, 3, and 20 hours (39°C, 5% CO_2) on the percentage of motile (**A**), progressively motile (**B**), and viable sperm (**C**). Within treatment (fresh, cryopreserved), different letters (a, b, c) indicate significant differences due to duration of incubation in SOF ($P < .05$). Within each time in SOF (0, 3, 20 hours), an asterisk indicates a significant difference as a result of cryopreservation ($P < .05$). Values are means ± SEM (n = 12 ejaculates from 5 different rams).

percentage of pattern AR (acrosome-reacted). In addition, cryopreservation led to a decrease ($P < .05$) in the percentage of sperm with pattern F and an increase ($P < .05$) percentage of sperm with pattern AR at 0 and 3 hours of incubation in SOF.

Correlations Among SCSA Variables and Classical Semen Quality Parameters

The correlations among SCSA variables and classical semen quality parameters, calculated separately at the different times of incubation in SOF, varied in magnitude from low to moderate and from positive to negative (Table 3). These correlations were rarely significant. In order to derive a general conclusion for such relationships, we pooled the data of the 3 incubation periods (0, 3, and 20 hours) within each treatment (fresh and cryopreserved) to augment the accuracy of estimates by increasing the number of observations.

Table 4 shows significant ($P < .05$ to $P < .001$) negative correlations among SCSA variables and most classical semen quality parameters from both fresh and cryopreserved semen. Values of these correlations varied from low to moderate and were greater for cryopreserved than

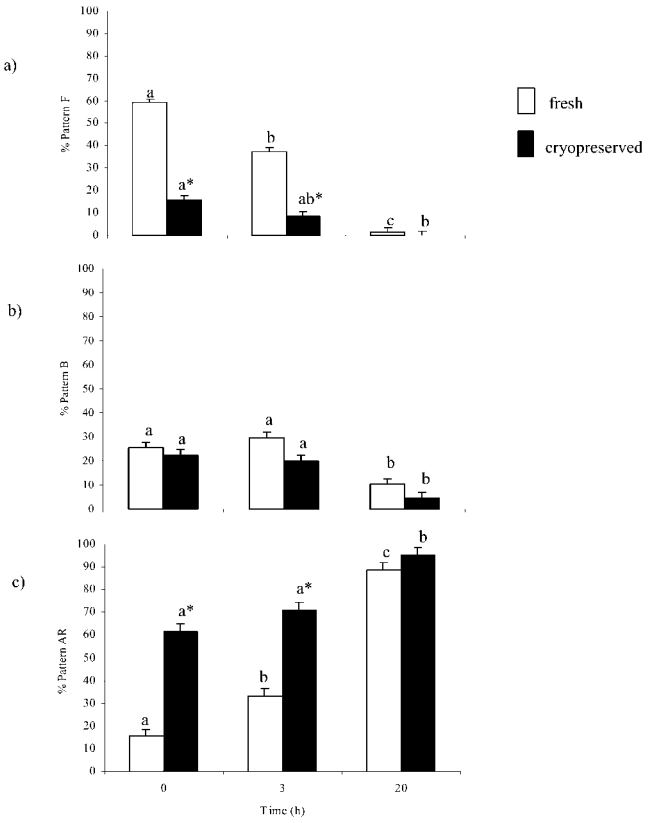

Figure 4. Effect of cryopreservation and subsequent incubation in synthetic oviductal fluid (SOF) for 0, 3, and 20 hours (39°C, 5% CO_2) on the percent sperm displaying the chlortetracycline fluorescent F (**A**) (non-capacitated), (**B**) B (capacitated), and (**C**) AR (acrosome-reacted). Within treatment (fresh, cryopreserved), different letters (a, b, c) indicate significant differences due to duration of incubation in SOF ($P < .05$). Within each time in SOF (0, 3, 20 hours), an asterisk indicates a significant difference as a result of cryopreservation ($P < .05$). Values are means ± SEM (n = 12 ejaculates from 5 different rams).

for fresh semen in most cases. The strongest correlations were observed between %COMPα_t and classical semen quality parameters compared with those obtained from Xα_t and SDα_t and classical semen quality parameters.

No significant correlations were obtained among Xα_t and any of the motility parameters for the fresh semen, but for the cryopreserved semen, there were statistically significant negative correlations with most motility parameters (ranging from −0.32, $P < .05$, to −0.41, $P < .001$). As shown in Table 4, correlations between either SDα_t or %COMPα_t and total or progressive motility were nearly the same and ranged from (−0.39, $P < .01$) to (−0.59, $P < .001$).

In the fresh semen, correlations between Xα_t, SDα_t, or %COMPα_t and viability as presented in Table 4 were weak but significant (−0.36, $P < .05$; −0.40 and −0.46, $P < .01$, respectively). However, in the cryopreserved semen, there were no significant correlations.

The relationship between the SCSA variables and the percentages of sperm with the different CTC patterns also

Table 3. *Correlation coefficients between sperm chromatin structure assay (SCSA) variables and classical semen quality parameters from fresh (F) and cryopreserved (C) ram semen at different durations of incubation in synthetic oviductal fluid (0, 3, 20 hours)*

Classical Semen Quality Parameters†	SCSA Variables																	
	Xα_t						SDα_t						% COMPα_t					
	0		3		20		0		3		20		0		3		20	
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
% Motile	0.25	0.10	−0.02	0.06	−0.15	−0.33	−0.08	−0.33	−0.08	−0.06	0.12	−0.40	0.22	−0.18	−0.26	0.11	0.19	−0.32
% Prog Mot	0.30	0.12	0.00	−0.13	0.60*	−0.42	0.05	−0.26	−0.19	−0.26	0.54	−0.44	0.43	−0.42	−0.39	−0.03	0.81**	−0.29
VAP	0.30	−0.13	0.18	0.18	0.36	−0.40	0.26	0.14	0.23	0.54	0.70**	−0.23	0.23	−0.42	−0.05	0.53	0.49	−0.29
VSL	0.30	−0.09	0.02	0.18	0.23	−0.36	0.42	0.15	0.30	0.52	0.59*	−0.14	0.34	−0.45	−0.21	0.55	0.35	−0.22
VCL	0.30	−0.17	0.24	0.11	0.34	−0.33	0.10	0.13	0.19	0.51	0.73**	−0.27	0.12	−0.51	0.07	−0.42	0.46	−0.29
ALH	0.22	−0.26	0.54	0.26	0.17	−0.58	0.34	−0.05	0.11	0.36	0.40	−0.46	0.14	−0.49	0.45	0.01	0.34	−0.47
BCF	0.20	−0.52	−0.26	0.08	0.19	−0.26	0.84**	−0.06	0.43	0.11	0.75**	−0.22	0.49	−0.47	0.04	0.19	0.28	−0.37
STR	−0.31	0.08	−0.67*	0.37	0.01	−0.18	0.30	0.10	0.41	0.16	0.31	−0.04	0.10	0.23	−0.41	0.25	−0.04	−0.16
LIN	−0.31	0.25	−0.50	0.44	−0.04	−0.24	−0.06	0.10	0.31	0.42	0.03	−0.03	0.07	0.52	−0.35	0.54	−0.17	−0.17
% Viable	−0.12	−0.16	−0.31	0.15	−0.43	−0.09	0.06	−0.35	−0.23	0.02	−0.36	−0.11	−0.01	0.03	−0.28	0.31	−0.26	0.11
% F	0.38	0.16	−0.63*	0.62	−0.19	−0.35	0.11	0.13	0.02	0.49	0.09	−0.31	0.26	0.23	−0.63*	0.43	−0.50	−0.37
% B	0.25	0.64*	0.40	0.25	−0.02	0.11	0.34	0.28	0.42	0.16	0.23	0.16	0.30	0.22	0.48	0.03	−0.17	0.12
% AR	−0.48	−0.53	0.15	−0.37	0.07	−0.03	−0.34	−0.25	−0.40	−0.26	−0.24	−0.10	−0.42	−0.24	0.08	−0.15	0.28	−0.04

Values are significant at * $P < .05$; ** $P < .01$.

† % Motile indicates percentage of motile spermatozoa; % Prog Mot, percentage of rapidly progressively motile spermatozoa; VSL, straight-line velocity; VAP, average path velocity; VCL, curvilinear velocity; LIN, linearity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; % Viable, percentage of viable spermatozoa; % F, % B, % AR, percentages of spermatozoa displaying chlortetracycline fluorescent patterns F, B, and AR, respectively.

Table 4. *Correlation coefficients between sperm chromatin structure assay variables and classical semen quality parameters for ram semen (pooled from the synthetic oviductal fluid incubations at 0, 3, and 20 hours) for each treatment (fresh and cryopreserved)*

Semen Quality Parameter†	$X\alpha_t$		$SD\alpha_t$		% $COMP\alpha_t$	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
% Motile	−0.18	−0.30	−0.39**	−0.43**	−0.50***	−0.57***
% Prog Mot	−0.14	−0.31	−0.39**	−0.43**	−0.49**	−0.59***
VAP	−0.06	−0.41**	−0.21	−0.33*	−0.41**	−0.62***
VSL	−0.11	−0.41**	−0.21	−0.34*	−0.45**	−0.64***
VCL	−0.02	−0.41**	−0.19	−0.34*	−0.36*	−0.62***
ALH	−0.03	−0.41**	−0.11	−0.40**	−0.14	−0.2***
BCF	0.03	−0.40**	0.27	−0.39**	−0.00	−0.61***
STR	−0.21	−0.32*	−0.08	−0.29	−0.39**	−0.52***
LIN	−0.28	−0.27	−0.28	−0.25	−0.51***	−0.46**
% Viable	−0.36*	−0.18	−0.40**	−0.28	−0.46**	−0.24
% Pattern F	−0.24	−0.23	−0.39**	−0.30	−0.60***	−0.58***
% Pattern B	0.01	−0.07	−0.02	−0.18	−0.33*	−0.45**
% Pattern AR	0.18	0.14	0.30	0.25	0.57***	0.54***

Values are significant at * $P < .05$; ** $P < .01$; *** $P < .001$.

† % Motile indicates percentage of motile spermatozoa; % Prog Mot, percentage of rapidly progressively motile spermatozoa; VSL, straight-line velocity; VAP, average path velocity; VCL, curvilinear velocity; LIN, linearity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; % Viable, percentage of viable spermatozoa; % F, % B, % AR, percentages of spermatozoa displaying chlortetracycline fluorescent patterns F, B, and AR, respectively.

was studied (Table 4). The %$COMP\alpha_t$ correlated positively with the percentage of pattern AR ($P < .001$) and correlated negatively with the percentages of patterns F and B (−0.33 to −0.60, $P < .05$ to $P < .001$). None of the other SCSA variables were correlated with CTC pattern distribution, except for $SD\alpha_t$, which was correlated with the percentage of pattern F only in fresh semen (−0.39, $P < .05$; Table 4).

Discussion

The major objective of this study was to test the hypothesis that cryopreservation disrupts the DNA structure of ram spermatozoa. Although not addressed in this study, it is possible that such DNA damage might account for the poor fertility of frozen-thawed ram semen relative to fresh (Salamon and Maxwell, 1995.)

It is well known that mature mammalian sperm nuclei are stable structures, with highly condensed and organized DNA (Ward and Coffey, 1991). Chromatin stabilization occurs by intermolecular and intramolecular covalent disulfide bonds between the protamines that replace histones during spermatogenesis (Poccia, 1986). This study used the SCSA to evaluate the susceptibility of ram sperm DNA to denaturation resulting from cryopreservation and whether there was any effect of incubation in SOF on sperm DNA damage. The SCSA has been used previously to evaluate sperm DNA in bulls (Ballachey et al, 1988; Karabinus et al, 1990), boars (Evenson et al, 1994), stallions (Love et al, 2002), mice (Evenson et al, 1993), and humans (Evenson et al, 1999; Saleh et al, 2002).

As expected and previously documented (reviewed by Bailey et al, 2000), the classical andrological sperm parameters were reduced following cryopreservation. More importantly, this study demonstrates that SCSA variables ($X\alpha_t$ and $SD\alpha_t$) were different ($P < .05$ and .001, respectively) between fresh and cryopreserved spermatozoa after 20 hours of incubation in conditions that were intended to mimic the female reproductive tract (in SOF), but not earlier (0 and 3 hours). The elevated SCSA values for cryopreserved spermatozoa after 20 hours in SOF suggest both an increased susceptibility of sperm nuclear DNA to denaturation and heterogeneity of sperm nuclear chromatin in comparison with the fresh spermatozoa. Previous studies (Ballachey et al, 1986, 1987; Karabinus et al, 1990, 1991) demonstrated that elevated heterogeneity of sperm nuclear chromatin is related to spermatogenic disturbances, morphological abnormalities of spermatozoa, and decreased fertility in vivo. In a previous study with boar semen, sperm chromatin structure was not altered by freezing directly on dry ice or in liquid nitrogen in different types of extenders, as determined by the SCSA (Evenson et al, 1994). Similar results were obtained with mouse (Evenson et al, 1989, 1993), bull (Van der Schans et al, 2000), and human spermatozoa (Duru et al, 2001). Furthermore, it was concluded that cryopreservation does not significantly damage sperm DNA immediately or within 30 minutes of thawing, as assessed by an immunochemical assay in bull sperm (Van der Schans et al, 2000) and TUNEL for human sperm (Duru et al, 2001). In contrast, studies on human semen assessed by the SCSA (Spano et al, 1999) or a modified alkaline single-cell gel electrophoresis (comet) assay (Donnelly et al,

2001) showed that sperm DNA integrity deteriorates after cryopreservation. In the current investigation, it is difficult to compare our results with those in the previous studies because of the differences in species, cryopreservation procedure, and time of evaluation postthaw. However, the freeze-thaw processes did not have an immediate or early influence on the degree of DNA damage during incubation in SOF (0 and 3 hours), but damage was apparent later, after 20 hours.

The incubation in SOF, a physiological type of medium, was intended to mimic the ewe genital tract, thereby allowing us to monitor sperm DNA structure within a time frame relevant to the interval between thawing, AI, and fertilization in vivo. In sheep, ovulation usually occurs several hours postinsemination (Walker et al, 1989). Thus, the observation that cryopreserved ram spermatozoa have more susceptible and heterogeneous chromatin than do fresh, suggests that the poorer fertilizing efficiency of frozen ram semen might be at least partly due to abnormal sperm DNA structure, despite having a normal appearance soon after thawing. The increased susceptibility to DNA denaturation in situ during incubation in SOF is in agreement with other reports. Incubation of bull sperm in cryopreservation extenders at temperatures comparable with those of the female genital tract augmented the susceptibility of sperm DNA to denaturation within 30 minutes (Karabinus et al, 1990, 1991). Also, Estop et al (1993) showed that in vitro incubation of mouse spermatozoa induced a sharp increase in abnormal chromatin structure during the first hour (half of the cells were altered), followed by a continual rise to 70% at 12 hours and about 95% at 48 hours. In contrast, undiluted human semen does not show any significant increase of DNA damage when kept at room temperature for up to 4 hours (Alvarez et al, 2002). It seems that the level of sperm DNA damage depends on the composition of the incubation medium, which might contain agents that encourage or inhibit this damage, and this must be considered in any sperm incubation protocol (Estop et al, 1993). Moreover, Karabinus et al (1991) found that frozen-thawed bull spermatozoa incubated at 38.5°C in milk extender were more susceptible to DNA denaturation than in egg yolk extender. The authors suggested that milk components might either possess actively detrimental agents on chromatin quality or lack a protective effect that is present in egg yolk.

Although the mechanism of DNA damage in cryopreserved sperm after 20 hours is unknown, it is tempting to speculate that thawed cells are more susceptible to oxidative stress. Reactive oxygen species can induce sperm DNA damage (Barroso et al, 2000), and cryopreservation is known to reduce sperm antioxidant levels (Bilodeau et al, 2000). This hypothesis implies that the stability of sperm nuclear chromatin might be achieved via the addition of antioxidants to the semen extender. Alternatively, trying to inseminate at a closer time to ovulation might also improve sperm competence.

A secondary objective of this study was to determine whether SCSA parameters and classical criteria of ram sperm quality are correlated. DNA damage and abnormal chromatin structure can occur in bovine spermatozoa classified as normal (Karabinus et al, 1997). However, we show here significant negative correlations, ranging from poor to moderate, among SCSA variables and most classical semen quality parameters from both fresh and cryopreserved semen (Table 4). For example, correlations between either $SD\alpha_t$ or $\%COMP\alpha_t$ and total or progressive motility were similar and varied from -0.39 ($P < .01$) to -0.59 ($P < .001$), indicating that as the percentage of ram spermatozoa with good motility declined, susceptibility to chromatin denaturation rose. These results are supported by other studies with bull (Ballachey et al, 1988; Januskauskas et al, 2001) and human spermatozoa (Evenson et al, 1991, 1999; Saleh et al, 2002). In addition, Januskauskas et al (2001) reported negative relationships between SCSA variables and sperm viability parameters and speculated that abnormal chromatin structure might impede the survival of bull spermatozoa during freezing and thawing. On the other hand, no significant correlations were detected between SCSA variables and viability traits also with bovine spermatozoa (Karabinus et al, 1991).

The negative correlations observed in this study were expected because higher values for SCSA variables mean a greater level of DNA denaturation and, consequently, lower semen quality, whereas for the other semen quality parameters, higher values indicate better semen quality. The low and moderate values of the correlation coefficients observed in this study between SCSA and the other semen quality parameters suggest that, together, both types of assays (ie, classical semen quality parameters and SCSA variables) are better predictors of semen quality and male fertility potential than each separately.

The positive correlations between $\%COMP\alpha_t$ and CTC pattern AR in both fresh and frozen semen indicates that acrosome-reacted spermatozoa are more susceptible to sperm nuclear DNA denaturation. Negative correlations also were observed between SCSA variables and acrosomal integrity of cryopreserved bovine spermatozoa (Karabinus et al, 1990). They added that the mechanism behind this relationship is not clear. Perhaps the chromatin of acrosome-reacted spermatozoa is less tightly coiled, thereby facilitating DNA decondensation that normally occurs after oocyte penetration. Moreover, a direct relationship has been observed between chromatin condensation and the capacity of sperm to fertilize. Human spermatozoa that have incomplete chromatin condensation fertilize a very low percentage of ova in vitro (Ham-

madeh et al, 1998) or fail to fertilize, even after direct injection of spermatozoa into the ovum (Rosenbusch, 2000).

Of interest in this study was the observation that the variation between the ejaculates within the individual rams was significant ($P < .01$) for all SCSA variables during the period of the experiment, which was 1 breeding season (October–January). In contrast, SCSA parameters in men (Evenson et al, 1991) and bulls (Ballachey et al, 1987) were constant within individuals. These differences suggest that defects in chromatin structure might be a variable trait (Bochenek et al, 2001). Previous studies reported changes in the frequency of abnormal sperm chromatin structure during extended periods because of disrupted spermatogenesis or external factors such as heat stress (Sailer et al, 1997), ionization radiation (Sailer et al, 1995a), toxic chemicals such as triethlenemelamine and methyl methanesulfonate (Evenson et al, 1989, 1993), and semen extenders (Karabinus et al, 1991). In our study, all rams were apparently in good health, without stress, and no chemical treatments were used during this period. Therefore, other reasons, perhaps genetic or otherwise, must be responsible for the variation between ejaculates within ram.

Semen cryopreservation had little or no effect on the susceptibility of ram sperm DNA to denature in situ when measured immediately at thawing or after 3 hours of incubation, but significant DNA damage appeared later in physiological conditions. This finding supports our hypothesis that freezing and thawing disrupts the stability of ram sperm chromatin, suggesting that the reduced fertilization efficiency of cryopreserved semen in vivo might partly be due to abnormal DNA structure. Furthermore, the low to moderate correlations between the SCSA variables and conventional semen quality parameters corroborate the utility of the SCSA in providing additional information on sperm quality and competence in the AI and andrology laboratories.

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References

Alvarez JG, Sharma RK, Ollero M, Saleh RA, Lopez MC, Thomas AJ Jr, Evenson DP, Agarwal A. Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. *Fertil Steril.* 2002;78:319–329.

Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl.* 2000; 21:1–7.

Ballachey BE, Evenson DP, Saacke RG. The sperm chromatin structure assay. Relationship with alternate tests of semen quality and heterospermic performance of bulls. *J Androl.* 1988;9:109–115.

Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biol Reprod.* 1987;36:915–925.

Ballachey BE, Miller HL, Jost LK, Evenson DP. Flow cytometry evaluation of testicular and sperm cells obtained from bulls implanted with zeranol. *J Anim Sci.* 1986;63:995–1004.

Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod.* 2000;15:1338–1344.

Barth AD, Oko RG. *Abnormal Morphology of Bovine Spermatozoa.* Ames, IA: Iowa State University Press; 1989.

Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology.* 2001;56:275–286.

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev.* 2000;55:282–288.

Bochenek M, Smorag Z, Pilch J. Sperm chromatin structure assay of bulls qualified for artificial insemination. *Theriogenology.* 2001;56:557–567.

Canadian Council on Animal Care. Guide to the care and use of experimental animals. In: Olfert ED, Cross BM, McWilliams AA, eds. Vol 1. Ottawa, Canada: CCAC; 1993.

Collin S, Sirard MA, Dufour M, Bailey JL. Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. *J Androl.* 2000;21:938–943.

Darzynkiewicz Z. Probing nuclear chromatin by flow cytometry. In: Melamed MR, ed. *Flow Cytometry and Sorting.* New York, NY: Wiley-Liss; 1990:315–340.

Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. *Exp Cell Res.* 1975;90:411–428.

Davis RO, Siemers RJ. Derivation and reliability of kinematic measures of sperm motion. *Reprod Fertil Dev.* 1995;7:857–869.

Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. *Fertil Steril.* 2001;76:892–900.

Duru NK, Morshedi MS, Schuffner A, Oehninger S. Cryopreservation-thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *J Androl.* 2001;22:646–651.

Estop AM, Munne S, Jost LK, Evenson DP. Studies on sperm chromatin structure alterations and cytogenetic damage of mouse sperm following in vitro incubation. Studies on in vitro-incubated mouse sperm. *J Androl.* 1993;14:282–288.

Evenson DP, Baer RK, Jost LK. Long-term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. *Environ Mol Mutagen.* 1989;14:79–89.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science.* 1980;210:1131–1133.

Evenson DP, Higgins PJ, Grueneberg D, Ballachey BE. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. *Cytometry.* 1985a;6:238–253.

Evenson DP, Jost LK, Baer RK. Effects of methyl methanesulfonate on mouse sperm chromatin structure and testicular cell kinetics. *Environ Mol Mutagen.* 1993;21:144–153.

Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol.* 1991;5:115–125.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay

as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod.* 1999;14:1039–1049.

Evenson DP, Lee J, Darzynkiewicz Z, Melamed MR. Rhodamine 123 alters the mitochondrial ultrastructure of cultured L1210 cells. *J Histochem Cytochem.* 1985b;33:353–359.

Evenson DP, Melamed MR. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. *J Histochem Cytochem.* 1983;31:248–253.

Evenson DP, Thompson L, Jost LK. Flow cytometric eveluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology.* 1994;41:637–651.

Garner DL, Pinkel D, Johnson LA, Pace MM. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. *Biol Reprod.* 1986;34:127–138.

Gillan L, Evans G, Maxwell WM. Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reprod Fertil Dev.* 1997;9:481–487.

Graham EF, Schmehl KL, Nelson DS. Problems with laboratory assays. In: *Proc 8th Tech Conf AI and Reprod.* Milwaukee, Wis: NAAB; 1980:1–8.

Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol Reprod.* 1990;43:55–64.

Hammadeh ME, Stieber M, Haidl G, Schmidt W. Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. *Andrologia.* 1998;30:29–35.

Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl.* 1990;11:73–88.

Januskauska A, Johannisson A, Rodriguez-Martinez H. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology.* 2001;55:947–961.

Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biol Reprod.* 1989;41:199–203.

Johnson LA, Flook JP, Look MV, Pinkel D. 1987. Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. *Gamete Res.* 1987;16:1–9.

Karabinus DS, Evenson DP, Jost LK, Baer RK, Kaproth MT. Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. *J Dairy Sci.* 1990;73:2364–2371.

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.

Karabinus DS, Vogler CJ, Saacke RG, Evenson DP. Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. *J Androl.* 1997;18:549–555.

Love CC, Thompson JA, Lowry VK, Varner DD. Effect of storage time and temperature on stallion sperm DNA and fertility. *Theriogenology.* 2002;57:1135–1142.

Maxwell WMC. Current problems and future potential of artificial insemination programmes. In: Lindsay DR, Pearce DT, eds. *Reproduction in Sheep.* Canberra: Australian Academy of Science and Australian Wool; 1984:291–298.

Morrier A, Castonguay F, Bailey JL. Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. *Can J Anim Sci.* 2002; 82:347–356.

Nath J. Correlative biochemical and ultrastructural studies on the mechanism of freezing damage to ram semen. *Cryobiology.* 1972;9:240–246.

Poccia D. Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. *Int Rev Cytol.* 1986;105:1–65.

Rosenbusch BE. Frequency and patterns of premature sperm chromosome condensation in oocytes failing to fertilize after intracytoplasmic sperm injection. *J Assist Reprod Genet.* 2000;17:253–259.

Rowe PJ, Comhaire FH, Hargreave TB, Mellows HJ. *WHO Manual for the Standard Investigation and the Diagnosis of the Infertile Couple.* Cambridge, United Kingdom: Cambridge University Press; 1993.

Sailer BL, Jost LK, Erickson KR, Tajiran MA, Evenson DP. Effects of X-irradiation on mouse testicular cells and sperm chromatin structure. *Environ Mol Mutagen.* 1995a;25:23–30.

Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl.* 1995b;16:80–87.

Sailer BL, Sarkar LJ, Bjordahl JA, Jost LK, Evenson DP. Effects of heat stress on mouse testicular cells and sperm chromatin structure. *J Androl.* 1997;18:294–301.

Salamon S, Maxwell WM. Frozen storage of ram semen. 1. Processing, freezing, thawing and fertility after cervical insemination. *Anim Reprod Sci.* 1995;37:185–249.

Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, Alvarez JG, Thomas AJ Jr, Sharma RK. Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil Steril.* 2002;78:313–318.

Shapiro HM. *Practical Flow Cytometry.* 2nd ed. New York, NY: Alan R. Liss Inc; 1988.

Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A, Gandini L. Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. *Mol Hum Reprod.* 1999;5:29–37.

Van der Schans GP, Haring R, van Dijk-Knijnenburg HC, Bruijnzeel PL, den Daas NH. An immunochemical assay to detect DNA damage in bovine sperm. *J Androl.* 2000;21:250–257.

Walker SK, Smith DH, Frensham A, Ashman RJ, Seamark RF. The use of synthetic gonadotropin releasing hormone treatment in the collection of sheep embryos. *Theriogenology.* 1989;31:741–752.

Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod.* 1991;44: 569–574.

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.