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Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls

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Abstract

The aim of this study was to identify different motile sperm subpopulations in ejaculates from an autochthonous bull breed (Bos taurus) and to determine possible modifications in these subpopulations resulting from cryopreservation. Ejaculates were collected and cryopreserved following a conventional protocol. The overall sperm motility and the kinematic parameters of individual spermatozoa were evaluated in fresh ejaculates, after 4 h at 5 °C, and at 0 and 2 h postthaw. A multivariate clustering procedure separated 23,585 motile spermatozoa into four subpopulations: Subpopulation 1 showed medium velocity (VCL: 99.4 ± 17.8 μm/sec) and high progressiveness (LIN: 65.1 ± 14.0%); Subpopulation 2 included spermatozoa with high velocity (VCL: 148.7 ± 25.6 μm/sec) but a nonprogressive trajectory (LIN: 33.1 ± 10.5%); Subpopulation 3 represented slowly motile (VCL: 58.3 ± 24.3 μm/sec) and nonprogressive sperm (LIN: 39.6 ± 18.3%); and Subpopulation 4 included very rapid (VCL: 152.8 ± 25.7 μm/sec) and highly progressive sperm (LIN: 70.9 ± 13.7%). Subpopulation 4 was present in the greatest quantity in fresh ejaculates (36%), but after cooling, it significantly decreased (21%) concomitantly with an increase ($P < 0.001$) in Subpopulation 2 (from 21% in fresh to 34% in postcooled semen). After freezing and thawing, the overall sperm motility was reduced, mainly due to Subpopulation 2 decreasing from 34% after cooling to 14% after thawing. Differences among bulls in the frequency distribution of spermatozoa within subpopulations were evidenced after thawing by different proportions of spermatozoa in Subpopulations 2 and 4. The current results indicate that a structure of four sperm subpopulations may be a common characteristic of bovine ejaculates and that the cooling phase of cryopreservation seems to be the determinant of postthaw semen quality.
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1. Introduction

Semen cryopreservation has been successfully used in dairy cattle for decades, but the fertility outcomes are not as robust as those seen with fresh semen [1]. Reduced fertility of frozen semen is largely attributed to altered membrane structure and function during cryopreservation that may cause damage to spermatozoa [2,3]. During conventional cryopreservation protocols, bull spermatozoa are submitted to a number of potentially damaging stresses: the rapid change in temperature during cooling that could cause cold shock [4], the addition of molar concentrations of

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cryoprotectants associated with osmotic and toxic stress [5], and the formation and dissolution of ice crystals in the extracellular environment associated with solution effects [6]. Furthermore, numerous observations suggest that spermatozoa surviving freezing and thawing have altered membrane properties that may render them functionally similar to capacitated or acrosome-reacted cells [7].

The existence of well-defined motile sperm subpopulations within mammalian ejaculates is now widely accepted by the scientific community [8–14]. In several species, semen cryopreservation has been shown to modify the different subpopulations of motile sperm originally present in the fresh ejaculates, and such modifications can affect either the motility parameters defining each subpopulation or the frequency distribution of spermatozoa within them [14–17].

The existence of four motile sperm subpopulations within a single ejaculate has recently been described in fresh and frozen-thawed semen from Holstein bulls [18]. The physiologic significance of such subpopulational structure is not known. However, the proportion of spermatozoa surviving after thawing and their longevity during postthaw incubation were found to be significantly correlated with the incidence of a specific sperm subpopulation in the fresh ejaculates. Whether this well-defined subpopulational structure is a breed-specific characteristic or a general finding in bovine ejaculates is not known, as it has not been investigated in other bull breeds.

It is likely that each of the different steps of a cryopreservation protocol have a specific influence on a determinate sperm subpopulational structure and that, in some instances, the impaired fertility of frozen-thawed semen may be due not only to low overall sperm motility but also, and probably more importantly, to insufficient numbers of spermatozoa in a specific subpopulation. Thus, a detailed study of the motile sperm subpopulational structure along the different steps of cryopreservation might help to improve the protocols currently used for bovine semen.

Therefore, the purpose of this study was to (a) determine the existence of distinguishable motile sperm subpopulations in fresh ejaculates from beef bulls belonging to an autochthonous breed from the northwest of Spain and named Asturiana de los Valles; (b) determine potential changes in the motile sperm subpopulation structure identified in fresh ejaculates during the main steps of a conventional semen cryopreservation protocol (after cooling at 5 °C, immediately after thawing, and after 2 h of postthaw incubation at 37 °C); (c) evaluate the individual variability in the frequency distribution of spermatozoa within subpopulations either in fresh semen or at the different steps of the cryopreservation protocol.

2. Materials and methods

2.1. Animals

A total of 60 ejaculates were collected from 10 Asturiana de los Valles bulls Bos taurus with ages between 14 and 24 mo. The bulls were in a regular artificial insemination (AI) service housed at the Centro de Inseminación Artificial de Somió (SERIDA), Asturias, and they were subjected to six semen collections on the same experimental day, twice a week for three successive weeks. The overall 90-day nonreturn rates of the bulls, as obtained from previous records including only first inseminations of nulliparous heifers, were in the range of 60% to 65%. It should be mentioned that for some of the younger bulls, the number of inseminated heifers was still low (25 to 35).

2.2. Experimental design

Upon collection, semen was initially evaluated for the following variables: volume, sperm concentration, sperm morphology, and sperm motility by subjective assessment. Afterwards, a semen aliquot was removed for computer-assisted sperm analysis (CASA) of motility (fresh sample), and the rest of the ejaculate was diluted and cooled at 5 °C for 4 h. After the cooling, a second aliquot was removed for computer-aided motility evaluation (postcooled sample), and the rest of the diluted semen was packaged and frozen, as described below. After 1 mo of storage, the frozen semen was thawed, and the CASA-derived motility characteristics were analyzed after 0 and 2 h of postthaw incubation at 37 °C. The assessment of the overall sperm motility, as well as of the kinematic parameters of individual motile spermatozoa, were carried out using a CASA system (Sperm Class Analyzer 5.0; Microptic, Barcelona, Spain) to investigate the existence of separate motile sperm subpopulations in fresh ejaculates and to evaluate the influence of the cooling, freezing, and postthaw incubation on the frequency distribution of spermatozoa within the different subpopulations.

2.3. Semen collection, cooling, and freezing

Ejaculates were collected using an artificial vagina (initial temperature of the water: 45 °C) and evaluated

for volume, sperm concentration using a photometer (Accucell; IMV, L'Aigle, France), sperm morphology, and the proportion of total motile spermatozoa by subjective visual assessment. Sperm motility was evaluated after dilution of a semen aliquot with Bioxcell (IMV) to reach a sperm concentration of approximately 40×10^6 spermatozoa (spz)/mL. The same aliquot of diluted semen was then used to determine the sperm motility and the kinematic parameters of individual spermatozoa by means of a CASA system.

To assess sperm morphology, a semen aliquot was diluted and fixed in prewarmed (37 °C) 2% glutaraldehyde in PBS. Sperm cells (n = 200) were counted in wet mounts under oil immersion using a phase-contrast microscope at ×1000 magnification.

Mean (±SD) values for volume of ejaculates, sperm concentration, total sperm motility (determined subjectively), and percentage of abnormal spermatozoa were 5.0 ± 2.0 mL, $1327.1 \pm 307.7 \times 10^6$ spz/mL, $94.7 \pm 13.4\%$, and $9.7 \pm 4.3\%$, respectively.

The rest of the ejaculate was diluted with Bioxcell at room temperature (22 °C) at a final sperm concentration of about 92×10^6 spz/mL. The extended semen was cooled from 22 °C to 5 °C over a period of 1.5 h (at a cooling rate of approximately 0.2 °C/min) and then left at 5 °C for an additional 2.5 h. At the end of the cooling period (4 h), an aliquot of equilibrated semen was taken to determine the sperm motility and the kinematic parameters of individual spermatozoa by means of a CASA system. Then, it was packaged in 0.25-mL straws (23×10^6 spz/straw) and frozen in liquid nitrogen vapors in a programmable freezer following the IMV Digit-cool standard curve for bovine semen (5 °C/min from +4 °C to −10 °C; 40 °C/min from −10 °C to −100 °C; and 20 °C/min from −100 °C to −140 °C).

Thawing was done in a water bath at 37 °C for 40 sec. Three straws per ejaculate were simultaneously thawed, their contents were pooled in a 5-mL Falcon tube, and the thawed semen was incubated at 37 °C in the dark for 2 h.

2.4. Sperm motility evaluation by using a CASA system

The CASA system used was based on the analysis of 16 consecutive, digitized photographic images, which were taken in a time lapse of 0.64 sec, which implied a velocity of image-capturing of one photograph every 40 msec. Images were taken from 5-μL semen aliquots, which were placed on slides and covered with 20 × 20 mm coverslips. Three microscopic fields were analyzed in each sample using a phase-contrast microscope supplied with a prewarmed stage at 37 °C and at ×200 magnification. The number of spermatozoa analyzed per sample ranged between 100 and 200, including the immotile sperm. Objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. Total motility was defined as the percentage of spermatozoa with mean velocity (VAP) above 10 μm/sec.

The kinematic parameters recorded for each spermatozoon, as described by Mortimer [19,20], were curvilinear velocity (VCL, μm/sec: the average path velocity of the sperm head along its actual trajectory); straight-line velocity (VSL, μm/sec: the average path velocity of the sperm head along a straight line from its first to its last position); average path velocity (VAP, μm/sec: the average velocity of the sperm head along its average trajectory); percentage of linearity (LIN, %: the ratio between VSL and VCL); percentage of straightness (STR, %: the ratio between VSL and VAP); wobble coefficient (WOB, %: the ratio between VAP and VCL); mean amplitude of lateral head displacement (ALH, μm: the average value of the extreme side-to-side movement of the sperm head in each beat cycle); and beat cross-frequency (BCF, Hz: the frequency with which the actual sperm trajectory crosses the average path trajectory).

2.5. Statistical analysis

Data from all the motile spermatozoa obtained in evaluations of 60 fresh (10 bulls × 6 ejaculates) and 120 thawed (10 bulls × 6 ejaculates × 2 evaluation times) semen samples were imported into a single data set that represented 23,585 spermatozoa, each one defined by the 8 motility descriptors specified above. A multivariate k-means cluster analysis was carried out to classify the 23,585 spermatozoa into a reduced number of subpopulations according to their patterns of movement so that every spermatozoon belonged to one and only one cluster. Spermatozoa that were very close to each other were assigned to the same cluster, whereas spermatozoa that were far apart were put into different clusters. The k-means clustering model used Euclidean distances computed from the eight quantitative variables after normalization of the data so that the cluster centers were the means of the observations assigned to each cluster. The specified number of clusters was based on the previous analysis of hierarchical dendrograms [8] constructed on individual ejaculates using the Ward method [21]. For each

ejaculate from each bull, contingency tables were used to determine the percentages of spermatozoa assigned to the different clusters at the different time points. The effects of cooling, freezing-thawing, and postthaw incubation on the relative distribution frequency of spermatozoa within subpopulations were analyzed using a general linear model (GLM) procedure. The GLM procedure was also used to evaluate the influence of the independent variables on the mean kinematic parameters defining the different sperm subpopulations (i.e., the cluster centers). Differences between means were analyzed by Tukey's test. All the statistical analyses were performed using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA), and differences were considered significant at $P < 0.05$ level.

3. Results

3.1. Overall sperm motility in fresh, cooled, and frozen-thawed bull spermatozoa

The mean total sperm motility in the fresh semen samples collected from the 10 bulls was $95.0 \pm 4.4\%$, and after semen cooling, it was $91.6 \pm 6.6\%$. After the semen was frozen and thawed, total sperm motility decreased to $78.8 \pm 16.7\%$, and after 2 h of postthaw incubation at 37 °C, it was further decreased to $76.9 \pm 16.7\%$. Postthaw sperm motility at 0 and 2 h of incubation was lower ($P < 0.05$) than that recorded for fresh and postcooled semen, but there was no significant difference between fresh and postcooled samples nor between thawed samples evaluated at 0 and 2 h postthaw.

There were significant differences between bulls in total sperm motility (Fig. 1a–d), but when data were pooled across bulls, there were no differences in total motility of fresh, cooled, or thawed semen between the different experimental days.

3.2. Motile sperm subpopulations

Four sperm subpopulations were defined after multivariate cluster analysis of the 23,585 individual motile spermatozoa. The motility characteristics of those subpopulations are shown in Table 1, and their patterns of movement can be described as follows.

Subpopulation 1 represented spermatozoa with relatively low velocity (medium VCL, VSL, and VAP) but with high progressiveness (high LIN, STR, WOB, BCF and low ALH). This population included about 28% of the total motile spermatozoa.

Subpopulation 2 contained highly active but non-progressive spermatozoa, as indicated by the high

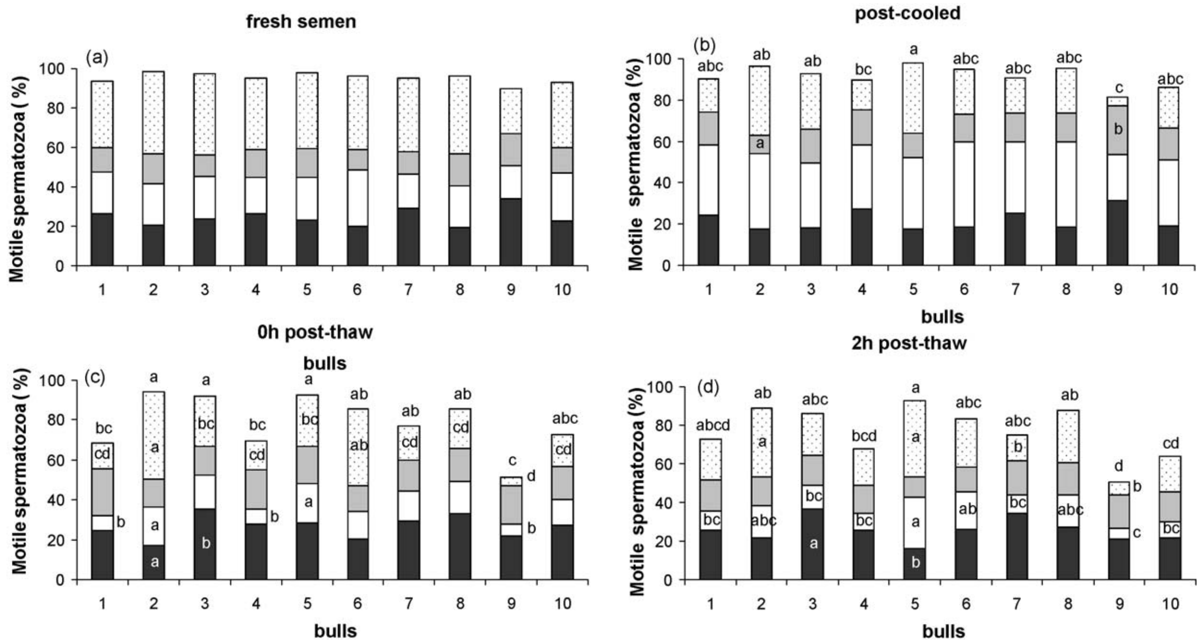

Fig. 1. Relative frequency distribution of motile spermatozoa within subpopulations (Subpopulation 1, black columns; Subpopulation 2, white columns; Subpopulation 3, gray columns; Subpopulation 4, dotted columns) between bulls in (a) fresh semen, (b) postcooled semen, and frozen-thawed semen after (c) 0 h and (d) 2 h of postthaw incubation at 37 °C. [a–d]Different letters inside or beside columns indicate significant differences within subpopulations between bulls. [a–d]Different letters over the columns indicate significant differences in total sperm motility between bulls.

Table 1
Mean values (±SD) of the kinematic parameters defining the four subpopulations identified in fresh, postcooled, and frozen-thawed bull semen samples.

	Sperm subpopulations			
	1	2	3	4
Number of spermatozoa (%)	6700 (28.4)	5811 (24.6)	4133 (17.5)	6941 (29.4)
Kinematic parameters				
VCL, μm/sec	99.4 ± 17.8^a	148.7 ± 25.6^b	58.3 ± 24.3^c	152.8 ± 25.7^d
VSL, μm/sec	63.7 ± 14.3^a	49.0 ± 16.6^b	21.8 ± 10.6^c	106.6 ± 20.7^d
VAP, μm/sec	74.6 ± 14.1^a	90.3 ± 19.3^b	34.3 ± 14.7^c	120.7 ± 19.3^d
LIN, %	65.1 ± 14.0^a	33.1 ± 10.5^b	39.6 ± 18.3^c	70.9 ± 13.7^d
STR, %	85.4 ± 10.5^a	55.2 ± 18.0^b	64.7 ± 20.2^c	88.4 ± 9.4^d
WOB, %	75.9 ± 11.7^a	61.3 ± 11.7^b	59.6 ± 14.2^c	79.9 ± 11.1^d
ALH, μm	3.4 ± 1.1^a	5.9 ± 1.4^b	2.6 ± 1.1^c	4.6 ± 1.6^d
BCF, Hz	8.7 ± 3.8^a	7.5 ± 3.3^b	5.7 ± 3.1^c	9.1 ± 3.5^d

[a–d]Different letters indicate significant differences between subpopulations.

values of VCL and ALH together with low values of LIN and STR and with moderate BCF. This population might be considered as having an ‘‘hyperactivated-like’’ movement, and about 24% of the total motile sperm were assigned to this subpopulation.

Subpopulation 3 included about 17% of the total spermatozoa, which were poorly motile and nonprogressive, as indicated by the lowest values of VCL, VSL, VAP, ALH, and BCF together with a low LIN, STR, and WOB.

Subpopulation 4, containing about 29% of the total population, consisted of those spermatozoa that moved most rapidly and progressively, as indicated by the highest values of VCL, VSL, VAP, and BCF together with the highest LIN, STR, and WOB and moderate ALH.

3.3. Frequency distribution of spermatozoa within subpopulations before and after semen cryopreservation

The proportions of spermatozoa assigned to the four subpopulations significantly changed during the cryopreservation process (Fig. 2). In fresh semen samples, 36% of the total motile spermatozoa moved very rapidly and progressively (Subpopulation 4), but after 4 h of cooling at 5 °C, this percentage decreased to 21%, with no further variation after thawing. In contrast, the proportion of spermatozoa assigned to Subpopulation 2 significantly increased from 21% in fresh samples to 34% after cooling, and after freezing and thawing, this subpopulation decreased to 14%.

The poorly motile nonprogressive sperm (Subpopulation 3) that represented 13% of all the fresh motile sperm slightly increased to 15% after cooling and to 17% after thawing. Finally, the proportion of spermatozoa assigned to Subpopulation 1 (moderately slow but progressive sperm) did not significantly change during the process of cryopreservation (25%, 22%, 26%, and 25%, respectively, for fresh, postcooled, and 0 and 2 h postthaw).

3.4. Effect of individual variability on the frequency distribution of spermatozoa within subpopulations

In fresh semen samples, the proportions of spermatozoa assigned to the four subpopulations were not different for the 10 bulls (Fig. 1a).

After cooling (Fig. 1b), Subpopulation 2 increased in the 10 bulls in relation to that observed in fresh semen, and no between-bull variations were found. However, the proportion of spermatozoa assigned to Subpopulation 4, which decreased in the 10 bulls in relation to fresh semen,

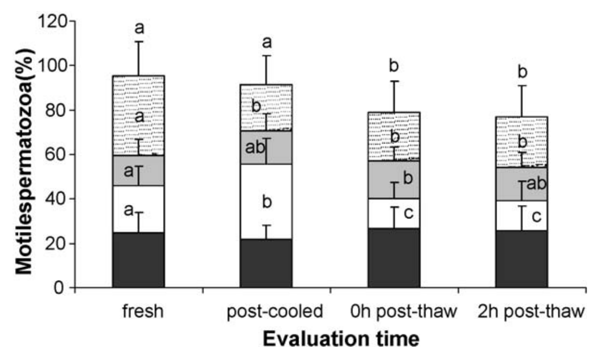

Fig. 2. Relative frequency distribution of motile spermatozoa within subpopulations (Subpopulation 1, black columns; Subpopulation 2, white columns; Subpopulation 3, gray columns; Subpopulation 4, dotted columns) in fresh semen, postcooled semen, and frozen-thawed bull semen samples after 0 and 2 h of postthaw incubation at 37 °C. [a–c]Different letters inside columns indicate significant differences within subpopulations between evaluation time points. [a,b]Different letters over the columns indicate significant differences in total sperm motility between different evaluation time points.

Table 2
Motility characteristics (means ± SD) for Subpopulation 1 (moderately slow and progressive sperm) as identified in bull semen samples during the cryopreservation process.

	Fresh semen	Postcooled	0 h postthaw	2 h postthaw
Number of spermatozoa (%)	1879 (25.8)	1488 (23.7)	1649 (33.4)	1684 (33.1)
Sperm motility descriptors				
VCL, μm/sec	97.8 ± 18.0[c]	103.2 ± 17.6[a]	97.6 ± 18.0[c]	99.8 ± 17.0[b]
VSL, μm/sec	63.9 ± 14.7[b]	60.9 ± 13.4[c]	64.1 ± 14.0[b]	65.7 ± 14.6[a]
VAP, μm/sec	75.6 ± 14.8[a]	72.6 ± 13.7[b]	75.3 ± 14.1[a]	74.7 ± 13.6[a]
LIN, %	66.3 ± 14.5[a]	59.8 ± 12.6[b]	66.8 ± 13.7[a]	66.6 ± 13.9[a]
STR, %	84.6 ± 11.11[bc]	84.0 ± 9.8[c]	85.4 ± 10.5[b]	87.8 ± 10.0[a]
WOB, %	78.1 ± 11.6[a]	71.1 ± 11.6[c]	78.0 ± 11.2[a]	75.5 ± 11.0[b]
ALH, μm	3.2 ± 1.1[c]	3.8 ± 1.0[a]	3.2 ± 1.0[c]	3.3 ± 1.0[b]
BCF, Hz	6.8 ± 3.4[d]	8.4 ± 3.5[c]	9.3 ± 3.4[b]	10.3 ± 3.7[a]

[a–d]Different letters indicate significant differences between columns within a row.

Table 3
Motility characteristics (means ± SD) for Subpopulation 2 (hyperactivated-like spermatozoa) as identified in bull semen samples during the cryopreservation process.

	Fresh semen	Postcooled	0 h postthaw	2 h postthaw
Number of spermatozoa (%)	1698 (29.2)	2331 (40.1)	867 (15.0)	915 (15.7)
Sperm motility descriptors				
VCL, μm/sec	146.2 ± 24.3[b]	153.4 ± 26.8[a]	144.2 ± 24.3[b]	145.7 ± 23.9[b]
VSL, μm/sec	47.5 ± 16.4[c]	49.9 ± 16.5[a]	49.2 ± 17.4[ab]	49.1 ± 16.6[ab]
VAP, μm/sec	95.9 ± 20.6[a]	86.3 ± 18.2[c]	90.4 ± 17.7[b]	89.8 ± 18.3[b]
LIN, %	32.7 ± 10.7[b]	32.7 ± 10.2[b]	34.1 ± 11.0[a]	33.8 ± 10.5[ab]
STR, %	50.6 ± 17.4[c]	58.5 ± 17.9[a]	54.9 ± 18.3[b]	55.2 ± 17.4[b]
WOB, %	66.0 ± 11.9[a]	56.8 ± 10.7[d]	63.4 ± 11.6[b]	62.0 ± 10.1[c]
ALH, μm	5.6 ± 1.5[b]	6.3 ± 1.4[a]	5.6 ± 1.4[b]	5.7 ± 1.3[b]
BCF, Hz	6.3 ± 2.8[c]	7.6 ± 3.3[b]	8.5 ± 3.5[a]	8.4 ± 3.5[a]

[a–d]Different letters indicate significant differences between columns within a row.

showed significant differences between bulls. Subpopulations 1 and 3 did not differ significantly among bulls.

After thawing (Fig. 1c, d), differences among bulls in the sperm subpopulation distribution were mainly due to Subpopulations 2 and 4 ($P < 0.001$), whereas Subpopulations 1 and 3 did not differ significantly among bulls.

3.5. Influence of the cryopreservation process on the kinematic parameters defining the four sperm subpopulations

For each one of the four sperm subpopulations (Tables 2 to 5), the mean kinematic parameters significantly varied along the main steps of the

Table 4
Motility characteristics (means ± SD) for Subpopulation 3 (poorly motile nonprogressive sperm) as identified in bull semen samples during the cryopreservation process.

	Fresh semen	Postcooled	0 h postthaw	2 h postthaw
Number of spermatozoa (%)	997 (24.1)	1039 (25.1)	1073 (25.9)	1024 (24.7)
Sperm motility descriptors				
VCL, μm/sec	61.8 ± 22.7[a]	64.4 ± 23.7[b]	52.8 ± 24.2[b]	54.5 ± 24.7[b]
VSL, μm/sec	24.1 ± 10.0[a]	23.2 ± 9.8[a]	20.0 ± 10.9[b]	20.2 ± 11.0[b]
VAP, μm/sec	38.2 ± 13.9[a]	36.6 ± 13.5[a]	31.3 ± 14.9[b]	31.3 ± 15.1[b]
LIN, %	41.9 ± 18.5[a]	38.4 ± 17.4[b]	39.9 ± 19.1[ab]	38.2 ± 18.0[b]
STR, %	64.8 ± 20.6	64.7 ± 19.6	64.8 ± 20.5	64.6 ± 20.2
WOB, %	63.1 ± 13.3[a]	58.0 ± 13.9[c]	59.8 ± 14.7[b]	57.5 ± 14.2[c]
ALH, μm	2.7 ± 1.1[b]	2.9 ± 1.1[a]	2.3 ± 1.1[c]	2.4 ± 1.2[c]
BCF, Hz	4.9 ± 3.0[b]	5.1 ± 2.9[b]	6.5 ± 3.1[a]	6.3 ± 3.0[a]

[a–c]Different letters indicate significant differences between columns within a row.

Table 5
Motility characteristics (means ± SD) for Subpopulation 4 (rapid and progressive sperm) as identified in bull semen samples during the cryopreservation process.

	Fresh semen	Postcooled	0 h postthaw	2 h postthaw
Number of spermatozoa (%)	2711 (39.0)	1423 (20.5)	1350 (19.4)	1457 (20.9)
Sperm motility descriptors				
VCL, μm/sec	150.8 ± 25.3^{c}	159.6 ± 27.0^{a}	148.9 ± 23.3^{c}	153.5 ± 26.0^{b}
VSL, μm/sec	110.8 ± 23.9^{a}	100.6 ± 16.3^{d}	104.1 ± 18.0^{c}	107.2 ± 18.7^{b}
VAP, μm/sec	126.3 ± 21.3^{a}	115.4 ± 16.0^{c}	117.5 ± 17.0^{b}	118.4 ± 18.0^{b}
LIN, %	74.4 ± 14.6^{a}	64.3 ± 12.4^{c}	70.8 ± 12.2^{b}	70.8 ± 12.3^{b}
STR, %	87.7 ± 10.5^{c}	87.3 ± 8.6^{c}	88.6 ± 8.5^{b}	90.6 ± 8.5^{a}
WOB, %	84.4 ± 10.3^{a}	73.5 ± 11.2^{d}	79.7 ± 10.3^{b}	77.9 ± 9.6^{c}
ALH, μm	4.1 ± 1.7^{c}	5.5 ± 1.6^{a}	4.5 ± 1.4^{b}	4.7 ± 1.4^{b}
BCF, Hz	7.3 ± 2.7^{d}	9.2 ± 3.3^{c}	10.0 ± 3.2^{b}	11.3 ± 3.4^{a}

$^{a–d}$Different letters indicate significant differences between columns within a row.

cryopreservation protocol. In general, in the four subpopulations, the cooling phase of cryopreservation induced a more oscillatory pattern of sperm movement, as indicated by significant increases of VCL and ALH, whereas VAP and LIN significantly decreased. However, after thawing, these parameters were corrected in the four subpopulations, showing similar or lower values than in fresh semen.

4. Discussion

The results of the current study indicate that four distinct sperm subpopulations were present in fresh ejaculates from Asturiana de los Valles bulls. The kinematic properties of these four subpopulations, as well as the frequency distribution of spermatozoa within them, were nearly the same as those previously found in ejaculates from Holstein bulls [18]. Moreover, for the two bull breeds, the overall sperm subpopulation structure showed little variation among individuals. This suggests that such a specific subpopulational structure, depicted by spermatozoa with different physiologic and metabolic characteristics, may be a constant finding in bovine ejaculates. The presence of three or four well-defined motile sperm subpopulations has been demonstrated in numerous species [8,9,11,14], and, as indicated by our current and previous [18] results, the bovine species does not seem to be an exception.

The cryopreservation process induced substantial changes in the distribution of sperm subpopulations, and these changes were already evident after the cooling phase of the protocol. A significant increase in the proportion of spermatozoa assigned to the subpopulation of hyperactivated-like spermatozoa (Subpopulation 2), together with a significant decrease in the proportion of most rapid and progressive sperm (Subpopulation 4), was seen after semen cooling, and this change was consistent for all the bulls and ejaculates. Moreover, the four sperm subpopulations showed higher VCL and ALH and lower VAP and LIN after semen cooling.

Because a rapid cooling of semen between 30 °C and 0 °C is known to induce cold shock injury in bull spermatozoa [4], this step of the cryopreservation protocol was done carefully at a low cooling rate. Yet, it seems that even with slow cooling, this change in temperature induced a certain degree of cellular damage, probably related to thermotropic phase transitions of lipids and altered permeability of membranes [3]. The increase in the subpopulation of hyperactivated-like spermatozoa observed in this study might be a symptom of deficient intracellular calcium regulation, an ion that is known to play a major role in regulating hyperactivated motility [22]. Likewise, a clear increase in the subpopulation of hyperactivated-like spermatozoa was also found after the slow cooling of boar spermatozoa [16], a species that is known to be extremely sensitive to cold shock [4]. In this context, it would be interesting to see if bull spermatozoa also would show an increase of Subpopulation 2 after slow cooling if they were cryopreserved in an egg yolk–based extender instead of in the soy bean–derived extender (Bioxcell) used in this study. A recent work showed that choline phospholipids from egg yolk and soy beans are equally effective in preventing membrane damage during cryopreservation of stallion spermatozoa [23]. However, for bull sperm cryopreservation, the use of extenders containing phospholipids of vegetal origin may be less effective than use of egg yolk–based extenders [24–27].

After freezing and thawing, the reduction in overall sperm motility was mainly due to a decrease in Subpopulation 2, whereas the other three subpopulations did not significantly change. This indicated that

many of the spermatozoa showing hyperactivated-like movement after cooling suffered irreversible damage [28] and were unable to survive to freezing and thawing. To improve the quality of frozen-thawed bull semen, perhaps a first step should be the reevaluation of extenders, cooling methods, or cooling rates so that the sperm subpopulational structure of fresh ejaculates after the cooling period remains as unaltered as possible.

Forward progressive motility, among other sperm attributes, is believed to be required for mammalian spermatozoa to be able to traverse the uterotubal junction and enter the oviduct, whereas nonprogressive patterns of movement seem to make it difficult for sperm to gain access to the fertilization site [29–31]. Therefore, the sperm reservoir within the oviductal isthmus would be expected to be colonized preferably by spermatozoa selected from subpopulations showing forward progressive motility in fresh ejaculates. In this sense, a recent study [32] has shown that boar spermatozoa from separate motile subpopulations responded differently to in vitro capacitation. Whereas subpopulations containing progressive sperm reacted with significant increases in velocity and linearity, those containing nonprogressive spermatozoa did not practically modify their motility parameters. Moreover, the subpopulation with the most rapid and progressive sperm seemed to be the most prone to undergo the acrosome reaction after progesterone treatment [32].

Concerning the current results, such findings might indicate that the fertilizing capacity of bull semen might preferentially reside in the subpopulation of most active and progressive sperm. In fact, this subpopulation was the most variable among bulls, especially in cooled and frozen-thawed semen, a pattern that is in agreement with previous observations in Holstein bulls [18]; perhaps this pattern could also determine differences among bulls in their in vivo fertility.

On the other hand, it is interesting to note that a subpopulation of less rapid but progressive spermatozoa (Subpopulation 1) remained constantly present in fresh, cooled, and frozen-thawed semen from all the bulls. The spermatozoa included in this subpopulation probably were being continuously added from Subpopulation 4, and as they became senescent, they switched to Subpopulations 2 and 3. Whether this sperm subpopulation merely represents an early stage of cell aging or is a ''healthy'' population with a lower metabolic rate that could be activated in the female genital tract and be part of the fertilizing population is not known. It is noteworthy that in thawed semen, the proportion of spermatozoa included in Subpopulation 4 significantly varied among bulls. However, the previously recorded nonreturn rates were very similar for all the bulls, but this, of course, may be due to the use of insemination doses sufficiently high to maintain above-threshold sperm numbers [33].

In conclusion, the current results confirm that four sperm subpopulations with specific patterns of movement are present in ejaculates from Asturiana de los Valles bulls. The motility characteristics of the four sperm subpopulations, as well as the frequency distribution of spermatozoa within subpopulations, are virtually the same as those previously observed in Holstein bulls. The cryopreservation process significantly modified the subpopulational structure originally present in the fresh ejaculates; the most remarkable change, observed after the cooling phase of the protocol, was an increase in the subpopulation of hyperactivated-like spermatozoa, concomitant with a decline in the subpopulation of most rapid and progressive sperm. Such modification during cooling is believed to be responsible for a reduction in the sperm subpopulation with the highest velocity and progressiveness after thawing. In agreement with previous observations in Holstein bulls, individual variability was mainly due to differences among bulls in the proportion of the most rapid and progressive sperm, especially in frozen-thawed semen. Further studies should be aimed to determine whether the quality of cryopreserved bovine semen can be improved by minimizing the impact of the cooling step on the original subpopulational structure of the fresh ejaculates and whether this could be reflected in the fertility of cryopreserved semen.

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