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Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation

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Abstract

The aims of the present study were: (1) to determine the existence of sperm subpopulations with specific motility characteristics in fresh ejaculates from Holstein bulls, (2) to investigate the effects of semen cryopreservation and post-thaw incubation on the distribution of spermatozoa within the different subpopulations, and (3) to evaluate the existence of between-bull variation in the sperm subpopulations structure of fresh and frozen-thawed semen. Six ejaculates were collected from each of 9 Holstein bulls and cryopreserved following a standard protocol. Overall sperm motility and the individual kinematic parameters of motile spermatozoa, determined using a CASA system, were evaluated before freezing and after 0, 2 and 4 h of post-thaw incubation at 37 °C. Data from 16,740 motile spermatozoa, defined by VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF, were analysed using a multivariate clustering procedure to identify and quantify specific subpopulations within the semen samples. The statistical analysis clustered all the motile spermatozoa into four separate subpopulations with defined patters of movement: Subpopulation (Subp. 1) moderately slow but progressive spermatozoa (23.2%), (Subp. 2) highly active but non-progressive spermatozoa (16.0%), (Subp. 3) poorly motile non-progressive sperm (35.5%), and (Subp. 4) highly active and progressive sperm (25.3%). Subpopulations 2 and 4 significantly ($P < 0.01$) decreased during cryopreservation and post-thaw incubation (Subp. 2: 21.1%, 18.1%, 8.7% and 5.9%; and Subp. 4: 34.1%, 20.6%, 15.2% and 7.3%, respectively, for fresh, 0, 2 and 4 h post-thaw) whereas Subp. 3 significantly ($P < 0.01$) increased (10.7%, 27.2%, 27.2% and 30.7%, respectively, for fresh, 0, 2 and 4 h post-thaw). The frequency distribution of spermatozoa within subpopulations was quite similar for the 9 bulls, either in fresh or frozen–thawed semen, and differences among bulls were mainly due to differences in the Subp. 4. Significant correlations ($P < 0.01$)

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were found between the proportions of spermatozoa assigned to Subp. 4 in the fresh ejaculates and those in frozen-thawed semen after 0 ($r = 0.473$), 2 ($r = 0.513$) and 4 h post-thaw ($r = 0.450$). This indicated that the ejaculates with the highest subpopulations of rapid and progressive sperm were also the most resistant to cryopreservation and showed the best post-thaw sperm longevity.

Keywords: Bull semen; Cryopreservation; Kinematic parameters; Sperm subpopulations

1. Introduction

Substantial amount of data supports the hypothesis that any mammalian ejaculate constitutes a heterogeneous population of spermatozoa within which functionally different subpopulations coexist (Curry and Watson, 1994; Harrison, 1996; Holt, 1996; Abaigar et al., 1999). Such heterogeneity makes it possible for the female reproductive tract to exert multiple selective processes which will finally reduce a population of several millions to a few competent spermatozoa (Holt and Van Look, 2004). This concept of sperm heterogeneity has been taken into consideration by many researchers, and various computerized and laboratory methods for sperm quality assessment have been developed, in an attempt to distinguish the subpopulation of potentially competent spermatozoa among the whole sperm population (Evenson et al., 1980; Sailer et al., 1996; Thomas et al., 1997; Abaigar et al., 1999; Thurston et al., 2001; Peña et al., 2005; Rubio-Guillén et al., 2007).

Different sperm subpopulations have been identified in mammalian ejaculates on the basis of the motility characteristics displayed by individual spermatozoa. Studies carried out by several researchers on fresh and frozen-thawed semen from species as diverse as marmosets (Holt, 1996), gazelles (Abaigar et al., 1999, 2001), boars (Abaigar et al., 1999; Quintero-Moreno et al., 2004; Cremades et al., 2005; Rivera et al., 2005, 2006), stallions (Quintero-Moreno et al., 2003), dogs (Nuñez-Martínez et al., 2006a,b) or rabbits (Quintero-Moreno et al., 2007) have demonstrated that, using CASA systems, it is possible to identify and quantify different sperm subpopulations with specific patterns of movement. This can be achieved by using different procedures of multivariate clustering analysis applied to the CASA-derived kinematic parameters obtained for each individual spermatozoon in a semen sample.

In mammals, sperm motility is important for sperm transport within the female reproductive tract and for egg penetration. Distinct sperm populations showing forward progressive motility or, in contrast, non-progressive patterns of movement will have different probability to cross the utero-tubal junction and enter the oviduct (Gaddum-Rosse, 1981; Olds-Clarke, 1986; Shalgi et al., 1992; Scott, 2000). Furthermore, the number of spermatozoa able to traverse the barriers of the female reproductive tract to reach the fertilization site has been demonstrated to be possitively associated with the fertility of several domestic species (Overstreet and Adams, 1971; Hunter and Wilmut, 1984; Weitze et al., 1988; DeJarnette et al., 1992; Nadir et al., 1993). Therefore, the identification of sperm subpopulations with a preferential ability to reach the female oviducts might be of utmost importance to improve the accuracy of the sperm quality assessments, and proportions of motile but probably ineffective spermatozoa (non progressive, poorly motile or hyperactivated sperm) could be precisely quantified.

Artificial inseminations in dairy cattle are mainly done with frozen-thawed semen. The cryopreservation process not only induces a loss of sperm viability but also impairs the functionality of the surviving spermatozoa, which accounts for the lower fertilizing capacity of the frozen-thawed

spermatozoa (Watson, 2000). Individual variation in semen freezability is recognized to exist in most domestic species, including the bovine (Parkinson and Whitfield, 1987), and such variation has been related to the incidence of motile (Davis et al., 1995; Nuñez-Martínez et al., 2006a,b) and morphologically (Thurston et al., 2001; Esteso et al., 2006; Nuñez-Martínez et al., 2007; Rubio-Guillén et al., 2007) distinct sperm subpopulations present in the fresh ejaculates.

The purpose of this study was to (1) determine the existence of distinct motile sperm subpopulations, as well as their incidence, in fresh bull ejaculates, (2) evaluate the effects of cryopreservation on the frequency distribution of spermatozoa within the different subpopulations, and (3) evaluate between-bull variations in the sperm subpopulations structure of fresh and frozen-thawed semen.

2. Materials and methods

2.1. Animals

A total of 54 ejaculates were collected from 9 Holstein bulls (six ejaculates per bull), with ages between 14 and 24 months. The bulls were in regular AI service housed at the Centro de Inseminación Artificial de Somió (SERIDA), Gijón, Asturias. The overall 90 day non-return rates of the bulls, as obtained from previous records including all the inseminations (not only first but also second, third and subsequent inseminations), were in the range 45–51%.

2.2. Experimental design

The 9 bulls were collected on the same experimental days, twice a week for 3 successive weeks. After collection and initial evaluation of each ejaculate (to determine its volume, sperm concentration and motility by subjective assessment) an aliquot was removed for computer-assisted evaluation of sperm motility, and the rest of the ejaculate was frozen. The overall sperm motility as well as the kinematic parameters of individual spermatozoa were determined by using a CASA system (Sperm Class Analyzer, Microptic; Barcelona, Spain) to investigate the existence of separate motile subpopulations in fresh bull semen samples. After 2 weeks of storage, the frozen semen was thawed and the CASA-derived motility characteristics were analysed after 0, 2 and 4 h of post-thaw incubation at 37 °C, to evaluate the effect of cryopreservation and post-thaw thermal stress on the distribution of spermatozoa into the different subpopulations.

2.3. Semen collection and freezing

Ejaculates were collected by using an artificial vagina (initial temperature of the water: 45 °C) and evaluated for volume, sperm concentration by using a photometer (Accucell, IMV, L'Aigle, France), and the proportion of total motile spermatozoa by subjective visual assessment. The sperm motility was evaluated after dilution of a semen aliquot with Bioxcell® (IMV, L'Aigle, France) to reach a sperm concentration of approximately 40×10^6 spz/ml. All the ejaculates collected had at least 80% of total motile spermatozoa, as determined subjectively. 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.

Mean (±S.D.) values for volume of ejaculates, sperm concentration and total sperm motility (determined subjectively) were: 5.11 ± 1.86 ml, $1183 \pm 343 \times 10^6$ spz/ml and 87 ± 6%, respectively.

The rest of the ejaculate was diluted with Bioxcell® at room temperature (22 °C), at a final sperm concentration of 92×10^6 spz/ml. Extended semen was left to equilibrate in a cooler at 5 °C for 4 h, then it was packaged in 0.25 ml French-straws (23×10^6 spz/straw), and frozen in liquid nitrogen vapours 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 waterbath at 37 °C for 40 s. Three straws per ejaculate were simultaneously thawed, their content was pooled in a 5 ml falcon tube and the thawed semen was incubated at 37 °C in the dark for 4 h.

2.4. Sperm motility evaluation by using a CASA system

The CASA system used was based on the analysis of 16 consecutive, digitalized photographic images which were taken in a time lapse of 0.64 s, which implied a velocity of image-capturing of 1 photograph every 40 ms. Images were taken from 5-μl semen aliquots, which were placed on slides and covered with 20 × 20 mm coverslips. Three microscopic fields were analysed in each sample using a phase-contrast microscope supplied with a prewarmed stage at 37 °C and at 200× magnification. The number of spermatozoa analysed per sample ranged between 100 and 200, including the inmotile 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/s.

The kinematic parameters recorded for each spermatozoon, as described by Mortimer (1997, 2000) were: curvilinear velocity (VCL, μm/s): the average path velocity of the sperm head along its actual trajectory; straight-line velocity (VSL, μm/s): the average path velocity of the sperm head along a straight line from its first to its last position; average path velocity (VAP, μm/s): 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 54 fresh (9 bulls × 6 ejaculates) and 162 thawed (9 bulls × 6 ejaculates × 3 evaluation times) semen samples were imported into a single data set that represented 16,740 spermatozoa, each one defined by the 8 motility descriptors specified above. A multivariate k-means cluster analysis was carried out to classify the 16,740 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 8 quantitative variables, after standardisation of the data (arcsin transformation), 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 dendograms (Holt, 1996) constructed on individual ejaculates using the Ward method. 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 (pre-freezing, 0, 2 and 4 h post-thaw). The effects of cryopreservation, post-thaw incubation time, bull and ejaculate, on the relative distribution frequency of spermatozoa within subpopulations were analyzed using a 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 analysed 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 determined by CASA in fresh and frozen-thawed bull spermatozoa

The mean (±S.D.) total sperm motility in the fresh semen samples collected from the 9 bulls (6 ejaculates per bull) was 82.3 ± 9.8%, and after semen cryopreservation, it decreased to 75.8 ± 12.8%, 69.8 ± 16.3% and 59.4 ± 20.9%, respectively, at evaluations done after 0, 2 and 4 h of incubation post-thaw at 37 °C. Post-thaw sperm motility after 2 h of incubation was lower ($P < 0.05$) than that recorded pre-freezing, but it was not different from that obtained immediately after thawing (Fig. 1). After 4 h of incubation post-thaw, total sperm motility was lower ($P < 0.05$) than that in fresh and frozen-thawed semen at 0 and 2 h post-thaw.

There were significant differences ($P < 0.01$) between bulls in total sperm motility. In fresh semen (Fig. 2a), bulls 3 and 7 had higher sperm motility than bull 4. However, just after thawing (Fig. 2b), bulls 1, 2 and 9 had significantly higher sperm motilities than bull 7; and after 4 h of incubation post-thaw (Fig. 2d), bull 1 had a significantly higher sperm motility than bulls 3, 6, 7 and 8.

There were no differences in total sperm motility between the different experimental days (when including pooled data across bulls) neither in fresh semen nor immediately after thawing or after 2 or 4 h of post-thaw incubation.

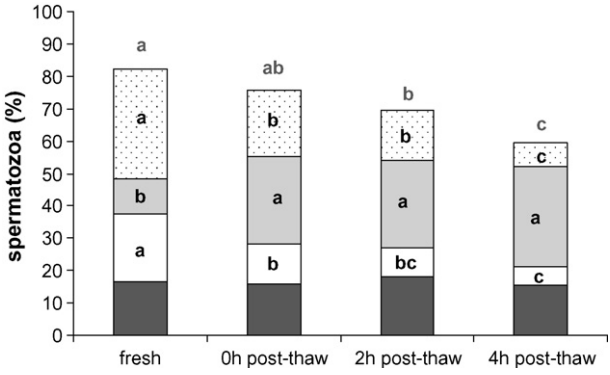

Fig. 1. Relative frequency distribution of motile spermatozoa (mean percentages; $n = 54$) within subpopulations (1: black columns, 2: white columns, 3: grey columns, 4: dotted columns) in fresh and frozen-thawed bull semen samples. Different letters (a–c) inside columns indicate significant differences within subpopulations between evaluation time points (fresh, 0, 2 or 4 h post-thaw). Letters over the columns (a–c) indicate significant differences in total sperm motility between different evaluation time points.

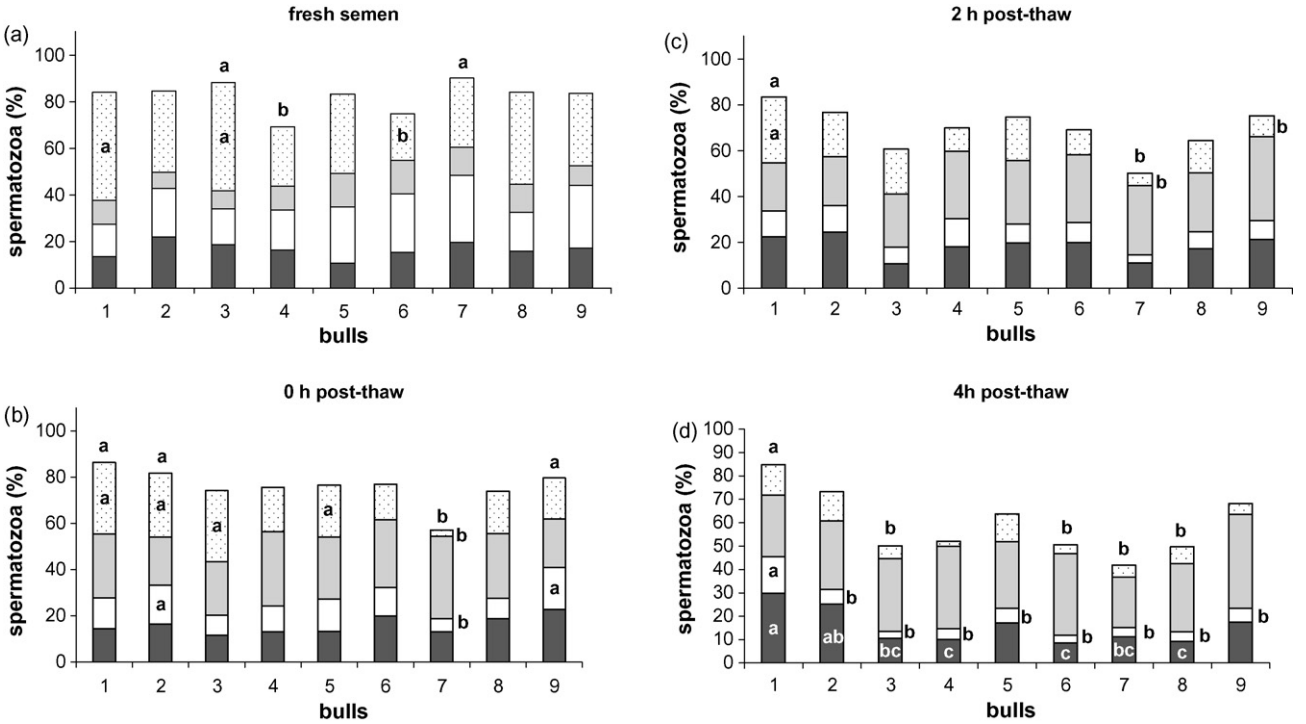

Fig. 2. (a–d) Relative frequency distribution of motile spermatozoa (mean percentages; $n = 6$) within subpopulations (1: black columns, 2: white columns, 3: grey columns, 4: dotted columns) between bulls in fresh (a) and frozen-thawed semen after 0 (b), 2 (c) and 4 h (d) of post-thaw incubation at 37 °C. Different letters (a, b and c) inside or by side columns indicate significant differences within subpopulations between bulls. Different letters (a and b) over the columns indicate significant differences in total motility between bulls.

3.2. Motile sperm subpopulations

Four sperm subpopulations were defined after multivariate cluster analysis of the 16,740 individual motile spermatozoa. The motility characteristics of those subpopulations are shown in Table 1, and their pattern of movement can be described as follows.

Subpopulation 1 represented those 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 23% of the total motile spermatozoa.

Subpopulation 2 contained highly active but non-progressive spermatozoa, as indicated by the high values of VCL and ALH together with low values of LIN and STR, and moderate BCF. This population might be considered as having an "hyperactivated-like" movement, and about 16% of the total motile spermatozoa were assigned to this subpopulation.

Subpopulation 3 included about 35% of the total spermatozoa, and those were poorly motile and non-progressive, as indicated by the lowest values of VCL, VSL, VAP, ALH and BCF together with the lowest LIN, STR and WOB.

Subpopulation 4, containing about 25% of the total population, represented those spermatozoa which moved most rapidly and progressively, as indicated by the highest values of VCL, VSL, VAP and BCF together with the highest LIN, STR, WOB and moderate ALH.

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

The proportions of spermatozoa assigned to the 4 subpopulations significantly changed ($P < 0.01$) during cryopreservation (Fig. 1). In fresh semen samples, 34.1% of the total spermatozoa moved very rapidly and progressively (subpopulation 4), but immediately after thawing, only 20.6% of the total spermatozoa showed this type of movement, and after 2 and 4 h of incubation, this subpopulation further decreased to 15.2% and 7.3%, respectively. In contrast, the poorly motile non-progressive sperm (subpopulation 3), that represented 10.7% of all the fresh spermatozoa, increased to 27.2% immediately after thawing, and then it did not significantly vary during post-thaw incubation. In fresh semen samples, 21.1% of the spermatozoa showed a

Table 1
Mean values (and ranges) of the kinematic parameters defining the four subpopulations identified in fresh and frozen-thawed bull semen samples

Kinematic parameters	Sperm subpobpulations			
	1	2	3	4
No. spz (%)	3880 (23.2)	2688 (16.0)	5937 (35.5)	4235 (25.3)
VCL (μm/s)	70.1a (10.1–109.3)	118.4 b (66.0–227.2)	29.5 c (10.0–93.0)	131.0d (86.1–254.0)
VSL (μm/s)	48.3a (9.9–82.3)	44.6b (8.5–72.1)	8.9c (1.2–26.7)	95.6d (60.1–201.0)
VAP (μm/s)	54.3a (10.3–89.9)	76.8b (27.6–168.0)	15.0c (1.9–57.9)	107.5d (73.3–205.2)
LIN (%)	69.8a (32.9–100.0)	38.1b (10.0–65.7)	31.4c (10.0–93.4)	73.9d (30.7–99.5)
STR (%)	88.9a (43.7–100.0)	59.4b (11.7–97.7)	59.2b (13.4–100.0)	89.1a (42.6–99.9)
WOB (%)	78.2a (42.2–100.0)	65.4b (21.1–98.1)	51.5c (10.6–100.0)	82.8d (44.8–99.8)
ALH (μm)	2.3a (0.3–6.3)	4.6b (1.0–11.8)	1.5c (0.3–5.1)	3.7d (0.6–12.0)
BCF (Hz)	9.0a (0.4–22.0)	7.6b (0.8–20.3)	5.0c (0.0–15.4)	9.5d (1.0–21.7)

Different letters (a–d) indicate significant differences between subpopulations ($P < 0.05$).

"hyperactivated-like" pattern of movement (subpopulation 2), and after thawing, this proportion decreased to 18.1% to further decrease to 8.7% and 5.9%, respectively, after 2 and 4 h of incubation post-thaw. Finally, the proportion of spermatozoa assigned to subpopulation 1 (moderately slow but progressive sperm) did not significantly change during cryopreservation or incubation post-thaw (16.5%, 15.9%, 18.3% and 15.5%, respectively, for fresh, 0, 2 and 4 h post-thaw).

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

In fresh semen samples, the proportion of spermatozoa assigned to subpopulation 4 was the only one that significantly varied among bulls ($P < 0.05$), whereas subpopulations 1, 2 and 3 were not different for the 9 bulls (Fig. 2a).

Immediately after thawing (Fig. 2b), subpopulations 2 and 4 had decreased in the 9 bulls in relation to prefreezing values, but such decrease was different ($P < 0.05$) for the different bulls. Subpopulations 1 and 3, however, did not differ among the 9 bulls.

After 2 h of incubation post-thaw (Fig. 2c), differences among bulls in the sperm subpopulations distribution were only due to the proportion of spermatozoa assigned to subpopulation 4. At the end of the incubation period (Fig. 2d), however, subpopulation 4 had decreased considerably in all bulls, and individual differences were mainly due to differences in subpopulation 1.

As described, the bull had a significant effect on the subpopulations distribution of spermatozoa in fresh and frozen–thawed semen samples, however, when data were pooled across bulls, there was no difference between replicates nor significant interaction between bull and replicate.

There was no significant correlation between the total sperm motility in fresh samples and that in frozen–thawed semen either immediately after thawing or after post-thaw incubation. However, significant correlations ($P < 0.01$) were found between the proportions of spermatozoa assigned to subpopulation 4 in the fresh ejaculates and those in frozen–thawed semen after 0 ($r = 0.473$), 2 ($r = 0.513$) and 4 h post-thaw ($r = 0.450$). This subpopulation in fresh semen, was the only one significantly correlated with itself in frozen–thawed semen. Moreover, the percentage of spermatozoa assigned to subpopulation 4 in the fresh ejaculates was significantly correlated ($P < 0.01$) with the total motility ($r = 0.477$) and with the proportion of spermatozoa included in subpopulation 1 ($r = 0.423$) after 4 h of incubation post-thaw.

3.5. Kinematic parameters of spermatozoa within the different subpopulations before and after semen cryopreservation

There were significant differences between fresh and frozen-thawed semen samples in the kinematic parameters defining the four sperm subpopulations (data not shown). In general, such differences indicated that, in the four subpopulations, the sperm velocity parameters (VCL, VSL and VAP) decreased after cryopreservation and also during post-thaw incubation. However, the trajectory of the sperm movement changed in a different way depending on the sperm subpopulation. In subpopulations 2 and 4, the sperm trajectories were more progressive after thawing and during post-thaw incubation than before freezing (higher LIN, STR and BCF), whereas in subpopulations 1 and 3, the sperm trajectories were less progressive immediately after thawing than before freezing, and became even more oscillatory during post-thaw incubation.

The magnitude of the changes in the sperm kinematic parameters was quite similar for the four sperm subpopulations. For example, for spermatozoa assigned to subpopulation 4, mean values (and ranges) of VCL, VSL and VAP changed from 134.6 (86.1–254.0), 97.2 (60.1–201.0) and

111.8 (74.1–205.2) μm/s, respectively, before freezing to 118.6 (86.4–196.2), 90.2 (65.0–147.0) and 96.9 (75.2–148.7) μm/s, respectively, after 4 h of post-thaw incubation. And mean values (and ranges) of LIN, STR, ALH and BCF changed from 73.3 (30.7–99.5)%, 87.1 (42.6–99.9)%, 3.8 (0.6–11.1) μm and 7.2 (2.0–17.5) Hz, respectively, before freezing to 76.9 (37.0–97.6)%, 93.1 (51.5–99.6)%, 3.3 (0.7–8.3) μm and 12.6 (2.1–21.0) Hz, respectively, after 4 h of post-thaw incubation. Changes of a similar magnitude were observed in the kinematic parameters defining the other three subpopulations.

3.6. Effect of individual variability on the sperm kinematic parameters defining the four sperm subpopulations

There were significant differences among bulls ($P < 0.05$) on the sperm kinematic parameters defining the four subpopulations. For example, for spermatozoa assigned to subpopulation 4, bulls 3 and 9 showed the highest differences. Bull 3 had significantly ($P < 0.05$) higher VCL, VSL, VAP, LIN, WOB and significantly ($P < 0.05$) lower values of ALH and BCF than bull 9 [mean values (and ranges) were VCL: 133.9 (89.7–228.2) versus 127.1 (88.5–206.1) μm/s; VSL: 101.5 (63.0–201.0) versus 87.8 (64.0–181.0) μm/s; VAP: 113.9 (75.6–202.6) versus 98.2 (73.7–190.8) μm/s, LIN: 76.6 (36.3–99.5) versus 70.5 (35.5–95.6)%; WOB: 85.5 (46.5–99.6) versus 78.5 (44.9–99.2)%; ALH: 3.4 (0.9–10.4) versus 3.9 (0.9–9.6) and BCF: 9.2 (1.8–21.0) versus 11.0 (1.7–21.3) Hz].

Differences were also observed among the other bulls in some, but not all, of the kinematic parameters defining the sperm velocity or the trajectory of subpopulation 4. Similar individual variability was observed in the kinematic parameters defining the other 3 subpopulations.

4. Discussion

The results of the present study indicated that four sperm subpopulations were distinguishable in fresh and frozen-thawed bull semen samples, as determined by the 8 kinematic parameters studied. The most interesting finding in this study was that the four sperm subpopulations were quite similarly distributed in the nine bulls, either in fresh or frozen-thawed semen, and differences among bulls were almost exclusively established by the proportion of spermatozoa assigned to the subpopulation of most active and progressive sperm.

From studies in several species, it seems that the presence of three or four well-defined sperm populations is a common finding among mammalian ejaculates (Quintero-Moreno et al., 2003). To our knowledge, the existence of separate motile subpopulations in fresh or frozen-thawed bovine semen has not been previously investigated.

In semen from boars (Quintero-Moreno et al., 2004; Rivera et al., 2005, 2006), rabbits (Quintero-Moreno et al., 2007) and dogs (Nuñez-Martínez et al., 2006a,b), significant differences in the sperm subpopulations structure have been described between-breeds within species. And in a recent study, objective differences in the percentages of total and progressive motility, as well as in the velocity and straightness of spermatozoa, were found in semen from Belgian Blue and Holstein bulls (Hoflack et al., 2007). The authors suggested that a genetic component could be the basis for the breed differences in sperm motility, probably as a result of the world-wide long-term selection of highly fertile Holstein AI bulls (Söderquist et al., 1991). Although in the present study the frequency distribution of spermatozoa within subpopulations was very consistent among bulls and ejaculates, it is likely that different subpopulations could be defined when using bulls of different breeds.

The cryopreservation process had significant effects on the frequency distribution of spermatozoa within subpopulations (Fig. 1). Although different mortality rates in the four sperm subpopulations may account for a change in their frequency distribution, it was obvious that the cryopreservation process modified the motility patterns of the surviving spermatozoa, some spermatozoa being assigned to a different subpopulation. A possible sequence for the progression of spermatozoa through the four subpopulations, similarly to that proposed for gazelle sperm (Abaigar et al., 2001), can be hypothesized. Spermatozoa in subpopulation 4 represent the most rapid and progressive sperm, probably the pattern movement most suitable for being part of the fertilizing population, and this was the majoritary population in fresh ejaculates. After cryopreservation and during post-thaw incubation, some of those spermatozoa might reduce their flagellar activity, probably as a consequence of sublethal cryoinjury, and they would be included into subpopulation 1. Some of the spermatozoa from subpopulation 1, constituted by relatively slow but progressive spermatozoa, might lose the ability to control the semipermeability of their membranes and thereby experience a sort of false hyperactivation (Yanagimachi, 1994), being assigned then to subpopulation 2. Finally, subpopulation 3, constituted by poorly motile non-progressive spermatozoa, could represent a late stage of cell deterioration, probably contributed to by spermatozoa from the other three subpopulations, but most importantly by those from subpopulation 2.

The changes in the sperm subpopulations structure induced by cryopreservation were quite similar for the nine bulls, and differences between bulls were mainly due to subpopulation 4, either in fresh semen, just after thawing or after 2 h post-thaw. Only at the end of the incubation period, it was the subpopulation 1 which established differences among bulls. It is interesting to note that the proportion of spermatozoa included in subpopulation 4 in the fresh ejaculates was significantly correlated with that in thawed semen (at 0 and 2 h post-thaw) and with subpopulation 1 after 4 h of incubation post-thaw. However, the overall motility of the fresh ejaculates showed no correlation at all with total post-thaw motility or with any of the sperm subpopulations obtained after thawing. This indicated that the ejaculates with the highest subpopulations of rapid and progressive sperm were also the most resistant to cryopreservation and showed the best post-thaw sperm longevity. Similar results were also found in human (Davis et al., 1995) and dog ejaculates (Nuñez-Martínez et al., 2006a) and it has been proposed that sperm belonging to clusters with high velocities and more progressive movement can be considered the sperm with the highest fertilizing potential (Quintero-Moreno et al., 2003; Cremades et al., 2005; Nuñez-Martínez et al., 2006a).

As differences among bulls were mainly evidenced by the proportion of spermatozoa included into subpopulation 4, perhaps this subpopulation could also determine differences in their *in vivo* fertility. However, to provide valid measurements of fertility in which a bull accounts for a substantial proportion of the total variance, insemination doses of $0.8–1.5 \times 10^6$ sperm should be used, to place the results on the dose–responsive rather than on the asymptotic part of the curve (Amann, 1989; Christensen et al., 2005). Insemination doses of about 23×10^6 sperm would largely compensate differences among bulls in the proportions of spermatozoa assigned to subpopulation 4. In such a situation, bull and ejaculate effects would account for less than 1% of the random variation in NRR (Stålhammar et al., 1994; Amann and Hammerstedt, 2002; Christensen et al., 2005).

To evaluate the importance of identifying concrete motile subpopulations in the ejaculates of different bulls, perhaps a first approach should be an *in vitro* estimation of the quality of sperm at the site of fertilization. For example, by using a swim-up procedure (Zhang et al., 1998), differences in the recovered sperm populations after swim-up separation could reflect those

among the 'competent' motile subpopulations. In addition, the use of IVF tests could demonstrate differences in the fertilizing capacity of bulls with different proportions of the most interesting subpopulation. Then, if the initial results were positive, a low-dose insemination trial could be carried out to try to establish a threshold number of spermatozoa in subpopulation 4 above which an optimal fertility should be expected. This information could be used to adjust the insemination doses to the minimum threshold required, especially from highly demanded bulls.

In conclusion, the results of the present study indicated that four well-defined motile sperm subpopulations were present in fresh and frozen-thawed semen from Holstein bulls. The cryopreservation process significantly modified the distribution of spermatozoa within subpopulations, and these changes were consistent among bulls and ejaculates. Differences among bulls were almost exclusively established by the subpopulation of spermatozoa with the highest velocity and progressiveness; the magnitude of this subpopulation in fresh ejaculates was positively correlated with their resistance to cryopreservation. Further studies should be conducted to determine the relative importance of the different subpopulations of motile spermatozoa coexisting in a bull semen sample when it comes to predict its *in vivo* fertility.

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