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Effects of holding semen for 4 h at 15 °C in different extenders prior to cryopreservation on sperm characteristics and subpopulation kinematics in INRA180 sheep

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Abstract The aim of the present study was to evaluate the effects of holding semen for 4 h at 15 °C in different extenders prior to cryopreservation on sperm characteristics and subpopulation kinematics in INRA180 sheep. Semen samples were collected using an artificial vagina from two animal groups. Group 1: the rams kept next to the lab (G1) considered as a control and group 2: the rams kept in a farm faraway (170 km) from the lab (G2). The semen was extended in Milk egg yolk, Andromed® and Tryladil® extenders to reach 0.2 x 10^9 spermatozoa/mL. Before freezing, the samples from G1 were directly cooled to 5 °C during 2 h. While those for G2 were transported within 4 h to the laboratory in a temperature-regulated cooler box at 15 °C then cooled to 5 °C. Differences in sperm motility, viability and abnormality in pre- and post-thaw steps and subpopulation kinetic parameters in post thaw step were detected between the two groups, and between extenders within the groups. Evaluation of the kinetic data obtained with a CASA (Computer-Assisted Sperm Analysis), applying a cluster analysis, yielded in INRA180 rams two post thaw sperm subpopulations (SPs). The SP1 and SP2 included spermatozoa characterized by low and high kinetic parameters, respectively. Disregarding the extender type, the proportional size of SP1 was lower than SP2 in G1, Nevertheless, in G2 the percentage of SP1 was higher than SP2. In conclusion, changing conditions during the equilibration time (from 2 h at 5 °C to 4 h at 15 °C) can extend the total process duration and causes a decrease in post-thawed semen quality in INRA180 rams.

Keywords: ram, spermatozoa, extenders, sperm subpopulation, cryopreservation

Introduction

Handling semen requires that animals of interest are within the reasonable proximity of laboratories. Nevertheless, in many cases, those animals are kept on farms far from laboratory facilities and with little infrastructure that makes semen processing and cryopreservation very difficult. Consequently, it is necessary to collect and transport semen samples from the place of collection to the laboratory while maintaining its quality before and after cryopreservation. This kind of handling has been largely developed in cooled stallion semen (Backman et al., 2004) as well as in boar cryopreserved semen (Torres et al., 2019). The adoption of

such scheme would facilitate ram semen collection and freezing without being obliged to transport animals to a collection facility, and without requiring equipment for cryopreservation at farms.

Incubating sperm for a short time period prior to cryopreservation, was shown to be beneficial for boar (Guthrie and Welch, 2005), bull (Foote and Kaprotht, 2002) and stallion (Backman et al., 2004) sperm. Similar beneficial effects were obtained in ram semen held at 5 °C for 24 h prior to cryopreservation (Purdy et al., 2010). However, documentation concerning ram sperm cryopreservation following incubations at 15 °C of up to 4 h has never been reported.

It is well known that the duration of the equilibration step and the extender composition are key requirements for successful semen cryopreservation. On one hand, the length of the equilibration step can affect sperm quality and subsequently alters the cryoprotectant penetration into cells. It affects the balance of intra- and extracellular concentrations of glycerol and other smotically active elements of the extender (Evans et al., 1987). Moreover, the chemical composition of extenders through their basic composition (milk, egg yolk, soy lecithin, liposome etc.) is also critical for successful ram semen storage (Salamon and Maxwell, 2000; Forouzanfar et al., 2010; Benmoula et al., 2018). Egg yolk and milk are the most common additives of animal origin used for sperm cryopreservation (Amirat et al., 2004). However, molecules of plant origin were also tested in different studies. In fact, lecithin (or phosphatidylcholine) was shown to have an important role in the regulation of animal cell bio-membranes (Evans et al., 1987; Üstüner et al., 2014). Similarly, soy lecithin-based diluents are also employed as an alternative to the animal-based diluents (milk and egg yolk), and has been shown to have good outcomes in sperm cryopreservation (Aires et al., 2003).

There are relatively few studies targeting the effect of cryopreservation on ram sperm motility using the CASA (Gil et al., 2003; Peris et al., 2004). Besides, the majority of these studies reported the mean values of predetermined parameters of motility based on biological criteria, without subjecting the data to different available analysis (Dorado et al., 2010). The use of only mean values to describe sperm parameters derived from the CASA systems is of limited value (Dorado et al., 2010; Martínez-Pastor et al., 2011) and no longer prescribed (Holt et al., 2007; Ledesma et al., 2017; van der Horst et al., 2018). The development of CASA systems has improved the ability to analyze sperm motility, allowing assessment of individual cells. This leads to identification and quantification of different sperm subpopulations with specific patterns of movement. In the last 20 years, multivariate statistics have been applied to identify sperm subpopulations, making it possible to determine how they are affected by specific treatments. Therefore, different sperm subpopulations have been identified in mammalian ejaculates based on the single sperm cell motility characteristics (Quintero-Moreno et al., 2003; Martínez-Pastor et al., 2011).

The purpose of the present work was to investigate the effects of holding semen for 4 h at 15 °C prior to cryopreservation in different extenders on sperm characteristics and subpopulation kinematics in INRA180 sheep.

Materials and methods

Semen collection and processing

Semen samples were collected using an artificial vagina once a week during 12 weeks from two groups of animals. Group 1: the rams were housed next to the laboratory (G1) (n = 6) considered as a control and group 2: the rams were housed 170 km far from the laboratory (G2) (n = 8) which is the tested group. The rams in both groups had the same age and were fed in the same manner. Ejaculates were assessed for volume, sperm concentration using a spectrophotometer (Photometer SDM6, Minitube, Germany), total motility (%) and mass motility (based on a 5-point scale : 0 = no motility; 5 = excellent forward motile sperm). Microscopic assessments were conducted by the same experienced technician and the initial quality control cutoff values were defined as fellows: a mass motility > 3; individual motility > 70%, and a concentration > 3 × 10^9 spermatozoa/mL. Any ejaculate which did not meet these criteria was rejected. Table 1 shows means and the standard errors of the mean for sperm parameters. No significant difference was recorded between ram groups, in terms of semen volume, concentration, mass

Table 1. Semen characteristics in two INRA180 ram groups before processing (mean ±SEM)

Ram group	Semen volume (mL)	Concentration (10^9spz/mL)	Mass motility	Individual motility (%)
G1 (n = 6)	1.58±0.06	4.07±0.17	4.64±0.06	91.97±0.97
G2 (n = 8)	1.50±0.04	4.22±0.012	4.39±0.11	92.97±0.08

G1: Rams were kept close to the lab; G2: Rams were kept 170 km away from the lab

and total motilities ($P > 0.05$). Following the initial semen assessment, each acceptable ejaculate was diluted in pre-warmed (37 °C) extenders, namely, Tryladil® (Minitube, Germany), Andormed® (Minitube, Germany) and milk-egg yolk to a final concentration 0.2×10^9 spermatozoa/mL. The commercial extenders were prepared as indicated in the manufacturer's instructions. The milk-egg yolk extender was made by mixing 10.3 g lactose, 100 mL sterilized double-distilled water, 25 mL egg yolk, 25 mg gentamycin, 50,000 IU penicillin (fraction 1), 100 mL skim milk UHT, 10 mL glycerol (fraction 2) (Colas, 1975). The first fraction is added when the temperature reached 5 °C and then the second fraction was added after 45 minutes.

The ejaculate collected from G1 rams was cooled directly to 5 °C over 2 h for equilibration. The ejaculates collected from G2 rams were placed into a temperature-regulated cooler box at 15 °C (Box, IMV, France) and transported to the laboratory within approximately 4 h, and then cooled to 5 °C over 2 h for equilibration. In both cases, French mini-straws (0.25 mL) (IMV, L'Aigle France) were filled and sealed with polyvinyl alcohol as per routine procedures and then gradually cooled during 15 minutes to static nitrogen vapor 4 cm above liquid nitrogen. Then, they were submersed and stored in liquid nitrogen at −196 °C. After 1 months of storage, the straws were thawed for 30 s at 37 °C using a special thaw unit (Minitube, Ref. 15043/0636).

Computer-assisted sperm analysis (CASA)

For motility and kinematic parameters, a computer-assisted sperm motility analysis (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) was used. It allows the assessment of sperm total motility (TM, %) and progressive motility (PM, %) as well as the kinematic parameters for each spermatozoa. The latter includes the curvilinear velocity (VCL, $\mu m/s^{-1}$), the straight line velocity (VSL, $\mu m/s^{-1}$), the average path velocity (VAP, $\mu m/s^{-1}$), the linearity index (LIN %), the straightness index (STR, %), the mean amplitude of lateral head displacement (ALH, µm) and mean of the beat cross frequency (BCF, Hz). For each sample, the semen was diluted in phosphate-buffered saline supplemented with bovine serum albumin (1 mg/mL) to achieve 20×10^6 spermatozoa/mL. Then, a 3-µL portion of semen was placed onto a pre-warmed chamber slide (37 °C) for analyzing the sperm parameters.

Sperm viability

Sperm viability was evaluated by the nigrosine eosin staining. Semen suspension smears were prepared by mixing 2.5 µL of semen sample with 2.5 µL of stain on a warm slide and uniform smear (spreading the mixture with a cover slide). After drying, 200 sperm cells were microscopically evaluated (400 ×, light microscopy). Spermatozoa showing partial or complete purple stain were considered non–viable, and only spermatozoa showing strict exclusion of the stain were considered to be alive.

Sperm abnormalities

The percentage of abnormal cells was assessed using a Diff–Quik staining (Automatic Diagnostic Systems S.L., Barcelona, Spain). Briefly, a smear of 3 µL of diluted semen was prepared on a slide and air–dried for 4 minutes. Then, the slide was dipped for 1 minute in Diff–Quik fixative solution (0.002 g/L of Fast Green in methanol) prior to staining with Diff–Quik solution 1 (1.22 g/L of Eosin Y in phosphate buffer at pH 6.6 and 0.1 % (w/v) sodium azide as preservative) for 50 seconds and with Diff–Quik solution 2 (1.1 g/L of tiazine day in phosphate buffer at 6.6) for 50 seconds. At each step, the excess stain was dried by placing the slide vertically on absorbent paper. Sperm abnormality was assessed by counting at least 200 cells (400 ×, light microscopy).

Statistical analysis

Statistical analyses were performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The data obtained from the analysis of motility, viability, abnormality and all kinematic parameters were first tested for normality and homogeneity using the Shapiro–Wilk and Kolmogorov–Smirnov tests, respectively. Clustering procedures were performed to identify sperm subpopulations from the complete set of post-thaw kinetic data. The first step was to perform a principal component analysis (PCA). The number of principal components (PC) that should be used in the next step of the analysis was determined using the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted for that particular PC) > 1. The second step was to perform a cluster procedure with the sperm-derived indices obtained after the PCA.

For each holding time process, all post-thawed sperm cells within each ram group and extender were clustered. For that, a nonhierarchical cluster analysis using the selected principal components as variables was performed as a second step. This procedure performs a disjoint cluster analysis on the basis of distances computed from one or more quantitative varia-

bles, using Euclidean distances (k-means model) to calculate cluster centers. This clustering method is often used with large data sets before trying a hierarchical one, to reduce the data to a few initial clusters and then pass them to the hierarchical procedure. We selected a maximum number of clusters of 15 as this number represents a fairly high number of initial clusters for the subsequent hierarchical procedure. The third step of the clustering analysis was carried out using the cluster procedure, which performed a hierarchical clustering on the clusters obtained by the previous step, using Ward's averaging method. To determine the final number of clusters, we studied the evolution along the clustering process of the cubic clustering criterion (CCC) (local peaks of the CCC).

The data of fresh and thawed semen progressive and total motility, viability and abnormality and the effects of clusters between treatments for post thaw measuring CASA parameters were analyzed by a factorial design ANOVA. The statistical model included the fixed effect of ram group and the extender. When statistically significant differences were found, the Student's t and Tukey's post hoc tests were applied to compare the means at $P < 0.05$. Data are expressed as mean ± SEM.

Results

Effect of ram group and extender

After the equilibration step, progressive and total motilities, viability and abnormality were significantly affected by ram group, extender and group × extender interaction ($P < 0.05$; Table 2).

In G1, the greatest semen progressive and total motilities were recorded in Tryladil® followed by Andromed® ($P < 0.05$) and then milk-egg yolk extender ($P < 0.05$). The viability was higher in Andromed® and lowest in milk-egg yolk and Tryladil® extenders ($P < 0.05$). The sperm abnormality increased significantly in milk-egg yolk extender compared to Andromed® and Tryladil® (Table 2). In G2, the greatest semen progressive and total motilities and viability were recorded in milk-egg yolk extender and the lowest one in Andromed® followed by Tryladil® ($P < 0.05$). The abnormality increased significantly in Tryladil® and Andromed® compared to milk-egg yolk extender (Table 2). At pre-thaw step, Andromed® and Tryladil® revealed the highest TM, PM and viability, but the lowest abnormality in G1 compared to G2 (Table 2).

Similarly, after thawing, PM, TM, viability and abnormality were also significantly affected by ram group,

Table 2. Effects of ram group (G1 and G2) and extender (Andromed®, milk-egg yolk, and Tryladil®) on pre- and post-thaw semen characteristics in INRA180 sheep (Mean ± SEM)

		Pre-thaw				Post-thaw			
Ram Group	Extenders	PM (%)	TM (%)	Abn (%)	Via (%)	PM (%)	TM (%)	Abn (%)	Viab (%)
G1 (n = 6)	Andromed®	$54.91±1.87^{Ba}$	$85.91±1.54^{Ba}$	$7.00±0.56^{Bb}$	$93.29±0.82^{Aa}$	$38.22±1.72^{Aa}$	$65.22±1.27^{Aa}$	$24.57±1.39^{Ba}$	$73.11±2.48^{Aa}$
	Milk-EY	$50.22±1.59^{Cb}$	$81.24±1.33^{Cb}$	$9.83±0.25^{Aa}$	$85.08±0.98^{Cb}$	$35.38±1.54^{Bb}$	$54.13±1.55^{Ca}$	$29.24±1.75^{Aa}$	$62.88±2.51^{Cb}$
	Tryladil®	$59.48±1.69^{Aa}$	$88.67±1.37^{Aa}$	$7.16±1.19^{Bb}$	$89.72±1.65^{Ba}$	$24.14±1.22^{Ca}$	$64.24±1.34^{Aa}$	$23.82±3.98^{Bb}$	$74.89±2.49^{Aa}$
G2 (n = 8)	Andromed®	$51.81±1.09^{Bb}$	$79.77±1.64^{Bb}$	$8.93±0.42^{Aa}$	$86.52±2.46^{Bb}$	$39.56±1.49^{Aa}$	$54.13±1.41^{Ab}$	$25.26±1.27^{Aa}$	$64.63±2.03^{Ab}$
	Milk-EY	$59.49±1.08^{Aa}$	$84.39±1.23^{Aa}$	$5.36±0.52^{Bb}$	$92.49±2.57^{Aa}$	$40.08±1.58^{Aa}$	$54.56±1.64^{Aa}$	$25.99±1.88^{Ab}$	$65.85±2.12^{Aa}$
	Tryladil®	$47.68±1.07^{Cb}$	$66.45±1.66^{Cb}$	$8.18±0.71^{Aa}$	$72.65±2.72^{Bb}$	$24.81±1.76^{ba}$	$44.88±1.19^{Bb}$	$25.24±1.97^{Aa}$	$47.21±2.09^{Bb}$

G1: Rams (INRA180 sheep) were kept close to the lab; G2: Rams were kept 170 km away from the lab; Milk-EY : milk-Egg yolk, Via : viability, Abn: abnormality; PM: progressive motility, TM: Total motility.
A, B, C. Different superscripts within columns indicate an effect of extender in each ram semen group at each step ($P<0.05$).
a, b. Different superscripts within columns indicate an effect of ram group semen for each extender at each step ($P<0.05$).

extender and interaction ($P < 0.05$). Table 2 shows that in G1, TM followed the same tendency recorded after the equilibration step. Progressive motility and viability were improved in Tryladil® and Andromed® compared to milk-egg yolk extender. The latter recorded the highest abnormality compared to Tryladil® and Andromed®. In G2, TM, PM and viability were significantly increased in Andromed® and milk-egg yolk extender compared to Tryladil®. However, no significant difference was recorded between the extenders in sperm abnormality (Table 2). Concerning the effect of extenders, milk-egg yolk extender recorded the highest PM in G2 compared to G1 ($P < 0.05$). While, Tryladil® and Andromed® did not show any significant differences between the two groups. Total motility was improved in Tryladil® and Andromed® for G1 compared to G2. The milk-egg yolk recorded similar results for the two groups ($P > 0.05$). Milk-egg yolk, decreased significantly the abnormality in G2 compared to G1. However, Tryladil® decreased the latter parameter in G1 compared to G2. Andromed® and Tryladil® recorded the highest viability in G1 compared to G2 (Table 2).

Effect of ram group and extender on semen, pre-thaw kinetic parameters

After equilibration and as reported above the kinetic variables were significantly affected by ram group, extender and interaction ($P < 0.05$; Table 3).

In G1, Tryladil®, recorded the greatest VCL, VSL and VAP compared to Andromed® and milk-egg yolk extender. The lowest VAP was obtained in milk-egg yolk extender. ALH and BCF increased significantly by milk-egg yolk and Tryladil® extenders compared to Andromed®. However, LIN and SRT were not affected by the extender type. For the G2, Tryladil® as well as milk-egg yolk extender increased VCL, VSL, VAP, LIN and WOB compared to Andromed® ($P < 0.05$). Milk-egg yolk extender increased ALH and decreased LIN compared to Tryladil® and Andromed®. SRT and BCF were not influenced by the extender type. The highest VCL, VSL, VAP and WOB were recorded in G1 compared to G2 whatever the extended was. In G1, linearity was increased significantly in Andromed® and milk-egg yolk extender compared to those in G2, while Tryladil increased LIN in G2 compared to the G1. SRT was increased by Andromed® and Tryladil® in G2 compared to those in G1, while milk-egg yolk extender gave the same SRT value in G1 and G2. Tryladil® in G1 increased significantly ALH compared to Tryladil® in G2. However, Andromed® in G2 increased ALH compared to Andromed® in G1 while milk-egg yolk extender resulted in the same ALH values in both ram groups. Milk-egg yolk extender and Tryladil® increased BCF in G1 compared to those in G2 ($P < 0.05$), and Andromed® recorded the same BCF value whatever the ram group was.

PCA and subpopulations

At post-thaw step, PCA identified 8 principal components. Among them, two are with an eigenvalues (Table 4) accounting for 81.4% of the variance. Looking at the highest eigenvectors (> 0.30), the first principal component was positively related to VCL, VAP, VSL, LIN and WOB and the second principal component was positively related to VCL and ALH and negatively related to LIN and SRT. These two PCs were used to identify two subpopulations (Table 4). SP1 included

Table 3. Effects of extender type on pre-thaw CASA motility variables in INRA180 sheep for two ram groups (G1 and G2) (Mean ± SEM)

	G1 (n = 6)			G2 (n = 8)		
Parameters	Andromed®	Milk-EY	Tryladil®	Andromed®	Milk-EY	Tryladil®
VCL	187.15 ± 1.41^{Ba}	189.00 ± 1.72^{Ba}	199.31 ± 1.04^{Aa}	151.31 ± 1.08^{Bb}	156.33 ± 0.94^{Ab}	152.07 ± 1.18^{Ab}
VSL	153.19 ± 1.49^{Ba}	150.81 ± 1.58^{Ba}	159.72 ± 1.16^{Aa}	116.59 ± 0.71^{Bb}	114.82 ± 0.54^{Bb}	120.81 ± 0.91^{Ab}
VAP	177.71 ± 1.21^{Ba}	172.21 ± 1.34^{Ca}	183.83 ± 0.93^{Aa}	130.92 ± 0.75^{Bb}	129.89 ± 0.58^{Bb}	134.90 ± 1.00^{Ab}
LIN	81.12 ± 0.22^{Aa}	80.84 ± 0.32^{Aa}	80.99 ± 0.13^{Ab}	78.55 ± 0.11^{Bb}	75.45 ± 0.12^{Cb}	81.21 ± 0.01^{Aa}
STR	86.11 ± 0.26^{Ab}	88.08 ± 0.23^{Aa}	87.00 ± 0.21^{Ab}	89.31 ± 0.21^{Aa}	89.29 ± 0.24^{Aa}	89.33 ± 0.23^{Aa}
WOB	95.15 ± 0.13^{Aa}	91.18 ± 0.11^{Ba}	92.20 ± 0.15^{Ba}	87.19 ± 0.13^{Bb}	84.22 ± 0.14^{Cb}	89.24 ± 0.13^{Ab}
ALH	3.74 ± 0.08^{Bb}	4.23 ± 0.08^{Aa}	4.05 ± 0.05^{Aa}	3.88 ± 0.08^{Ba}	4.37 ± 0.07^{Aa}	3.63 ± 0.06^{Cb}
BCF	8.99 ± 0.22^{Ba}	11.07 ± 0.25^{Aa}	11.34 ± 0.15^{Aa}	9.19 ± 0.14^{Aa}	9.23 ± 0.11^{Ab}	9.15 ± 0.11^{Ab}

G1: Rams (INRA180 sheep) were kept close to the lab; G2: Rams were kept 170 km away from the lab

A, B, C: Different superscripts within rows indicate an effect of the extender for ram group ($P<0.05$).

a, b: Different superscripts within rows indicate an effect of ram group for each extender ($P<0.05$).

RM, Ram group; VCL (μm/s^{-1}), curvilinear velocity; VSL (μm/s^{-1}), straight velocity; VAP (μm/s^{-1}), velocity average path; LIN (%), linearity; STR (%), straightness; ALH (μm), lateral head displacement; BCF (Hz), beat cross frequency; WOB (%), Wobble.

the spermatozoa characterized by low velocity (VCL, VSL and VAP), low linear trajectories (LIN and STR), and low ALH, WOB and BCF. This subpopulation represented 36.67% of the entire sample. SP2 included spermatozoa characterized by high speed (VCL, VSL and VAP), high linear trajectories (LIN and STR) and high ALH, WOB and BCF (Table 4). This subpopulation represented 63.33% of the total sample (Table 4). The percentage of spermatozoa in each subpopulation according to ram groups and extenders is shown in Table 4. More precisely, in G1, the proportional size of SP1 was lower than SP2 whatever the extender (5.36% vs 94.64% in Andromed®, 11.19% vs 88.81% in milk-egg yolk extender and 9.61% vs 90.39% in Tryladil®). In G2, the percentage of SP1 was higher than SP2 whatever the extender (91.35% vs 8.47% in Andromed®, 85.82% vs 14.18% in milk-egg yolk extender and 84.23 % vs 15.77% in Tryladil®).

In Sp1, the eight post-thaw CASA-Mot variables were not affected by the extender type for the G1. Yet, for G2; milk-egg yolk extender increased significantly the VSL, VAP, LIN, SRT, WOB, ALH and BCF compared to Andromed® and Tryladil®. The VCL was higher in Andromed® compared to milk-egg yolk extender and Tryladil® ($P < 0.05$). Andromed® increased VAP and BCF as well as milk-egg yolk extender, while Tryladil®, decreased significantly these parameters. Tryladil and milk-egg yolk extender significantly increased ALH. The lowest LIN, SRT and WOB were recorded in Andromed®. Whatever the extender was, the highest CASA variables were recorded in G1 compared to G2 (Table 5).

In Sp2; 7 CASA variables (VCL, VSL, VAP, LIN, SRT, WOB, and BCF) were highest in Tryladil®, intermediate in milk-egg yolk extender and lowest in Andromed but ALH was not affected by the extender type ($P > 0.05$). For G2, VCL was significantly higher in Andromed® compared to milk-egg yolk extender and Tryladil®. VSL, LIN, SRT, WOB and ALH were increased by milk-egg yolk extender and Tryladil® ($P < 0.05$) and decreased by Andromed®; BCF and VAP, they were not affected by the extender type (Table 5).

The highest VCL, VSL, VAP, ALH and BCF were recorded in G1 compared to G2 in all extenders. On the other hand, LIN and SRT were significantly higher in G2 compared to G1 whatever the extender was. Andromed® and Tryladil® in G1 recorded the highest WOB compared to those in G2 while milk-egg yolk extender showed the same WOB in both G1 and G2 groups (Table 5).

Discussion

In this work, we investigated the effects of holding semen for 4 h at 15°C prior to cryopreservation on viability, abnormality and kinematic of post-thaw sperm subpopulations of INRA180 rams. The rams in both groups were of the same age and were fed identical diets; which explains the lack of significant difference of semen characteristics before processing. However, this pattern was not found after the equilibration step. It is known that the latter is expected to be beneficial, as it allows a better osmotic balance after an interaction between sperm and the cryoprotectant. However, the effectiveness of this step depends on the extender composition. In the present study, the data analysis from the eight pre-thaw CASA-Mot variables revealed that Tryladil® improved most of the CASA variables compared to Andromed® and milk-egg yolk extenders in both groups. Moreover, PM, TM, viability and ab-

Table 4. Values of the two principal components used in the clustering procedure and the analytical statistics for eight post-thaw CASA-Mot variables for each determining sperm subpopulation (Sp1 and Sp2) obtained in post-thaw sperm CASA descriptors in INRA180 sheep

	Principal components		Subpopulations	
Parameters	PC 1	PC 2	Sp1	Sp2
VCL	0.39	0.38	40.28 ± 0.46^{b}	155.63 ± 0.54^{a}
VSL	0.45	-0.05	14.92 ± 0.25^{b}	88.86 ± 0.66^{a}
VAP	0.43	0.24	25.65 ± 0.33^{b}	132.63 ± 0.56^{a}
LIN	0.35	-0.45	33.55 ± 0.28^{b}	61.47 ± 0.16^{a}
STR	0.28	-0.47	58.64 ± 1.09^{a}	70.77 ± 0.32^{a}
WOB	0.37	-0.19	68.99 ± 0.43^{b}	84.30 ± 0.01^{a}
ALH	0.18	0.57	2.07 ± 0.02^{b}	4.11 ± 0.02^{a}
BCF	0.29	0.11	4.41 ± 0.04^{b}	7.90 ± 0.05^{a}

a, b: Different superscripts within rows indicate an effect of cluster group ($P<0.05$).
VCL (µm/s-1), curvilinear velocity; VSL (µm/s-1), straight velocity; VAP (µm/s-1), velocity average path; LIN (%), linearity; STR (%), straightness; ALH (µm), lateral head displacement; BCF (Hz), beat cross frequency; WOB (%), Wobble.

Table 5. Effect of extender on eight post-thaw CASA-Mot variables for each sperm subpopulation (Sp1 and Sp2) and each ram group (Mean± SEM)

Sp	Groupe	Extender	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
Sp1	G1	Andromed®	112.25±1.56^{Aa}	48.73±3.59^{Aa}	78.58±1.29^{Aa}	43.22±0.30^{Aa}	62.88±1.41^{Aa}	69.54±0.24^{Aa}	4.25±0.21^{Aa}	4.62±0.55^{ABa}
		Milk-egg yolk	113.45±2.16^{Aa}	44.32±4.08^{Aa}	77.51±1.96^{Aa}	39.35±0.31^{Ab}	59.56±1.05^{Ab}	69.89±0.45^{Aa}	4.13±0.29^{ABa}	6.07±0.57^{Aa}
		Tryladil®	116.59±2.79^{Aa}	50.29±2.71^{Aa}	92.90±1.87^{Aa}	43.41±0.29^{Aa}	59.36±0.85^{Aa}	80.36±0.64^{Aa}	3.3±0.31^{Aa}	3.06±0.27^{Ab}
	G2	Andromed®	44.21±0.66^{Ab}	12.46±0.35^{Bb}	25.51±0.45^{Ab}	28.11±0.19^{Cb}	47.28±0.91^{Cb}	58.57±0.54^{Cb}	2.36±0.03^{Bb}	4.62±0.07^{Aa}
		Milk-egg yolk	37.59±0.71^{Bb}	17.55±0.43^{Ab}	25.97±0.49^{Ab}	49.00±0.21^{Aa}	65.64±0.92^{Aa}	71.23±0.36^{Aa}	1.86±0.03^{Ab}	4.78±0.08^{Ab}
		Tryladil®	33.92±0.80^{Cb}	13.32±0.47^{Bb}	22.26±0.62^{Bb}	37.19±0.22^{Bb}	57.09±1.01^{Bb}	64.35±0.45^{Bb}	1.80±0.03^{Ab}	3.69±0.08^{Ba}
Sp2	G1	Andromed®	149.54±0.77^{Ca}	78.00±1.19^{Ca}	122.87±0.87^{Ca}	52.33±0.15^{Cb}	61.21±0.21^{Cb}	82.20±0.41^{Ca}	4.26±0.04^{ABa}	7.75±0.09^{Ba}
		Milk-egg yolk	160.29±0.86^{Ba}	89.03±1.32^{Ba}	134.34±0.95^{Ba}	56.31±0.14^{Bb}	65.34±0.11^{Bb}	84.34±0.22^{Ba}	4.32±0.05^{Aa}	7.81±0.11^{Ba}
		Tryladil®	170.60±0.75^{Aa}	102.16±1.04^{Aa}	150.01±0.76^{Aa}	60.18±0.12^{Ab}	67.32±0.09^{Ab}	88.35±0.13^{Aa}	4.26±0.03^{Ba}	8.13±0.08^{Aa}
	G2	Andromed®	92.82±1.22^{Ab}	53.90±2.71^{Bb}	74.62±1.92^{Ab}	59.28±0.13^{Ba}	69.15±0.23^{Ba}	80.14±0.25^{Bb}	3.09±0.12^{Bb}	7.04±0.30^{Ab}
		Milk-egg yolk	83.39±1.35^{Bb}	55.49±1.92^{ABb}	70.73±1.40^{Ab}	68.21±0.22^{Aa}	78.21±0.19^{Aa}	85.33±0.21^{Aa}	2.61±0.08^{Ab}	7.52±0.22^{Ab}
		Tryladil®	86.45±1.24^{Bb}	61.78±1.75^{Ab}	73.32±1.29^{Ab}	72.51±0.41^{Aa}	83.36±0.17^{Aa}	85.45±0.19^{Ab}	2.66±0.08^{Ab}	7.09±0.29^{Aa}

G1: Rams (INRA180 sheep) were kept close to the lab; G2: Rams were kept 170 km away from the lab.
A, B, C: Different superscripts within columns indicate an effect of extender for ram group in each subpopulation ($P<0.05$).
a, b: Different superscripts within columns indicate an effect of ram group for each extender in each subpopulation ($P<0.05$).
VCL (µm/s-1), curvilinear velocity; VSL (µm/s-1), straight velocity; VAP (µm/s-1), velocity average path; LIN (%), linearity; STR (%), straightness; ALH (µm), lateral head displacement; BCF (Hz), beat cross frequency; WOB (%), Wobble.

normality in G1 were improved by Tryladil®, while these results were achieved for milk-egg yolk extender in G2. Likewise, Andromed® and Tryladil® were more appropriate for G1 than G2. On the other hand, milk-egg yolk extender was more suitable for G2 than G1. Similarly, Benmoula et al. (2018) reported that extenders containing skim milk allowed better preservation of INRA180 ram semen quality at 15 °C than those containing egg yolk or soya lecithin at 5 °C.

In artificial insemination centers, where daily collection schedules involve a large number of rams and/or where semen is being transported over long distances, a prolonged period of equilibration is necessary. In both cases, cooling the samples within the first 4 h after semen collection leads to reduce chilling injury. Our findings showed that the effects of prolonged holding time on extended semen at 15 °C during transport were less marked than expected. In previous study, Purdy et al. (2010) indicated that ram sperm can be held at 5 °C for 24 h prior to cryopreservation without altering sperm quality and fertility. However, controversial results are reported for many species concerning spermatozoa held for long time at different temperatures. In bull, holding sperm for up to 18 h at 5 °C and from 6 to 72 h at 4 °C prior to cryopreservation did not decrease the post-thaw motile sperm proportions (Foote and Kaprotht, 2002; Murphy et al., 2018), nor its fertility (Graham et al., 1957). In addition, boar sperm are frequently held for periods of time up to 24 h at reduced temperatures (15 °C) prior to cryopreservation without showing any differences in *in vitro* oocyte penetration (Eriksson et al., 2001). However, pregnancy rates determined after insemination with frozen-thawed boar sperm held for either 3 or 24 h at 15 °C declined overtime and the longer holding time resulted in a decrease in embryo number from 15 to 9 (Guthrie and Welch, 2005). Similarly, preserving stallion semen at low temperatures (5 or 15 °C) for 18 h prior to cryopreservation significantly reduced the total and progressive motility after thawing compared to samples frozen immediately after cooling to 5 °C (Backman et al., 2004). According to Leboeuf et al. (2000) , rapid cooling of the extended semen from 30 to 15° C may not affect sperm survival. However, Watson (2000) reported that the fast cooling from 30 to 10, 5 or 0° C, caused injuries to some sperm cells, called "cold shock". This phenomenon is more pronounced in boar, but it also occurs in ruminant's spermatozoa. During cooling process, the temperature range of 5–15 °C is the most critical for cell damage. This is more related to changes in plasma membrane fatty acid composition and lipid class ratios (Dorbnis et

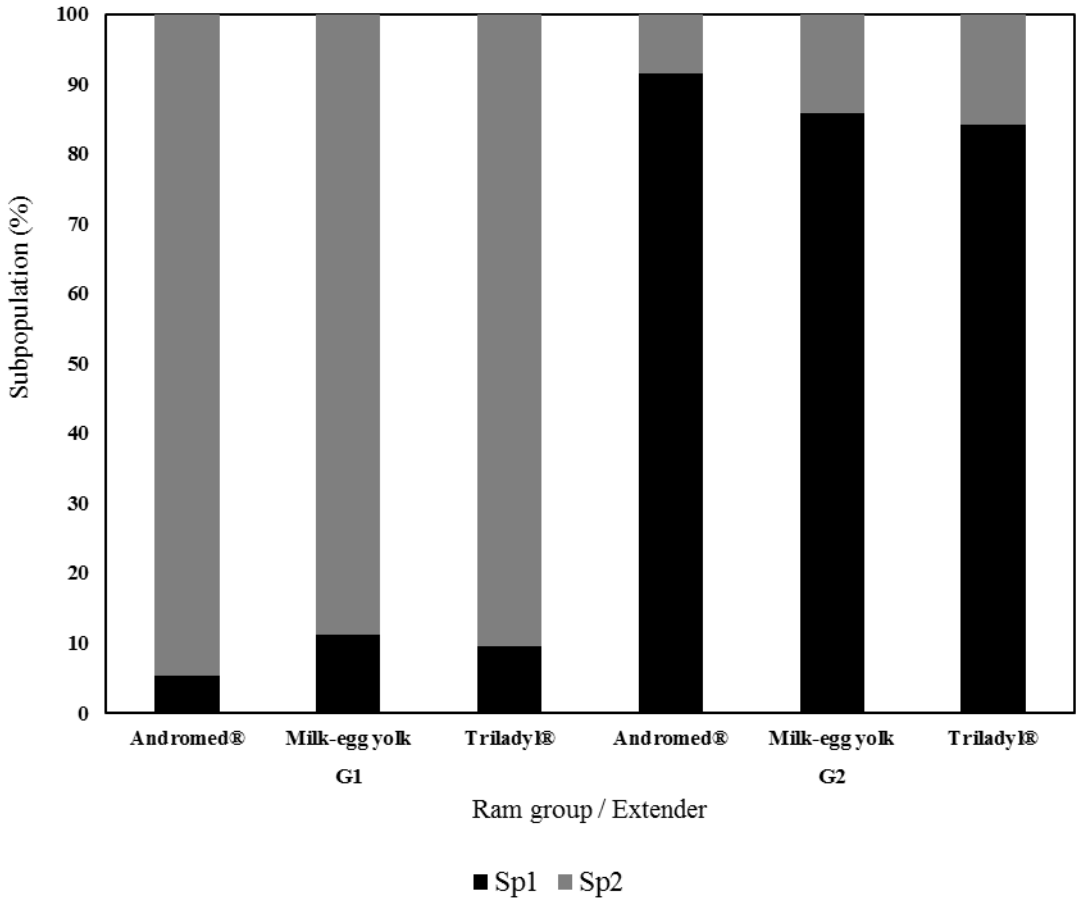

Figure 1. The percentage of sperm subpopulations (Sp1, Sp2) in each ram group (G1 vs G2) extended in Andromed®, Colas and Tryladil®.
G1: Rams (INRA180 sheep) were kept close to the lab; G2: Rams were kept 170 km away from the lab.

al., 1993). In addition, the transport might be another factor impacting semen cryopreservation. It is known that when sperm is transported in a vehicle, it is exposed to continuous agitation leading to oxygen incorporation into the medium. This will probably cause lipid peroxidation and a decline in semen quality (Vichas et al., 2018). Thus, the choice of a good extender remains a crucial step in reducing the undesirable effects of sperm transport.

Our findings showed that in the first subpopulation all extenders had the same efficiency in maintaining the motility of sperm in G1 group. However, milk-egg yolk extender recorded the best CASA variables compared to Andromed® and Tryladil® in G2 rams. In the second subpopulation, Tryladil® preserved the G1 sperm more efficiently, and milk-egg yolk extender and Tryladil were superior in G2 rams. The PM, TM, viability and abnormality in G1 were improved by Andromed® compared to milk-egg yolk extender and Tryladil®, while in G2, milk-egg yolk extender and Andromed® were superior to Tryladil. Extender composition leads to stabilization of the cell membrane during freezing and thawing processes (Nur et al., 2010). Several researchers have developed different extender compositions and protocols for freezing ram semen (Soylu et al., 2007). Gil et al. (2003) recommended that egg yolk concentration not higher than 5-10% supports frozen-thawed ram semen characteristics. However, taking into account that soybean lecithin is safer than egg yolk concerning, the risk of microbial contamination (Zhang et al., 2009) and the absence of tris cytotoxicity effects, it is widely used as an alternative to egg yolk in ram semen diluents (Benmoula et al., 2018). In both cases, it has been suggested that phospholipids from egg yolk or soybean lecithin might integrate in the sperm membrane. It forms a protective film against the formation of intracellular ice crystals which protects the sperm membrane from

mechanical damages during the freeze-thawing process (Amirat et al., 2004). Moreover, milk-based extenders are also used, but to avoid above cited contaminations, Bioexcell® (free extender of animal origin; IMV, L' Aigle, France) was succefully used as an. Nonetheless, there was no difference in pregnancy rates after intracervical insemination of ewes with frozen-thawed semen in milk-egg yolk and BioXcell® extenders (Gil et al., 2003). In addition, other studies did not demonstrate any significant variation among pregnancy rates of ewes inseminated with semen stored in Andromed®, milk, and egg yolk extenders (Fukui et al., 2008).

The multivariate cluster analysis of data from the eight post-thaw CASA-Mot variables in the present study allowed us to distinguish tow subpopulations. The first subpopulation (SP1) included spermatozoa characterized by low velocity (VCL, VSL and VAP), low linear trajectories (LIN and STR) and low ALH, WOB and BCF. This subpopulation represented 36.67% of the total sample. The second subpopulation (SP2) included spermatozoa showing high speed (VCL, VSL and VAP) and characterized with high linear trajectories (LIN and STR) and high ALH, WOB and BCF. The cluster approach confirmed that post-thawed samples were heterogeneous as they contained spermatozoa with different motility patterns (Contri et al., 2012; Kanuga et al., 2012). In G1, whatever the extender, the relative size of SP1 was lower than SP2. While, in G2 the percentage of SP1 was higher than SP2. This means that holding semen for 4 h at 15°C prior to cryopreservation had caused a loss of movement for most post-thawed spermatozoa.

Conclusions

The present study shows that changing the equilibration conditions (from 2 h at 5 °C to 4 h at 15 °C) can extend the total processing time and causes a decrease in post-thawed semen quality. Furthermore, the choice of extender was found to have a great influence on sperm subpopulation distribution depending on the equilibration methods. The conventional semen cryopreservation (2 h at 5 °C prior to cryopreservation) leads to subpopulations dominated with rapid motility cells. However, the modified storage procedure (4 h at 15 °C prior to cryopreservation) resulted in dominating cells with slow motility. Based on the findings of this work, it has to be emphasized that the combination of the CASA system parameters and the multivariate cluster analysis can provide a greater understanding of the sperm behavior during cryopreservation. Further studies are needed for determining the contribution of each sperm subpopulation on ram semen fertility.

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