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RESEARCH ARTICLE

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Effect of short-term conservation temperature, with or without centrifugation, on the survival and motility of Catalonian donkey spermatozoa

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Abstract

Aim of study: To analyze the effect of three short-term storage temperatures with or without removing seminal plasma on the survival and motility of donkey sperm and the response to refrigeration and centrifugation of the different spermatozoa subpopulations.

Area of study: North-eastern Spain (Catalonia).

Material and methods: Semen from seven Catalonian jackasses was diluted with a skimmed milk-based (Kenney) extender and different treatments were obtained: FRESH semen, FRESH semen immediately centrifuged to remove the seminal plasma before resuspension in Kenney extender (FRESH+CENTRIFUGATION), FRESH semen stored at 5/15/20ºC for 2 h (STORAGE 5/15/20ºC), and STORAGE 5/15/20ºC semen then centrifuged (STORAGE 5/15/20ºC+CENTRIFUGATION). Survival was examined using eosin-nigrosin stained smears. Motion was assessed by means of a computer-assisted sperm analyzer (CASA).

Main results: The spermatozoa of the STORAGE 5ºC and 20ºC showed an overall motility similar to that seen in FRESH samples. However, the STORAGE 15ºC led to an important motility reduction. No differences were seen between the FRESH and STORAGE 5/15/20ºC with respect to progressive motility. However, STORAGE 5/15/20ºC+CENTRIFUGATION all reduced total motility, and STORAGE 15ºC+CENTRIFUGATION led to reduced survival. The sperm motile subpopulations structure of donkey semen was maintained after STORAGE 5/15/20ºC+CENTRIFUGATION, although STORAGE 15ºC+CENTRIFUGATION led to important changes. STORAGE 5/20ºC+CENTRIFUGATION, in contrast, only induced slight changes. STORAGE 20ºC+CENTRIFUGATION was associated with no change in the percentage of sperm cells belonging to each Subpopulation compared to FRESH sperm.

Research highlights 2 h of storage at 20ºC followed by centrifugation is suitable for the short-term storage of donkey semen.

Additional key words: jackass, semen preservation, semen storage.

Abbreviations used: ALH (mean lateral head displacement); BCF (frequency of head displacement); LIN (linearity coefficient); spz (spermatozoa); STR (straightness coefficient); VAP (average path velocity); VCL (curvilinear velocity); VSL (linear velocity); WOB (Wobble coefficient)

Authors' contributions: Both authors contributed in the design, experimental phase, data and results analysis, and writing of the manuscript.

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Introduction

The interest on donkey knowledge has showed an important increase in recent years. New uses, such as onotherapy, ecotourism, silviculture, or as companion animals, are increasing. On the other hand, there has been an increased global demand for donkey products, donkey milk and milk products as alternative in milk allergic infants or adults, donkey milk-based cosmetics, meat, or skin to obtain a donkey-hide gelatin (ejiao) used in the traditional Chinese medicine.

The Catalonian donkey breed has suffered a substantial reduction in its numbers, a consequence of the intense mechanization of agriculture (Aranguren *et al*., 2001). This could lead to high levels of inbreeding, which would only increase the risk of the breed's extinction. The use of reproductive technologies and the setting up of gene banks can, however, contribute to

the preservation of endangered species, and might help the Catalonian donkey. In this respect, the optimization of the cooling of collected semen for transport would facilitate the involvement of animals far from facilities where artificial insemination or semen cryopreservation are performed.

Cooling semen reduces the metabolic activity of the spermatozoa it contains, reduces microbial growth, and helps to extend the time over which the viability of sperm is maintained. However, cooling between 18 and 8ºC is a critical step that can lead to "cold shock", a problem associated with damage to sperm cell plasma membranes (Amann & Pickett, 1987). Indeed, semen processing involves a number of factors that can cause such damage, including the addition of semen extender, centrifugation and storage (Aurich, 2005). Damage to the plasma membrane results in the irreversible loss of its functions. A loss of motility and fertilizing capacity can be caused, at least in part, by the peroxidation of lipids in the membrane (Papas *et al.*, 2019). Injuries can be reduced if the cooling rate is slow (<0.3ºC/min), but there is no real consensus regarding the optimal final storage temperature for the liquid preservation of equine spermatozoa. Temperatures as low as 4-6°C have been reported to provide better environments for the maintenance of motility (Varner *et al*., 1989; Moran *et al*., 1992) and fertility (Squires *et al*., 1988), but some authors (Province *et al*., 1985) indicate temperatures of 15 or even 20ºC to better maintain motility and fertility (if storage is no longer than 12 h).

The centrifugation of equine semen is necessary to limit the harmful effects of seminal plasma on sperm motility during storage (Miró & Papas, 2018). It is also necessary for the addition of cryoprotectants and the adjustment of sperm concentrations before freezing (Vidament *et al*., 2000). Centrifugation has to be performed with great care since damage can result from the mechanical forces induced by the close packing of the spermatozoa. This can be manifested as structural damage of the sperm acrosome and an important loss of motility and enzymatic activity (Matás *et al*., 2007). Some studies have assessed the use of different extenders, storage temperatures and the elimination of seminal plasma by centrifugation as means of better preserving donkey semen (Mello *et al*., 2000; Cottorello *et al*., 2002; Serres *et al*., 2002; Rota *et al*., 2008; Miró *et al*., 2009).

The aim of the present study was to determine the effect of three storage temperatures (5, 15 and 20ºC for 2 h) with and without centrifugation on donkey sperm survival and motility, and to study the changes induced by these treatments in the different sperm subpopulations.

Material and methods

Animals and semen collection

A total of 35 ejaculates were obtained from seven healthy, mature Catalonian donkeys aged 4-6 years, all of which were previously reported as fertile (5 ejaculates/donkey). Animals were housed at the Experimental Farm and Countryside Service of the School of Veterinary Medicine of the Autonomous University of Barcelona (Bellaterra, Spain). Semen was collected using an artificial vagina (Hannover model) with an in-line gel filter to allow the collection of gel-free semen. Ejaculates were obtained at 2-3 day intervals in the presence of an ovariectomized female donkey. Immediately after collection, the gel-free semen was diluted (proportion 1:5, v/v) with dry skimmed milk extender (24 mg/mL dry skimmed milk and 49 mg/mL glucose) kept at 37ºC in a water bath. The diluted semen was immediately cooled in a water bath to 20ºC before all other treatments.

Storage conditions and centrifugation

One aliquot of freshly collected semen was immediately analyzed (rewarmed to 37ºC) for sperm survival and motility (FRESH). A further aliquot was immediately centrifuged at 660 *g* for 15 min at 20ºC to remove the seminal plasma. The pellet obtained was resuspended with skimmed milk-based (Kenney) extender (skimmed milk + glucose) to a final concentration of 200×10^6 live spz (spermatozoa) per mililiter, incubated at 37ºC for 5 min and reanalyzed for sperm survival and motility (FRESH+CENTRIFUGATION). Other aliquots of each sample were maintained at 5ºC, 15ºC or 20ºC under anaerobic conditions for 2 h. The average cooling rate was 0.25ºC/min. They were then re-evaluated at 37ºC for viability and motility (STORAGE 5/15/20ºC) before being centrifuged at 660 *g* for 15 min at the corresponding storage temperature in a programmable refrigerated centrifuge (Medifriger BL-S; JP Selecta; Barcelona, Spain). The supernatant was eliminated and the sperm re-suspended in Kenney extender to a final concentration of 200×10^6 live spz/mL. These centrifuged samples were then incubated in a water bath at 37ºC for 15 min and viability and motility re-evaluated once more (STORAGE 5/15/20ºC+CENTRIFUGATION).

Analysis of semen quality variables

Fresh semen was subjected to standard analysis to determine the sperm concentration, total sperm number,

sperm survival, morphological abnormalities and motility. After the different treatments each sample was analyzed to determine sperm cell survival and motility. Sperm concentration and total sperm number were determined using a Neubauer hemocytometer. Sperm survival and total morphological abnormalities were determined by eosin-nigrosin staining as described by Bamba (1988). Viable spermatozoa show uniform white staining over the entire cell; the presence of a partial or totally pinkish stain is indicative of non-viable sperm cells. This determination was made after examining a minimum of 200 spermatozoa/sample by light microscopy (magnification: 1000x).

The motion characteristics of the samples were determined using a computer-assisted sperm analyzer (CASA, ISAS v1.0; Proiser SL, Valencia, Spain). An aliquot of each sample of treated semen was incubated for 5 min in a water bath at 37ºC. Drops (5 µL) of each sample were observed using a phase contrast microscope with a heatable stage (37ºC). Three fields per drop were analyzed. The CASA system is based on the analysis of 50 consecutive, digital images of a single field at a magnification of 200x (dark ground). The settings for the system were: 50 images acquired over 1 s (1 every 20 ms), minimum contrast 80, and minimum cell size 4 pixels. Total motility was defined as the percentage of spermatozoa with an average path velocity (VAP) of >10 µm/s. Progressive motility was defined as the percentage of spermatozoa with a VAP of >90 µm/s plus a straightness coefficient (STR) of >75%.

The sperm motility descriptors obtained by the CASA system are indicated in Table 1.

Statistical analysis

The results were analyzed using the SAS statistical package (SAS Inst., 2000). Normality was assessed by the Shapiro-Wilks test (W) included in the UNIVARIATE procedure. The FASTCLUS clustering procedure (which performs a disjointed cluster analysis based on Euclidean distances calculated from one or more quantitative variables - in this case the sperm motility variables measured by the CASA system) was then used to separate the spermatozoa into subpopulations. Sperm cells were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster. The PROC GLM procedure was used to detect differences between the values for the sperm motility descriptors of these subpopulations. The LSMEANS procedure was used to determine the degree of significance of these differences. A Chi-squared test was used to examine the percentage of sperm cells belonging to each cell subpopulation after each treatment. New PROC GLM and LSMEANS procedures were used to determine and list, respectively, any differences in the number of sperm cells belonging to the different subpopulations after the different treatments. The total number of spermatozoa analyzed following this protocol was 14,860.

Results

Quality of refrigerated and centrifuged donkey semen

Table 2 shows the quality of the fresh semen and the characteristics of the semen samples processed under the different conservation and centrifugation conditions. No differences were seen between the FRESH and FRESH+CENTRIFUGATION sperm in terms of progressive motility or survival. However, the LIN (linearity coefficient) and STR (straightness coefficient); values of the FRESH samples were significantly smaller, and the VCL (curvilinear velocity) value significantly higher than those of the FRESH+CENTRIFUGATION samples ($p<0.05$).

No significant differences were seen in sperm survival or progressive or total motility between the STORED 5/15/20ºC and FRESH treatments. The CASA system showed the overall motility associated with the

Table 1. The sperm motility descriptors obtained by the CASA system are:

Curvilinear velocity (VCL)	µm/s	Measures the sequential progression along the true trajectory
Linear velocity (VSL)	µm/s	Measures the straight trajectory of the spermatozoa per unit time
Average path velocity (VAP)	µm/s	Measures the mean trajectory of the spermatozoa per unit time
Linearity coefficient (LIN)	%	(VSL/VCL) × 100
Straightness coefficient (STR)	%	(VSL/VAP) × 100
Wobble coefficient (WOB)	%	(VAP/VCL) × 100
Mean lateral head displacement (ALH)	µm	Measures the mean head displacement along the curvilinear trajectory
Frequency of head displacement (BCF)	Hz	Frequency with which the sperm trajectory crosses the average path trajectory

Table 2. Semen quality analysis before and after centrifugation at different storage temperatures. Results are expressed as means ± SEM of 34 different experiments with a total analyzed sperm number of 14,860.

	FRESH	FRESH+ CTR	STORAGE					
			5ºC	5ºC+CTR	15ºC	15ºC+CTR	20ºC	20ºC+CTR
Concentration (cells × 10/mL)	598.44±229.40							
Abnormalities (%)	15.91±5.73							
Viability (%)	71.0±3.8^{a}	69.7±4.0^{ab}	71.9±4.1^{a}	65.2±4.7^{ab}	68.0±3.7^{ab}	63.1±4.0^{b}	73.3±3.7^{a}	69.0±4.1^{ab}
Total motility (%)	94.8±1.4^{a}	88.6±2.7^{ab}	86.4±3.5^{ab}	78.0±4.5^{b}	88.0±1.9^{ab}	82.1±3.1^{bc}	92.6±2.4^{ac}	82.6±3.2^{bc}
Progressive motility (%)	50.4±2.6	44.0±3.0	41.1±3.1	38.6±3.7	41.8±2.6	41.3±3.2	48.8±3.2	38.5±2.8
VCL (µm/sec)	114.2±0.5^{ae}	112.2±0.5^{b}	112.8±0.7^{ab}	114.1±0.7^{abe}	109.2±0.5^{c}	107.4±0.6^{c}	117.8±0.6^{d}	115.4±0.6^{e}
VSL (µm/sec)	72.1±0.4^{a}	72.3±0.5^{a,c}	68.8±0.6^{b}	70.7±0.6^{a,b}	74.1±0.5^{c}	65.6±0.6^{d}	72.0±0.5^{a,e}	70.2±0.5^{b,e}
VAP (µm/sec)	91.2±0.4^{a}	89.6±0.5^{a}	89.5±0.6^{a}	91.0±0.6^{a,c}	89.9±0.5^{a}	84.6±0.5^{b}	93.0±0.5^{c}	91.1±0.5^{a}
LIN (%)	58.3±0.4^{a,c}	60.1±0.5^{b}	56.6±0.6^{a,d}	59.3±0.6^{b,c,e}	61.0±0.5^{b}	58.0±0.5^{a,d,e}	57.0±0.5^{a,d}	56.5±0.5^{d}
STR (%)	74.6±0.4^{a,c}	76.8±0.5^{b}	72.8±0.6^{c,d}	75.6±0.6^{a,b}	76.7±0.5^{b}	74.8±0.5^{a,c,d}	73.6±0.5^{c,d}	73.4±0.5^{d}
WOB (%)	75.7±0.3^{a,c}	76.2±0.3^{a,b}	75.2±0.4^{a,c}	76.5±0.4^{a,b}	77.2±0.3^{b}	75.3±0.4^{a,c}	75.3±0.4^{a,c}	74.9±0.4^{c}
Mean ALH (µm)	3.50±0.02^{a}	3.44±0.03^{a}	3.48±0.03^{a}	3.50±0.04^{a,d}	3.20±0.03^{b}	3.40±0.03^{a}	3.66±0.03^{c}	3.61±0.03^{c,d}
BCF (Hz)	6.72±0.07^{a,c,d}	7.01±0.08^{a,b}	6.96±0.10^{a,b,c}	7.15±0.10^{b}	6.48±0.08^{d}	6.65±0.10^{c,d}	6.67±0.09^{c,d}	6.66±0.09^{c,d}

VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement, BCF: frequency of head displacement; CTR, centrifugation. Different superscripts between rows indicate significant differences (p<0.05).

FRESH and STORAGE 5ºC treatments to be very similar; in fact, only the VSL (linear velocity) showed a significant reduction in the latter treatment (from 72.1 µm/s in FRESH semen to 68.8 µm/s). The STORED 15ºC treatment was associated with significant reductions in the VCL and mean ALH values (VCL falling from 114.2 µm/s in FRESH semen to 109.2 µm/s, and mean ALH (mean lateral head displacement) falling from 3.50 µm to 3.20 µm). The same treatment was also associated with an overall motility more linear than that seen at other temperatures, as the increases in the VSL, LIN, STR and WOB (Wobble coefficient) values show. The STORAGE 20ºC treatment was associated with a significant increase in VCL, VAP and mean ALH.

After 2 h of storage at the different temperatures, centrifugation had different effects on viability and progressive motility. No differences were seen in terms of these variables between the STORED 5ºC+CENTRIFUGATION and FRESH treatments, nor between the STORED 20ºC+CENTRIFUGATION and FRESH treatments. However, the STORED 15ºC+CENTRIFUGATION treatment was associated with a significant reduction in survival (p<0.05). In addition, all the STORED 5/15/20ºC+CENTRIFUGATION treatments were associated with a significant reduction in total motility compared to the FRESH treatment, although no differences were seen with respect to progressive motility. No differences were seen between the STORED 5ºC+CENTRIFUGATION and FRESH treatments in terms of sperm motion characteristics, except for BCF (frequency of head displacement), which was significantly increased. In contrast, the STORED 15ºC+CENTRIFUGATION treatment was associated with important changes in velocity characteristics. The values of all the velocity variables were significantly lower than in FRESH sperm. Finally, the STORED 20ºC+CENTRIFUGATION treatment was associated with significant reductions in the VSL, LIN and STR values compared to FRESH samples, while a significant increase was observed in mean ALH.

Sperm Subpopulation structure

The CASA system revealed that the FRESH samples showed the typical four-Subpopulation structure.

—*Subpopulation 1:* This Subpopulation showed the highest VCL value (170.51±0.84 µm/s) and the highest velocity and linearity characteristics, as indicated by the VCL, VSL, VAP, LIN and STR values. The oscillatory movement of the spermatozoa was also very notable, as indicated by the high WOB, mean ALH and BCF values. This Subpopulation made up 28.0 ± 2.6% of all sperm cells (Table 3).

—*Subpopulation 2:* This Subpopulation had a high VCL value (160.52±0.95 µm/s). Its cells showed high velocity and low linearity, as indicated by the low LIN

and STR values. They also showed quite notable oscillatory movement, as indicated by the WOB, mean ALH and BCF values. This Subpopulation made up 20.3 ± 2.1% of all sperm cells (Table 3).

—*Subpopulation 3:* This subpopulation had medium VCL values (93.76±0.87 µm/s). The cells showed a medium velocity (as indicated by the VCL, VSL and VAP values), medium linearity, and notable oscillatory movement (as indicated by their WOB, mean ALH and BCF values). This Subpopulation made up 23.6 ± 1.5% of all sperm cells (Table 3).

—*Subpopulation 4:* This Subpopulation showed the lowest VCL values (31.89 ± 0.78 µm/s), as well as low VSL and VAP values, reduced oscillatory movement (as indicated by the WOB, mean ALH and BCF values) and low linearity (as indicated by the values for LIN and STR). This subpopulation made up 28.1 ± 3.4% of all sperm cells (Table 3).

Table 3. Motility variables of the different sperm subpopulations. Results are expressed as means ± SEM of 34 different experiments with a total number of analyzed spermatozoa of 14,860.

	FRESH	FRESH+CTR	STORAGE 5ºC	STORAGE 5ºC+CTR	STORAGE 15ºC	STORAGE 15ºC+CTR	STORAGE 20ºC	STORAGE 20ºC+CTR
Subpopulation 1								
VCL (µm/s)	170.51±0.84^{a}	167.92±0.97^{a}	167.07±1.16^{ab}	167.14±1.29^{ab}	177.03±1.18^{c}	161.65±1.06^{b}	172.38±0.94^{ac}	167.84±1.06^{a}
VSL (µm/s)	139.73±0.76^{a}	138.77±0.87^{ab}	134.95±1.05^{bc}	136.16±1.17^{ac}	146.61±1.07^{d}	130.74±0.96^{c}	140.26±0.85^{a}	135.77±0.96^{ab}
VAP (µm/s)	153.14±0.72^{ab}	150.76±0.83^{abc}	148.56±0.99^{cd}	148.33±1.10^{acd}	160.32±1.01^{e}	143.67±0.91^{d}	153.91±0.80^{b}	148.80±0.90^{c}
LIN (%)	82.55±0.72	83.43±0.83	81.66±1.00	81.99±1.11	83.60±1.01	81.96±0.91	81.88±0.81	81.52±0.91
STR (%)	91.33±0.72	92.30±0.83	90.65±1.00	92.01±1.11	91.53±1.02	91.23±0.92	91.14±0.81	91.32±0.91
WOB (%)	90.20±0.53	90.25±0.61	89.05±0.73	88.95±0.81	90.99±0.74	89.55±0.66	89.62±0.59	89.02±0.66
Mean ALH (µm)	3.89±0.04	3.81±0.05	3.82±0.06	3.94±0.07	3.82±0.06	3.78±0.06	3.89±0.05	3.98±0.06
BCF (Hz)	8.32±0.13	8.44±0.15	8.93±0.18	8.87±0.20	8.25±0.18	8.04±0.16	8.44±0.15	8.39±0.17
Subpopulation 2								
VCL (µm/s)	160.52±0.95^{a}	153.88±1.23^{bc}	157.25±1.57^{ac}	159.95±1.85^{ac}	148.00±1.01^{d}	147.15±1.57^{bd}	163.01±1.24^{a}	164.64±1.33^{a}
VSL (µm/s)	77.38±0.86^{a}	75.00±1.11^{ab}	68.40±1.42^{bc}	65.28±1.67^{ce}	87.13±0.91^{d}	59.79±1.42^{e}	72.08±1.12^{abc}	71.80±1.20^{bc}
VAP (µm/s)	119.87±0.81^{a}	113.35±1.05^{b}	117.23±1.34^{ab}	117.34±1.58^{ab}	117.47±0.86^{ab}	105.93±1.34^{c}	120.15±1.05^{a}	122.15±1.14^{a}
LIN (%)	49.23±0.81^{a}	49.36±1.06^{a}	44.15±1.35^{ab}	41.21±1.58^{b}	61.03±0.87^{c}	41.31±1.34^{b}	45.05±1.06^{ab}	44.00±1.14^{ab}
STR (%)	65.52±0.82^{ad}	66.91±1.06^{a}	59.14±1.35^{b}	56.48±1.59^{b}	75.41±0.87^{c}	57.94±1.35^{b}	61.19±1.07^{bd}	59.47±1.15^{b}
WOB (%)	75.07±0.59^{a}	74.30±0.77^{a}	74.72±0.98^{a}	73.66±1.16^{a}	80.29±0.63^{b}	72.09±0.98^{a}	74.11±0.77^{a}	74.55±0.83^{a}
Mean ALH (µm)	5.31±0.05^{a}	5.25±0.06^{a}	5.23±0.08^{a}	5.41±0.10^{a}	4.55±0.05^{b}	5.20±0.08^{a}	5.52±0.06^{a}	5.47±0.07^{a}
BCF (Hz)	6.56±0.15	7.03±0.19	6.84±0.25	6.67±0.29	6.95±0.16	6.21±0.24	6.25±0.19	6.33±0.21
Subpopulation 3								
VCL (µm/s)	93.76±0.87^{a}	95.20±0.90^{a}	96.45±1.06^{ab}	100.58±1.01^{ab}	85.14±0.94^{b}	90.14±0.94^{a}	99.53±0.97^{c}	97.61±0.96^{a}
VSL (µm/s)	60.39±0.79^{ab}	63.25±0.81^{a}	61.84±0.96^{ab}	69.99±0.91^{ab}	53.58±0.85^{b}	60.72±0.85^{ab}	62.84±0.87^{a}	62.86±0.87^{ab}
VAP (µm/s)	73.51±0.74^{ab}	75.13±0.77^{a}	74.73±0.90^{ab}	80.64±0.86^{ab}	66.17±0.81^{b}	70.56±0.81^{ab}	76.71±0.83^{a}	75.47±0.82^{ab}
LIN (%)	65.51±0.75^{a}	67.29±0.77^{b}	65.20±0.91^{a}	70.36±0.87^{c}	64.30±0.81^{a}	68.67±0.81^{b}	63.84±0.83^{ab}	65.89±0.82^{a}
STR (%)	82.08±0.75^{a}	83.95±0.77^{bc}	82.60±0.91^{a}	86.67±0.87^{c}	81.24±0.81^{a}	86.28±0.81^{b}	81.48±0.83^{ab}	83.37±0.83^{a}
WOB (%)	79.09±0.55^{ad}	79.45±0.56^{bc}	78.37±0.66^{ab}	80.68±0.63^{c}	78.51±0.59^{ab}	79.14±0.59^{bcd}	77.71±0.61^{ab}	78.39±0.60^{a}
Mean ALH (µm)	3.26±0.05^{ab}	3.19±0.05^{ac}	3.36±0.06^{ac}	3.17±0.05^{a}	3.07±0.05^{a}	3.13±0.05^{ac}	3.51±0.05^{b}	3.40±0.05^{bc}
BCF (Hz)	6.62±0.14^{ab}	7.15±0.14^{a}	6.96±0.16^{ab}	7.75±0.16^{ab}	5.88±0.15^{b}	7.30±0.15^{ab}	6.43±0.15^{a}	6.96±0.15^{ab}
Subpopulation 4								
VCL (µm/s)	31.89±0.78^{ad}	31.77±0.69^{ae}	30.25±0.86^{ab}	28.62±0.76^{b}	26.66±0.73^{c}	30.65±0.66^{d}	36.34±0.83^{be}	31.68±0.71^{ab}
VSL (µm/s)	10.74±0.70^{a}	12.29±0.63^{a}	9.86±0.78^{a}	11.53±0.68^{b}	9.03±0.66^{c}	11.07±0.60^{a}	12.65±0.75^{a}	10.31±0.64^{a}
VAP (µm/s)	18.38±0.66^{ad}	19.32±0.59^{a}	17.30±0.74^{ad}	17.63±0.65^{b}	15.49±0.62^{c}	18.11±0.56^{d}	21.33±0.71^{ab}	18.05±0.61^{a}
LIN (%)	35.85±0.67^{ac}	40.10±0.60^{ab}	35.33±0.74^{ac}	43.43±0.65^{b}	35.22±0.62^{a}	39.96±0.57^{bc}	37.31±0.71^{a}	34.71±0.61^{ac}
STR (%)	59.63±0.67^{a}	63.96±0.60^{ab}	58.96±0.75^{ab}	67.04±0.66^{b}	58.68±0.63^{a}	63.73±0.57^{b}	60.70±0.72^{a}	59.29±0.62^{ab}
WOB (%)	58.45±0.49	60.95±0.43	58.60±0.54	62.74±0.48	58.79±0.46	60.59±0.41	59.74±0.52	57.63±0.45
Mean ALH (µm)	1.55±0.04^{ab}	1.51±0.04^{abd}	1.52±0.05^{ac}	1.38±0.04^{abc}	1.36±0.04^{b}	1.50±0.03^{ab}	1.74±0.04^{cd}	1.58±0.04^{d}
BCF (Hz)	5.40±0.12^{ad}	5.41±0.11^{abd}	5.13±0.14^{abd}	5.29±0.12^{b}	4.86±0.11^{c}	5.04±0.10^{ab}	5.55±0.13^{cd}	4.97±0.11^{ad}

CTR = centrifugation. Different superscripts between rows indicates significant (p<0.05) differences.

Refrigeration and later centrifugation induced different changes in the mean motion characteristics of each Subpopulation:

—*Subpopulation 1:* This subpopulation showed modifications only in terms of velocity variables, mainly after the STORAGE 15ºC+CENTRIFUGATION treatment. The STORAGE 15ºC treatment actually led to increases in the VCL, VSL and VAP values compared to the FRESH treatment, but later centrifugation led to their reduction. The STORAGE 5ºC treatment led to reduced VSL and VAP values, while the STORAGE 5ºC+CENTRIFUGATION treatment led to no significant differences in the values of velocity variables compared to the FRESH treatment. No difference in any motility variable was seen between the STORAGE 20ºC and FRESH treatments. Finally, the STORAGE 20ºC+CENTRIFUGATION treatment had no effect on the velocity variables except for VAP, which was significantly reduced.

—*Subpopulation 2:* This subpopulation suffered the most important changes in terms of motility variables. The STORAGE 5ºC treatment led to reductions in the VSL and STR values compared to the FRESH treatment. The STORAGE 5ºC+CENTRIFUGATION treatment was associated with the same changes plus a reduction in the LIN value. The STORAGE 15ºC treatment was associated with important changes in motility. The VCL and mean ALH values were reduced while the VSL, LIN, STR and WOB values increased. The STORAGE 15ºC+CENTRIFUGATION treatment led to a reduction in the values of VCL, VSL, VAP, LIN and STR. The STORAGE 20ºC treatment led to no changes in any variable compared to the FRESH treatment, while the STORAGE 20ºC+CENTRIFUGATION treatment was associated with reduced VSL and STR values.

—*Subpopulation 3:* No differences were seen in any motility variable between the STORAGE 5ºC and FRESH treatments. The STORAGE 5ºC+CENTRIFUGATION treatment, however, led to increases in the LIN, STR and WOB values. The STORAGE 15ºC treatment led to a reduction in VCL, while the STORAGE 15ºC+CENTRIFUGATION treatment induced an increase in sperm linearity, as indicated by the LIN and STR values. The STORAGE 20ºC treatment induced an increase in VCL values. The STORAGE 20ºC+CENTRIFUGATION treatment led to no changes in motility variables.

—*Subpopulation 4:* Changes in motility variables were induced in this Subpopulation, especially by storage at 5ºC and 15ºC. The STORAGE 15ºC treatment led to important reductions in all velocity variables (VCL, VSL and VAP) and in the mean ALH and BCF values compared to the FRESH treatment. However, in the STORAGE 15ºC+CENTRIFUGATION treatment only the STR values increased significantly. After the STORAGE 5ºC+CENTRIFUGATION treatment the motility characteristics of the sperm were similar to those of FRESH sperm. However, this treatment led to a reduction of all velocity variables and the BCF value, along with an increase in the LIN and STR values. The STORAGE 20ºC treatment led to increased VCL and mean ALH values; the STORAGE 20ºC+CENTRIFUGATION treatment also led to increased mean ALH values.

The percentage of sperm cells belonging to each Subpopulation experienced slight changes after the different treatments, with only Subpopulation 4 experiencing significant differences. Figure 1 shows that

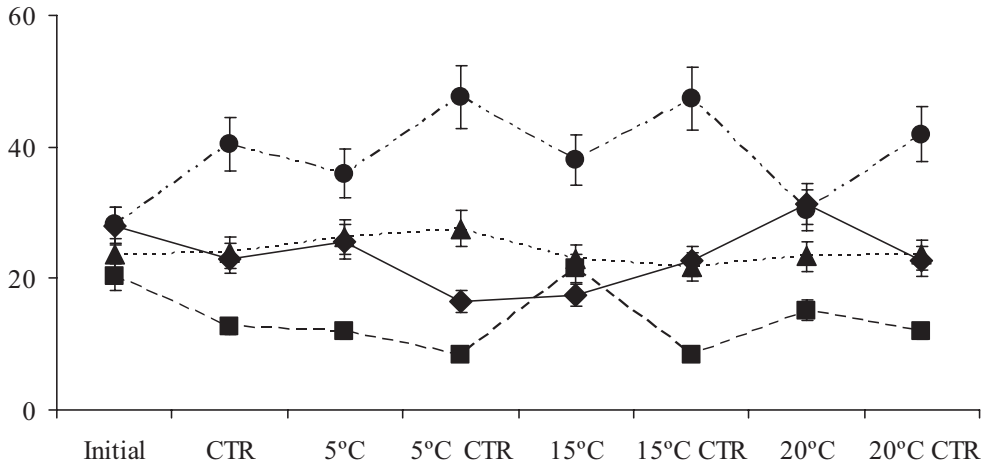

Figure 1. Number of cells belonging to each subpopulation before and after refrigeration and centrifugation. Results are means ± SEM for 34 different experiments. Superscripts indicate significant (p<0.05) differences between FRESH samples and after refrigeration/centrifugation treatment. ◆: Subpopulation 1. ■: Subpopulation 2. ▲: Subpopulation 3. ●: Subpopulation 4.

the STORAGE 5%+CENTRIFUGATION and STORAGE 15%+CENTRIFUGATION treatments significantly ($p<0.05$) increased the percentage of sperm cells in Subpopulation 4 (from 28.1±3.4% in FRESH samples to 47.6±4.7% and 47.4±3.5% respectively).

Discussion

Equine semen is commonly cooled to reduce the metabolic activity of the spermatozoa, to reduce microbial growth, and to maintain the viability of the sperm for extended periods of time. However, semen processing is known to damage the plasma membrane, contributing significantly to a loss of motility and fertilizing ability. Sometimes only a short storage period is required before artificial insemination or semen freezing, but no data are available on the best short-term storage conditions for donkey semen. Success in the use of cooled/stored semen depends on damage being avoided as far as possible since mature spermatozoa can no longer call upon repair mechanisms (Eddy & O'Brien, 1994). The decision was therefore made to evaluate sperm motility and survival after short-term storage at different temperatures followed by centrifugation (or not) at the same temperatures.

The optimum storage temperature for maintaining the motility and fertility of horse semen has previously been reported as 4-6ºC (Varner *et al*., 1989; Moran *et al*., 1992). However, other authors have reported 15 or 20ºC to be better than 5ºC for maintaining motility and fertility – although the duration of storage was between 4 and 12 h (Francl *et al*., 1987; Province *et al*., 1995). Cottorello *et al*. (2002) concluded that a 5ºC maintenance temperature and the use of modified Baken extender (10% egg yolk) was more appropriate than a 10ºC or 0ºC storage temperature for the preservation of donkey sperm motility *in vitro*, while Serres *et al*. (2002) reported it to be better preserved at 4ºC or 15ºC in the presence of INRA82 extender than at 20ºC. The latter authors also observed that the storage of donkey semen diluted with INRA 82 under aerobic conditions at 15ºC best maintained the integrity of the plasma membrane. The results of the present study indicate that, after 2 h of cool storage, survival, total motility and progressive motility were maintained at all the temperatures investigated, although 5ºC or 20ºC seem to be the best for maintaining the mean motion characteristics as determined by the CASA system. The discrepancies between the present results and those of other authors might be due to the use of different storage times, different diluents, aerobic conditions, or differences in the accuracy of sperm motility assessments.

Stallion semen is routinely centrifuged to reduce the harmful effect of seminal plasma and to adjust the sperm cell concentration before freezing. Certainly, an improvement in the maintenance of sperm motility in cooled stallion and indeed donkey semen has been observed after reducing the concentration of seminal plasma (Varner *et al*., 1987; Pruitt *et al*., 1993; Serres *et al*., 2002; Miró *et al*., 2009). However, Rota *et al*. (2008) reported higher total motility values and percentages of rapid spermatozoa in non-centrifuged donkey samples. These authors also observed that the removal of seminal plasma increased the number of spermatozoa showing high progressive motility, a consequence of the greater straightness of their tracks after 48 h of storage. In the present study, the FRESH+CENTRIFUGATION treatment had a significant, positive effect on sperm linearity and reduced the curvilinear velocity, in agreement with other authors (Rota *et al*., 2008).

Several authors (Cochran *et al*., 1984; Vidament *et al*., 2000; Crockett *et al*., 2001) have reported better post-thaw motility and fertility for stallion semen centrifuged at 22-25ºC and then cooled to 5ºC before freezing than for semen centrifuged at 5ºC. Backman *et al*. (2004) compared the post-thaw motility of ejaculates first cooled to 5ºC for 18 h and then centrifuged to that of ejaculates centrifuged at room temperature and then cooled to 5ºC for 18 h, and reported no differences with respect to post-thaw total and progressive motility. The present results show that after all STORAGE+CENTRIFUGATION treatments, total motility was significantly reduced, while progressive motility was not affected. Moreover, sperm viability decreased in the STORAGE 15ºC+CENTRIFUGATION treatment. The sperm in the STORAGE 5ºC+CENTRIFUGATION treatment showed mean motion values more similar to those of FRESH samples. These results are not comparable with those of other authors since, in the present study, post-thaw motility was not evaluated (Backman *et al*., 2004). Further studies are needed to determine the effect of storing and centrifuging donkey sperm at different temperatures on post-thaw motility and fertility.

Motile sperm subpopulations have been described in a large number of mammals, including the donkey (Abaigar *et al*., 1999; Rigau *et al*., 2001; Quintero-Moreno *et al*., 2003; Martínez-Pastor *et al*., 2005; Miró *et al*., 2005; Flores *et al*., 2008). Previous studies have established that motility changes induced by centrifugation, cooling or freezing/thawing procedures are linked to changes in specific motion variables and the percentage of sperm cells belonging to each Subpopulation (Flores *et al*., 2008; Miró *et al*., 2009). In the present study, the Subpopulation 4 structure of donkey semen was maintained after cooling and centrifugation at all the temperatures investigated. The STORAGE

15ºC+CENTRIFUGATION treatment was associated with important changes in the sperm motion characteristics, especially in Subpopulations 2 and 4. However, the STORAGE 5/20ºC+CENTRIFUGATION treatments induced only slight changes on the mean motion characteristics of each Subpopulation. No differences were seen with respect to FRESH sperm in terms of the percentage of sperm cells belonging to each Subpopulation when the semen was subjected to the STORAGE 20ºC+CENTRIFUGATION treatment; significant changes were only seen in the percentage of spermatozoa belonging to Subpopulation 4 after the STORAGE 5/15ºC+CENTRIFUGATION treatments. In conclusion, fresh donkey semen can be maintained in adequate condition for around 2 h if kept at 20ºC and then centrifuged. However, 5ºC would also appear to be an adequate storage temperature before centrifugation if, for some reason, such conditions were necessary or, possibly, if more semen transportation time were needed.

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