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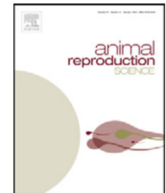

Undiluted or extended storage of ram epididymal spermatozoa as alternatives to refrigerating the whole epididymes

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ABSTRACT

The effect of storage procedure at 5 °C on the quality of ram spermatozoa from the cauda epididymis was analyzed. Two strategies were tested at 0, 24, 48 and 72 h post-mortem: (1) spermatozoa held in the epididymal fluid and stored either in the cauda epididymis (In-EPID) or in vitro (Ex-EPID), (2) epididymal spermatozoa extended in three media at 320, 370 and 420 mOsm/kg (D320, D370, D420). Analyzed parameters were: osmolality, pH, motility, acrosomal status and viability. In experiment 1, osmolality of the In-EPID samples, but not in Ex-EPID, increased with post-mortem time. Motility of In-EPID spermatozoa in samples, after 24 h post-mortem, was higher compared to the Ex-EPID samples, although differences decreased at 48 and 72 h. In experiment 2, total (TM) and progressive motility (PM) were not significantly affected by storage time for D320 and In-EPID samples. However, the motility of D370 and D420 samples significantly decreased with time. TM and PM of D320 were significantly higher than D370 and D420 at 72 h. At 24 h, sperm viability was higher for In-EPID (80.7 ± 3.4%) than for the extended samples (44.8 ± 2.9%, 37.7 ± 3.9% and 48.6 ± 6.0% for D320, D370 and D420, respectively), which also decreased faster with time. At 24 h, the percentage of damaged acrosomes was low and similar for the four methods of storage, but damaged acrosomes increased with time for D320 and D370. Storing the spermatozoa in the epididymis is a good strategy for maintaining sperm quality in ram, at least for 48 h. The D320 extender preserve motility of epididymal spermatozoa but does not protect the status of the acrosome.

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1. Introduction

In the last decade the conservation of rare breeds of domesticated species has been very difficult and many of them have disappeared or are going to become extinct (Canali, 2006). This loss of biodiversity has been due to a poor management policy of genetic resources. In Spain, there is a wide range of animal genetic resources due to its specific geographical and climatic characteristics. These resources are threatened by the introduction of alien breeds with high production rates, and as an example we can cite the 34 sheep breeds officially declared endangered. This situation has motivated the implementation of a National Programme for conservation, improvement and promotion of livestock breeds (Royal Decree 2129/2008). This programme recommended establishing a germplasm bank for certain sheep breeds with increased risk of losses of genetic variability. This bank would ideally contain germplasm systematically collected from living animals. However, genetically valuable animals may die unexpectedly, calling for a methodology to collect their germplasm

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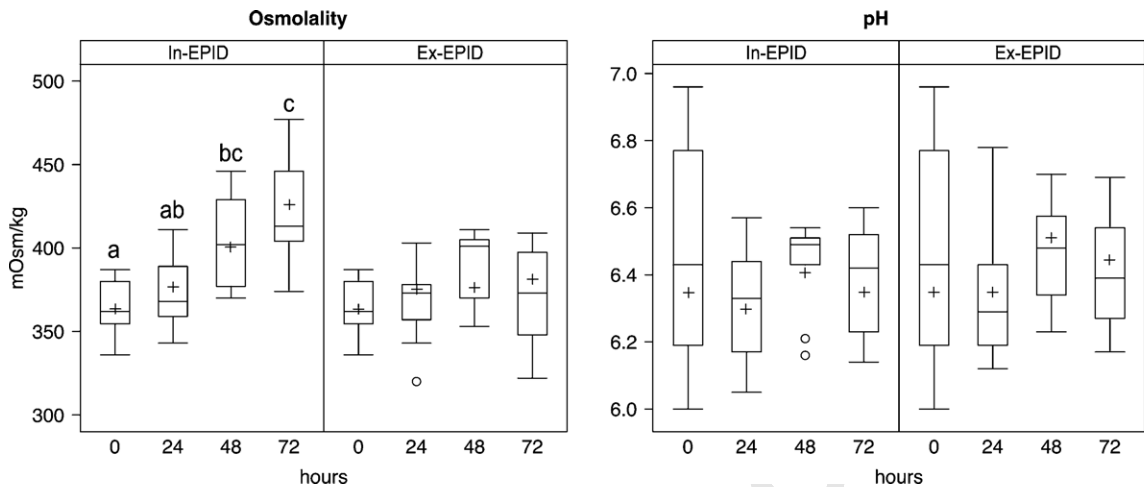

Fig. 1. Osmolality and pH of the epididymal samples, Intra-epididymal (In-EPID) and Extra-epididymal undiluted (Ex-EPID), at each sampling time (0, 24, 48 and 72 h). Lower and upper limits of the boxes indicate the first and third quartiles, respectively, and the horizontal line inside indicates the median. The whiskers reach the maximum and the minimum values of the range. The mean is shown with a cross. Within each treatment, sampling times not sharing letters differ significantly ($P < 0.05$).

post-mortem. In that event, the post-mortem collection of epididymal spermatozoa allows the conservation of valuable genetic material that would otherwise be lost (Saragusty et al., 2006). Epididymal spermatozoa provide a sufficient quantity of viable spermatozoa to be used to fertilize oocytes with the resulting zygotes being able to develop into live young (Songsasen et al., 1998; Sankai et al., 2001; Soler et al., 2003a; Martins et al., 2009).

However, sperm in the epididymis are viable only for a certain period of time and then degenerate rapidly (Hishinuma et al., 2003; Kaabi et al., 2003; Soler et al., 2003b; Yu and Leibo, 2002). Previous studies in several species have indicated that storage of epididymides at 5 °C may be an appropriate way to maintain sperm motility and fertilizing capacity for several days (mice: An et al., 1999; cat: Ganan et al., 2009; boar: Kikuchi et al., 1998; mouse: Kishikawa et al., 1999; red deer: Martinez-Pastor et al., 2005a; bull: Martins et al., 2009; dog: Yu and Leibo, 2002).

Kaabi et al. (2003) preserved ram epididymides at 5 °C, finding good sperm viability until 48 h post-mortem, although their in vitro fertility potential declined significantly after 24 h. Similarly, Martinez-Pastor et al. (2005b) found that deer sperm quality obtained from the epididymis was mostly maintained for the first two days.

In studies examining the conservation of epididymal sperm, the epididymides are usually transported to the laboratory from the site of slaughter at 5 °C. In this period, alterations in the physical environment of the epididymal sperm may cause a loss in sperm quality. Histological examination of mouse epididymides revealed that distinct degenerative changes did not occur until 12 h postmortem (Songsasen et al., 1998) when the epithelial cells became pyknotic and released their intracellular contents into the lumen of the epididymides. By 24 h postmortem, the structure of the epididymal tubule appeared to be breaking down. Martinez-Pastor et al. (2005b) found that osmolality of the epididymal media in red deer increased with post-mortem time.

If the epididymis changes its structure after 12 h postmortem, it is possible that the epididymal microenvironment then begins to be harmful to sperm. We hypothesized that extracting the spermatozoa in the field, refrigerating them either undiluted or extended, and keeping them so for several days post-mortem might represent an advantage, comparing to the traditional methodology of storing the whole epididymes. Therefore, these could be alternative strategies for maintaining the quality of post-mortem samples, when immediate cryopreservation is not possible (Sankai et al., 2001; Fernandez-Santos et al., 2009).

The objective of this study was to analyse the effect of storage at 5 °C on the quality of ram epididymal sperm by: (1) evaluation of spermatozoa held in the epididymal fluid and stored either in the organ or in vitro, (2) analysis of epididymal spermatozoa extended in the same medium at different osmolalities.

2. Materials and methods

2.1. Reagents

All the products were obtained from Sigma (Madrid, Spain), except for the SYBR-14 (LIVE/DEAD Sperm Viability Kit) and YO-PRO-1 fluorescence probes, which were acquired from Invitrogen (Barcelona, Spain).

2.2. Animal and sample collection

Testes were collected from fifteen rams (Churra breed) after slaughter, and transported in a refrigerated cooler (5 °C) to the laboratory (University of León) within the first 2–4 h post-mortem. In a cold room, the caudae epididymides were isolated, the superficial vessels were cut to reduce blood contamination and the organ was kept at 5 °C.

2.2.1. Experimental design

Two experiments were carried out. In experiment 1, we compared sperm quality, osmolality and pH among intra-epididymal and undiluted extra-epididymal storage (9 + 9 epididymides). In experiment 2, we tested the effect of dilution of the epididymal spermatozoa in three extenders of increasing osmolality (320, 370 and 420 mOsm/kg), using intra-epididymal stored samples as the control group (6 epididymes for control and 6 for dilution in the three extenders). These three osmolalities were chosen according to previous experiment and represent a hypo-osmotic, iso-osmotic and hyper-osmotic medium, respectively, comparing to the physiological osmolality of the caudal epididymal fluid (experiment 1: the mean value is 365 (isotonic value), with a range of distribution of 355 (25th percentile) to 381 (75th percentile)).

2.2.2. Sample collection

In each cauda epididymes, two symmetrical parts were isolated by a clamp. Experiment 1: one part was maintained without manipulation and the sperm mass was obtained at the different sampling times (0, 24, 48 and 72 h) performing sequential cuts in defined areas of the surface with a scalpel (Intra epididymal sample, In-EPID). The other portion was processed by cuts to obtain the total sperm mass in a glass tube where it was kept undiluted (Extra epididymal sample, Ex-EPID) and samples were obtained sequentially in different time periods (24, 48 and 72 h).

Experiment 2: one part of each cauda was kept as the control (In-EPID) and was manipulated as described in the previous experiment. In the other portion, the total sperm mass was obtained by cuts and this sample was aliquoted and diluted with the same volume of each TTF media (TES-Tris-fructose, pH 7.2, 20% egg yolk and 8% glycerol) with different osmolalities (320, 370 and 420 mOsm/kg, obtained by varying fructose). These diluted aliquots were coded as D320, D370, D420, respectively. Each aliquot was sampled sequentially for analysis each 24 h (24, 48 and 72 h). TTF media containing glycerol, since the epididymal spermatozoa were prepared for freezing.

Between sampling times, the portions of epididymides used for the control samples (In-EPID) were wrapped with gauze moistened with saline, and put inside a plastic bag, which was stored in a refrigerator at 5 °C. At each sampling time (at 24, 48 and 72 h), we recorded the osmolality and the pH of the different samples obtained from the cauda epididymis (In-EPID and Ex-EPID). Tubes with semen were sealed with Parafilm and kept at 5 °C in a refrigerator until sampling.

2.3. Evaluation of sperm quality

Immediately after collection, volume was recorded and sperm concentration was assessed using Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) and evaluating the information through CASA (Sperm Class Analyzer; Microptic, Barcelona, Spain). In experiment 1, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany) and the pH value was determined by a CG 837 pHmeter (Schott Instruments, Main, Germany).

Motility: the motility was analyzed out using a computer-assisted sperm analysis system (CASA) (Sperm Class Analyzer; Microptic, Barcelona, Spain). A 5-μL drop was taken from each of the different sampling tubes (In-EPID, Ex-EPID, D320, D370, D420), and placed in a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at 10× (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard settings were set at 25 frames/s, 20–90 μm^2 for head area and VCL > 10 μm/s to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analyzed afterwards. Reported parameters were curvilinear velocity (VCL, μm/s) and linearity (LIN, %). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 μm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 μm/s and STR > 80% (straightness, also provided by the system).

2.4. Viability and acrosome status

These parameters were assessed simultaneously using fluorescence probes and flow cytometry. Briefly, samples were diluted in PBS at 5×10^6 spermatozoa/mL and incubated for 15 min with 24 μM of propidium iodide (PI) and 1 μg/mL of PNA-FITC (peanut agglutinin). PI stains membrane-damaged spermatozoa red, whereas PNA-FITC stains the acrosome green if it is damaged or reacted. Thus, we obtained four different subpopulations: red (non-viable sperm, intact acrosoma), green (viable sperm, damaged acrosome) red and green (non-viable sperm, damaged acrosome) or not-stained (viable sperm, intact acrosome).

To evaluate sperm viability, the double stain SYBR-14/PI was used (LIVE/DEAD Sperm Viability Kit: Invitrogen, Barcelona, Spain). Sperm samples were diluted with PBS down to 5×10^6 spermatozoa/ml, and incubated with 24 μM PI and 1.5 100 nM SYBR-14. The tubes were kept at 37 °C for 20 min in the dark. We detected three populations corresponding to live spermatozoa (green), moribund spermatozoa (red + green) and dead spermatozoa (red).

Evaluation of flow cytometer parameters was carried out using a FACScalibur flow cytometer (Becton Dicknson System, San Jose, CA, USA) equipped with standard optics and an argon-ion laser, tuned at 488 nm, and running at 200 mV. Data corresponding to the red (FL-3 photodetector) and green (FL-1 photodetector) fluorescence of 10,000 spermatozoa were recorded.

2.5. Statistical analysis

Statistical analyses were carried out using the R statistical package (http://www.r-project.org). Data were fitted to linear mixed-effect models by maximizing the log-likelihood (ML method). Male was always included as random effect and time (covariate) and treatment (factor with levels: storage in the epididymis and in extender with different osmolalities) as fixed effects. Results are given as mean ± SEM.

3. Results

3.1. Experiment 1: comparison of intra-epididymal versus undiluted extra-epididymal conservation

3.1.1. Osmolality and pH of epididymal samples

The osmolality of the In-EPID sample increased with post-mortem time (Fig. 1). At 0 h, osmolality was 365 ± 6 mOsm/kg, which increased to 375 ± 8, 403 ± 10 and 421 ± 12 at 24, 48 and 72 h, respectively ($P < 0.0001$). However, the Ex-EPID samples stored undiluted in a glass tube did not show any significant differences with post-mortem time (365 ± 5 mOsm/kg at 0 h and 369 ± 6 at 72 h). Similarly, pH did not vary significantly in the In-EPID samples (6.29 at 6.40,) or in the undiluted Ex-EPID ones (6.34 at 6.48) (Fig. 1).

3.1.2. Spermatozoa quality

The results of this experiment are shown in Table 1. After 24 h post-mortem, the In-EPID samples yielded higher motility (TM, PM, VCL and LIN) than the Ex-EPID ones stored undiluted in a glass tube ($P < 0.05$). These differences were not observed at 48 and 72 h. Linearity (LIN) among these storage methods at any time. The spermatozoa stored in the epididymis showed higher viability and lower damaged acrosomes, being significantly different to the samples stored in glass tubes at 48 h ($P < 0.05$).

For the Ex-EPID sample, TM, PM and VCL decreased significantly at 24 h in comparison with the control, VIAB decreased at 48 h, and LIN at 72 h; ACR increased significantly at 48 h in comparison with the control. In the In-EPID samples, TM decreased significantly at 48 h, and PM, VCL and VIAB decreased only at 72 h in comparison with the control. LIN and ACR did not significantly differ in these samples.

3.2. Experiment 2: effect of extender osmolality in diluted epididymal spermatozoa

The total motility of the spermatozoa in the diluted samples was higher than in the other treatments at 24 h (Fig. 2); thus, the TM of D370 was significantly higher than in the control sample stored in epididymis. There were no significant differences among extenders for other motility parameters at 24 h. At 48 h post-mortem, only LIN was different among treatments, with the control samples being significantly higher (54.3 ± 3.5%) than D370 (41.8 ± 3.7%) and D420 (38.1 ± 3.6%). At 72 h post-mortem, motility (TM, PM and VCL) in the samples stored in D320 extender was significantly higher than in the other extenders.

The time of post-mortem storage had no significant effect on TM and PM for D320 and control (epididymis-stored) samples. However, the motility of the spermatozoa stored in D370 or D420 extender decreased significantly in time. The kinetics parameters VCL and LIN showed a reduction only in D370 and D420, being significantly lower at 72 h.

At 24 h, the proportion of damaged acrosomes has no differences, but sperm viability was considerably higher for epididymis-stored samples (79.8 ± 5.1%) than for the extended samples (between 37.7 ± 3.9 to D370 and 48.6 ± 6.0% to D420). At 48 h and 72 h, the proportion of damaged acrosomes increased for the extended samples, D370 and D420 being significantly higher than the control samples. The viability of spermatozoa declined in the extended samples with increasing storage time.

4. Discussion

Two main conclusions can be extracted from our results. First, quality of ram epididymal sperm is kept storing the spermatozoa in the epididymis at 5 °C, at least for 48 h. Second, an extender of 320 mOsm/kg can preserve the motility of sperm extracted of epididymides but damage to acrosomes is observed.

In this study, we observed that the osmolality of freshly obtained samples, but not the pH, increased in successive sampling times. Therefore, extracting the sample as soon as possible and storing it, could prevent these deleterious changes. Previously Martinez-Pastor et al. (2005b) observed that the osmolality and pH of the semen samples obtained from the epididymides of red deer and roe deer was increased with time post-mortem. Moreover, while collecting the sample from the epididymis, tissue, blood and other fluids can be mixed with the spermatozoa, decreasing their quality (Martinez-Pastor et al., 2006a). This suggest that the presence of tissue debris resulting from the collection of semen may speed up the process of deterioration of stored cells, although this process is equalized after 48 h in our test.

We observed in epydidmal storage a sustained decrease of motility at 48 h and 72 h and a reduction in cell viability at 72 h. Others authors have observed that motility of spermatozoa was adversely affected by post-mortem storage in the epididymis (ram: Kaabi et al., 2003; red deer: Martinez-Pastor et al., 2005c; dog: Yu and Leibo, 2002). However, unlike what was observed in our study, several authors conclude that post-mortem storage does not affect sperm membrane integrity (red deer: Soler et al., 2003b and Martinez-Pastor et al., 2005c; dog: Yu and Leibo, 2002).

The loss of epididymal sperm quality may be related to the fertilizing capacity of sperm. A loss of fertilizing ability of epididymal spermatozoa with post-mortem time has been demonstrated in a few studies. Thus, Kaabi et al. (2003) showed that the in vitro fertilizing ability of epididymal spermatozoa was similar at 2 h (53%) and 24 h (45%) post-mortem, but decreased significantly at 48 h post-mortem (38%). However, other authors have observed that the fertilizing ability of epididymal spermatozoa decreased quickly with post-mortem time (red deer: Soler and Garde, 2003; mice: Songsasen et al., 1998; mouse: Sankai et al., 2001).

The extension of spermatozoa in a buffered medium could prevent some negative effects after collection, diluting unwanted fluids such as blood or interstitial fluid. In fact, the diluted spermatozoa showed better motility at 24 h post-mortem than the samples stored in the epididymis. Medium osmolality is crucial for the performance of spermatozoa (Yeung et al., 2006), and therefore we expected hyperosmotic media (370 or 420 mOsm/kg) to be more appropriate for storing ram epididymal spermatozoa (with a mean osmolality of 365 mOsm/kg in our

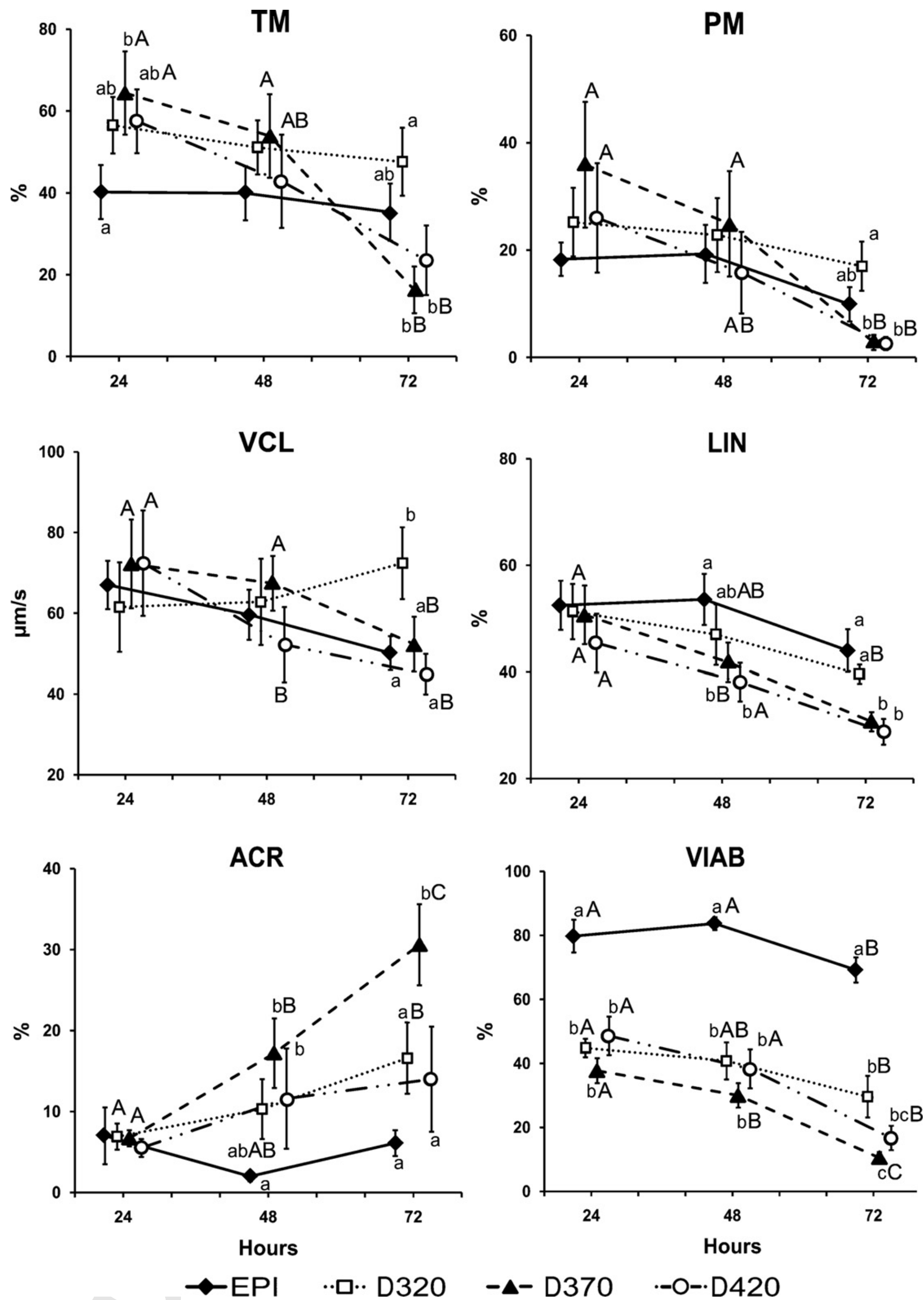

Fig. 2. Effects of extenders (D320, D370 or D420) on epididymal sperm quality stored at 5 °C for 24 and 48 and 72 h (mean ± SEM), applying semen from the cauda epididymis (In-EPID) as control. TM: total motility; PM: progressive motility; VCL: curvilinear velocity; LIN: linearity index; ACR: damaged acrosome; VIAB: viability. ab: different letters indicate differences ($P < 0.05$) between extenders for each time period (24 and 48 and 72 h). AB: different letters indicate differences ($P < 0.05$) between storing periods (24 and 48 and 72 h) for each extender.

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Table 1
Comparison of the two storage methods at 5 °C (Intra-epididymal -In.EPID- versus undiluted extra-epididymal -Ex-EPID-) during 24, 48 or 72 h post-mortem in comparison with a control (0 h). Results are shown as mean ± SEM.

	0 h	24 h		48 h		72 h	
		In-EPID	Ex-EPID	In-EPID	Ex-EPID	In-EPID	Ex-EPID
TM	67.0 ± 8.6^{A}	49.2 ± 7.6^{aAB}	27.6 ± 6.5^{bB}	42.2 ± 9.4^{B}	25.7 ± 7.6^{B}	32.8 ± 7.1^{B}	23.9 ± 4.3^{B}
PM	32.2 ± 5.3^{A}	22.0 ± 5.1^{aAB}	11.1 ± 3.6^{bB}	20.2 ± 6.6^{AB}	10.1 ± 5.7^{B}	11.3 ± 4.0^{B}	5.7 ± 1.2^{B}
VCL	68.8 ± 9.7^{A}	67.2 ± 6.0^{aA}	53.2 ± 6.3^{bB}	57.6 ± 6.4^{AB}	51.1 ± 9.0^{B}	49.1 ± 3.5^{B}	52.9 ± 8.5^{B}
LIN	55.3 ± 4.2^{A}	50.0 ± 3.5^{A}	48.0 ± 2.9^{AB}	50.4 ± 3.7^{A}	44.2 ± 3.7^{AB}	46.2 ± 3.0^{A}	41.7 ± 4.5^{B}
ACR	3.8 ± 0.7^{A}	5.3 ± 2.4^{A}	6.6 ± 1.9^{AB}	3.2 ± 1.2^{aA}	7.7 ± 1.3^{bB}	5.7 ± 1.2^{A}	7.4 ± 2.2^{B}
VIAB	79.7 ± 2.1^{A}	80.7 ± 3.4^{A}	75.6 ± 4.1^{A}	82.3 ± 1.9^{aA}	68.7 ± 4.0^{bB}	68.2 ± 2.6^{B}	62.3 ± 7.0^{B}

TM, total motility (%); PM, % progressive motility; VCL, curvilinear velocity (μm/s); LIN, linearity index (%); ACR, damaged acrosomes (%); VIAB, viability spermatozoa (%).
ab, different letters in the same row indicate that the storage method (In-EPID and Ex-EPID) differs significantly within each storage time.
AB, different letters in the same row indicate that the values for storage time (0, 24, 48 and 72 h) differ significantly within each storage method.

study) that a medium isotonic with epididymal fluid. For instance, Si et al. (2009) obtained higher fertility when collecting murine spermatozoa in hyperosmotic medium (415 mOsm/kg, isoosmotic with the epididymal fluid) compared with spermatozoa collected in 290 mOsm/kg as assessed by IVF. Other authors have reported better sperm quality after freezing spermatozoa using moderately hyperosmotic extenders for epididymal spermatozoa (deer: Martinez-Pastor et al., 2006b and Fernandez-Santos et al., 2007; bear: Anel et al., 2010). However, we have observed that D320 extender maintains motility better and induces less acrosomal damage than the hyperosmotic extenders at prolonged post-mortem times (72 h).

The epididymal spermatozoa incubated into a extender showed a higher degree of membrane and acrosomal damage than those stored into the epididymides. Moreover, the loss of sperm viability does not correspond to a reduction in motility at the same storage time and extender. These results suggest that sperm motility and viability are affected by different mechanisms during the cold storage of epididymides.

The ability of extenders to modify sperm membranes, especially when containing egg yolk or milk, has been described previously (Bergeron et al., 2007). While conveying some advantages, storing the spermatozoa in extenders could be triggering changes in membranes or in the general physiology of spermatozoa, which might increase their vulnerability to osmotic stress. It is noteworthy that, in our study, epididymal spermatozoa extended in D370 showed the lower results for sperm viability and acrosomes status, but D420 yielded similar results than D320. This suggests that extender osmolality could ameliorate or worsen the osmotic vulnerability of spermatozoa and confirms that the osmolality appears to exert a complex effect, as stated by Fernandez-Santos et al. (2007). Therefore, the hypothesis that a slightly hyperosmotic extender could provide the best environment for epididymal ram spermatozoa seems to be disproved and we note that a medium hypotonic to caudal epididymal plasma (D320) might be the best option for the conservation of diluted epididymal spermatozoa in ram. This medium can reduce cellular swelling caused by hypo-osmotic stress when epididymal spermatozoa endure the freezing process.

In conclusion, storing the spermatozoa in the epididymis at 5 °C is a good strategy for maintaining sperm quality in ram, at least for 48 h, and is a better option than extracting the sperm mass and keeping it undiluted. We have also shown that extracting the sperm mass and diluting it in an extender of 320 mOsm/kg can preserve sperm motility at least as well as maintaining it in the epididymis. Nevertheless, the reduction of viability and damage to acrosomes in this extender must be analyzed and remediated improving the extender composition.

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