Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection

ARTICLE *in* THERIOGENOLOGY · APRIL 2009

Impact Factor: 1.8 · DOI: 10.1016/j.theriogenology.2009.02.002 · Source: PubMed

9 AUTHORS, INCLUDING:

Felipe Martínez-Pastor
Universidad de León
145 PUBLICATIONS **1,395** CITATIONS
SEE PROFILE

Julian Garde
University of Castilla-La Mancha
153 PUBLICATIONS **2,394** CITATIONS
SEE PROFILE

Manuel Ramon
Health Sciences Institute, Castile-La Mancha
43 PUBLICATIONS **210** CITATIONS
SEE PROFILE

Ana J. Soler
University of Castilla-La Mancha
88 PUBLICATIONS **1,387** CITATIONS
SEE PROFILE

Elsevier Editorial System(tm) for Theriogenology
Manuscript Draft

Manuscript Number: THERIO-D-08-00481R1

Title: Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection

Article Type: Original Research Article

Section/Category:

Keywords: Electroejaculated spermatozoa; Epididymal spermatozoa; Germoplasm banking; Ram; Heterologous in vitro fertilization

Corresponding Author: Ms ana josefa soler, ph D

Corresponding Author's Institution: UCLM

First Author: Olga Garcia-Alvarez

Order of Authors: Olga Garcia-Alvarez; Alejandro Maroto-Morales; Felipe Martinez-Pastor; J.Julian Garde; M.Rocio Fernandez-Santos; Milagros C. Esteso; M.Dolores Perez-Guzman; ana josefa soler, ph D

Manuscript Region of Origin:

Abstract: The aim of this study was to assess two models of sperm collection on the quality and fertility of thawed spermatozoa from Black Manchego rams, a threatened breed. Sperm samples were collected by electroejaculation (EE) and postmortem (PM) from each male. Samples were diluted with Biladyl® and frozen. Motility (subjective and CASA), membrane integrity and acrosomal status (microscopy) were assessed on fresh semen; plasmalemma integrity, mitochondrial membrane potential, DNA integrity and acrosomal status were evaluated by flow cytometry on thawed semen. Thawed spermatozoa were used in a heterologous in vitro fertilization test. After

thawing, the proportion of live spermatozoa with intact membrane (YO-PRO-1-/PI-) was higher for PM samples ($P < 0.001$), although the ratio of YO-PRO-1- spermatozoa within the PI- population was higher for ejaculated samples ($P = 0.007$). Likewise, the proportion of live spermatozoa having high mitochondrial membrane potential (Mitotracker+) and intact acrosomes (PNA-) was higher for PM samples ($P < 0.001$ and $P < 0.001$, respectively). Considering only live spermatozoa, the ratio of Mitotracker +, PNA- cells was higher for EE samples ($P = 0.026$ and $P = 0.003$). Both EE and PM samples fertilized oocytes. Nevertheless, EE samples yielded a higher percentage of hybrid embryos ($P = 0.041$). In conclusion, although PM spermatozoa had better sperm quality after thawing, EE spermatozoa showed higher ratios for sperm quality when only the live population was considered. EE and PM samples might be used for germplasm banking of this threatened breed, but the fertility of PM spermatozoa might be lower.

Revised highlighted

Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection

Short Title: Effect of sperm collection method

O. García-Álvarez[a], A. Maroto-Morales[a], F. Martínez-Pastor[b], J.J. Garde[b], M.R. Fernández-Santos[a], M.C. Esteso[c], M.D. Pérez-Guzmán[a], A.J. Soler[a,b].

[a] Regional Center of Animal Selection and Reproduction (CERSYRA) JCCM, 13300 Valdepeñas, Spain; [b] Biology of Reproduction Group, National Wildlife Research Institute (IREC) UCLM-CSIC-JCCM, 02071 Albacete, Spain; [c] Animal Reproduction and Obstetrics, University of León, 24071 León, Spain.

*Corresponding author:

Ana J. Soler Valls

Ciencia y Tecnología Agroforestal y Genética, ETSIA, Universidad de Castilla-La Mancha

Av. España s/n, 02071-Albacete (España)

Phone: +34 967 599 200+2552

Fax: +34 967 599 238

e-mail: anajosefa.soler@uclm.es

Abstract

The aim of this study was to assess two models of sperm collection on the quality and fertility of thawed spermatozoa from Black Manchego rams, a threatened breed. Sperm samples were collected by electroejaculation (EE) and postmortem (PM) from each male. Samples were diluted with Biladyl® and frozen. Motility (subjective and CASA), membrane integrity and acrosomal status (microscopy) were assessed on fresh semen; plasmalemma integrity, mitochondrial membrane potential, DNA integrity and acrosomal status were evaluated by flow cytometry on thawed semen. Thawed spermatozoa were used in a heterologous in vitro fertilization test. After thawing, the proportion of live spermatozoa with intact membrane (YO-PRO-1-/PI-) was higher for PM samples ($P < 0.001$), although the ratio of YO-PRO-1- spermatozoa within the PI- population was higher for ejaculated samples ($P = 0.007$). Likewise, the proportion of live spermatozoa having high mitochondrial membrane potential (Mitotracker+) and intact acrosomes (PNA-) was higher for PM samples ($P < 0.001$ and $P < 0.001$, respectively). Considering only live spermatozoa, the ratio of Mitotracker +, PNA- cells was higher for EE samples ($P = 0.026$ and $P = 0.003$). Both EE and PM samples fertilized oocytes. Nevertheless, EE samples yielded a higher percentage of hybrid embryos ($P = 0.041$). In conclusion, although PM spermatozoa had better sperm quality after thawing, EE spermatozoa showed higher ratios for sperm quality when only the live population was considered. EE and PM samples might be used for germplasm banking of this threatened breed, but the fertility of PM spermatozoa might be lower.

Keywords: Electroejaculated spermatozoa; Epididydimal spermatozoa; Germplasm banking; Ram; Heterologous in vitro fertilization

1. Introduction

The Manchega sheep is an autochthonous dairy breed from Spain, with a white and a black variety. Currently, there are few herds of Black Manchega sheep (around 2000 animals), and in Spain it is considered an endangered breed of special interest (RD 1682/1997). As recommended by the Food and Agriculture Organization of the United Nations (FAO), endangered breeds must be preserved by *ex situ* and *in situ* means.

One of the *ex situ* procedures consists in the development of Genetic Resource Banks, cryopreserving gametes and embryos, thus allowing to store genetic resources indefinitely [1]. Assisted Reproductive Technologies have demonstrated to be successful tools to reproduce endangered species, being sperm cryopreservation, in combination with artificial insemination, the method that has been most extensively applied.

Semen can be collected from live animals by artificial vagina [2], but this technique requires a previous training period [3]. Electroejaculation is an alternative when males are not trained to artificial vagina. This method allowed the collection of high quality semen in Guirra ram without noticeable differences from semen collected by artificial vagina [4]. In that study, sperm quality was higher for electroejaculated samples even after cryopreservation.

Postmortem recovery of spermatozoa and their utilization has received much less attention in sheep than in other domestic species [5,6]. Collection and cryopreservation of epididymal spermatozoa is a useful method to rescue germplasm of dead animals which would be otherwise lost, and use it to preserve endangered breeds. This kind of samples have been successfully cryopreserved and used for artificial insemination or in vitro fertilization in other ungulates, such as red deer [7-13].

Ejaculated spermatozoa have a lower cryosurvival and osmotic resistance than epididymal ones [14]. Nevertheless, it has been showed that adding seminal plasma to epididymal spermatozoa protects against the effects of cooling and freezing [15,16]. However, seminal plasma could also have detrimental effects on cryosurvival [17].

In vitro fertility of fresh semen obtained by artificial vagina and postmortem has been studied in sheep [5]. However, so far any studies have been conducted in this species for evaluating the effect of collection method (electroejaculation or postmortem recovery) on post-thawing sperm quality and fertility.

Considering this background, the objective of this study was to evaluate the post-thawing sperm quality and in vitro fertility of sperm samples obtained both by electroejaculation and by postmortem collection from Black Manchega rams, in order to assess its suitability for germplasm banking, and the differences between both methodologies.

2. Materials and Methods

2.1. Animals and reagents

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2003/65. Six rams of Black Manchega breed (age > 3 years) were used. Males were maintained and managed at Regional Center of Animal Selection and Reproduction, placed in Valdepeñas (Spain). These males had not been trained to artificial vagina.

Chemicals were of reagent grade and were purchased from Sigma (Madrid, Spain). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) and peanut agglutinin conjugated with fluoresceine

isothyocianate (PNA-FITC), bought from Sigma. Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA).

2.2. Semen collection

Semen samples were collected by electroejaculation and from epididymis postmortem from the same males. Previous to electroejaculation (15 minutes before), males received an intramuscular injection of xylazine (0.2mg kg^{-1} body weight; Rompun 2%, Bayer S.A. Barcelona, Spain). Rectum was cleaned from faeces and the prepucial area was shaved and washed with physiological saline serum. Electroejaculation was carried out using a three-electrode probe (250 mm x 25 mm) connected to a power source that allowed voltage and amperage control (P. T. Electronics, Boring, OR). Semen was collected into a graduated collection vial at 37 ºC.

For the postmortem collection, the testicles of each male were obtained at slaughterhouse and transported at room temperature, arriving at the laboratory between 0.5 and 1 hour afterwards. Testicles with epididymes attached were isolated from scrotum. Epididymal spermatozoa were collected performing several incisions on each cauda epididymis with a surgical blade, placing the sperm suspension in 1 mL PBS (phosphate buffer saline, pH: 7.5, 310 mOsm/kg).

After sperm collection, sperm motility, percentage of spermatozoa with intact acrosome assessed by phase-contrast microscopy (%, NAR) and percentage of live spermatozoa assessed by nigrosin–eosin staining (%; Viability) were assessed as described by Soler et al. [9]. Motility was also assessed by Computer Assisted Semen Analysis (CASA) as described Martínez et al. [18]. A 5 µL drop was put on a slide at 37 ºC, covered with a 22 x 22 coverslip and examined (3 fields at least) with a phase contrast microscope (Nikon 80i, negative contrast optics) with a warming state at 37 ºC. Analysis were carried out using the Sperm Class Analyzer software (SCA2002,

Microptic, Barcelona, Spain) and the following parameters were used for the study: average path velocity (μm/s; VAP), linearity (%, LIN), amplitude of the lateral movement of the head (μm; ALH) and beat cross frequency (Hz; BCF).

2.3. Cryopreservation

After initial semen evaluation, sperm samples obtained by electroejaculation and postmortem collection were diluted with freezing extender Biladyl® (Minitüb, Germany) with 20% egg yolk. Semen was diluted to 400 x 10^6 spermatozoa/mL with Biladyl®, Fraction A, at 30 ºC. Diluted semen was cooled to 5 ºC for 2 hours and then was further diluted with the same volume of Bildadyl®, Fraction B. Sperm samples were allowed to equilibrate at 5 ºC for 2 h and packed in 0.25 ml plastic straws. Finally, they were frozen in a programmable biofreezer (IceCube 14S-Ver. 1.30 ©2004 SY-LAB Geräte GmbH Minitüb®) at 20 ºC/min to -100 ºC, and at 10 ºC/min from -100 ºC to -140 ºC and then plunged into liquid nitrogen.

2.4. Assessment of frozen-thawed spermatozoa

Thawing was performed by dropping the straws (two per male and per collection method) in a water bath at 37 ºC for 20 s. Thawed spermatozoa were layered on a Percoll discontinuous density gradient (45%/90%) and centrifuged at 700 x g for 10 min in order to eliminate the particles of egg yolk and enrich the sample in highly motile spermatozoa. After centrifugation, the supernatant was carefully removed. Sperm samples were evaluated for sperm motility, acrosomal status by phase-contrast (%; NAR), viability by nigrosin-eosin (%; Viability) and motility by CASA in the same way that in fresh sperm samples. After this evaluation, thawed samples were incubated at 37 ºC for 2 h, and then sperm motility, acrosomal status (%; NAR) and viability (%; Viability) were assessed as previously described.

In addition, two more straws were thawed for the assessment of the seminal quality by flow cytometry. We assessed the membrane status with YO-PRO-1, the mitochondrial membrane potential with Mitotracker Deep Red, the acrosome integrity with peanut agglutinin conjugated with fluorescein isothiocyanate, (FITC-PNA), and the viability (membrane integrity) with propidium ioide (PI).

We prepared two staining solution using flow cytometer sheath fluid (BD FACSFlow^{TM}). One of them was prepared with 3 nM Hoechst 33342 (stock: 9 µM in milli-Q water), 50 nM YO-PRO-1 (stock: 100 µM in DMSO), 15 µM PI (stock: 7.5 mM in milli-Q water) and 100 nM of Mitotracker Deep Red (stock: 1 mM in DMSO). The other solution was prepared with 3nM Hoechst 33342, 15 µM PI, and 10 µg/mL of PNA-FITC (stock of 0.2 mg/mL in dH_2O). We diluted the sample in 0.5 mL of each staining solution in polypropylene tubes for flow cytometry (5 x 10^6 spermatozoa/mL). The tubes were allowed to rest for 15 min in the dark and then analyzed using a Becton Dickinson LSR-I flow cytometer (BD Biosciences, San José, CA, USA). We used the three lasers of the cytometer to excite the different fluorochromes. A 325 nm Helium-Cadmium UV laser for exciting the Hoechst 33342, a 488 nm Argon-Ion laser for exciting YO-PRO-1, PNA-FITC and PI, and a 633 nm Helium-Neon laser for exciting Mitotracker Deep Red. We acquired the FSC (forward-scatter light) and SSC (side-scatter light) signals plus the fluorescence light of each fluorochrome using four photodetectors. FL1 was used for YO-PRO-1 and PNA-FITC (530/28BP filter), FL3 for propidium ioide (670LP filter), FL5 for Hoechst 33342 (424/44BP filter) and FL6 for Mitotracker Deep Red (670/40BP filter). The acquisition was controlled using the Cell Quest Pro 3.1 software (BD Biosciences). All the parameters were read using logarithmic amplification. We set up an acquisition template in the software which allowed us first to discriminate spermatozoa from debris within the events acquired.

FSC/SSC and FL5/FL3 (Hoechst 33342 vs. PI) dot-plots were used to discard debris. The filtered events were displayed in dot-plots showing either FL1/FL3 (YO-PRO-1 vs. PI or PNA-FITC vs. PI) and FL6/FL3 (Mitotracker Deep Red vs. PI). We acquired 10000 spermatozoa from each sample, saving the data in FCS v. 2 files. The analysis of the flow cytometry data was carried out using WinMDI v. 2.8 (The Scripps Research Institute, La Jolla, CA, USA).

YO-PRO-1-/PI- spermatozoa were considered as viable (non-"apoptotic", intact membrane), Mitotracker+/PI- spermatozoa as viable with high mitochondrial membrane potential and PNA-/PI- spermatozoa as viable with intact acrosome. Also, we calculated the ratios of YO-PRO-1-, Mitotracker+ and PNA- within live spermatozoa population (PI-).

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics, Inc., Brookings, SD, USA) [19]. This technique is based in the susceptibility of the sperm DNA to acid-induced denaturation in situ and in the metachromatic staining Acridine Orange (AO). This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). Thawed spermatozoa were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH=7.4) to 2 x 10^6 cells/mL. Samples were flash frozen in LN2 and stored at -80 ºC until analysis. For the analysis, the samples were thawed on crushed ice and 200 µl were put on a cytometry tube. Then, we added 400 µl of an acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH=1.2). Exactly 30 sec after adding the acid-detergent solution, we added 1.2 mL of staining solution (6 µg/mL of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH=6). After 3 min, the sample was run through a Becton Dikinson LSR-1 flow cytometer. Acridine orange was excited

using an argon laser providing 488 nm light. The red fluorescence was detected using a long pass (670LP) filter (FL-3) and the green one using a band pass (530/28BP) filter (FL-1). Sample acquisition was carried out with a CellQuest v. 3 software. Flow Cytometry data (FCS files) were processed and saved as tabbed text using WinMDI v. 2.8 (The Scripps Research Institute, La Jolla, California). We calculated the DNA fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red+green), expressed as a percentage. High values of DFI, indicates chromatin abnormalities. We also calculated %DFI, as the percentage of spermatozoa with DFI > 25, and High DNA Stainability (HDS) as the percentage of the spermatozoa with green fluorescence higher than channel 600 (of 1024 channels).

Thawed semen was incubated at 37 ºC for 2 h in the same freezing extender and all sperm parameters previously cited, except chromatin stability, were assessed by flow cytometry.

2.5. Heterologous in vitro fertilization (IVF)

We carried out a heterologous IVF test to assess the fertilizating potential of electroejaculated and postmortem sperm samples, since this assay is highly related with the in vivo fertility as described by García-Álvarez et al. [20]. We performed heterologous IVF four times per male and method collection, and a minimum of twenty oocytes per well was used. Ovaries were collected at slaughterhouse from heifers about 1 year old, and transported to our laboratory in saline solution (30 ºC) between 1-2 h after removal. Immature oocytes were collected from the ovaries using 19-gauge needle, in TCM-199 supplemented with HEPES (2.39 mg/mL), heparin (2 µL/mL) and gentamycin (40 µg/mL). Aspirated cumulus oocyte complexes (COC) were washed in TCM-199 gentamycin (40 µg/mL), selecting those with dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells. These COC were placed in four-well

plates containing 500 µL of TCM-199 supplemented with cysteamine (100 µM) and epidermal growth factor (EGF) (10 ng/ml) and matured at 38.5 ºC in 5% CO_2. After 24 h, COC were washed in synthetic oviduct fluid (SOF) supplemented with essential and non-essential amino acids [21], and cumulus cells were removed by gentle pipetting. Oocytes (minimum 20 oocytes per well) were transferred to four-well plates with 400 µL of fertilization medium (SOF supplemented with 10% of estrous sheep serum and 40 µg/mL gentamycin) under mineral oil.

Thawed samples (electroejaculated and epididymary) were centrifuged through a Percoll discontinuous density gradient (45%/90%) and were capacitated in the fertilization medium for 10 min. Sperm was co-incubated with oocytes at a final concentration of 10^6 spermatozoa/mL at 38.5 ºC in 5% CO_2.

Oocytes were evaluated visually with an inverted microscope (Nikon Eclipse TE2000-U) 40 h later for cleavage (two to eight cells). Then, the oocytes were fixed and stained with Hoechst 33342 to confirm the fertilization by the presence of 2 or more nuclei (%; cleavage).

2.6. Statistical Analysis

Statistical analyses were carried out using SPSS®, version 15.0 (SPSS Inc., Chicago, IL, USA). A GLM-ANOVA tested the effect of collection method (electroejaculation and postmortem recovery) on sperm quality for fresh, thawed and incubated sperm samples, and on heterologous in vitro fertility for thawed sperm samples. Data were split into two groups according to collection method (electroejaculation or postmortem recovery) and Pearson rank correlations were carried out to study the relationships between % cleavage and sperm quality. Also, stepwise multiple regression analyses were carried out in order to predict the % cleavage from quality analyses for fresh, thawed and incubated samples. Significance was set at $P < 0.05$.

3. Results

Sperm quality of fresh samples was not different between collection methods, except for sperm viability, which was higher in postmortem samples ($P < 0.001$) (Table 1).

After thawing, sperm motility and velocity were higher in postmortem samples ($P = 0.043$ and $P = 0.001$, respectively) (Table 1). There were significant differences between electroejaculated and postmortem samples for most of the parameters evaluated by flow cytometry, with lower values for electroejaculated spermatozoa (YO-PRO-1-/PI-;$P < 0.001$, PNA-/PI-; $P < 0.001$, Mitotracker+/PI-; $P < 0.001$) (Table 2). However, considering the live spermatozoa population (PI-) the electroejaculated samples yielded a higher ratio of YO-PRO-1-, ($P = 0.007$), PNA- ($P = 0.003$) and Mitotracker+ ($P = 0.026$) spermatozoa (Table 3). Moreover, the cleavage rate after the heterologous IVF was higher for electroejaculated spermatozoa (32.69% *vs* 21.84%, $P = 0.041$) (Fig. 1).

After incubation, sperm motility and the percentage of spermatozoa with intact acrosome (subjectively assessed) were higher for postmortem samples (34.16 ± 3.87% *vs*. 10.00 ± 4.74%, $P = 0.004$, and 74.33 ± 6.35% *vs*. 19.25 ± 7.78%, $P = 0.001$, respectively). We did not find significant differences between the collection methods for sperm viability (EE: 33.83 ± 5.92% and PM: 24.00 ± 7.25%, $P = 0.324$). The percentages of YO-PRO-1-/PI-, PNA-/PI- and Mitotracker+/PI- were higher in postmortem samples ($P = 0.001$, $P = 0.001$ and $P = 0.032$, respectively) (Table 2). However, considering only the PI- ("live") spermatozoa population the ratios of PNA- and Mitotracker+ were higher for electroejaculated samples ($P = 0.005$ and $P < 0.001$, respectively) (Table 3).

We also studied the relationships between sperm quality parameters and cleavage rate for samples obtained by electroejaculation and those obtained from the epididymis postmortem. For fresh sperm samples, any parameter was correlated with the % cleavage for both kinds of samples (electroejaculation and postmortem). However, for thawed sperm samples recovered from the epididymis postmortem, there was a correlation between LIN and cleavage rate ($r = 0.85$, $P = 0.029$). Incubated samples displayed a relationship between the ratio of YO-PRO-1- within PI- spermatozoa subpopulation and the fertility in postmortem samples ($r = 0.83$, $P = 0.039$).

Stepwise multiple regression analyses were used to select a set of sperm quality variables in fresh, thawed and incubated samples that best predicted fertility (cleavage rate) in the samples collected by electroejaculation and postmortem. For fresh sperm samples obtained either by electroejaculation or postmortem, any parameter was predictive of the fertility. For thawed epididymal samples, LIN was the only predictor of fertility ($r^2 = 0.73$, $P = 0.029$) (Fig. 2A). After incubation, the ratio of YO-PRO-1- within PI- subpopulation in postmortem samples was the only predictor of fertility ($r^2 = 0.69$, $P = 0.039$) (Fig. 2B).

4. Discussion

In this study, we assessed the effect of collection method (electroejaculation and postmortem), on sperm quality for fresh, thawed and incubated samples and on in vitro fertility from black Manchego rams. Both collection methods provided good sperm quality. Electroejaculation is an excellent choice for animals that have not been trained to artificial vagina, whereas postmortem collection can be used as an emergency method when valuable males die suddenly.

Our results showed that sperm quality for fresh samples was similar for electroejaculated and epididymal spermatozoa, although the viability was higher for epididymal spermatozoa (postmortem recovery). Other authors have studied the effect of collection method in other species. Blash et al. [22] compared the quality of epididymal spermatozoa and semen obtained by artificial vagina from goat bucks. These authors showed that for fresh samples the viability was higher in epididymal spermatozoa, results that agree with the finds out of this study. However, in other species such as red deer, the viability was higher in electroejaculated samples that in those obtained postmortem [18]. It is difficult to explain why these studies differed with ours, since different methodologies (collection and analyses) were used in these studies.

Cryopreservation, as expected, substantially reduced sperm viability in both kinds of samples (electroejaculated and postmortem), yielding better results for PM samples. Recent studies showed that electroejaculated samples had lower resistance than epididymal spermatozoa when submitted to cryobiologicaly stressful conditions such as chilling, osmotic stress and addition and removal of cryoprotective agents [14]. In our study, sperm quality of electroejaculated samples was worse than that of epididymal samples, after thawing. Nevertheless, the ratios for YO-PRO-1-, PNA- and Mitotracker+ spermatozoa, considering live spermatozoa subpopulation, were lower for epididymal spermatozoa, suggesting a higher heterogeneity, which may have contributed to their lower fertility results.

With respect to the IVF results, our outcomes do not agree with those obtained by Kaabi et al. [5], who found that ram epididymal and ejaculated spermatozoa rendered similar results in an IVF test. Our results may differ because Kaabi et al. [5] obtained ejaculated spermatozoa using artificial vagina, not electroejaculation, and because of rearing or breed differences. Furthermore, Blash et al. [22] did not find differences after

316 an IVF assay using ejaculated and epididymal spermatozoa from goat. However, they
317 found a higher pregnancy rate for ejaculated samples. Other authors showed that
318 ejaculated bull spermatozoa had a higher binding ability to zona pellucida than those
319 obtained postmortem from the same animals [23].

320 Spermatozoa complete their maturation during their transport along epididymis
321 [24]. Evenson and Wixon, [25] described that lack of appropriate sperm maturation
322 resulted in increased DNA stainability (HDS). Our results showed that there were not
323 differences in HDS between electroejaculated and epididymal spermatozoa. Likewise,
324 %DFI, indicative of DNA fragmentation, was not different for both collection methods
325 (electroejaculation and postmortem, recovery). García-Macías et al. [26] found in ram
326 that spermatozoa obtained of cauda epididymis had highly condensed chromatin, similar
327 to ejaculated samples obtained by artificial vagina. Our data confirm that
328 electroejaculated and postmortem samples have similar maturity indexes and that,
329 although epididymal spermatozoa are more resistant to cryopreservation, this procedure
330 affects similarly to both types of samples regarding to chromatin status.

331 The differences in IVF could be explained considering that electroejaculated and
332 epididymal spermatozoa respond differently to the capacitation treatments of the IVF
333 protocol used in this study. A very important difference between both kinds of samples
334 is that ejaculated spermatozoa contact with the seminal plasma. Recent papers have
335 revealed important roles for a number of proteins found in seminal plasma, which act in
336 the regulation of in vitro capacitation [27]. Thus, Maxwell et al. [28], suggested that
337 components of seminal plasma were responsible for the improved pregnancy rate
338 following the resuspension of frozen-thawed ram spermatozoa in a medium containing
339 seminal plasma. However, in recent studies, the addition of seminal plasma to
340 ejaculated semen did not improve the pregnancy rate in cervicaly inseminated ewes

[29], and the addition of seminal plasma to epididymal spermatozoa accelerated cell death in other cases [30]. In the present study is impossible to know if the higher fertility of ejaculated sperm samples was due to the action of seminal plasma or to the intrinsic characteristics of ejaculated cells.

We obtained correlations between several quality parameters and fertility only for the samples obtained postmortem, possibly due to their higher heterogeneity. These correlations were not found in fresh samples, but appear after thawing with LIN (linearity) and after incubation with the ratio of YO-PRO-1- in the PI- subpopulation. Both LIN [31] and membrane integrity [32, 33] have been found to be good predictors of fertilization and pregnancy rate. It is possible that epididymal sperm samples with a lower ratio of YO-PRO-1-, therefore with a predominant subpopulation of "apoptotic" spermatozoa (YO-PRO-1+/PI-) in the PI- subpopulation, were announcing a lower fertilizing ability.

Our results allow us concluding that it is possible to obtain ram spermatozoa of good quality and freezability both by electroejaculation and postmortem. Therefore, these methods might be used to preserve semen of endangered breeds, either when males have not been trained for artificial vagina (electroejaculation), in case of sudden death, or as a last resource in the case that males must be removed from herds (postmortem recovery). Nevertheless, in vitro fertility was higher for thawed spermatozoa recovery by electroejaculation. Further studies are necessary in order to explain this difference, and to improve IVF protocols for epididymal spermatozoa.

ACKNOWLEDGEMENTS:

This work was supported by the Education and Science Council (PBI-05-011), by Spanish Ministry of Education and Science (RZ2006-00006-C3) and by Agriculture

366 Council (PREG-05-004) of Junta de Comunidades de Castilla-La Mancha (JCCM). The
367 authors thank to Frimancha Industrias Cárnicas S.A. for their collaboration in the
368 collection of the ovaries used in this work. Olga García-Álvarez and Alejandro Maroto
369 Morales were recipients of scholarships from INIA and JCCM, respectively. Felipe
370 Martínez-Pastor, María Rocío Fernández-Santos and Milagros C. Esteso were supported
371 by the Juan de la Cierva program from the Spanish Ministry of Education and Science.
372 We are grateful to Alfonso Bisbal for his help revising the language of the manuscript.

373 **References**

374 [1] Watson PF, Holt WV. Organizational issues concerning the establishment of a
375 genetic resource bank. In: Cryobanking the Genetic Resource. Wildlife Conservation
376 the Future?, Watson PF, Holt WV (Eds.), Taylor and Francis, London; 2001. p. 86-112.
377 [2] Salamon S, Evans G, Maxwell WMC. Inseminación artificial de ovejas y cabras.
378 Editorial ACRIBIA, S.A. Zaragoza (España); 1990. p. 87–95.
379 [3] Wulster-Radcliffe MC, Williams MA, Stellflug JN, Lewis GS. Technical note:
380 artificial vagina vs. a vaginal collection vial for collecting semen from rams. J Anim Sci
381 2001;79:2964-2967.
382 [4] Marco-Jiménez F, Puchades S, Gadea J, Vicente JS, Viudes-de-Castro MP. Effect of
383 semen collection method on pre- and post-thaw Guirra ram spermatozoa.
384 Theriogenology 2005;64:1756-1765.
385 [5] Kaabi M, de Paz P, Álvarez M, Anel E, Boixo JC, Rouissi H, Herraez P, Anel L.
386 Effect of epididymis handling conditions on the quality of ram spermatozoa recovered
387 postmortem. Theriogenology 2003;60:1249-1259.
388 [6] Ehling C, Rath D, Struckmann C, Frenzel A, Schindler L, Niemann H. Utilization of
389 frozen-thawed epididymal ram semen to preserve genetic diversity in Scrapie
390 susceptible sheep breeds. Theriogenology 2006;66:2160-2164.
391 [7] Soler AJ, García AJ, Fernández-Santos MR, Esteso MC, Garde JJ. Effects of
392 thawing procedure on post-thawed in vitro viability and in vivo fertility of red deer
393 epididymal spermatozoa cryopreserved at -196ºC. J Androl 2003;24:746-756.
394 [8] Martínez-Pastor F, García-Macías V, Ávarez M, Herráez P, Anel L, de Paz P. Sperm
395 subpopulation in Iberian red deer epididymal sperm and their changes through the
396 cryopreservation process. Biol Reprod 2005;72:316-327.

[9] Soler AJ, Esteso MC, Fernández-Santos MR, Garde JJ. Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5ºC in the epididymis for several days. Theriogenology 2005;64:1503-1517.

[10] Fernández-Santos MR, Esteso MC, Montoro V, Soler AJ, Garde JJ. Cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa: effects of egg yolk, glycerol and cooling rate. Theriogenology 2006;66:1931-1942.

[11] Martínez-Pastor F, García-Macías V, Esteso MC, Anel E, Fernández-Santos MR, Soler AJ, de Paz P, Garde JJ, Anel L. A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology 2006;66:1165-1172.

[12] Gomendio M, Malo AF, Soler AJ, Fernández-Santos MR, Esteso MC, García AJ, Roldán ER, Garde JJ. Male fertility and sex ratio at birth in red deer. Science 2006;314:1445-1447.

[13] Soler AJ, Poulin N, Fernández-Santos MR, Cognie Y, Esteso MC, Garde JJ, Mermillod P. Heterologous in vitro fertility evaluation of cryopreserved Iberian red deer epididymal spermatozoa with zona-intact sheep oocytes and its relationship with the characteristics of thawed spermatozoa. Reprod Domest Anim 2008;43:293-298.

[14] Varisli O, Uguz C, Agca C, Agca Y. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperaturas. Anim Reprod Sci 2009;110:256-268.

[15] Barrios B, Pérez-Pe R, Gallego M, Tato A, Osada J, Muiño-Blanco T. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod 2000;63:1531-1537.

422 [16] Martínez-Pastor F, Anel L, Guerra C, Álvarez M, Soler AJ, Garde JJ, Chamorro C,
423 de Paz P. Seminal plasma improves cryopreservation of Iberian red deer epididymal
424 sperm. Theriogenology 2006;66:1847-1856.
425 [17] Kawano N, Shimada M, Terada T. Motility and penetration competence of frozen-
426 thawed miniature pig spermatozoa are substantially altered by exposure to seminal
427 plasma before freezing. Theriogenology 2004;61:351-364.
428 [18] Martínez F, Martínez-Pastor F, Álvarez M, Fernández-Santos MR, Esteso MC, de
429 Paz P, Garde JJ, Anel L. Sperm parameters on Iberina red deer: Electroejaculation and
430 postmortem collection. Theriogenology 2008;70:216-226.
431 [19] Evenson DP, Higgins PJ, Grueneberg D, Ballachey BE. Flow cytometric analysis
432 of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry
433 1985;6:238-253.
434 [20] García-Alvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos MR,
435 Esteso MC, Pérez-Guzmán MD, Soler AJ. Heterologous in vitro fertilization is a good
436 procedure to assess the fertility of thawed ram sperm. Theriogenology 2008 (doi:
437 10.1016/j.theriogenology.2008.09.036).
438 [21] Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and
439 development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of
440 serum and somatic cells: amino acids, vitamins, and culturing embryos in groups
441 stimulate development. Biol Reprod 1994;50: 390-400.
442 [22] Blash S, Melican D, Gavin W. Cryopreservation of epididymal sperm obtained at
443 necropsy from goats. Theriogenology 2000;54:899-905.
444 [23] Ellington JE, Wilker CW, Hillman RB, Ball BA. Ability of epididymal or
445 ejaculated bull spermatozoa to bind to cow zonae before and after oviduct epithelial cell
446 co-culture. Theriogenology 1993;39:213.

447 [24] Yanagimachi R. Mammalian fertilization. In: The physiology of reproduction,
448 Knobil E, Neil J (Eds.), Plenum Press, New York, 1988. p. 135-185.
449 [25] Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection
450 and male infertility. Theriogenology 2006;65:979-991.
451 [26] García-Macias V, Martínez-Pastor F, Álvarez M, Garde JJ, Anel E, Anel L, de Paz
452 P. Assessment of chromatin status (SCSA®) in epididymal and ejaculated sperm in
453 Iberian red deer, ram and domestic dog. Theriogenology 2006;66:1921-1930.
454 [27] Fraser LR, Adeoya-Osiguwa SA, Baxendale RW, Gibbons R. Regulation of
455 mammalian sperm capacitation by endogenous molecules. Front Biosci 2006;11:1636-
456 1645.
457 [28] Maxwell WM, Evans G, Mortimer ST, Gillan L, Gellatly ES, McPhie CA. Normal
458 fertility in ewes after cervical insemination with frozen-thawed spermatozoa
459 supplemented with seminal plasma. Reprod Fert and Dev 1999; 11: 123-126.
460 [29] O'Meara CM, Donovan A, Hanrahan JP, Duffy P, Fair S, Evans ACO, Lonergan P.
461 Resuspending ram spermatozoa in seminal plasma after cryopreservation does not
462 improve pregnancy rate in cervically inseminated ewes. Theriogenology 2007;67:1262-
463 1268.
464 [30] Way AL, Griel LC, Killian GJ. Effects of accessory sex gland fluid on viability,
465 capacitation, and the acrosome reaction of cauda epididymal bull spermatozoa. J Androl
466 2000;21:213-219.
467 [31] Bollendorf A, Check JH, Lurie D. Evaluation of the effect the absence of sperm
468 with rapid and lineal progressive motility on subsequent pregnancy rates following
469 intrauterine insemination and in vitro fertilization. J Androl 1996;17:550-557.

[32] Tartaglione CM, Ritta MN. Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen-thawed bull semen. Theriogenology 2004;62:1245-1252.

[33] Selvaraju S, Ravindra JP, Ghosh J, Gupta PSP, Suresh KP. Evaluation of sperm functional attributes in relation to in vitro sperm-zona pellucida binding ability and cleavage rate in assessing of frozen thawed buffalo (*Bubalus bubalis*) semen quality. Anim Reprod Sci 2008;106:311-321.

484 Figure 1. Cleavage rate for thawed sperm samples obtained by electroejaculation and
485 postmortem (epididymis) from Black Manchego rams. Bars with different letters
486 indicate statistically significant differences ($P = 0.041$).
487

Figure 2. Relationship between cleavage rate and different sperm parameters for samples obtained postmortem (epididymis) from Black Manchego rams

(A) Relationship between cleavage rate and linearity (LIN) in thawed sperm samples

(B) Relationship between cleavage rate and YO-PRO-1- within PI- spermatozoa subpopulation in incubated sperm samples

Table 1. Effect of collection method (electroejaculation and postmortem recovery) on sperm quality in fresh and thawed samples from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**						
		SM (%)	NAR (%)	Viability (%)	VAP (µm/s)	LIN (%)	ALH (µm)	BCF (Hz)
Fresh	Electroejaculation	74.17±3.96	94.33±0.66	82.00±1.69	194.58±13.99	66.86±4.65	5.93±0.80	10.0±0.59
	Postmortem	76.67±2.10	93.50±1.05	94.67±0.61	175.47±6.62	60.95±3.11	8.32±1.09	8.38±0.46
	P	0.590	0.520	<0.001	0.245	0.316	0.108	0.058
Thawed	Electroejaculation	36.67±7.60	63.00±15.35	45.00±7.13	76.29±4.80	61.24±5.53	4.37±0.50	7.67±0.47
	Postmortem	57.50±4.78	93.17±1.01	60.50±7.09	117.26±8.14	62.64±2.57	5.48±0.51	6.78±0.28
	P	0.043	0.078	0.154	0.001	0.823	0.156	0.138

Data are means ± SEM. SM: sperm motility; NAR: spermatozoa with intact acrosome; VAP: average path velocity; LIN: linearity; ALH: amplitude of the lateral movement of the head; BCF: beat cross frecuency

Table 2. Effect of collection method (electroejaculation and postmortem recovery) on parameters evaluated by flow cytometry in thawed and incubated sperm samples from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**				
		YO-PRO-1-/PI- (%)	PNA-/PI- (%)	Mitotracker+/PI- (%)	% DFI	HDS
Thawed	Electroejaculation	24.91±6.08	23.92±5.39	24.49±6.11	21.42±3.29	0.26±0.02
	Postmortem	65.98±1.53	65.28±1.90	62.71±1.90	15.86±1.83	0.28±0.06
	P	<0.001	<0.001	<0.001	0.171	0.729
Incubated	Electroejaculation	11.11±3.10	11.72±3.19	18.82±3.34	-	-
	Postmortem	28.55±1.68	29.35±1.96	29.65±2.79	-	-
	P	0.001	0.001	0.032	-	-

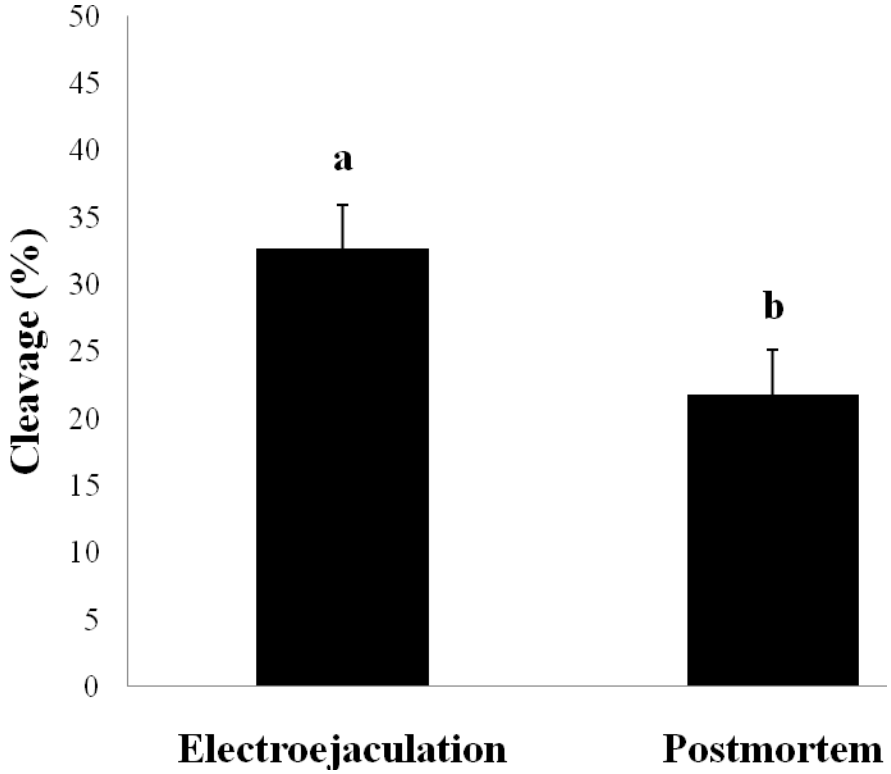
Data are means ± SEM. YO-PRO-1-/PI-: live spermatozoa with intac membrane; PNA-/PI-: live spermatozoa with intact acrosome; Mitotracker+/PI-: live spermatozoa with high mitochondrial membrane potential; % DFI: percentage of spermatozoa with DFI (DNA fragmentation index) higher than 25%; HDS: spermatozoa with high DNA stainability (green fluorescence higher than channel 600)

Table 3. Ratios of YO-PRO-1-, PNA- and Mitotracker+ (considering PI- subpopulation) in thawed and incubated sperm samples recovery by electroejaculation and from epididymis postmortem from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**		
		YO-PRO-1- (%)	PNA- (%)	Mitotracker+ (%)
Thawed	Electroejaculation	93.45±0.57	97.92±0.52	92.32±1.69
	Postmortem	90.58±0.62	93.95±0.88	86.59±1.40
	P	0.007	0.003	0.026
Incubated	Electroejaculation	54.97±5.32	89.29±2.32	77.62±1.27
	Postmortem	48.22±2.83	79.33±1.52	49.20±3.94
	P	0.290	0.005	<0.001

Data are means ± SEM. All data considering only the PI- spermatozoa subpopulation: YO-PRO-1-: spermatozoa with intact membrane; PNA-: spermatozoa with intact acrosome; Mitotracker+: spermatozoa with high mitochondrial membrane potential.

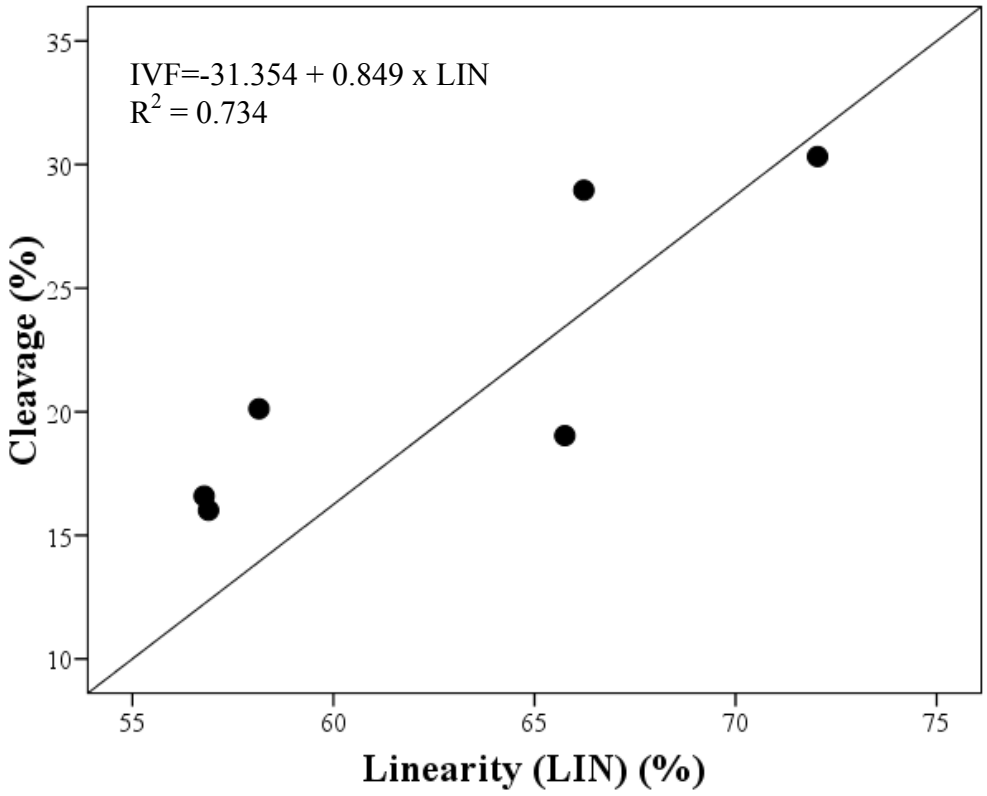
523 Figure 1. García-Álvarez et al.



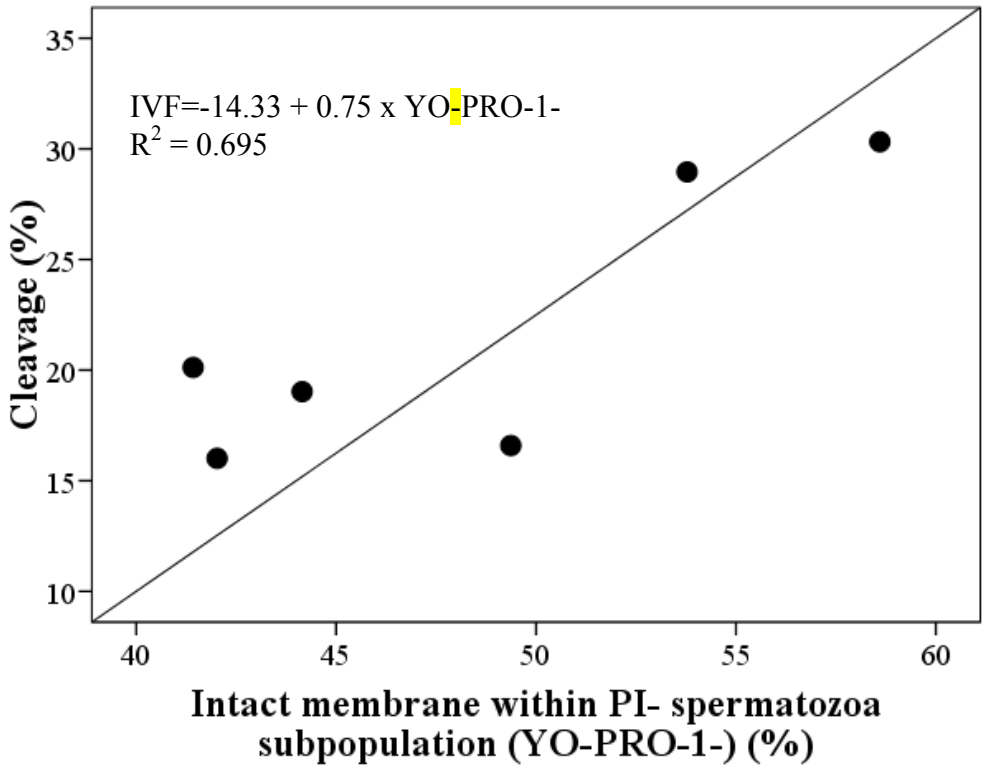
524

Figure 2. García-Álvarez et al.

(A)



(B)



Revised

Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection

Short Title: Effect of sperm collection method

O. García-Álvarez[a], A. Maroto-Morales[a], F. Martínez-Pastor[b], J.J. Garde[b], M.R. Fernández-Santos[a], M.C. Esteso[c], M.D. Pérez-Guzmán[a], A.J. Soler[a,b].

[a] Regional Center of Animal Selection and Reproduction (CERSYRA) JCCM, 13300 Valdepeñas, Spain; [b] Biology of Reproduction Group, National Wildlife Research Institute (IREC) UCLM-CSIC-JCCM, 02071 Albacete, Spain; [c] Animal Reproduction and Obstetrics, University of León, 24071 León, Spain.

*Corresponding author:

Ana J. Soler Valls

Ciencia y Tecnología Agroforestal y Genética, ETSIA, Universidad de Castilla-La Mancha

Av. España s/n, 02071-Albacete (España)

Phone: +34 967 599 200+2552

Fax: +34 967 599 238

e-mail: anajosefa.soler@uclm.es

Abstract

The aim of this study was to assess two models of sperm collection on the quality and fertility of thawed spermatozoa from Black Manchego rams, a threatened breed. Sperm samples were collected by electroejaculation (EE) and postmortem (PM) from each male. Samples were diluted with Biladyl® and frozen. Motility (subjective and CASA), membrane integrity and acrosomal status (microscopy) were assessed on fresh semen; plasmalemma integrity, mitochondrial membrane potential, DNA integrity and acrosomal status were evaluated by flow cytometry on thawed semen. Thawed spermatozoa were used in a heterologous in vitro fertilization test. After thawing, the proportion of live spermatozoa with intact membrane (YO-PRO-1-/PI-) was higher for PM samples ($P < 0.001$), although the ratio of YO-PRO-1- spermatozoa within the PI- population was higher for ejaculated samples ($P = 0.007$). Likewise, the proportion of live spermatozoa having high mitochondrial membrane potential (Mitotracker+) and intact acrosomes (PNA-) was higher for PM samples ($P < 0.001$ and $P < 0.001$, respectively). Considering only live spermatozoa, the ratio of Mitotracker +, PNA- cells was higher for EE samples ($P = 0.026$ and $P = 0.003$). Both EE and PM samples fertilized oocytes. Nevertheless, EE samples yielded a higher percentage of hybrid embryos ($P = 0.041$). In conclusion, although PM spermatozoa had better sperm quality after thawing, EE spermatozoa showed higher ratios for sperm quality when only the live population was considered. EE and PM samples might be used for germplasm banking of this threatened breed, but the fertility of PM spermatozoa might be lower.

Keywords: Electroejaculated spermatozoa; Epididydimal spermatozoa; Germplasm banking; Ram; Heterologous in vitro fertilization

1. Introduction

The Manchega sheep is an autochthonous dairy breed from Spain, with a white and a black variety. Currently, there are few herds of Black Manchega sheep (around 2000 animals), and in Spain it is considered an endangered breed of special interest (RD 1682/1997). As recommended by the Food and Agriculture Organization of the United Nations (FAO), endangered breeds must be preserved by *ex situ* and *in situ* means.

One of the *ex situ* procedures consists in the development of Genetic Resource Banks, cryopreserving gametes and embryos, thus allowing to store genetic resources indefinitely [1]. Assisted Reproductive Technologies have demonstrated to be successful tools to reproduce endangered species, being sperm cryopreservation, in combination with artificial insemination, the method that has been most extensively applied.

Semen can be collected from live animals by artificial vagina [2], but this technique requires a previous training period [3]. Electroejaculation is an alternative when males are not trained to artificial vagina. This method allowed the collection of high quality semen in Guirra ram without noticeable differences from semen collected by artificial vagina [4]. In that study, sperm quality was higher for electroejaculated samples even after cryopreservation.

Postmortem recovery of spermatozoa and their utilization has received much less attention in sheep than in other domestic species [5,6]. Collection and cryopreservation of epididymal spermatozoa is a useful method to rescue germplasm of dead animals which would be otherwise lost, and use it to preserve endangered breeds. This kind of samples have been successfully cryopreserved and used for artificial insemination or in vitro fertilization in other ungulates, such as red deer [7-13].

Ejaculated spermatozoa have a lower cryosurvival and osmotic resistance than epididymal ones [14]. Nevertheless, it has been showed that adding seminal plasma to epididymal spermatozoa protects against the effects of cooling and freezing [15,16]. However, seminal plasma could also have detrimental effects on cryosurvival [17].

In vitro fertility of fresh semen obtained by artificial vagina and postmortem has been studied in sheep [5]. However, so far any studies have been conducted in this species for evaluating the effect of collection method (electroejaculation or postmortem recovery) on post-thawing sperm quality and fertility.

Considering this background, the objective of this study was to evaluate the post-thawing sperm quality and in vitro fertility of sperm samples obtained both by electroejaculation and by postmortem collection from Black Manchega rams, in order to assess its suitability for germplasm banking, and the differences between both methodologies.

2. Materials and Methods

2.1. Animals and reagents

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2003/65. Six rams of Black Manchega breed (age > 3 years) were used. Males were maintained and managed at Regional Center of Animal Selection and Reproduction, placed in Valdepeñas (Spain). These males had not been trained to artificial vagina.

Chemicals were of reagent grade and were purchased from Sigma (Madrid, Spain). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) and peanut agglutinin conjugated with fluoresceine

93 isothyocianate (PNA-FITC), bought from Sigma. Chromatographically purified acridine
94 orange was purchased from Polysciences Inc. (Warrington, PA, USA).
95 *2.2. Semen collection*
96 Semen samples were collected by electroejaculation and from epidydimis postmortem
97 from the same males. Previous to electroejaculation (15 minutes before), males received
98 an intramuscular injection of xylazine (0.2mg kg^{-1} body weight; Rompun 2%, Bayer
99 S.A. Barcelona, Spain). Rectum was cleaned from faeces and the prepucial area was
100 shaved and washed with physiological saline serum. Electroejaculation was carried out
101 using a three-electrode probe (250 mm x 25 mm) connected to a power source that
102 allowed voltage and amperage control (P. T. Electronics, Boring, OR). Semen was
103 collected into a graduated collection vial at 37 ºC.
104 For the postmortem collection, the testicles of each male were obtained at
105 slaughterhouse and transported at room temperature, arriving at the laboratory between
106 0.5 and 1 hour afterwards. Testicles with epididymes attached were isolated from
107 scrotum. Epididymal spermatozoa were collected performing several incisions on each
108 cauda epididymis with a surgical blade, placing the sperm suspension in 1 mL PBS
109 (phosphate buffer saline, pH: 7.5, 310 mOsm/kg).
110 After sperm collection, sperm motility, percentage of spermatozoa with intact
111 acrosome assessed by phase-contrast microscopy (%, NAR) and percentage of live
112 spermatozoa assessed by nigrosin–eosin staining (%; Viability) were assessed as
113 described by Soler et al. [9]. Motility was also assessed by Computer Assisted Semen
114 Analysis (CASA) as described Martínez et al. [18]. A 5 µL drop was put on a slide at 37
115 ºC, covered with a 22 x 22 coverslip and examined (3 fields at least) with a phase
116 contrast microscope (Nikon 80i, negative contrast optics) with a warming state at 37 ºC.
117 Analysis were carried out using the Sperm Class Analyzer software (SCA2002,

Microptic, Barcelona, Spain) and the following parameters were used for the study: average path velocity (μm/s; VAP), linearity (%, LIN), amplitude of the lateral movement of the head (μm; ALH) and beat cross frequency (Hz; BCF).

2.3. Cryopreservation

After initial semen evaluation, sperm samples obtained by electroejaculation and postmortem collection were diluted with freezing extender Biladyl® (Minitüb, Germany) with 20% egg yolk. Semen was diluted to 400 x 10^6 spermatozoa/mL with Biladyl®, Fraction A, at 30 ºC. Diluted semen was cooled to 5 ºC for 2 hours and then was further diluted with the same volume of Bildadyl®, Fraction B. Sperm samples were allowed to equilibrate at 5 ºC for 2 h and packed in 0.25 ml plastic straws. Finally, they were frozen in a programmable biofreezer (IceCube 14S-Ver. 1.30 ©2004 SY-LAB Geräte GmbH Minitüb®) at 20 ºC/min to -100 ºC, and at 10 ºC/min from -100 ºC to -140 ºC and then plunged into liquid nitrogen.

2.4. Assessment of frozen-thawed spermatozoa

Thawing was performed by dropping the straws (two per male and per collection method) in a water bath at 37 ºC for 20 s. Thawed spermatozoa were layered on a Percoll discontinuous density gradient (45%/90%) and centrifuged at 700 x g for 10 min in order to eliminate the particles of egg yolk and enrich the sample in highly motile spermatozoa. After centrifugation, the supernatant was carefully removed. Sperm samples were evaluated for sperm motility, acrosomal status by phase-contrast (%; NAR), viability by nigrosin-eosin (%; Viability) and motility by CASA in the same way that in fresh sperm samples. After this evaluation, thawed samples were incubated at 37 ºC for 2 h, and then sperm motility, acrosomal status (%; NAR) and viability (%; Viability) were assessed as previously described.

142 In addition, two more straws were thawed for the assessment of the seminal
143 quality by flow cytometry. We assessed the membrane status with YO-PRO-1, the
144 mitochondrial membrane potential with Mitotracker Deep Red, the acrosome integrity
145 with peanut agglutinin conjugated with fluorescein isothiocyanate, (FITC-PNA), and
146 the viability (membrane integrity) with propidium ioide (PI).
147 We prepared two staining solution using flow cytometer sheath fluid (BD
148 FACSFlow^{TM}). One of them was prepared with 3 nM Hoechst 33342 (stock: 9 µM in
149 milli-Q water), 50 nM YO-PRO-1 (stock: 100 µM in DMSO), 15 µM PI (stock: 7.5 mM
150 in milli-Q water) and 100 nM of Mitotracker Deep Red (stock: 1 mM in DMSO). The
151 other solution was prepared with 3nM Hoechst 33342, 15 µM PI, and 10 µg/mL of
152 PNA-FITC (stock of 0.2 mg/mL in dH_2O). We diluted the sample in 0.5 mL of each
153 staining solution in polypropylene tubes for flow cytometry (5 x 10^6 spermatozoa/mL).
154 The tubes were allowed to rest for 15 min in the dark and then analyzed using a Becton
155 Dickinson LSR-I flow cytometer (BD Biosciences, San José, CA, USA). We used the
156 three lasers of the cytometer to excite the different fluorochromes. A 325 nm Helium-
157 Cadmium UV laser for exciting the Hoechst 33342, a 488 nm Argon-Ion laser for
158 exciting YO-PRO-1, PNA-FITC and PI, and a 633 nm Helium-Neon laser for exciting
159 Mitotracker Deep Red. We acquired the FSC (forward-scatter light) and SSC (side-
160 scatter light) signals plus the fluorescence light of each fluorochrome using four
161 photodetectors. FL1 was used for YO-PRO-1 and PNA-FITC (530/28BP filter), FL3 for
162 propidium ioide (670LP filter), FL5 for Hoechst 33342 (424/44BP filter) and FL6 for
163 Mitotracker Deep Red (670/40BP filter). The acquisition was controlled using the Cell
164 Quest Pro 3.1 software (BD Biosciences). All the parameters were read using
165 logarithmic amplification. We set up an acquisition template in the software which
166 allowed us first to discriminate spermatozoa from debris within the events acquired.

FSC/SSC and FL5/FL3 (Hoechst 33342 vs. PI) dot-plots were used to discard debris. The filtered events were displayed in dot-plots showing either FL1/FL3 (YO-PRO-1 vs. PI or PNA-FITC vs. PI) and FL6/FL3 (Mitotracker Deep Red vs. PI). We acquired 10000 spermatozoa from each sample, saving the data in FCS v. 2 files. The analysis of the flow cytometry data was carried out using WinMDI v. 2.8 (The Scripps Research Institute, La Jolla, CA, USA).

YO-PRO-1-/PI- spermatozoa were considered as viable (non-"apoptotic", intact membrane), Mitotracker+/PI- spermatozoa as viable with high mitochondrial membrane potential and PNA-/PI- spermatozoa as viable with intact acrosome. Also, we calculated the ratios of YO-PRO-1-, Mitotracker+ and PNA- within live spermatozoa population (PI-).

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics, Inc., Brookings, SD, USA) [19]. This technique is based in the susceptibility of the sperm DNA to acid-induced denaturation in situ and in the metachromatic staining Acridine Orange (AO). This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denatured). Thawed spermatozoa were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM EDTA; pH=7.4) to 2 x 10^6 cells/mL. Samples were flash frozen in LN2 and stored at -80 ºC until analysis. For the analysis, the samples were thawed on crushed ice and 200 µl were put on a cytometry tube. Then, we added 400 µl of an acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH=1.2). Exactly 30 sec after adding the acid-detergent solution, we added 1.2 mL of staining solution (6 µg/mL of acridine orange in a buffer containing 37 mM citric acid, 126 mM Na_2HPO_4, 1.1 mM disodium EDTA and 150 mM NaCl; pH=6). After 3 min, the sample was run through a Becton Dikinson LSR-1 flow cytometer. Acridine orange was excited

using an argon laser providing 488 nm light. The red fluorescence was detected using a long pass (670LP) filter (FL-3) and the green one using a band pass (530/28BP) filter (FL-1). Sample acquisition was carried out with a CellQuest v. 3 software. Flow Cytometry data (FCS files) were processed and saved as tabbed text using WinMDI v. 2.8 (The Scripps Research Institute, La Jolla, California). We calculated the DNA fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red+green), expressed as a percentage. High values of DFI, indicates chromatin abnormalities. We also calculated %DFI, as the percentage of spermatozoa with DFI > 25, and High DNA Stainability (HDS) as the percentage of the spermatozoa with green fluorescence higher than channel 600 (of 1024 channels).

Thawed semen was incubated at 37 ºC for 2 h in the same freezing extender and all sperm parameters previously cited, except chromatin stability, were assessed by flow cytometry.

2.5. Heterologous in vitro fertilization (IVF)

We carried out a heterologous IVF test to assess the fertilizating potential of electroejaculated and postmortem sperm samples, since this assay is highly related with the in vivo fertility as described by García-Álvarez et al. [20]. We performed heterologous IVF four times per male and method collection, and a minimum of twenty oocytes per well was used. Ovaries were collected at slaughterhouse from heifers about 1 year old, and transported to our laboratory in saline solution (30 ºC) between 1-2 h after removal. Immature oocytes were collected from the ovaries using 19-gauge needle, in TCM-199 supplemented with HEPES (2.39 mg/mL), heparin (2 µL/mL) and gentamycin (40 µg/mL). Aspirated cumulus oocyte complexes (COC) were washed in TCM-199 gentamycin (40 µg/mL), selecting those with dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells. These COC were placed in four-well

plates containing 500 µL of TCM-199 supplemented with cysteamine (100 µM) and epidermal growth factor (EGF) (10 ng/ml) and matured at 38.5 ºC in 5% CO_2. After 24 h, COC were washed in synthetic oviduct fluid (SOF) supplemented with essential and non-essential amino acids [21], and cumulus cells were removed by gentle pipetting. Oocytes (minimum 20 oocytes per well) were transferred to four-well plates with 400 µL of fertilization medium (SOF supplemented with 10% of estrous sheep serum and 40 µg/mL gentamycin) under mineral oil.

Thawed samples (electroejaculated and epididymary) were centrifuged through a Percoll discontinuous density gradient (45%/90%) and were capacitated in the fertilization medium for 10 min. Sperm was co-incubated with oocytes at a final concentration of 10^6 spermatozoa/mL at 38.5 ºC in 5% CO_2.

Oocytes were evaluated visually with an inverted microscope (Nikon Eclipse TE2000-U) 40 h later for cleavage (two to eight cells). Then, the oocytes were fixed and stained with Hoechst 33342 to confirm the fertilization by the presence of 2 or more nuclei (%; cleavage).

2.6. Statistical Analysis

Statistical analyses were carried out using SPSS®, version 15.0 (SPSS Inc., Chicago, IL, USA). A GLM-ANOVA tested the effect of collection method (electroejaculation and postmortem recovery) on sperm quality for fresh, thawed and incubated sperm samples, and on heterologous in vitro fertility for thawed sperm samples. Data were split into two groups according to collection method (electroejaculation or postmortem recovery) and Pearson rank correlations were carried out to study the relationships between % cleavage and sperm quality. Also, stepwise multiple regression analyses were carried out in order to predict the % cleavage from quality analyses for fresh, thawed and incubated samples. Significance was set at $P < 0.05$.

242

243 **3. Results**

244 Sperm quality of fresh samples was not different between collection methods, except for
245 sperm viability, which was higher in postmortem samples ($P < 0.001$) (Table 1).

246 After thawing, sperm motility and velocity were higher in postmortem samples
247 ($P = 0.043$ and $P = 0.001$, respectively) (Table 1). There were significant differences
248 between electroejaculated and postmortem samples for most of the parameters
249 evaluated by flow cytometry, with lower values for electroejaculated spermatozoa (YO-
250 PRO-1-/PI-;$P < 0.001$, PNA-/PI-; $P < 0.001$, Mitotracker+/PI-; $P < 0.001$) (Table 2).
251 However, considering the live spermatozoa population (PI-) the electroejaculated
252 samples yielded a higher ratio of YO-PRO-1-, ($P = 0.007$), PNA- ($P = 0.003$) and
253 Mitotracker+ ($P = 0.026$) spermatozoa (Table 3). Moreover, the cleavage rate after the
254 heterologous IVF was higher for electroejaculated spermatozoa (32.69% *vs* 21.84%, $P =$
255 0.041) (Fig. 1).

256 After incubation, sperm motility and the percentage of spermatozoa with intact
257 acrosome (subjectively assessed) were higher for postmortem samples (34.16 ± 3.87%
258 *vs*. 10.00 ± 4.74%, $P = 0.004$, and 74.33 ± 6.35% *vs*. 19.25 ± 7.78%, $P = 0.001$,
259 respectively). We did not find significant differences between the collection methods
260 for sperm viability (EE: 33.83 ± 5.92% and PM: 24.00 ± 7.25%, $P = 0.324$). The
261 percentages of YO-PRO-1-/PI-, PNA-/PI- and Mitotracker+/PI- were higher in
262 postmortem samples ($P = 0.001$, $P = 0.001$ and $P = 0.032$, respectively) (Table 2).
263 However, considering only the PI- ("live") spermatozoa population the ratios of PNA-
264 and Mitotracker+ were higher for electroejaculated samples ($P = 0.005$ and $P < 0.001$,
265 respectively) (Table 3).

We also studied the relationships between sperm quality parameters and cleavage rate for samples obtained by electroejaculation and those obtained from the epididymis postmortem. For fresh sperm samples, any parameter was correlated with the % cleavage for both kinds of samples (electroejaculation and postmortem). However, for thawed sperm samples recovered from the epididymis postmortem, there was a correlation between LIN and cleavage rate ($r = 0.85$, $P = 0.029$). Incubated samples displayed a relationship between the ratio of YO-PRO-1- within PI- spermatozoa subpopulation and the fertility in postmortem samples ($r = 0.83$, $P = 0.039$).

Stepwise multiple regression analyses were used to select a set of sperm quality variables in fresh, thawed and incubated samples that best predicted fertility (cleavage rate) in the samples collected by electroejaculation and postmortem. For fresh sperm samples obtained either by electroejaculation or postmortem, any parameter was predictive of the-fertility. For thawed epididymal samples, LIN was the only predictor of fertility ($r^2 = 0.73$, $P = 0.029$) (Fig. 2A). After incubation, the ratio of YO-PRO-1- within PI- subpopulation in postmortem samples was the only predictor of fertility ($r^2 = 0.69$, $P = 0.039$) (Fig. 2B).

4. Discussion

In this study, we assessed the effect of collection method (electroejaculation and postmortem), on sperm quality for fresh, thawed and incubated samples and on in vitro fertility from black Manchego rams. Both collection methods provided good sperm quality. Electroejaculation is an excellent choice for animals that have not been trained to artificial vagina, whereas postmortem collection can be used as an emergency method when valuable males die suddenly.

Our results showed that sperm quality for fresh samples was similar for electroejaculated and epididymal spermatozoa, although the viability was higher for epididymal spermatozoa (postmortem recovery). Other authors have studied the effect of collection method in other species. Blash et al. [22] compared the quality of epididymal spermatozoa and semen obtained by artificial vagina from goat bucks. These authors showed that for fresh samples the viability was higher in epididymal spermatozoa, results that agree with the finds out of this study. However, in other species such as red deer, the viability was higher in electroejaculated samples that in those obtained postmortem [18]. It is difficult to explain why these studies differed with ours, since different methodologies (collection and analyses) were used in these studies.

Cryopreservation, as expected, substantially reduced sperm viability in both kinds of samples (electroejaculated and postmortem), yielding better results for PM samples. Recent studies showed that electroejaculated samples had lower resistance than epididymal spermatozoa when submitted to cryobiologicaly stressful conditions such as chilling, osmotic stress and addition and removal of cryoprotective agents [14]. In our study, sperm quality of electroejaculated samples was worse than that of epididymal samples, after thawing. Nevertheless, the ratios for YO-PRO-1-, PNA- and Mitotracker+ spermatozoa, considering live spermatozoa subpopulation, were lower for epididymal spermatozoa, suggesting a higher heterogeneity, which may have contributed to their lower fertility results.

With respect to the IVF results, our outcomes do not agree with those obtained by Kaabi et al. [5], who found that ram epididymal and ejaculated spermatozoa rendered similar results in an IVF test. Our results may differ because Kaabi et al. [5] obtained ejaculated spermatozoa using artificial vagina, not electroejaculation, and because of rearing or breed differences. Furthermore, Blash et al. [22] did not find differences after

316 an IVF assay using ejaculated and epididymal spermatozoa from goat. However, they
317 found a higher pregnancy rate for ejaculated samples. Other authors showed that
318 ejaculated bull spermatozoa had a higher binding ability to zona pellucida than those
319 obtained postmortem from the same animals [23].

320 Spermatozoa complete their maturation during their transport along epididymis
321 [24]. Evenson and Wixon, [25] described that lack of appropriate sperm maturation
322 resulted in increased DNA stainability (HDS). Our results showed that there were not
323 differences in HDS between electroejaculated and epididymal spermatozoa. Likewise,
324 %DFI, indicative of DNA fragmentation, was not different for both collection methods
325 (electroejaculation and postmortem, recovery). García-Macías et al. [26] found in ram
326 that spermatozoa obtained of cauda epididymis had highly condensed chromatin, similar
327 to ejaculated samples obtained by artificial vagina. Our data confirm that
328 electroejaculated and postmortem samples have similar maturity indexes and that,
329 although epididymal spermatozoa are more resistant to cryopreservation, this procedure
330 affects similarly to both types of samples regarding to chromatin status.

331 The differences in IVF could be explained considering that electroejaculated and
332 epididymal spermatozoa respond differently to the capacitation treatments of the IVF
333 protocol used in this study. A very important difference between both kinds of samples
334 is that ejaculated spermatozoa contact with the seminal plasma. Recent papers have
335 revealed important roles for a number of proteins found in seminal plasma, which act in
336 the regulation of in vitro capacitation [27]. Thus, Maxwell et al. [28], suggested that
337 components of seminal plasma were responsible for the improved pregnancy rate
338 following the resuspension of frozen-thawed ram spermatozoa in a medium containing
339 seminal plasma. However, in recent studies, the addition of seminal plasma to
340 ejaculated semen did not improve the pregnancy rate in cervicaly inseminated ewes

[29], and the addition of seminal plasma to epididymal spermatozoa accelerated cell death in other cases [30]. In the present study is impossible to know if the higher fertility of ejaculated sperm samples was due to the action of seminal plasma or to the intrinsic characteristics of ejaculated cells.

We obtained correlations between several quality parameters and fertility only for the samples obtained postmortem, possibly due to their higher heterogeneity. These correlations were not found in fresh samples, but appear after thawing with LIN (linearity) and after incubation with the ratio of YO-PRO-1- in the PI- subpopulation. Both LIN [31] and membrane integrity [32, 33] have been found to be good predictors of fertilization and pregnancy rate. It is possible that epididymal sperm samples with a lower ratio of YO-PRO-1-, therefore with a predominant subpopulation of "apoptotic" spermatozoa (YO-PRO-1+/PI-) in the PI- subpopulation, were announcing a lower fertilizing ability.

Our results allow us concluding that it is possible to obtain ram spermatozoa of good quality and freezability both by electroejaculation and postmortem. Therefore, these methods might be used to preserve semen of endangered breeds, either when males have not been trained for artificial vagina (electroejaculation), in case of sudden death, or as a last resource in the case that males must be removed from herds (postmortem recovery). Nevertheless, in vitro fertility was higher for thawed spermatozoa recovery by electroejaculation. Further studies are necessary in order to explain this difference, and to improve IVF protocols for epididymal spermatozoa.

ACKNOWLEDGEMENTS:

This work was supported by the Education and Science Council (PBI-05-011), by Spanish Ministry of Education and Science (RZ2006-00006-C3) and by Agriculture

366 Council (PREG-05-004) of Junta de Comunidades de Castilla-La Mancha (JCCM). The
367 authors thank to Frimancha Industrias Cárnicas S.A. for their collaboration in the
368 collection of the ovaries used in this work. Olga García-Álvarez and Alejandro Maroto
369 Morales were recipients of scholarships from INIA and JCCM, respectively. Felipe
370 Martínez-Pastor, María Rocío Fernández-Santos and Milagros C. Esteso were supported
371 by the Juan de la Cierva program from the Spanish Ministry of Education and Science.
372 We are grateful to Alfonso Bisbal for his help revising the language of the manuscript.

373 **References**

374 [1] Watson PF, Holt WV. Organizational issues concerning the establishment of a
375 genetic resource bank. In: Cryobanking the Genetic Resource. Wildlife Conservation
376 the Future?, Watson PF, Holt WV (Eds.), Taylor and Francis, London; 2001. p. 86-112.
377 [2] Salamon S, Evans G, Maxwell WMC. Inseminación artificial de ovejas y cabras.
378 Editorial ACRIBIA, S.A. Zaragoza (España); 1990. p. 87–95.
379 [3] Wulster-Radcliffe MC, Williams MA, Stellflug JN, Lewis GS. Technical note:
380 artificial vagina vs. a vaginal collection vial for collecting semen from rams. J Anim Sci
381 2001;79:2964-2967.
382 [4] Marco-Jiménez F, Puchades S, Gadea J, Vicente JS, Viudes-de-Castro MP. Effect of
383 semen collection method on pre- and post-thaw Guirra ram spermatozoa.
384 Theriogenology 2005;64:1756-1765.
385 [5] Kaabi M, de Paz P, Álvarez M, Anel E, Boixo JC, Rouissi H, Herraez P, Anel L.
386 Effect of epididymis handling conditions on the quality of ram spermatozoa recovered
387 postmortem. Theriogenology 2003;60:1249-1259.
388 [6] Ehling C, Rath D, Struckmann C, Frenzel A, Schindler L, Niemann H. Utilization of
389 frozen-thawed epididymal ram semen to preserve genetic diversity in Scrapie
390 susceptible sheep breeds. Theriogenology 2006;66:2160-2164.
391 [7] Soler AJ, García AJ, Fernández-Santos MR, Esteso MC, Garde JJ. Effects of
392 thawing procedure on post-thawed in vitro viability and in vivo fertility of red deer
393 epididymal spermatozoa cryopreserved at -196ºC. J Androl 2003;24:746-756.
394 [8] Martínez-Pastor F, García-Macías V, Ávarez M, Herráez P, Anel L, de Paz P. Sperm
395 subpopulation in Iberian red deer epididymal sperm and their changes through the
396 cryopreservation process. Biol Reprod 2005;72:316-327.

[9] Soler AJ, Esteso MC, Fernández-Santos MR, Garde JJ. Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5ºC in the epididymis for several days. Theriogenology 2005;64:1503-1517.

[10] Fernández-Santos MR, Esteso MC, Montoro V, Soler AJ, Garde JJ. Cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa: effects of egg yolk, glycerol and cooling rate. Theriogenology 2006;66:1931-1942.

[11] Martínez-Pastor F, García-Macías V, Esteso MC, Anel E, Fernández-Santos MR, Soler AJ, de Paz P, Garde JJ, Anel L. A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology 2006;66:1165-1172.

[12] Gomendio M, Malo AF, Soler AJ, Fernández-Santos MR, Esteso MC, García AJ, Roldán ER, Garde JJ. Male fertility and sex ratio at birth in red deer. Science 2006;314:1445-1447.

[13] Soler AJ, Poulin N, Fernández-Santos MR, Cognie Y, Esteso MC, Garde JJ, Mermillod P. Heterologous in vitro fertility evaluation of cryopreserved Iberian red deer epididymal spermatozoa with zona-intact sheep oocytes and its relationship with the characteristics of thawed spermatozoa. Reprod Domest Anim 2008;43:293-298.

[14] Varisli O, Uguz C, Agca C, Agca Y. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperaturas. Anim Reprod Sci 2009;110:256-268.

[15] Barrios B, Pérez-Pe R, Gallego M, Tato A, Osada J, Muiño-Blanco T. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod 2000;63:1531-1537.

422 [16] Martínez-Pastor F, Anel L, Guerra C, Álvarez M, Soler AJ, Garde JJ, Chamorro C,
423 de Paz P. Seminal plasma improves cryopreservation of Iberian red deer epididymal
424 sperm. Theriogenology 2006;66:1847-1856.
425 [17] Kawano N, Shimada M, Terada T. Motility and penetration competence of frozen-
426 thawed miniature pig spermatozoa are substantially altered by exposure to seminal
427 plasma before freezing. Theriogenology 2004;61:351-364.
428 [18] Martínez F, Martínez-Pastor F, Álvarez M, Fernández-Santos MR, Esteso MC, de
429 Paz P, Garde JJ, Anel L. Sperm parameters on Iberina red deer: Electroejaculation and
430 postmortem collection. Theriogenology 2008;70:216-226.
431 [19] Evenson DP, Higgins PJ, Grueneberg D, Ballachey BE. Flow cytometric analysis
432 of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry
433 1985;6:238-253.
434 [20] García-Alvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos MR,
435 Esteso MC, Pérez-Guzmán MD, Soler AJ. Heterologous in vitro fertilization is a good
436 procedure to assess the fertility of thawed ram sperm. Theriogenology 2008 (doi:
437 10.1016/j.theriogenology.2008.09.036).
438 [21] Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and
439 development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of
440 serum and somatic cells: amino acids, vitamins, and culturing embryos in groups
441 stimulate development. Biol Reprod 1994;50: 390-400.
442 [22] Blash S, Melican D, Gavin W. Cryopreservation of epididymal sperm obtained at
443 necropsy from goats. Theriogenology 2000;54:899-905.
444 [23] Ellington JE, Wilker CW, Hillman RB, Ball BA. Ability of epididymal or
445 ejaculated bull spermatozoa to bind to cow zonae before and after oviduct epithelial cell
446 co-culture. Theriogenology 1993;39:213.

447 [24] Yanagimachi R. Mammalian fertilization. In: The physiology of reproduction,
448 Knobil E, Neil J (Eds.), Plenum Press, New York, 1988. p. 135-185.
449 [25] Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection
450 and male infertility. Theriogenology 2006;65:979-991.
451 [26] García-Macias V, Martínez-Pastor F, Álvarez M, Garde JJ, Anel E, Anel L, de Paz
452 P. Assessment of chromatin status (SCSA®) in epididymal and ejaculated sperm in
453 Iberian red deer, ram and domestic dog. Theriogenology 2006;66:1921-1930.
454 [27] Fraser LR, Adeoya-Osiguwa SA, Baxendale RW, Gibbons R. Regulation of
455 mammalian sperm capacitation by endogenous molecules. Front Biosci 2006;11:1636-
456 1645.
457 [28] Maxwell WM, Evans G, Mortimer ST, Gillan L, Gellatly ES, McPhie CA. Normal
458 fertility in ewes after cervical insemination with frozen-thawed spermatozoa
459 supplemented with seminal plasma. Reprod Fert and Dev 1999; 11: 123-126.
460 [29] O'Meara CM, Donovan A, Hanrahan JP, Duffy P, Fair S, Evans ACO, Lonergan P.
461 Resuspending ram spermatozoa in seminal plasma after cryopreservation does not
462 improve pregnancy rate in cervically inseminated ewes. Theriogenology 2007;67:1262-
463 1268.
464 [30] Way AL, Griel LC, Killian GJ. Effects of accessory sex gland fluid on viability,
465 capacitation, and the acrosome reaction of cauda epididymal bull spermatozoa. J Androl
466 2000;21:213-219.
467 [31] Bollendorf A, Check JH, Lurie D. Evaluation of the effect the absence of sperm
468 with rapid and lineal progressive motility on subsequent pregnancy rates following
469 intrauterine insemination and in vitro fertilization. J Androl 1996;17:550-557.

[32] Tartaglione CM, Ritta MN. Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen-thawed bull semen. Theriogenology 2004;62:1245-1252.

[33] Selvaraju S, Ravindra JP, Ghosh J, Gupta PSP, Suresh KP. Evaluation of sperm functional attributes in relation to in vitro sperm-zona pellucida binding ability and cleavage rate in assessing of frozen thawed buffalo (*Bubalus bubalis*) semen quality. Anim Reprod Sci 2008;106:311-321.

484 Figure 1. Cleavage rate for thawed sperm samples obtained by electroejaculation and
485 postmortem (epididymis) from Black Manchego rams. Bars with different letters
486 indicate statistically significant differences ($P = 0.041$).
487

Figure 2. Relationship between cleavage rate and different sperm parameters for samples obtained postmortem (epididymis) from Black Manchego rams

(A) Relationship between cleavage rate and linearity (LIN) in thawed sperm samples

(B) Relationship between cleavage rate and YO-PRO-1- within PI- spermatozoa subpopulation in incubated sperm samples

Table 1. Effect of collection method (electroejaculation and postmortem recovery) on sperm quality in fresh and thawed samples from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**						
		SM (%)	NAR (%)	Viability (%)	VAP (μm/s)	LIN (%)	ALH (μm)	BCF (Hz)
Fresh	Electroejaculation	74.17±3.96	94.33±0.66	82.00±1.69	194.58±13.99	66.86±4.65	5.93±0.80	10.0±0.59
	Postmortem	76.67±2.10	93.50±1.05	94.67±0.61	175.47±6.62	60.95±3.11	8.32±1.09	8.38±0.46
	P	0.590	0.520	<0.001	0.245	0.316	0.108	0.058
Thawed	Electroejaculation	36.67±7.60	63.00±15.35	45.00±7.13	76.29±4.80	61.24±5.53	4.37±0.50	7.67±0.47
	Postmortem	57.50±4.78	93.17±1.01	60.50±7.09	117.26±8.14	62.64±2.57	5.48±0.51	6.78±0.28
	P	0.043	0.078	0.154	0.001	0.823	0.156	0.138

Data are means ± SEM. SM: sperm motility; NAR: spermatozoa with intact acrosome; VAP: average path velocity; LIN: linearity; ALH: amplitude of the lateral movement of the head; BCF: beat cross frecuency

Table 2. Effect of collection method (electroejaculation and postmortem recovery) on parameters evaluated by flow cytometry in thawed and incubated sperm samples from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**				
		YO-PRO-1-/PI- (%)	PNA-/PI- (%)	Mitotracker+/PI- (%)	% DFI	HDS
Thawed	Electroejaculation	24.91±6.08	23.92±5.39	24.49±6.11	21.42±3.29	0.26±0.02
	Postmortem	65.98±1.53	65.28±1.90	62.71±1.90	15.86±1.83	0.28±0.06
	P	<0.001	<0.001	<0.001	0.171	0.729
Incubated	Electroejaculation	11.11±3.10	11.72±3.19	18.82±3.34	-	-
	Postmortem	28.55±1.68	29.35±1.96	29.65±2.79	-	-
	P	0.001	0.001	0.032	-	-

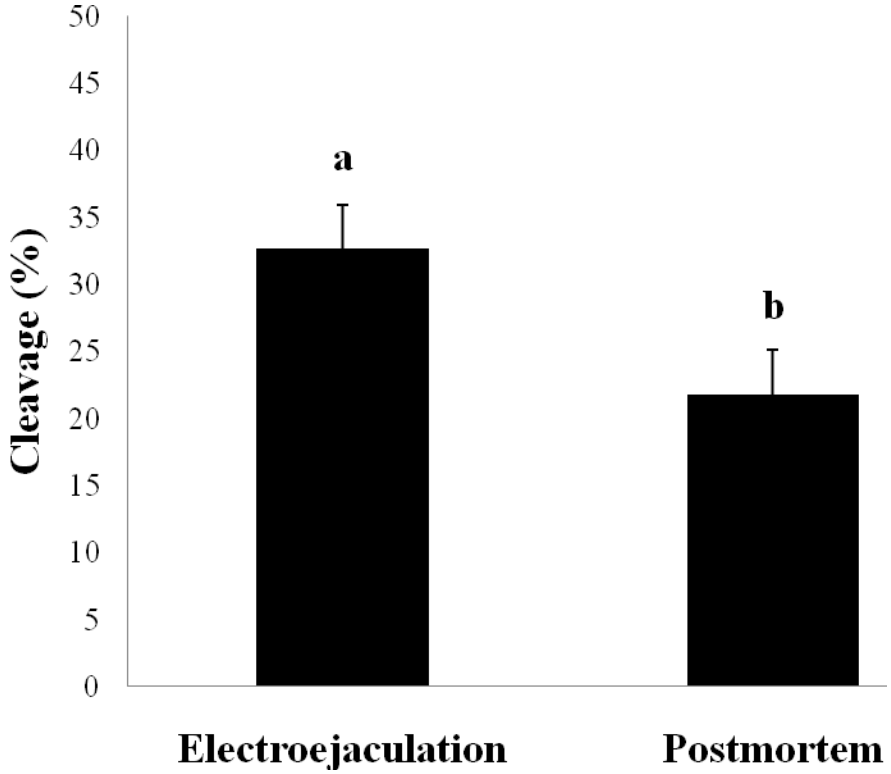
Data are means ± SEM. YO-PRO-1-/PI-: live spermatozoa with intac membrane; PNA-/PI-: live spermatozoa with intact acrosome; Mitotracker+/PI-: live spermatozoa with high mitochondrial membrane potential; % DFI: percentage of spermatozoa with DFI (DNA fragmentation index) higher than 25%; HDS: spermatozoa with high DNA stainability (green fluorescence higher than channel 600)

Table 3. Ratios of YO-PRO-1-, PNA- and Mitotracker+ (considering PI- subpopulation) in thawed and incubated sperm samples recovery by electroejaculation and from epididymis postmortem from six Black Manchego rams.

Sperm samples	**Collection method**	**Sperm parameter**		
		YO-PRO-1- (%)	PNA- (%)	Mitotracker+ (%)
Thawed	Electroejaculation	93.45±0.57	97.92±0.52	92.32±1.69
	Postmortem	90.58±0.62	93.95±0.88	86.59±1.40
	P	0.007	0.003	0.026
Incubated	Electroejaculation	54.97±5.32	89.29±2.32	77.62±1.27
	Postmortem	48.22±2.83	79.33±1.52	49.20±3.94
	P	0.290	0.005	<0.001

Data are means ± SEM. All data considering only the PI- spermatozoa subpopulation: YO-PRO-1-: spermatozoa with intact membrane; PNA-: spermatozoa with intact acrosome; Mitotracker+: spermatozoa with high mitochondrial membrane potential.

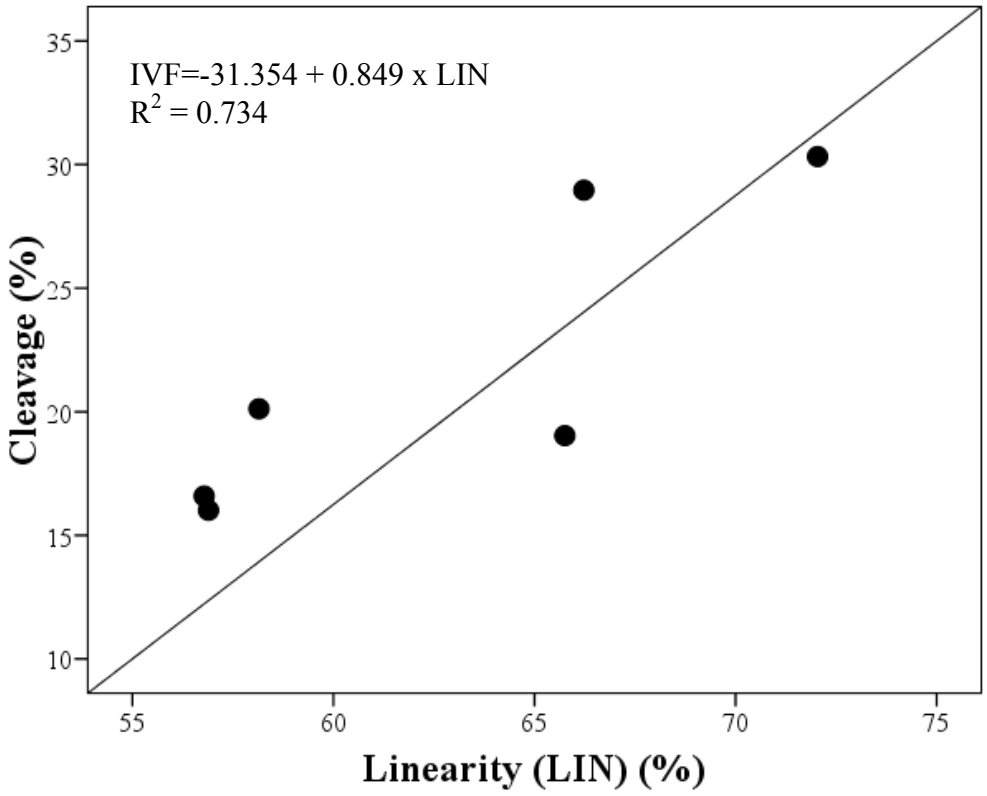
523 Figure 1. García-Álvarez et al.



524

Figure 2. García-Álvarez et al.

(A)



(B)