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Effect of Seminal Plasma on Post-Thaw Quality and Functionality of *Corriedale* Ram Sperm Obtained by Electroejaculation and Artificial Vagina

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Contents

We have already shown that seminal collection method affects seminal plasma composition and sperm quality in *Corriedale* rams. In this study, we evaluated the effect of seminal plasma collected by electroejaculation or artificial vagina on sperm resistance to cryodamage. Seminal plasma of five rams of the *Corriedale* breed collected by artificial vagina or electroejaculation was added before freezing to sperm cells collected by the two methods, and post-thaw quality parameters were evaluated. We found that seminal plasma has no effect on sperm resistance to cryodamage. However, we observed significantly higher percentages of sperm with intact and functional plasma membrane, intact acrosome and greater fertilizing potential after thawing in samples obtained by electroejaculation. This study demonstrates that sperm collected by electroejaculation are more resistant to damage caused by cryopreservation than those collected by artificial vagina.

Introduction

It is widely known that the cryopreservation process, involving cooling, freezing and thawing, produces serious detrimental changes in sperm function resulting in lowered fertilizing capacity and low lambing rates after cervical insemination (Mattner et al. 1969; Lightfoot and Salamon 1970). Among those changes, alterations in motility, motion characteristics and membrane integrity have been studied in multiple species such as ram (Holt and North 1994), boar (Buhr et al. 1994), bull (Gravance et al. 1998) and goat (Purdy 2006). At the cellular level, it is known that sperm cryodamage includes the premature induction of a capacitation-like status, also called cryocapacitation (Bailey et al. 2003). Capacitation is a physiological phenomenon by which the sperm acquire the fertilizing ability (Yanagimachi 1994) and occurs within the female reproductive tract through a series of events that alter the stability and permeability of the cell membrane and triggers internal signalling cascades ending with two important events known as acrosomal reaction (AR) and flagellar hyperactivation (Benoff 1993). These physiological events (capacitation and AR) are chained and end either with the egg fertilization or with sperm death. Therefore, if they occur prematurely, there is an asynchrony between the ovulation time and fertilization engaging (Yanagimachi 1994). Thus, cryocapacitated sperm acquire the ability to fertilize the egg too early, while reducing their half-life.

In ram, seminal collection techniques of wider use are the artificial vagina (AV) and electroejaculation (EE). The collection of semen with AV resembles natural service and is easy to apply, but usually requires a previous training period, which may extend up to 3 months depending on the male (Wulster-Radclife et al. 2001). The use of EE has some advantages over the AV because it is a quick and more convenient method that allows the collection of semen from a large number of animals and without any training. Ejaculates collected with EE have an increased volume compared with ejaculates collected with AV, due to the presence of a higher proportion of seminal plasma (SP) (Marco-Jiménez et al. 2005; Jiménez-Rabadán et al. 2012) as a result of an overstimulation of accessory sex glands (Mattner and Voglmayr 1962).

Semen quality after freeze also differs according to the collection method applied. Marco-Jiménez et al. (2005) observed that sperm collected with EE were more resistant to cryodamage than when obtained by AV. However, other studies provided the opposite evidence (Quinn et al. 1968; Jiménez-Rabadán et al. 2012) and hypothesized that freezability differences are related to intrinsic differences in SP. More recently, we have shown that the SP collection method significantly affects its protein composition, concentration and sperm quality. Electroejaculation allows to obtain ejaculates with a higher proportion of SP, total protein content and relative abundance of low molecular weight proteins including RSVP14 and RSVP22 (Ledesma et al. 2014).

It has been reported that both the whole SP as the isolated SP proteins increase sperm resistance to cryopreservation and cold-shock when added either before or after semen freezing (Graham 1994; Barrios et al. 2000; Pérez-Pé et al. 2001). Addition of SP improved motility, viability, acrosome integrity and mitochondrial respiration in frozen-thawed ram sperm (Domínguez et al. 2008; Bernardini et al. 2011). Moreover, SP increased sperm penetration of cervical mucus and the percentage of pregnant ewes after cervical insemination (Maxwell et al. 1999; Rickard et al. 2014). The beneficial effect of SP has been attributed to its protein components, especially to low molecular weight proteins (≤30 kDa) synthesized in the seminal vesicles. In addition, Pérez-Pé et al. (2001) reported that this protection effect was protein concentration dependent. Barrios

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et al. (2005) demonstrated that two SP proteins were capable of protect sperm from cryodamage. These two proteins were named according to their origin (seminal vesicles) and molecular weight as RSVP14 and RSVP20 (Fernández-Juan et al. 2006).

With this background, the aim of this study was to evaluate the effect of SP collected with EE and AV on post-thaw quality parameters of ram sperm collected with both semen collection methods.

Materials and Methods

Animals and management

All animals used in this study were handled in strict accordance with good animal practice and the conditions approved by the Animal Ethics Committee at INTA, Argentina. All efforts were made to minimize animals suffering. The experiment was carried out in the Experimental Station of Balcarce (Instituto Nacional de Tecnología Agropecuaria, INTA Argentina; 37°45′ south, 58°18′ west), during the natural breeding season (March-June; autumn). Five fertile mature (5 years old) *Corriedale* rams, with a mean body condition score of 2.9 ± 0.8 (scale 1–5), were used in the study. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Semen collection

Semen samples were obtained using AV and EE methods according to Marco-Jiménez et al. (2008). Briefly, ejaculates were routinely collected from all males using the AV and EE methods alternately, both on the same day, during 12 weeks, obtaining one pool collected by AV and one pool collected by EE per week. Males were maintained with an abstinence period of 2 days on the basis of results of Ollero et al. (1996). However, the samples processed corresponded to ejaculates obtained during the first day of each collection week. To avoid individual effects, ejaculates were pooled by method and collection week, conforming 12 pools collected by AV and 12 pools collected by EE. Pooled ejaculates of the first 6 weeks of the collection period were used for SP obtaining, and pooled ejaculates of the last 6 weeks of the collection period were used for sperm obtaining and treatment evaluation.

For the EE, Electrojac V® stimulator (Ideal instruments; Neogen Company, Lansing, MI, USA) was used, with a rectal probe of 22 cm long, 2.5 cm in diameter and three lineal electrodes. Animals received an intramuscular injection of xylazine (0.2 mg/kg Rompun® 2% i.m.; Bayer S.A., Buenos Aires, Argentina). The rectal probe was lubricated and gently inserted into rectum and orientated so that the electrodes were positioned ventrally. The device was used in automatic setting, applying cycles of stimuli of 2-s with 2-s rest intervals between stimuli. Voltage was increased by one cycle (0.5 V) at a time. According to the individual animal sensitivity to electrostimuli, the minimum voltage required to obtain an ejaculate was used, without exceeding the seven cycles. The penis was extended beyond the prepuce, and semen was collected into a graduated collection tube. For semen collection with the AV, rams were exposed to ovariectomized ewes, in the presence of a handler with an AV. Temperature of the water in the lining of the AV ranged from 40 to 44°C at the time of seminal collection.

Seminal plasma obtaining

Pooled ejaculates collected with AV and EE of the first 6 weeks of collection period were centrifuged twice (2000 x ***g*** for 15 min at 4°C). The clear supernatant was recovered, filtered (0.22 μm filter) and kept at −80°C until time of use (Fig. 1).

Sperm obtaining

Pooled ejaculates collected with AV and EE of the last 6 weeks of collection period were used for sperm obtaining and treatments conformation.

To evaluate the effect of the SP obtained with both collection methods over sperm post-thaw quality parameters, ejaculates were washed to eliminate SP that accompanying sperm and then it was replaced by SP collected with EE, SP collected with AV or Salamon's buffer (SB; 300 mm Tris [hydroxymethyl] aminomethane, 37.7 mm glucose, 94.7 mm citric acid, pH 7, 300 mOsm/ml). Briefly, pooled ejaculates collected by AV and EE of the last 6 weeks of collection period were separately diluted (1 : 9) with SB and centrifuged (700 x ***g*** for 15 min at 37°C) to eliminate SP. Then, the obtained sperm pellet was divided into three aliquots (20×10^7 sperm) to conform treatments. Each sperm aliquot was supplemented with SP collected with EE, SP collected with AV or SB. SP or SB was added in a proportion that represented the average volume of SP in an ejaculate collected by AV (Ledesma et al. 2014). The following treatments were established (Fig. 1):

- Sperm collected by AV + SP collected by AV
- Sperm collected by AV + SP collected by EE
- Sperm collected by AV + SB
- Sperm collected by EE + SP collected by AV
- Sperm collected by EE + SP collected by EE
- Sperm collected by EE + SB

Freezing and thawing protocol

Semen samples were diluted at 35°C in one step with a TRIS-glucose-citric acid extender supplemented with egg yolk (10% v/v) and glycerol (7% v/v) and cooled to 5°C for 2 h. Then, they were held at 5°C for 2 h more before freezing. Later, they were packaged in 0.25-mL plastic straws, sealed with polyvinyl alcohol and frozen in liquid nitrogen vapours, 5 cm above the liquid

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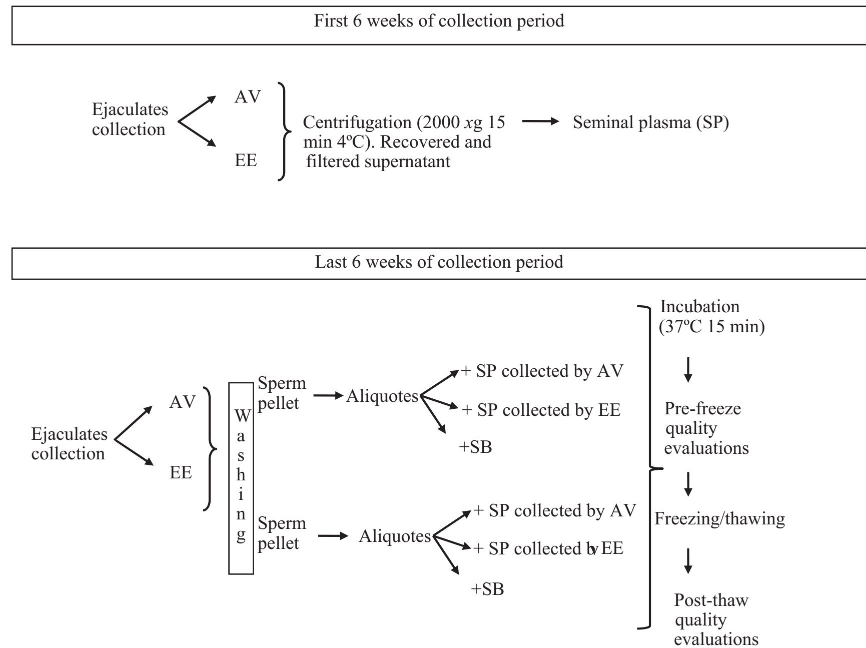

Fig. 1. Experimental design. Ram ejaculates were collected by artificial vagina (AV) and electroejaculation (EE) and pooled by method and collection week. Pooled ejaculates of the first 6 weeks were used for seminal plasma (SP) obtaining and pooled ejaculates of the last 6 weeks were washed to eliminate SP. Sperm pellet was divided into three aliquots and each aliquot was supplemented with SP collected by EE, SP collected by AV or Salamon's buffer (SB). Samples were assessed before and after cryopreservation

nitrogen level for 10 min, before being plunged into the liquid nitrogen for storage. Samples were frozen to a final concentration of 50×10^6 spermatozoa. Thawing was carried out by dropping the straws in a water bath at 37°C for 1 min and pouring the semen in dry tubes and incubated for 5 min at the same temperature. For each treatment and collection week, five straws were thawed and analysed.

Semen analysis

Seminal evaluations were performed in two stages: Pre-freezing: after 15 min of incubation in water bath at 37°C (motility, plasma membrane integrity, plasma membrane functionality, mitochondrial status) and post-thawing (motility, plasma membrane integrity, plasma membrane functionality, mitochondrial status, fertilizing potential and capacitation status) as described below:

Motility parameters

Total and progressive sperm motility were assessed by placing 5 μl of semen on a pre-warmed glass slide, covering with a cover slip and immediately observed in a phase contrast microscope (400×; Nikon Diaphot, Tokyo, Japan). The percentages of total motile and progressive motile sperm were estimated at 5% increments, and the same trained technician performed the evaluation throughout the study.

Plasma membrane integrity

The percentage of sperm cells with intact plasma membrane was measured according to Mortimer (1994) with modifications. Briefly, semen samples (6 μl) were mixed during 10 s with an eosin–nigrosin solution and then smeared onto a pre-warmed microscope slide and air-dried. At least 200 sperm were evaluated per slide using light microscopy (400×). Pink-coloured sperm were considered as sperm with a damaged plasma membrane, and unstained cells were recorded as sperm with intact plasma membrane.

Plasma membrane functionality

The functionality of sperm plasma membrane was evaluated using the hypo-osmotic swelling test (HOST) (Jeyendran et al. 1984; García Artiga 1994). A volume of 10 μl of semen was added to 1 ml of the hypo-osmotic solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) and incubated at 37°C for 30 min. After incubation, one drop of semen was placed on a glass slide, covered with a coverslip and evaluated under a phase contrast microscope (400×). At least 200 sperm were counted, and the proportion of sperm with swollen or coiled tail was calculated (HOST+).

Mitochondrial status

Mitochondrial functionality was evaluated by the specific probe rhodamine 123 (Rh 123), a cationic lipophilic fluorochrome that accumulates selectively inside of active mitochondria, coupled with propidium iodide (PI) stain to discriminate between living and dead sperm (Evenson et al. 1982). An aliquot of 250 μl of semen sample containing 6×10^9 cells was mixed with 1 ml of isotonic solution (140 mM ClNa, 10 mM glucose, 2.5 mM ClK, 20 mM Hepes, 0.5 mM polyvinyl alcohol,

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0.5 mm polyvinylpyrrolidone) and 3 μl of Rh 123 (0.2 mm) and incubated at 37°C for 30 min in the dark. After the addition of 25 μl PI solution (0.5 mm), the samples were incubated for 15 min and the reaction was stopped by the addition of 10 μl of a formalin isotonic solution. Cells were examined under a Nikon fluorescence microscope at 546 nm, and four subpopulations of cells were observed: cells with functional mitochondria with an intact (Rh+/PI−) or damaged plasma membrane (Rh+/PI+) and cells without functional mitochondria with an intact (Rh−/PI−) or damaged (Rh−/PI+) plasma membrane. At least 200 sperm were counted, and the proportion of sperm with functional mitochondria and intact plasma membrane was calculated.

Fertilizing potential

To evaluate sperm fertilizing potential, the heterologous *in vitro* fertilization test was used. It was assessed as described by García-Álvarez et al. (2009) with modifications. Three replicates per treatment were carried out. Bovine ovaries were collected from a local slaughterhouse and transported within 2 h in a thermic container to the laboratory at room temperature. COCs with homogeneous ooplasm and more than four complete layers of *cumulus* cells, corresponding to grade 1 and 2 according to De Loos et al. (1989), were selected under a stereomicroscope and washed three times in modified M199 supplemented with 0.5% HEPES (w/v). Cumulus–oocyte complexes (COCs) were cultured in TCM-199 supplemented with 50 μg/ml gentamycin, 5.5 mm Ca lactate, 2.3 mm Na pyruvate, 36 mm NaHCO3, 5 mm Hepes, 0.01 UI/ml rhFSH (Gonal F-75, Serono, UK). Maturation was performed in four-well plates (Nunc®, Roskilde, Denmark) in groups of 50 COCs in 400 μl of maturation media for 24 h at 38.5°C under 5% CO_2 in air with maximum humidity. Matured COCs were transferred to a new four-well plates containing 400 μl of fertilization medium (SOF supplemented with 10% of oestrous sheep serum and 40 μg/ml gentamycin). Thawed sperm were capacitated in the fertilization medium for 10 min. Sperm was co-incubated with oocytes at a final concentration of 1×10^6 ml at 38.5°C in 5% CO_2. Then, the oocytes were fixed and stained with Hoechst 33342. Fertilization rate was assessing 40 h later with an inverted microscope by the presence of cleaved oocytes (two to eight cells) or the presence of two or more nuclei.

Capacitation status

In vitro capacitation status was assessed with the chlortetracycline fluorescence assay or CTC staining as described by Pérez et al. (1996), with modifications (Gil et al. 2003). A CTC solution (750 μm) was freshly prepared in a buffer containing 20 mm TRIS, 130 mm NaCL and 5 mm DL-cysteine and pH 7.8. A 5 μl of semen was mixed with 20 μl of CTC-working solution. After 20 s, the reaction was stopped by the addition of 5 μl of 1% (v/v) glutaraldehyde in 1 m Tris-HCL pH 7.8. Smears were prepared in a clean microscope slide, covered with a coverslip, sealed with colourless nail varnish and stored in the dark at 4°C. Samples were examined under an epifluorescent inverted Eclipse TE-300 microscope (Nikon) within 12 h after staining, using green filters (380 nm excitation and emission at 420 nm). At least 200 stained sperm were classified into three categories, according to their CTC-staining patterns (Fraser et al. 1995) as follows: F pattern (uniform bright fluorescence in the head with a fluorescence band in the equatorial segment, non-capacitated sperm with an intact acrosome); B pattern (fluorescence-free band in the post-acrosomal region of the head, capacitated sperm with an intact acrosome); and AR pattern (uniform bright fluorescence over the entire sperm head and full fluorescence along the equatorial segment, sperm that had undergone an acrosome reaction).

Statistical analyses

All data were assessed for normality distribution using the Shapiro–Wilk test. Sperm parameters were compared by anova using the GLM procedure of the SAS statistic package (SAS 2000). Numeric results are expressed as least square means (LSM) ± standard error of the means (SEM). Data were considered statistically significant when $p \leq 0.05$.

For the analysis, a factorial design with three levels was conducted and the factors were as follows: the SP collection method (AV, EE and SB) and the sperm collection method (AV and EE levels). The fixed effects were the SP collection method, the sperm collection method and their interaction. Week of collection had initially been included in the model, but was excluded as no differences were shown. To evaluate frozen-thawed semen, data of fresh semen evaluation were used as a covariable.

Results

Pre-freeze sperm quality parameters

The interaction between SP and sperm collection method was significant ($p < 0.05$) for total and progressive sperm motility. The highest percentages of motility were observed in treatments conformed by sperm collected with EE, regardless whether SP was added or not. Treatment composed by sperm collected by EE had the highest percentage of sperm with intact and functional plasma membrane, while the percentage of sperm with functional mitochondria was not different (Table 1).

Post-thaw sperm quality parameters

Post-thaw sperm quality of semen samples treated with SP collected by AV, EE or SB is shown in Table 2. The

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Table 1. Effect of seminal plasma collected by artificial vagina or electroejaculation on quality parameters of pre-freeze ram sperm collected by artificial vagina or electroejaculation

	Treatments						
Collection method of seminal plasma	AV		EE		Salamon's buffer		
Collection method of sperm	AV	EE	AV	EE	AV	EE	p
Total motility	46.7 ± 4.7[a]	67.5 ± 4.7[b]	74.1 ± 4.7[b]	65.0 ± 4.7[b]	48.3 ± 4.7[a]	63.3 ± 4.7[b]	0.010
Progressive motility	52.0 ± 2.9[a]	76.0 ± 2.9[b]	78.5 ± 2.9[b]	70.0 ± 2.9[b]	53.5 ± 2.9[a]	72.0 ± 2.9[b]	0.018
Intact plasma membrane	47.7 ± 3.7[a]	68.0 ± 3.7[b]	49.3 ± 3.7[a]	65.2 ± 3.7[b]	53.5 ± 3.7[a]	66.8 ± 3.7[b]	0.001
Functional plasma membrane	43.5 ± 4.5[a]	53.2 ± 4.5[b]	39.7 ± 4.5[a]	52.3 ± 4.5[b]	48.3 ± 4.5[a]	57.2 ± 4.5[b]	0.009
Functional mitochondria	27.4 ± 5.0	33.2 ± 5.0	30.5 ± 5.0	29.2 ± 5.0	24.5 ± 5.0	30.7 ± 5.0	0.130

LSM, least square means; SEM, standard error means; AV, artificial vagina; EE, electroejaculation.
Values in the same row with different superscripts (ab) are statistically different ($p < 0.05$).

percentage of sperm with intact and functional plasma membrane and the *in vitro* fertilizing potential were significantly greater ($p < 0.005$) in treatments composed by sperm collected by EE, regardless of the SP added. The percentage of sperm with total and progressive motility and functional mitochondria were not different between treatments. Regarding to CTC-patterns, treatment composed by sperm collected by EE had the lesser percentage of sperm with acrosome reacted, while the percentage of sperm with CTC-F and CTC-B pattern were not different.

Discussion

Cryopreservation process produces serious detrimental changes in sperm function (Watson 1981). The plasma and acrosomal membranes of sperm are critical for their survival after thawing and are considered to be the primary site of the changes caused by cryopreservation (Maxwell and Watson 1996). It has been suggested that some aspects of the freezing/thawing procedure may advance the maturation of sperm membranes and increase the proportion of capacitated and acrosome reacted sperm (Watson 1995; Maxwell and Watson 1996). Sperm membrane changes shorten the lifespan of the cells and render them less fertile or incapable of fertilization.

As collection method is known to affect seminal parameters and based on the differences found by us and other authors in SP composition obtained by EE compared to AV (Marco-Jiménez et al. 2008; Ledesma et al. 2014), we designed a study to analyse whether SP is responsible for sperm resistance to cryopreservation. By changing the source of SP that accompanied sperm before freezing, we found that sperm collected by EE showed higher quality parameters when either fresh or cryopreserved semen was evaluated. Sperm collected by EE were more resistant to damage caused by cryopreservation than those collected by AV, evidenced by the higher percentage of sperm with intact and functional plasma membrane and intact acrosome. These results might explain the greater fertilizing potential observed by us in sperm cells collected by EE after thawing.

Sperm quality parameters evaluated after thawing were not affected by the pre-freezing addition of SP obtained by EE or AV, suggesting that sperm cells obtained by electrical stimulation were, by themselves, more resistant to cryodamage than sperm collected by AV. Accordingly, Marco-Jiménez et al. (2005) observed

Table 2. Effect of seminal plasma collected by artificial vagina or electroejaculation on quality parameters of post-thaw ram sperm collected by artificial vagina or electroejaculation

	Treatments						
Collection method of seminal plasma	AV		EE		Salamon's buffer		
Collection method of sperm	AV	EE	AV	EE	AV	EE	p
Total motility	36.4 ± 3.0	35.2 ± 3.0	36.0 ± 3.0	34.8 ± 3.0	36.0 ± 3.0	37.2 ± 3.0	0.280
Progressive motility	35.6 ± 4.0	32.1 ± 4.0	31.0 ± 4.0	36.2 ± 4.0	35.6 ± 4.0	36.0 ± 4.0	0.350
Intact plasma membrane	41.1 ± 2.7[ab]	50.0 ± 2.8[a]	34.7 ± 2.7[b]	43.3 ± 2.6[a]	35.2 ± 2.5[b]	43.2 ± 2.7[a]	0.010
Functional plasma membrana	42.0 ± 3.9[a]	37.4 ± 3.9[ab]	26.4 ± 4.2[b]	30.4 ± 3.9[b]	40.7 ± 3.9[a]	34.3 ± 4.1[ab]	0.020
Functional mitochondria	29.1 ± 6.3	27.5 ± 6.3	26.5 ± 6.3	20.6 ± 6.3	24.6 ± 6.3	30.4 ± 6.3	0.800
Fertilizing potential	36.6 ± 5.4[ab]	42.0 ± 5.4[a]	24.2 ± 5.4[b]	45.6 ± 5.4[a]	20.6 ± 5.4[b]	38.7 ± 5.4[a]	0.030
CTC-F pattern	33.4 ± 4.0	28.4 ± 4.0	28.4 ± 4.0	29.8 ± 4.0	39.4 ± 4.0	37.7 ± 4.0	0.600
CTC-B pattern	41.3 ± 2.9	43.3 ± 2.9	40.8 ± 2.9	42.3 ± 2.9	38.4 ± 2.9	42.1 ± 2.9	0.700
CTC-AR pattern	33.4 ± 2.1[ab]	30.6 ± 2.1[b]	36.0 ± 2.1[a]	29.8 ± 2.1[b]	39.4 ± 2.1[a]	29.6 ± 2.1[b]	0.002

LSM, least square means; SEM, standard error means; AV, artificial vagina; EE, electroejaculation.
Values in the same row with different superscripts (ab) are statistically different ($p < 0.05$).

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a higher number of acrosome intact frozen-thawed sperm cells collected by EE than by AV. We could hypothesize that maybe the previous contact upon ejaculation of sperm collected by EE with SP (previous to treatments formation) might be responsible for the higher resistance of these sperm cells to cryodamage. Thus, we have already shown that the electroejaculation favours the collection of SP with higher total protein content and relative abundance of low molecular weight proteins considered as a cryoprotectans (Ledesma et al. 2014). According with Dott et al. (1979), this is possible because the immediate contact of sperm with SP at ejaculation sets a particular conduct that is not affected by the subsequent washings. It has been previously demonstrated that ejaculated sperm are less capable to bind proteins from SP compared to epididymal sperm (Domínguez et al. 2008). This appears to be because sperm surface ligands are occupied by SP proteins upon the very first contact with accessory glands secretions.

Numerous studies have shown the protective effect of SP or their proteins on the damage caused by freezing/thawing (Ollero et al. 1997; Cardozo et al. 2009) or cold-shock in sperm cells (Barrios et al. 2000; Pérez-Pé et al. 2002). In our study, we could not observe that and this could be due to the semen washing procedure used. Centrifugation could have caused the loss of plasma membrane proteins avoiding the ability of sperm cells to recognize other ligands such as SP components (Risopatrón et al. 1996). In the studies cited above where demonstrated the protective effect of SP or its proteins sperm cells were obtained by a dextran/swim-up procedure without the use of centrifugation. In this regard, Jiménez-Rabadán et al. (2012) observed that the quality of cryopreserved Blanca-Celtibérica buck sperm collected by EE was improved when a sperm selection technique was carried out after thawing.

Although, there are many studies that demonstrated the effects of SP on sperm quality in different species (Ollero et al. 1998; Azeredo et al. 2001; Martínez-Pastor et al. 2006), to our knowledge, there are few available studies about its effect on semen fertility (Belibasaki et al. 2000; Maxwell et al. 2007; O′Meara et al. 2007). In this study, we performed the heterologous *in vitro* fertilization assay because García-Álvarez et al. (2009) showed high correlation between *in vitro* and *in vivo* male fertility in frozen/thawed ram sperm. As was mentioned previously, sperm collected by EE had greater fertilizing potential after freezing and thawing than sperm collected by AV.

In summary, the present study showed that seminal plasma has no effect on sperm resistance to cryodamage. However, we observed that sperm cells obtained by EE were more resistant to cryodamage than sperm collected by AV, evidenced by the higher percentage of sperm with intact and functional plasma membrane, intact acrosome and greater *in vitro* fertilizing potential observed after thawing.

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Conflict of interest

The authors have not declared any conflicts of interest.

Author contributions

The description of each individual's contribution to the research and its publication were statement in the first time the paper was sent.

References

Azeredo G, Esper C, Resende K, 2001: Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. Small Rum Res **41**, 257–263.

Bailey J, Morrier A, Cormier N, 2003: Semen cryopreservation: successes and persistent problems in farm species. Can J Anim Sci **83**, 393–401.

Barrios B, Pérez-Pé R, Gallego M, Tato A, Osada J, Muiño-Blanco T, Cebrián-Pérez J, 2000: Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod **63**, 1531–1537.

Barrios B, Fernández-Juan M, Muiño-Blanco T, Cebrián-Pérez J, 2005: Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold-shock. J Androl **26**, 539–549.

Belibasaki S, Amiridis G, Lymberopoulos A, Varsakeli S, Kouskoura T, 2000: Ram seminal plasma and fertility: results from an ongoing field study. Acta Vet Hung **48**, 335–341.

Benoff S, 1993: Preliminaries to fertilization: the role of cholesterol during capacitation of human spermatozoa. Hum Res **8**, 2001.

Bernardini A, Hozbor F, Sanchez E, Fornes M, Alberio R, Cesari A, 2011: Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. Theriogenology **76**, 436–447.

Buhr M, Curtis E, Kakuda N, 1994: Composition and behaviour of head membrane lipids of fresh and cryopreserved boar sperm. Cryobiology **31**, 224–238.

Cardozo J, Grasa P, Muiño-Blanco T, Cebrián-Pérez J, 2009: Adición de proteínas del plasma seminal ovino durante la congelación del espermatozoide y efectos sobre su motilidad y viabilidad. Revista Corpoica – Ciencia y Tecnología Agropecuaria **10**, 51–59.

De Loos F, van Vliet C, van Maurik P, Kruip TA, 1989: Morphology of immature bovine oocytes. Gamete Res **24**, 197–204.

Domínguez M, Falcinelli A, Hozbor F, Sanchez E, Cesari A, Alberio R, 2008: Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. Theriogenology **69**, 564–573.

Dott H, Harrison R, Foster G, 1979: The maintenance of motility and the surface properties of epididymal spermatozoa from bull rabbit and ram in homologous seminal and epididymal plasma. J Reprod Fertil **55**, 113–124.

Evenson D, Darzynkiewicz Z, Melamed M, 1982: Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem **30**, 279–280.

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Fernández-Juan M, Gallego M, Barrios B, Osada J, Cebrián J, Perez-Pé R, Muiño-Blanco T, 2006: Immunohistochemical localization of sperm-preserving proteins in the ram reproductive tract. J Androl **27**, 588–595.
Fraser L, Abeydeera L, Niwa K, 1995: Ca (2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev **40**, 233–241.
García Artiga C. Test de endósmosis en ovino. 7th International Meeting on Animal Reproduction AI; Murcia, España 1994, 77–81.
García-Álvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos M, Esteso M, Pérez-Guzmán M, Soler A, 2009: Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. Theriogenology **71**, 643–650.
Gil J, Lundeheim N, Soderquist L, Rodríguez-Martínez H, 2003: Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology **59**, 1241–1255.
Graham J, 1994: Effect of seminal plasma on the motility of epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process. Theriogenology **41**, 1151–1162.
Gravance C, Vishwanath R, Pitt C, Garner D, Casey P, 1998: Effects of cryopreservation on bull sperm head morphometry. J Androl **19**, 704–709.
Holt W, North R, 1994: Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol Reprod **51**, 414–424.
Jeyendran R, Vanderven H, Pérez-Peláez M, Crabo B, Zaneveld L, 1984: Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil **70**, 219–228.
Jiménez-Rabadán P, Ramón M, García-Álvarez O, Maroto-Morales A, Del Olmo E, Pérez-Guzmán M, Bisbal A, Fernández-Santos M, Gardel J, Soler A, 2012: Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. Anim Reprod Sci **132**, 88–95.
Ledesma A, Manes J, Cesari A, Alberio R, Hozbor F, 2014: Electroejaculation increases low molecular weight proteins in seminal plasma modifying sperm quality in *Corriedale* rams. Reprod Dom Anim **49**, 324–332.
Lightfoot R, Salamon S, 1970: Fertility of ram spermatozoa frozen by the pellet method. I. Transport and viability of spermatozoa within the genital tract of the ewe. J Reprod Fertil **22**, 385–398.
Marco-Jiménez F, Puchades S, Gadea J, Vicente J, Viudes de Castro M, 2005: Effect of semen collection method on pre and post-thaw Guirra ram spermatozoa. Theriogenology **64**, 1756–1765.
Marco-Jiménez F, Vicente J, Viudes de Castro M, 2008: Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. Reprod Dom Anim **43**, 403–408.
Martínez-Pastor F, Anel L, Guerra C, Alvarez M, Soler AJ, Garde JJ, Chamorro C, de Paz P, 2006: Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. Theriogenology **66**, 1847–1856.
Mattner P, Voglmayr J, 1962: A comparison of ram semen collection method by the artificial vagina and by electroejaculation. Aust J Exp Agric Anim Husb **2**, 78–81.
Mattner P, Entwistle K, Martin I, 1969: Passage, survival and fertility of deep-frozen ram semen in the genital tract of the ewe. Aust J Biol Sci **22**, 181–187.
Maxwell P, Watson P, 1996: Recent progress in the preservation of ram semen. Anim Reprod Sci **42**, 55–65.
Maxwell W, Evans G, Mortimer S, Gillan L, Gellaltly E, McPhie C, 1999: Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. Reprod Fertil Dev **11**, 123–126.
Maxwell W, de Graaf S, Ghaoui RE-H, Evans G, 2007: Seminal plasma effects on sperm handling and female fertility. Soc Reprod Fertil Suppl **64**, 13–38.
Mortimer D, 1994: Sperm recovery techniques to maximize fertilizing capacity. Reprod Fertil Dev **6**, 25–31.
O′Meara C, Donovan A, Hanrahan J, Duffy P, Fair S, Evans A, Lonergan P, 2007: Resuspending ram spermatozoa in seminal plasma after cryopreservation does not improve pregnancy rate in cervically inseminated ewes. Theriogenology **67**, 1262–1268.
Ollero M, Muiño-Blanco T, López-Pérez M, Cebrián-Pérez J, 1996: Viability of ram spermatozoa in relation to the abstinence period and successive ejaculations. Int J Androl **19**, 287–292.
Ollero M, Cebrián-Pérez J, Muiño-Blanco T, 1997: Improvement of cryopreserved ram sperm heterogeneity and viability by addition of seminal plasma. J Androl **18**, 732–739.
Ollero M, Pérez-Pé R, Muiño-Blanco T, Cebrián-Pérez J, 1998: Improvement of ram sperm cryopreservation protocols assessed by sperm quality parameters and heterogeneity analysis. Cryobiology **37**, 1–12.
Pérez L, Valcarcel A, de las Heras M, Moses D, Baldassarre H, 1996: Evidence that frozen/thawed ram spermatozoa show accelerated capacitation in vitro as assessed by chlortetracycline assay. Theriogenology **46**, 131–140.
Pérez-Pé R, Cebrián-Pérez J, Muiño-Blanco T, 2001: Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology **56**, 425–434.
Pérez-Pé R, Grasa P, Fernández-Juan M, Peleato M, Cebrián-Pérez J, Muiño-Blanco T, 2002: Seminal plasma proteins reduce protein tyrosine phosphorylation in the plasma membrane of cold-shocked ram spermatozoa. Mol Reprod Dev **61**, 226–233.
Purdy P, 2006: A review on goat sperm cryopreservation. Small Rum Res **63**, 215–225.
Quinn P, Salamon S, White I, 1968: The effect of cold shock and deep-freezing on ram spermatozoa collected by electrical ejaculation and by an artificial vagina. Aust J Agric Res **19**, 119–128.
Rickard J, Pini T, Soleilhavoup C, Cognie J, Bathgate R, Lynch G, Evans G, Maxwell W, Druart X, de Graaf S, 2014: Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. Reproduction **48**, 469–478.
Risopatrón J, Sánchez R, Sepúlveda N, Peña P, Villagran E, Miska W, 1996: Migration/sedimentation sperm selection method used in bovine in vitro fertilization: comparison with washing/centrifugation. Theriogenology **46**, 165–173.
SAS Statistical Analysis System, 2000: SAS User's Guide: Statistics. SAS Inst. Inc., Cary, NC.
Watson P, 1981: The roles of lipid and protein in the protection of ram spermatozoa at 5°C by egg-yolk lipoprotein. J Reprod Fertil **62**, 483–492.
Watson P, 1995: Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev **7**, 871–891.
Wulster-Radclife M, Williams M, Stellflug J, Lewis G, 2001: Technical note: artificial vagina vs. a vaginal collection vial for collecting semen from rams. J Anim Sci **79**, 2964–2967.
Yanagimachi R, 1994: Fertility of mammalian spermatozoa: its development and relativity. Zigote **3**, 371–372.

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