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Electroejaculation Increases Low Molecular Weight Proteins in Seminal Plasma Modifying Sperm Quality in *Corriedale* Rams

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Contents

This study was conducted to evaluate the effect of seminal collection method (artificial vagina or electroejaculation) on the protein composition of seminal plasma and sperm quality parameters in *Corriedale* rams. To address this question, we assessed the effect of seminal collection method on motility, plasma membrane integrity and functionality, mitochondrial functionality and the decondensation state of nuclear chromatin in sperm cells. Volume, pH, osmolarity, protein concentration, total protein content and protein profile using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and 2-D polyacrylamide electrophoresis of seminal plasma collected with artificial vagina and electroejaculation were also analysed. The main findings from this study were that ejaculates obtained with electroejaculation had (i) a higher number of spermatozoa with intact plasma membrane and functional mitochondria and (ii) a higher proportion of seminal plasma, total protein content and relative abundance of low molecular weight proteins than ejaculates obtained with artificial vagina. Five of these proteins were identified by mass spectrometry: binder of sperm 5 precursor; RSVP14; RSVP22; epididymal secretory protein E1 and clusterin. One protein spot with molecular weight of approximately 31 kDa and isoelectric point of 4.8 was only found in the seminal plasma from electroejaculation.

Introduction

Seminal plasma (SP) of mammals is a complex biological fluid composed of multiple secretions from the male reproductive glands (seminal vesicles, prostate and bulbourethral glands) and a smaller amount of epididymal fluid. During ejaculation, sperm is mixed with SP, increasing the volume of the ejaculate and facilitating their passage through the reproductive tract (Moura et al. 2006). It is known that SP contains proteins, enzymes, lipids, organic acids, minerals and other components that may play significant roles in the regulation of sperm function. Some proteins from SP (decapacitating factors) are adsorbed onto the surface of ejaculated sperm (Amann 1999) and maintain the stability of the membrane until the occurrence of the capacitation process in the female genital tract (Manjunath et al. 1993; Ollero et al. 1996) when their removal is a prerequisite for fertilization to take place (Yanagimachi 1994). Barrios et al. (2005) demonstrated that two SP proteins of low molecular weight synthesized in the seminal vesicles protect sperm from the damage that occurs during cryopreservation. A year later, Fernández-Juan et al. (2006) named these two proteins according to their origin (seminal vesicles) and molecular weight as RSVP14 and RSVP20. Conversely, detrimental effect of SP on sperm viability (Dott et al. 1979) and motility (Iwamoto and Cagnon 1988) was found. These controversial results could be related to SP composition that varies between males (Muiño-Blanco et al. 2008), season of the year (Domínguez et al. 2008) and seminal collection method applied (Marco-Jiménez et al. 2005).

Ram semen can be recovered with artificial vagina (AV) or electrical stimulation (EE). The collection of semen with AV resembles natural service, 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. Additionally, EE admits seminal collection of males with any physical injury that keeps them from performing mating. Besides, differences in sperm quality and SP composition between ejaculates obtained with AV and EE have been found (Pineda and Dooley 1991; Marco-Jiménez et al. 2008). Ejaculates collected with EE have an increased volume compared with ejaculates collected with AV, due to the presence of a higher proportion of 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). As ejaculates obtained by EE have a greater proportion of SP, we hypothesize that these ejaculates have a greater proportion of protective components for sperm that improve sperm quality. The aim of this study was to evaluate the effect of seminal collection method on (i) sperm quality parameters (motility, plasma membrane integrity and functionality, mitochondrial functionality and the decondensation state of nuclear chromatin) and (ii) seminal plasma parameters (volume, pH, osmolarity, protein concentration, total protein content and protein profile).

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 fields of Experimental Research Station of the Instituto Nacional de Tecnología Agropecuaria INTA Balcarce,

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Argentina (37°45′ south, 58°18′ west), during the natural breeding season (March–June; autumn). All the reagents were obtained from Sigma-Aldrich Co® (St Louis, MO, USA) and GE Healthcare® (Buenos Aires, Argentina). 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. During the experiment, the animal grazed raygrass (*Lolium perenne*), fescue (*Festuca arundinacea*) and white clover (*Trifolium repens*) pasture.

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 pooled 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 collect were used for the evaluation of seminal quality parameters, and pooled ejaculates of the last 6 weeks of collect were used for seminal plasma 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 body weight; 2%; Rompun, Bayer S.A., 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.

Semen evaluation

The proportion of SP and sperm in ejaculates collected with both methods was determined by *spermatocrit* (Abdel-Rahman et al. 2010). Briefly, haematocrit capillary tubes were filled with a semen sample and centrifuged for 15 min at 2000 × ***g***. Both phases of the ejaculates (SP/sperm) were established by measuring with a calliper (± 0.05 mm). Sperm quality was assessed according to the seminal parameters as described below.

Total and progressive sperm motility

Five microlitres of semen was placed on a pre-warmed glass slide, covered with a cover slip and assessed visually under a phase-contrast microscope (400×) (Nikon Diaphot, Japan) to assess mean percentage of total and progressive motile sperm. 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 functionality

The functionality of sperm plasma membrane was evaluated using the hypo-osmotic swelling test (HOS) (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 cover slip 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 (HOS+).

State of decondensation of nuclear chromatin

This test is an *in vitro* determination of the degree of maturation of sperm chromatin, based on the reduction of protamine disulphide bonds. Briefly, chromatin is exposed to a reducing agent, being the immature chromatin decondensation faster and more complete than mature chromatin. The percentage of sperm showing nuclear chromatin decondensation *in vitro* correlates with the percentage of immature sperm present in an ejaculate (Rodríguez et al. 1985). The degree of nuclear chromatin decondensation was evaluated according to Rodríguez et al. (1985). Semen and 1% w/v dithiothreitol (DTT) were incubated in equal parts (60 μl final volume) for 30 min at 22°C. Then, 6 μl of the sample was smeared and dried rapidly by heat platen. Smears were stained with toluidine blue for 20 min, washed with distilled water, allowed to air-dry and observed with an optical microscope (1000×) (Nikon). Sperm were classified into four groups according to the responsiveness to DTT. Group A: normal size and homogeneous faint stain; B: normal size and shape with intense colour and generally homogeneous; C: moderate vacuoles and loss of shape and D: decondensated, highly vacuolated and significant loss of shape. Groups C and D were considered as immature. At least 200 sperm were counted, and the proportion of immature sperm (C and D groups) was calculated.

Plasma membrane integrity

The percentage of sperm cells with intact plasma membranes 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 cells were evaluated per slide using light microscopy (400×).

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Pink-coloured sperm were considered as sperm with a damaged plasma membrane, and unstained cells were recorded as sperm with intact plasma membrane.

Mitochondrial functionality

It 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; 20 mM HEPES; 0.5 mM polyvinyl alcohol; 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.

Seminal plasma collection and processing

Pooled ejaculates collected with AV and EE of the last 6 weeks of collection period were centrifuged twice (2000 × ***g*** for 15 min at 4°C), and the clear supernatant was recovered, filtered (0.22-μm filter) and kept at −80°C.

Seminal plasma volume was determined with a graduated plastic tube, and osmolarity and pH value were measured using an osmometer and a pH meter, respectively. Protein concentration was assessed according to the method described by Bradford (1976) using BSA as a standard protein. Total protein content was estimated as the product between protein concentration and SP volume.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the molecular weight and relative content of various SP proteins. Samples were subject to SDS-PAGE according to the method previously described by Laemmli et al. (1970). An amount of 20 μg of proteins from SP collected by EE and AV was electrophoresed either on 10% or on 15% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS-PAGE) at room temperature (~18–24°C) and 20 mA/gel in a Hoefer mini VE device (Amersham GE, Buckinghamshire, UK). Samples were mixed with 5× Laemmli sample buffer under reducing conditions (100 mM DTT) and boiled. The molecular mass was estimated using protein full-range molecular weight standards (RPN800E, GE Healthcare, Uppsala, Sweden). After electrophoresis, gels were stained with Coomassie Brilliant blue R-250. Gel images were analysed to determine relative protein content by measuring the intensity of the bands using ImageJ 1.43 free software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

Two-dimensional electrophoresis (2D-GE)

Two-dimensional electrophoresis was performed to determine the molecular weight (MW) and isoelectric point (pI) of SP proteins contained in the 12–31 kDa and 3.0–7.0 pI rank.

The SP samples collected by EE and AV were thawed at room temperature, and three samples of each collection method were randomly chosen for electrophoresis. Samples were prepared as follows: 100 μg of acetone-precipitated SP proteins was diluted in 125 μl of rehydratation buffer containing 8 M urea, 2% [3-(3-(cholamidopropyl) dimethyl-ammonio)-1 propane sulphomate] (CHAPS), 40 mM dithiothreitol (DTT), 0.5% IPG buffer 3–10 NL (GE Healthcare, Uppsala Sweden) and 0.002% bromophenol blue. Passive rehydratation was performed onto 7-cm immobilized 3–10 NL pH gradients strips (IPGs GE Healthcare, Uppsala, Sweden) by placing the strips for 16 h in the channel of a rehydratation tray that contained the solution described above. Isoelectric focusing was performed using a Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare). The instrument was programmed as follows: 300 V 200 Vh (steady-state level), 1000 V 300 Vh (gradient), 5000 V 4500 Vh (gradient) and 5000 V 2000 Vh (steady-state level). Upon completion of the first dimension, IPG strips were incubated with gentle shaking in the equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue) containing 1% (w/v) DTT for 15 min and then in the same buffer containing 2.5% (w/v) iodoacetamide for another 15 min. The second dimension was performed on 12% polyacrylamide gels.

After electrophoresis, gels were stained with colloidal Coomassie Brilliant blue G-250. The two-dimensional gels were scanned at 300 dpi and analysed using PD-Quest software (Bio-Rad, Hercules, CA, USA). The two-dimensional maps obtained of SP samples collected by EE and AV were evaluated as a single match set and a master gel generated, based on a representative gel. Proteins in key regions of the master gel were used as landmarks, and the matching of spots was achieved after several rounds of extensive comparison. Also, final spot matches were performed by checking each spot in each gel with the respective pattern in the master. Protein quantities were reported as parts per million of the total integrated optical density of spots in the gels, according to PD-Quest.

Protein bands and spots of interest in one- and two-dimensional gels, respectively, were excised individually, transferred to clean tubes and sent to the CEQUIBIEM (Universidad de Buenos Aires, Argentina) for protein identification by MALDI-ToF/ToF mass spectrometry. The resulting tandem mass spectra were searched against the non-redundant protein sequence database from the National Center for Biotechnology Information (NCBI), using the Mascot MS/MS ion search tool

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(version 2.1; www.matrixscience.com). The search parameters used included no restrictions on the protein molecular mass and non-fixed modifications of cysteine (carbamidomethylation). The peptides were considered to be identified when the scoring value exceeds the identity or extensive homology threshold value, as calculated by Mascot, based on the MOWSE score. In cases of protein identification based on a single peptide, the minimum threshold of the probability was based on a Mascot score of 50.

Statistical analysis

All data were assessed for normality distribution using the Shapiro–Wilk test and homogeneity of variance using the general linear models procedure of the SAS statistic package (SAS 2000). The sperm parameters were analysed using ANOVA. The statistical model included the effects of collection method, collection week and their interaction. To evaluate the protein composition of SP, three experiments were performed and a representative image was analysed. Intensity values for each band were normalized to total protein within each lane, and statistical analysis was carried out using Student's *t*-test for unpaired data (two tailed). Statistically significant differences were determined at $p \leq 0.05$.

Results

Spermatocrit and sperm quality parameters

The proportion of ejaculate components was 56.04 ± 2.05% of SP and 43.9 ± 1.5 of sperm cells in ejaculates collected by AV and 87.08 ± 2.8% of SP and 13 ± 1.08% of sperm cells in ejaculates collected by EE ($p = 0.02$).

The results of quality parameters evaluation in pooled ejaculates obtained by AV and EE are shown in Table 1. The percentage of sperm with intact plasma membrane and functional mitochondria was significantly greater in ejaculates collected by EE, while total and individual progressive motile sperm, membrane functionality and the percentage of sperm with immature chromatin were not different for samples collected with both methodologies. The effect of collection week and the collection week-by-collection method interaction were not significant for any of the quality parameters evaluated ($p > 0.05$).

Seminal plasma parameters

In relation to the collection method volume, pH, osmolarity and total protein content in seminal plasma were affected by collection method ($p < 0.05$), showing those collected by EE the higher volume, pH and total protein content and the lesser osmolarity. Protein concentration was not influenced by collection method (Table 2). The effect of collection week and the collection week-by-collection method interaction were not significant for any of the seminal plasma parameters evaluated ($p > 0.05$).

Protein composition of seminal plasma

Seminal plasma proteins obtained by both collection methods were separated electrophoretically and analysed. Differential protein profile was observed between methods when high molecular proteins and low molecular proteins were analysed (Fig. 1a upper and lower panel, respectively). Two protein bands of high molecular mass were significantly increased ($p < 0.05$) in SP obtained by AV (approximately 102 and 52 kDa designated as *1 and *5, respectively; Fig. 1, upper panel), whereas five protein bands of low molecular mass were significantly increased ($p < 0.05$) in SP obtained by EE (approximately 25, 23 kDa and three bands below the 12-kDa marker, denoted as *10, *11, *13, *15 and *16, respectively; Fig. 1, lower panel). Protein band of approximately 38 kDa, designated as band *8, was also increased in EE compared with AV ($p = 0.05$). Three of the low molecular mass proteins that were found to be augmented in SP obtained by EE could be identified by tandem mass spectrometry (Table 3).

Based on the observation that SP obtained by EE was enriched in low molecular mass proteins, we focused the study on proteins within the 12–31 kDa rank by 2D-GE (Fig. 2). The PD-Quest analysis software detected 15 protein spots with isoelectric points (pI) ranging from 3.0 to 7.0. Quantitative differences were analysed on all spots between recovery methods (Fig. 2), showing 5 spots augmented in SP collected by AV (*2, *8, *9, *10

Table 1. Effect of semen collection method (artificial vagina or electroejaculation) on quality parameters of pooled ram sperm

Parameter (%)	Artificial Vagina n = 6	Electroejaculation n = 6	p-value
Sperm motility parameters			
Total motile	71.7 ± 16.0	81.7 ± 11.7	0.27
Progressively motile	78.3 ± 9.8	77.5 ± 10.4	0.63
Functional plasma membrane	44.7 ± 4.4	35.0 ± 11.8	0.18
Immature chromatin	33.4 ± 15.8	25.0 ± 9.3	0.51
Intact plasma membrane	49.5 ± 12.7[a]	71.3 ± 11.9[b]	0.001
Functional mitochondria	16.7 ± 10.7[a]	47.8 ± 4.2[b]	0.001

Data are means ± SEM. Values in the same row with different superscripts (a, b) are statistically different ($p \leq 0.05$).

Table 2. Effect of collection method (artificial vagina and electroejaculation) on ram seminal plasma volume, pH, osmolarity, protein concentration and total protein content

Parameter	Artificial vagina n = 6	Electroejaculation n = 6	p-value
Volume (μl)	1740 ± 474.1[a]	3994 ± 386[b]	0.0004
pH	6.3 ± 0.2[a]	6.8 ± 0.3[b]	0.02
Osmolarity (mOsm/l)	322.6 ± 11.0[a]	302.8 ± 2.9[b]	0.01
Protein concentration (mg/ml)	66.4 ± 21.1	48.6 ± 14.7	0.08
Total protein (mg)	127.1 ± 55.9[a]	216.3 ± 56.5[b]	0.03

Data are means ± SEM. Values in the same row with different superscripts (a, b) are statistically different ($p \leq 0.05$).

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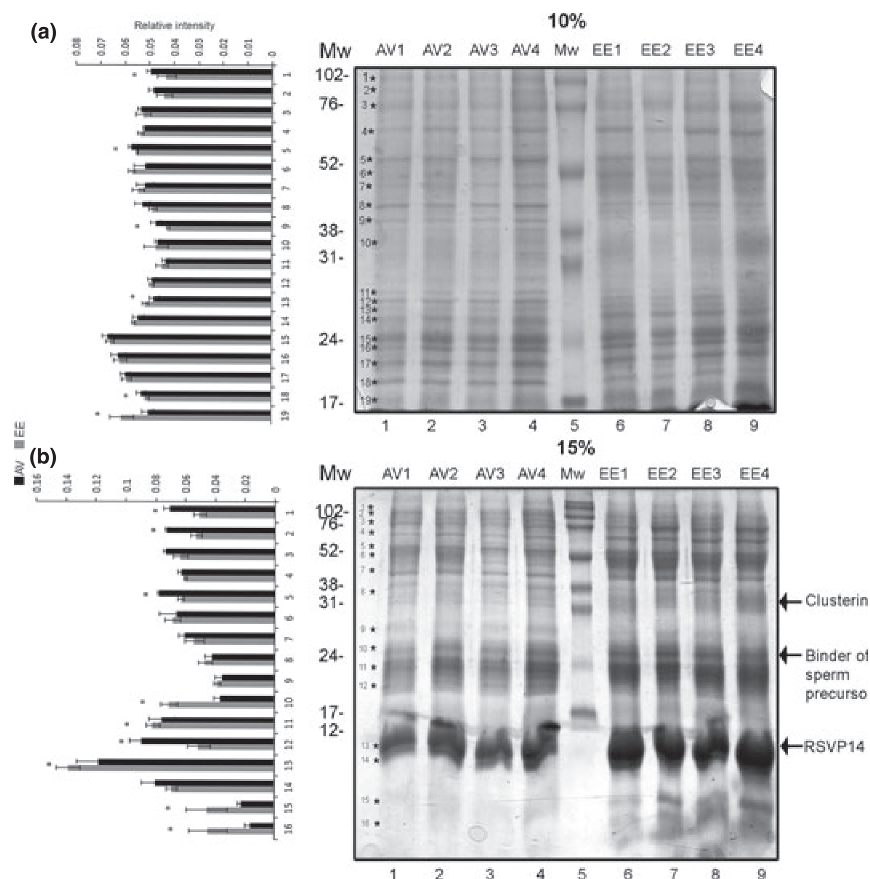

Fig. 1. Electrophoresis in polyacrylamide gels showing high molecular mass (a, 10% acrylamide) or low molecular mass (b, 15% acrylamide) protein profile (right panels) and scanning densitometry (left panels). Four different pools of SP (20 μg/lane) collected by artificial vagina ($AV_{1–4}$) or electroejaculation ($EE_{1–4}$) were electrophoretically analysed. The position of the molecular mass markers is indicated at the left. For each lane, all bands were quantified, and the intensity of each band (denoted by *) was normalized to the sum of the total protein in the corresponding lane. To analyse differences between methods, the average ± standard deviation values for each band were plotted and analysed by T-test (left panel). Significant differences are indicated by. Three of the proteins increased in SP collected by EE were identified by peptide mass fingerprinting as indicated at the right side of the gel image

Table 3. Proteins of the seminal plasma of *Corriedale* rams identified by one- and two-dimensional electrophoresis and mass spectrometry (MALDI-ToF/ToF)

Protein	Excised from	Experimental kDa/pI	NCBI accession number	MS/MS protein score	Sequence covered (%)	Matched peptides
Seminal vesicle 14-kDa protein precursor (RSVP 14) [Ovis aries]	1D-PAGE	17	219521810	51	15	DKSSEESHEDEECVFPFTYK SSEESHEDEECVFPFTYK
	2D-PAGE	15/4.6	219521810	258	15	DKSSEESHEDEECVFPFTYK SSEESHEDEECVFPFTYK
Binder of sperm 5 precursor [Ovis aries]	1D-PAGE	28	148225308	98	49	RIYYK ICSDGDRPK CHFPFIYR EYAWQYCDR WKICSDGDRPK EYAWQYCDRY EWCSLDEDYVGR CTSVNSEREWCSLDEDYVGR CTSVNSEREWCSLDEDYVGR
Clusterin isoform 1 [Ovis aries]	1D-PAGE	38	426220555	112	25	TQIEQTNEEK DSENKLK IDSLMENDREQSHVMDVMEDSFTR EQSHVMDVMEDSFTR ASSIMDELFQDR ASSIMDELFQDRFFPR RPQDTQYYSPFSSFPR GSLFFNPK TPYHFPVTEFTENNDR TPYHFPVTEFTENNDRTVCK TVCKEIR ALQQYR
Seminal vesicle protein precursor (RSVP22) [Ovis aries]	2D-PAGE	24/4.8	219521812	58	34	CHFPFIYR EYAWQYCDR EWCSLDEDYVGR CTTEGSAFGLAWCSLTEYFER
Epididymal secretory protein E1 precursor [Bos taurus]	2D-PAGE	12/7.0	27806881	80	15	SGIRCPIEK VVVEWELTDDKNQR

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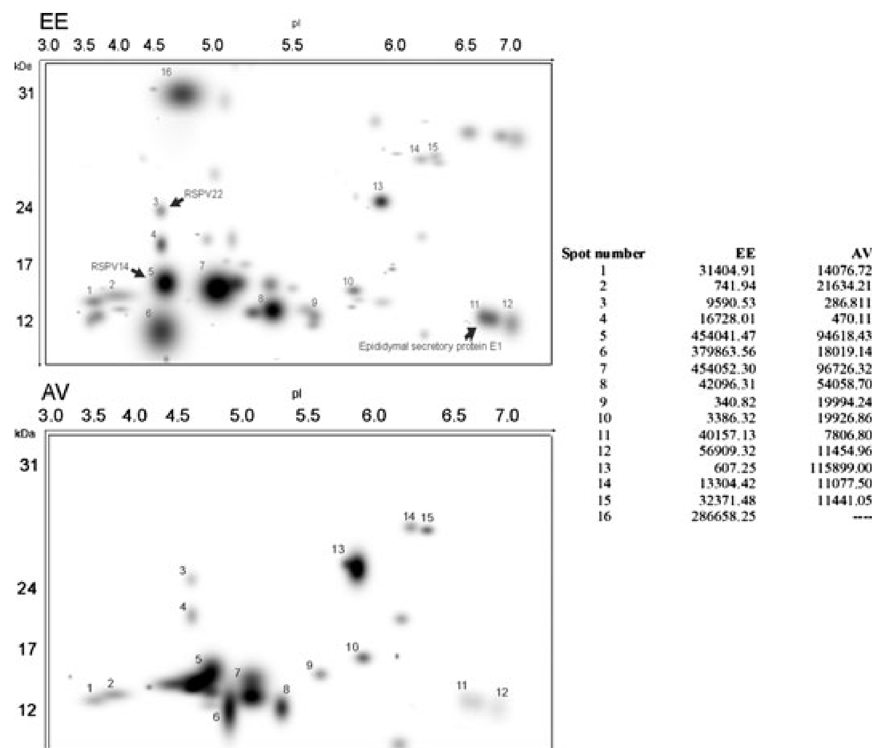

Spot number	EE	AV
1	31404.91	14076.72
2	741.94	21634.21
3	9590.53	286.811
4	16728.01	470.11
5	454041.47	94618.43
6	379863.56	18019.14
7	454052.30	96726.32
8	42096.31	54058.70
9	340.82	19994.24
10	3386.32	19926.86
11	40157.13	7806.80
12	56909.32	11454.96
13	607.25	115899.00
14	13304.42	11077.50
15	32371.48	11441.05
16	286658.25	---

Fig. 2. Master gels generated by PD-Quest software (Bio-Rad) and respective spot intensities of seminal plasma proteins from *Corriedale* rams obtained by electroejaculation (EE) and artificial vagina (AV). The experiment was focused on proteins between 12 and 31 kDa and 3.0–7.0 pI range. Molecular weight markers are indicated at the left and pH markers are indicated on top gels. The arrows show proteins that were identified by MALDI-ToF/ToF mass spectrometry

and *13) and 10 spots increased in EE (*1, *3, *4, *5, *6, *7,*11, *12, *14 and *15). One protein spot (*16) with molecular mass of approximately 31 kDa and pI 4.8 was exclusively observed in the SP recovered with EE. Three of these spots could be identified by tandem mass spectrometry (Table 3).

Discussion

The results of the present study show a higher number of sperm with intact plasma membrane and functional mitochondria in ejaculates collected with EE compared with that collected by AV. It was also observed that ejaculates collected with EE have a higher proportion of SP, which is in agreement with previous reports (Marco-Jiménez et al. 2005; Jiménez-Rabadán et al. 2012). As expected, total protein content was increased in SP collected by EE, probably due to the effect of the collection method over the secretory function of accessory sex glands. However, to our surprise, protein composition of SP obtained by EE differed from that obtained by AV. SP collected by EE had greater total protein content and relative abundance of low molecular mass proteins than ejaculates collected by AV.

The higher proportion of SP to sperm cells observed in ejaculates collected with EE can be explained by the overstimulation that electrical impulses exert on accessory sex glands (Dziuk et al. 1954; Quinn and White 1966; Pineda et al. 1987). SP of mammals plays an important role in sperm viability by preserving the integrity of plasma membrane that is crucial to maintain its fertilizing potential (Maxwell and Johnson 1999; Maxwell et al. 2007). Thus, a higher proportion of SP should be beneficial and might explain in part the best quality parameters observed in sperm collected with EE.

Sperm cells produce reactive oxygen species (ROS) as part of their normal metabolism, however, if produced in excess; these molecules are detrimental to the cells leading to decreased sperm viability and acrosomal integrity, DNA disruption and mitochondrial deterioration (Jeulin et al. 1989; Baumber et al. 2000; Aitken and Koppers 2011). Considering that spermatozoa have limited antioxidant capacity, the SP components are responsible for protecting sperm cells against oxidative stress. As ejaculates obtained with EE have higher proportion of SP, they might also contain higher proportion of antioxidant components, explaining the better quality of sperm cells in those ejaculates. Accordingly, Muiño et al. (Muiño-Blanco et al. 2008) hypothesized a potential antioxidant effect of two low molecular weight proteins (RSVP14 and RSVP20) that were found increased in SP from semen collected by EE in this work.

The recovery method changed the total protein content in SP, contrary to the results reported by Marco-Jiménez et al. (2008). Additionally, we found differences in the relative content of various proteins by SDS-PAGE and specially observed that SP collected by EE possesses greater abundance of low molecular weight proteins (≤31 kDa). Therefore, this molecular mass range was further analysed by 2D gel electrophoresis (2D-GE). The protein profile of seminal plasma using 2D-GE has previously been used for characterization of several proteins from SP of bovine (Desnoyers et al.1994), boar (Jonáková et al. 2007), equine (Brandon et al. 1999) and ram (Jobim et al. 2005). According to Bergeron et al. (2005), Jobim et al. (2005)

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and Cardozo et al. (2006), the major proteins in ram SP were a group of proteins of low molecular weight (12–30 kDa). In this study, the quantitative analysis of the 2D-GE protein maps detected remarkable differences between semen recovery methods in various protein spots, which increased significantly when semen was recovered using EE. In addition, one protein spot (Mr 31 kDa, pI 4.8) was present only in the SP when semen was obtained with EE. This protein spot may correspond to alpha-2-HS-glycoprotein, a broad-range protease inhibitor that regulates post-mating sperm function (Hedrich et al. 2010). However, their mechanism is still unknown (Souza et al. 2012).

By combining the information obtained by 1D SDS-PAGE, 2D-GE and mass spectrometry, five proteins that were increased in SP from EE ejaculates were identified. Ram *seminal vesicle proteins 22 and 14 (RSVPs)* and *binder of sperm 5 precursor* belong to the family of binder of sperm proteins (BSPs) that contain fibronectin type II domains as several SP proteins from other species, particularly bovine PDC-109 (Esch et al. 1983), GSP-14/15 kDa and RSP15 (Bergeron et al. 2005). This domain interacts with choline phospholipids on the sperm membrane, with high- and low-density lipoproteins and with heparin, playing major roles in membrane stabilization (decapacitation) and destabilization (capacitation) in the female genital tract (Manjunath and Thérien 2002). In addition, BSPs in bovine regulate sperm volume, helping to maintain cell viability after the osmotic shock that occurs during ejaculation (Shain et al. 2009). Several researchers (Pérez-Pé et al. 2001; Barrios et al. 2005; Domínguez et al. 2008) demonstrated that RSVP14 and RSVP20 could prevent/revert membrane damage in sperm cells exposed to freezing temperatures. *Epididymal secretory protein E1* is a cholesterol-binding protein originated in the mammalian epididymis and is the most abundant protein in the cauda epididymal fluid of bulls. It binds to sperm cells (Bernardini et al. 2011) and might help to a proper sperm maturation (Kirchhoff et al. 1996; Moura et al. 2010). *Clusterin* is the major glycoprotein in ram rete testis, and Sertoli cells may be the principal source of this protein (Blaschukz et al. 1983). This protein is present in the SP and sperm surface from several mammalian species, including humans (Sylvester et al. 1991). It is involved in sperm maturation (Howes et al. 1998) and possesses heparin-binding sites (Pankhurst et al. 1998). Jobim et al. (2005) suggested that this protein is related to high semen freezability in bulls.

In conclusion, the present study showed that seminal plasma obtained by electroejaculation is enriched with low molecular mass proteins, some of which have been demonstrated to be beneficial to the spermatozoa. Given that we also found that sperm obtained by EE had higher viability values and higher proportion of mitochondrial functionality than those obtained by AV, one might speculate that this could have been influenced by their differential seminal plasma composition. However, additional experimental work is needed to confirm this hypothesis.

It is known that ram SP proteins can prevent (Pérez-Pé et al. 2001, 2002) and repair (García-López et al. 1996; Ollero et al. 1997; Barrios et al. 2000) the cold-shock and cryopreservation (Bernardini et al. 2011) sperm membrane damage. Furthermore, a specific protein fraction containing RSVP14 and RSVP22 has been isolated from ram SP and identified as responsible for these protective effects (Barrios et al. 2000; Bernardini et al. 2011). The importance of our findings claims for more studies involving the utilization of SP obtained by EE to enhanced ram sperm quality after freezing and thawing procedures. The result of this work is a preliminary step to the development of strategies for improving the quality and the fertilizing ability of cryopreserved ovine semen.

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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.

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