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Evaluation of ram semen quality using polyacrylamide gel instead of cervical mucus in the sperm penetration test

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Abstract

Fertility is a very complex biological function that depends on several properties of the spermatozoa, including sperm motility. Two objectives are analyzed in this study: (1) Replace the cervical mucus by a synthetic medium in a sperm penetration test, and (2) evaluating the results of this test objectively analyzing the sperm number that migrates. In experiment 1, we have tested eight concentrations of acrylamide (1%–2%). Rheological properties of media were analyzed. The plastic straws, loaded with acrylamide, were placed vertically on the semen sample tube for 15 min at 39 °C. After, the acrylamides were placed, by segments of 5 mm, into wells of a 24-well plate, dyed with Hoechst 33342 and the number of spermatozoa were calculated by automated microscopy analysis. The 1.55% and 1.6% acrylamide gel showed a number of spermatozoa emigrating closer to that seen with natural mucus. In experiment 2, we applied the sperm penetration in acrylamide 1.6% and 1.55% using fresh semen and cooled semen at 15 °C and 5 °C. The spermatozoa counts were performed for each segment of 10 mm. Semen chilled at 15 °C presented intermediate values of sperm counts in comparison with fresh semen (higher) and 5 °C chilled semen. The sperm counts do not differ between acrylamides but the rheological properties of acrylamide 1.6% were more similar to those of the natural cervical mucus. In experiment 3, we have observed significant correlations between the number of spermatozoa and several sperm quality parameters (positive: progressive motility and velocity according to the straight path; negative: damaged acrosomes and apoptotic cells) in 1.6% acrylamide media. We conclude that the size of the cell subpopulation, objectively calculated, that migrate beyond 20 mm in 0.5-mL straws filled with acrylamide is a useful parameter in ram sperm quality assessment and further studies are needed to evaluate its relationship with field fertility.

Keywords: Ram; Sperm quality; Penetration test; Motility; Mucus

1. Introduction

The passage of sperm through the female reproductive tract is regulated to maximize the chance of fertilization and ensure that sperm with normal morphology and vigorous motility will be the ones to succeed [1]. Cervical mucus filters out sperm with poor morphology and motility and as such only a minority of ejaculated sperm actually enter the cervix [1]. Thus, mucus is considered a means of sperm selection in many species. Taking into account the effect of cervical mucus on sperm transport, the evaluation of the ability of spermatozoa to progress through natural mucus (cervical

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mucus penetration test [CMPT]) or mucus substitutes has been proposed as an analysis of sperm quality [2–9]. This penetration test has been applied in several animal species and is accepted by the World Health Organization [10] as a means of analyzing human semen.

Generally, the test is based on the visual assessment of the linear distance covered by the foremost sperm cell (vanguard spermatozoa) in the capillary tube. Another method using the number of spermatozoa accumulated in different segments of the capillary tube as a parameter of analysis. Visual sperm counts at certain distances (10, 20 mm, etc.) from the base of the tube in flat capillary tubes has been used for this kind of assessment [11,12]. Tas et al. [13] have developed a new CMPT technique in which transparent plastic straws are used instead of capillary tubes and the total number of spermatozoa penetrating to predetermined distances in cervical mucus are measured on slides. Ola et al. [7] reviewed the accuracy of in vitro sperm penetration into cervical mucus or substitutes in evaluating sperm motility in human semen, and they showed that vanguard distance as a diagnostic criterion has a low accuracy while sperm concentration is more accurate.

A number of diagnostic studies into the usefulness of the CMPT technique have been developed. Fertilizing capacity of spermatozoa has been shown to be strongly related to the parameters observed in the cervical mucus penetration test (human [14]; bull [13,15]). In other studies, the correlation between sperm migration capacity and fertility was not observed [2–4,16,17]. However, it is generally accepted that penetration of spermatozoa into cervical mucus in vitro provides important information predictive of sperm function [11].

The major problem with cervical mucus as a component of any test system is the difficulty encountered in standardizing the quality of this material. It is difficult to obtain large volumes of natural cervical mucus and the variation among lots of natural mucus is large, even between batches from the same female [18]. Thus, it is desirable to formulate a synthetic medium free of these problems, simple to prepare and with easily reproducible rheological properties. Acrylamide, methylcellulose, and hyaluronic acid have previously been used as a natural cervical mucus substitute for in vitro sperm penetration tests (human [11,12,19]; bull [20]; ram [21]).

In ram, few studies have been performed to analyze the relationship between the penetration test and sperm quality. A modified sperm penetration test was used by Suttiyotin et al. [22], noting that sperm penetration distance in Tris-glucose solution was correlated with a 48-day nonreturn rate and a 60-day conception rate. Robayo et al. [9] studied the relationship between sperm migration in ruminant cervical mucus (distance traveled by the vanguard spermatozoa) and motility patterns observed by computer assisted semen analysis (CASA). Continuous line velocity and average path velocity were the only kinematic parameters that presented significant positive correlations with the migration in sheep cervical mucus. O'Hara et al. [21] assessed the penetrating ability of fresh ram semen using flat capillary tubes and aiding visibility to cells with Hoechst 33342. These authors showed that the penetrating ability of fresh ram semen into artificial mucus was influenced by diluents and storage duration.

The aim of this study was to automate the quantitative analysis of the ram sperm population that migrates in a column of ovine cervical mucus or substitutes (acrylamide) into a plastic straw. We propose to evaluate the straw content by segments, placing each segment onto a slide or a plate, to stain spermatozoa with cell permeable nucleic acid stains and to analyze these samples automatically by a microscope to count the spermatozoa in each sample. This method is more objective than visually counting of the number of unstained spermatozoa in the straw and opens the possibility of assessing the physiological status of spermatozoa using other fluorescent probes.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: assessment of the suitability of eight synthetic media for in vitro evaluation of sperm progression by a mucus penetration test

To formulate a synthetic medium as an ovine cervical mucus substitute, eight concentrations of acrylamide (1%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.85%, and 2%) were compared in the sperm penetration test performed at 39 °C for 30 min. A test with ovine cervical mucus was used as a control assay. The sperm count of the migration assay was measured for each 5 mm of plastic straw (12 segments in total). The tests were conducted over 4 wk (in February and March). Ejaculates from four rams were collected twice a week by artificial vagina, and pooled. Two concentrations of acrylamide, the sperm count of which is more similar to that observed for the natural mucus, were selected for experiment 2.

2.1.2. Experiment 2: assessment of sperm progression in two synthetic media using semen samples stored in liquid state at a reduced temperature (5 °C or 15 °C)

The calculation of the population of sperm that migrate for each segment is instrumentally complex and thus we propose a more simple analysis. In this study we evaluated the sperm population that progresses for each 10 mm of plastic straw (6 segments in total) after 30 min at 39 °C. In a second analysis, these data are summarized in two variables: sperm count that progress beyond 20 mm or 30 mm (D20 or D30, respectively). This analysis was performed with four media: two acrylamides (1.55% and 1.6%), and two reference controls (sheep cervical mucus and 1.65% acrylamide). Ram semen was analyzed in three different physiological states: fresh semen, semen stored to 15 °C, and semen stored at 5 °C. The tests were conducted over 4 wk (during March and April). Ejaculates from four rams were collected twice a week by artificial vagina and pooled. The basic rheological properties of acrylamide gels prepared with four concentrations of reference were analyzed and these properties of natural cervical mucus were discussed.

2.1.3. Experiment 3: correlation between in vitro sperm progression and spermatozoa quality of fresh semen samples

The ability of the sperm penetration test to predict semen quality is evaluated by the relationship between quality parameters of ram spermatozoa and the sperm count observed in the sperm-mucus penetration test. This analysis was performed with acrylamide 1.6% and sheep cervical mucus, as reference control. We evaluated the sperm population of fresh ejaculates that progress beyond 20 or 30 mm after 30 min at 39 °C. The tests were conducted over 4 wk (during April and May). Ejaculates from four rams were collected twice a week by artificial vagina, and pooled.

2.2. Collection of cervical mucus

The ewes were synchronized using intravaginal sponges (Chronogest, Laboratorios Intervet SA, Madrid, Spain) impregnated with 20 mg of fluorogestone acetate. After 14 days, the sponges were removed, and the ewes received 500 IU of equine chorionic gonadotropin (Folligon, Laboratorios Intervet SA) intramuscularly. Cervical mucus was collected from 24 ewes during the induced estrus using plastic AI sheaths connected to a 20-mL syringe. Collected mucus samples were screened and only clear ones were used. Selected mucus samples were stored in 30-mL sterile tubes (pooling the mucus of several females) and were transported to the laboratory at 5 °C, where they were stored at −20 °C until use according to Memon and Gustafsson [23].

2.3. Preparation of synthetic media

Eight acrylamide gels (1%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.85%, and 2%) were prepared to test sperm progression. These synthetic media were obtained from 30% liquid acrylamide (30% ProtoGel, National Diagnostics, Atlanta, GA, USA), diluting it to the corresponding volume with 1.5 M Tris (pH 8.8) in water. Once mixed, a solution of 2% ammonium persulphate up to 1.5% of final volume and a 0.05% TEMED were added, to induce acrylamide polymerization [5]. The mixture (pH 7.2, 320 mOsm) was left at room temperature for 24 h and then stored at 4°C until use.

2.4. Evaluation of rheological properties of media

Rheological measurement of different media were made using a Rotovisco RV 12 viscometer (Haake Mess-Technik, GmbH, Co., Karlruhe, Germany) at 38 °C according to López-Gatius et al. [24].

2.5. Ram sperm collection

Semen from four Assaf rams was collected by means of an artificial vagina (40 °C) in the presence of a female decoy. The glass collection tube was placed in a thermoregulated bath at 34 °C, and a preliminary seminal evaluation was carried out (volume, mass motility, and concentration). Sperm concentration was assessed by Bürker hemocytometer (Marienfeld, GmbH, Marienfeld, Germany) using CASA (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). The ejaculates used in the experiment were those with a volume higher than 0.5 mL, mass motility ≥4 (determination by microscopy with warming stage at 37 °C, × 40; score: 0–5) and a sperm concentration greater than 3000×10^6 spermatozoa/mL. Ten min after collection, these ejaculates were diluted in TCF (0.27 M Tris, 90 mM citric acid, 53 mM fructose) at 1600×10^6 spermatozoa/mL, obtaining a sperm pool.

2.6. Sperm refrigeration

A proportion of the diluted semen was used for immediate experiment (fresh sample), and the remainder was divided into two fractions of 200 μL which were cooled: (1) to 15 °C (R15 sample) in an incubator (WTB Binder; REGO, Madrid, Spain); and (2) to 5 °C (R05 sample) in a refrigerator. The cooled semen sam-

ples were stored in the respective containers for 24 h and were then evaluated by the progression test.

2.7. Sperm motility evaluation

Sperm were diluted in TCF to 10 to 20 × 10^6 spermatozoa/mL and loaded into a Makler counting chamber (10-μm depth) at 37 °C. The CASA system consisted of a triocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) using a 10 × negative phase-contrast objective, equipped with a warming stage at 37 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured and analyzed using a computer-assisted motility analyzer (ISAS; Proiser) with specific settings to ram spermatozoa. The software rendered the following parameters: (1) percentage of motile spermatozoa (PM), and (2) for each spermatozoon, three velocity parameters (VCL, velocity according to the actual path; VSL, velocity according to the straight path; and VAP, velocity according to the smoothed path), three track linearity parameters (LIN, linearity; STR, straightness; WOB, wobble), the amplitude of the lateral displacement of the sperm head (ALH), and the head beat-cross frequency (BCF).

2.8. Assessment of sperm quality by cytometry

2.8.1. Acrosomal status

Double stain with PNA-FITC (Sigma-Aldrich, Madrid, Spain) and propidium iodide (PI; Sigma-Aldrich) were used. The staining was performed by diluting the sperm sample (1–2 million spermatozoa/mL) in 300 μL of PBS with 1 μg/mL of PNA-FITC and 1.5 μM of PI. After 10 min at room temperature and darkness, samples were analyzed by flow cytometry. Spermatozoa were classified in four sperm subpopulations: red fluorescence (not viable), green fluorescence (viable with damaged acrosome), double fluorescence (not viable with damaged acrosome), and no staining (viable with intact acrosome).

2.8.2. Cell viability

The analysis was performed with a double staining SYBR-14 and PI using the Sperm Viability Kit (LIVE/DEAD, Invitrogen, Barcelona, Spain). The sperm sample was diluted in 300 μL of PBS (1–2 million spermatozoa/mL) with 500 nM of SYBR-14 and 0.8 mg/mL of PI. After 10 min at room temperature and darkness, samples were analyzed by flow cytometry. This double staining classified the spermatozoa in three different cell groups: sperm with red fluorescence in the nucleus (nonviable), sperm with green fluorescence in the nucleus (viable), and sperm cells with double fluorescence (nonviable).

2.8.3. Mitochondrial status

Sperm samples were diluted (1–2 million spermatozoa/mL) and 100 nM of Mitotracker Deep Red (Invitrogen) stock solution 1 mM in DMSO and 100 nM YO-PRO-1 (Iodide 491/509, Invitrogen) were added. Samples were incubated in the dark for 15 min at 38 °C and then analyzed by flow cytometry. Viable spermatozoa with high fluorescence for Mitotracker Deep Red (Invitrogen) were interpreted as having active mitochondria.

2.8.4. Apoptotic cells

Double stain with YO-PRO-1 (Invitrogen) and PI were used. The staining was performed by diluting the sperm sample (1–2 million spermatozoa/mL) in 300 μL of PBS with 100 nM YO-PRO-1 (Invitrogen) and 1.5 μM of PI. After 10 min at room temperature and in darkness, samples were analyzed by flow cytometry. This double staining allows us to differentiate three populations of spermatozoa: sperm with red fluorescence (dead cells), spermatozoa with green fluorescence by YO-PRO-1 (Invitrogen) (living apoptotic cells), and unstained spermatozoa (viable cells).

2.9. Flow cytometry analysis

Cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser (488 nm) and red diode laser (635 nm). SYBR-14, YO-PRO-1 (Invitrogen), PNA-FITC, and PI were excited at 488 nm and Mitotracker Deep Red (Invitrogen) was excited at 635 nm. The fluorescence emitted by SYBR-14, YO-PRO-1 (Invitrogen) and PNA-FITC was analyzed using the FL1 photodetector (530/28 BP filter) and PI and Mitotracker Deep Red (Invitrogen) fluorescence using FL3 photodedector (670 LP filter). The signals, forward scatter/side scatter (FSC/SSC), were used to discriminate the sperm population from other events. For each sample, we have acquired 10 000 spermatozoa using Cell Quest Pro v. 3.1 (BD Biosciences) software. The analysis of flow cytometry data were performed using Weasel v.2.6 (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

2.10. Sperm progression test

This test was carried out using 0.5-mL plastic straws (0.3 × 13 cm) filled with ovine cervical mucus or with each of the acrylamide gels to test (9 replicates). Semen samples were first diluted to 25 × 10^6 motile spermato-

zoa/mL in TCF. Diluted samples were distributed in 2-mL microtubes (180 μL/tube), and the straws were placed perpendicularly by a clamping device over the semen sample. Straw position was adjusted so that only 1 mm of mucus (natural or synthetic), protruding from the straw, stayed in contact with the sperm sample. This device remained for 15 minutes at 39 °C. After this time, straws were emptied in segments of 5 mm (experiment 1) or 10 mm (experiment 2), placing each segment in a well of a 24-well plate. The first 5 mm of the straw content was discarded, because of high sperm concentration due to direct sample contact, was irrelevant to real sperm progression. To automatically obtain the image of the sperm under a microscope, 100 μL of 10 μg/mL Hoechst 33342 was added to each well. The plates were left to dry on a plate at 39 °C.

The plates were analyzed on an inverted microscope (T2000 U; Nikon) equipped with epifluorescence and motorized stage. Each well was photographed automatically (ORCA digital camera, Hamamatsu, Tokyo, Japan), capturing 96 images (10× objective). The motorized plate and the digital camera were automatically controlled by Metamorph v.7 software (Molecular Devices, Inc., USA). Subsequently, these images were analyzed with the image analysis software NIS Elements v.3 (Nikon), to objectively count the spermatozoa in each well. Specific adjustment to discriminate sperm with regard to debris, according to their shape and size, were applied in this analysis.

A negative control is performed to avoid an overestimation of sperm count in the cervical mucus, due to the presence of epithelial cells. In each trial, a straw filled only with ovine cervical mucus was emptied in segments of 5 mm or 10 mm (depending on the type of experiment), placing each segment in a well of a 24-well plate. As with the plates containing sperm, Hoechst 33342 was added to each well and finally the cells present are counted. This number was subtracted from the sperm counts that we obtained with the sperm progression test.

2.11. Statistical analysis

Results are presented as mean ± SEM (standard error of the mean). Data were analyzed with the Statistica v. 9 (StatSoft, Tulsa, OK, USA) program using general linear models (GLM) or, where appropriate, the Pearson correlation coefficient. In experiment 1, GLM was used to study the sperm progression in the straws by sperm counts in each straw segment (5 mm), and to compare the nine synthetic media with the cervical mucus control (post hoc comparison by Fisher LSD test). The rheological data (consistency index) were fitted to an exponential function. In experiment 2, a GLM was carried out using as factors the four synthetic media, the three types of semen samples, and the two distances (20 or 30 mm), with the spermatozoa counts as the dependent variable. In experiment 3, Pearson correlation coefficients were carried out between the spermatozoa counts and the individual and progressive motility, the kinematic parameters, the acrosomal status, the sperm viability, the mitochondrial status and the proportion of apoptotic cells.

3. Results

3.1. Experiment 1: assessment of the suitability of eight synthetic media for in vitro evaluation of sperm progression

Sperm migration of fresh semen samples into straws containing each of the eight concentrations of acrylamide tested or ovine cervical mucus is presented in Table 1. Eleven 5-mm segments (from S02 to S12) were quantified. Ovine cervical mucus, used as control, showed higher sperm numbers than synthetic media. The data were compared for pairs of successive segments and we found significant differences between the first three segments (S02–S03 and S03–S04) both for the natural mucus as for the synthetic media with an acrylamide concentration between 1% and 1.6%. In the different media evaluated, the concentration of spermatozoa declined exponentially in relation to penetration depth.

The existence of two groups of synthetic media defined by the acrylamide concentration could be appreciated. One group was composed of synthetic media containing an acrylamide concentration of between 1% and 1.6%, with sperm count values which are closer to those of the cervical mucus (Table 1). In this group, the consistency index of synthetic media with acrylamide 1% and 1.5% (k = 0.01 and 0.11, respectively) was different from that of the cervical mucus (k = 0.17, Table 2). The fitting curve obtained with rheological values of the different media allows us to conclude that acrylamide 1.55% and 1.6% are more similar to the characteristics of cervical mucus. The second group, composed of the acrylamide media from 1.65% to 2%, showed significantly lower sperm count values and their rheological characteristics differ greatly from those of cervical mucus.

3.2. Experiment 2: assessment of sperm progression in two synthetic media using semen samples with different types of preservation

The medium used as negative control (acrylamide 1.65%) showed the lowest cell density data in the three

Table 1
Number of spermatozoa (mean ± standard error) observed in each segment of 5 mm (S02–S12)* in a sperm penetration test using fresh semen samples and testing eight different synthetic media (A 1% to A 2%).

Segment	A 1%	A 1.5%	A 1.55%	A 1.6%	A 1.65%	A 1.7%	A 1.85%	A 2%	Mucus
S02	3227 ± 522^{aA}	4171 ± 702^{aA}	3756 ± 622^{aA}	3452 ± 194^{aA}	1161 ± 422^{aB}	1022 ± 329^{aB}	350 ± 148^{aC}	287 ± 95^{aC}	5630 ± 337^{aD}
S03	2241 ± 363^{bA}	3299 ± 436^{bA}	3126 ± 475^{bB}	2520 ± 573^{bAB}	603 ± 229^{aC}	306 ± 114^{bC}	58 ± 18^{bD}	108 ± 36^{bD}	3863 ± 279^{bB}
S04	1450 ± 159^{cA}	1939 ± 265^{cA}	1865 ± 203^{cA}	1102 ± 135^{cA}	201 ± 87^{bB}	108 ± 48^{bB}	66 ± 36^{bC}	52 ± 19^{bC}	3624 ± 375^{bD}
S05	1159 ± 152^{cA}	1738 ± 530^{cA}	1388 ± 146^{bA}	1247 ± 147^{cA}	116 ± 37^{bB}	109 ± 31^{bB}	37 ± 11^{bC}	31 ± 10^{bC}	2994 ± 286^{bD}
S06	1068 ± 151^{cA}	1219 ± 207^{cA}	1140 ± 135^{bA}	1141 ± 137^{cA}	79 ± 27^{bB}	86 ± 28^{bB}	18 ± 11^{bC}	43 ± 19^{bC}	2429 ± 290^{bD}
S07	964 ± 109^{cA}	1021 ± 134^{cA}	1414 ± 304^{bA}	1145 ± 205^{cA}	130 ± 48^{bB}	53 ± 13^{bC}	36 ± 18^{bC}	41 ± 12^{bC}	2306 ± 287^{bD}
S08	824 ± 156^{cA}	613 ± 111^{cA}	1255 ± 296^{bB}	822 ± 191^{cA}	69 ± 31^{bC}	54 ± 17^{bC}	33 ± 12^{bC}	37 ± 8^{bC}	2066 ± 357^{bD}
S09	413 ± 93^{cA}	448 ± 74^{cA}	547 ± 90^{cA}	447 ± 97^{cA}	63 ± 37^{bB}	73 ± 16^{bB}	24 ± 8^{bB}	26 ± 8^{bB}	2234 ± 354^{bC}
S10	394 ± 80^{cA}	411 ± 62^{cA}	586 ± 68^{cA}	428 ± 90^{cA}	54 ± 18^{bB}	38 ± 14^{bB}	19 ± 6^{bB}	17 ± 6^{bB}	1692 ± 208^{bC}
S11	359 ± 83^{cA}	512 ± 100^{cA}	551 ± 83^{cA}	194 ± 80^{cB}	24 ± 8^{bC}	15 ± 5^{bC}	12 ± 5^{bC}	9 ± 3^{bC}	1588 ± 233^{bD}
S12	329 ± 55^{cA}	495 ± 91^{cA}	556 ± 95^{cA}	133 ± 32^{cB}	16 ± 3^{bC}	13 ± 4^{bC}	14 ± 4^{bC}	15 ± 3^{bC}	1410 ± 145^{bD}

Ovine cervical mucus (Mucus) was control media (9 replicates).
Different superscript letters (ab) in the same column indicate that pair of segments [S02–S03], [S03–S04], [S04–S05], [S05–S06], [S06–S07], [S07–S08], [S08–S09], [S09–S10] differ significantly within each media ($P < 0.05$). Different superscript letters (AB) in the same row indicate that media differ significantly within each segment ($P < 0.05$).
A, acrylamide gel.
* The first segment is not valued.

Table 2
Rheological properties (mean ± standard error) of ovine cervical mucus (Mucus) and four substitutes based on acrylamide (A) 1% to A 2.5% at 38°C (10 replicates).

Media	Index of consistency (K)*	Flow behavior index (n)	*r*
A 1%	0.01 ± 0.01	0.99 ± 0.08	0.99
A 1.5%	0.13 ± 0.02	0.79 ± 0.04	0.98
A 2%	0.87 ± 0.06	0.45 ± 0.02	0.94
A 2.5%	1.69 ± 0.1	0.48 ± 0.03	0.96
Mucus	0.17 ± 0.03	0.75 ± 0.05	0.98

* K in Pascals per second^{n}. Pearson moment-correlation coefficient (*r*) ($P < 0.05$).

types of semen tested, whereas the highest values were observed for cervical mucus (Table 3). Semen chilled at 15 °C presented intermediate sperm count values with regard to those observed in fresh semen (higher) and 5 °C chilled semen. Segments S02 and S03 showed the highest sperm counts which are significantly different from the values observed in other analyzed segments, in both acrylamide 1.6% and acrylamide 1.55% media.

In order to evaluate the two synthetic media tested, we have obtained two single values: the sperm numbers that progress beyond 20 mm or 30 mm (D20 or D30, respectively; Fig. 2). We found that there were no significant differences between acrylamide 1.6% and acrylamide 1.55% media in any case. However, the acrylamide 1.6% medium showed a consistency index that was closer to the data presented in cervical mucus (Fig. 1).

3.3. Experiment 3: correlation between in vitro sperm progression of semen samples in different types of conservation and semen quality

Table 4 represents the correlation coefficients between the sperm count observed in a migration test with acrylamide 1.6% and ovine cervical mucus, in the two distances analyzed (D02 and D03), and the corresponding semen quality parameters. In both distances, we found that sperm count had a significant negative correlation with the percentage of apoptotic spermatozoa percentage (YO-PRO-1+/PI−) and the percentage of cells with damaged acrosome cells (PNA+), with both acrylamide 1.6% and mucus. However, for both media, we found no significant correlation with the percentage of viable cells (SYBR+/PI−) or the potential of mitochondrial membrane. The number of spermatozoa showed a positive correlation with PM and velocity according to the straight path (VSL) for both media. In the case of acrylamide 1.6%, LIN and WOB also showed a significant correlation with the number of

Table 3
Number of spermatozoa (mean ± standard error) in each 1 cm segment (S02–S06)* observed in three different synthetic media (A 1.55% to 1.65%) and ovine cervical mucus (Mucus), for semen samples preserved by two procedures (refrigerated at 5°C [R05] or 15 °C [R15]) and fresh semen (12 replicates).

Medium	Semen	S02	S03	S04	S05	S06
A 1.55%	Fresh	$5326 \pm 356^{\alpha Aa}$	$3422 \pm 237^{\alpha Ab}$	$1734 \pm 155^{\alpha Ac}$	$1434 \pm 216^{\alpha Ac}$	$1145 \pm 246^{\alpha Acc}$
	R15	$4688 \pm 630^{\alpha Aa}$	$2743 \pm 429^{\alpha Ab}$	$1558 \pm 295^{\alpha Ac}$	$1336 \pm 199^{\alpha Ac}$	$1032 \pm 273^{\alpha Ac}$
	R05	$3003 \pm 425^{\beta Aa}$	$1844 \pm 233^{\beta Ab}$	$1290 \pm 185^{\alpha Ab}$	$945 \pm 156^{\alpha Ab}$	$646 \pm 132^{\alpha Ab}$
A 1.6%	Fresh	$5932 \pm 653^{\alpha Aa}$	$3918 \pm 458^{\alpha Ab}$	$1857 \pm 213^{\alpha Ac}$	$1240 \pm 181^{\alpha Ac}$	$873 \pm 60^{\alpha Ac}$
	R15	$4153 \pm 527^{\beta Aa}$	2522 ± 269^{cAb}	$1362 \pm 201^{\alpha\beta Ab}$	$1023 \pm 148^{\alpha\beta ABc}$	$818 \pm 109^{\alpha Ac}$
	R05	$3228 \pm 270^{\beta Ba}$	$1328 \pm 174^{\beta Ab}$	$846 \pm 185^{\beta Ac}$	$472 \pm 83^{\beta Bb}$	$601 \pm 82^{\alpha Ab}$
A 1.65%	Fresh	$1189 \pm 178^{\alpha Ba}$	$391 \pm 59^{\alpha Bb}$	$264 \pm 56^{\alpha Bc}$	$110 \pm 31^{\alpha Ab}$	$156 \pm 36^{\alpha Bb}$
	R15	$457 \pm 158^{\beta Ba}$	$386 \pm 38^{\alpha Ba}$	$90 \pm 23^{\alpha Ba}$	$134 \pm 95^{\alpha Aa}$	$124 \pm 41^{\alpha Ba}$
	R05	$352 \pm 51^{\beta Ca}$	$202 \pm 28^{\alpha Bb}$	$77 \pm 13^{\alpha Bb}$	$79 \pm 27^{\alpha Ab}$	$64 \pm 12^{\alpha Bb}$
Mucus	Fresh	$6882 \pm 574^{\alpha Ca}$	$4672 \pm 416^{\alpha Cb}$	$3665 \pm 439^{\alpha Cb}$	$2307 \pm 367^{\alpha Ac}$	$1418 \pm 629^{\alpha Ac}$
	R15	$4501 \pm 488^{\beta Aa}$	$2999 \pm 428^{\beta Ab}$	$2212 \pm 438^{\beta Cb}$	$1394 \pm 180^{\beta Bb}$	$707 \pm 60^{\beta Ab}$
	R05	$3820 \pm 499^{\beta Ba}$	$2410 \pm 411^{\beta Cb}$	$1224 \pm 178^{\beta Ab}$	$1034 \pm 132^{\beta Bb}$	$629 \pm 78^{\beta Ab}$

Different superscript letters (a,b) in the same row indicate that the pair of segments (S02–S03), (S03–S04), (S04–S05), and (S05–S06) differ significantly within each medium and preservation procedure ($P < 0.05$). Different superscript letters (A,B) in the same column indicate that media differ significantly within each segment and preservation procedure ($P < 0.05$). Different superscript letters (α,β) in the same column indicate that the preservation procedure differ significantly within each segment and media ($P < 0.05$).
A, acrylamide gel.
* The first segment was discarded.

spermatozoa ($r = 0.59$, $P = 0.012$; $r = 0.54$, $P = 0.024$, respectively for D02). Straightness showed a significant correlation with sperm counts in cervical mucus. In this context, we must point out that, in general the correlation coefficients with the cervical mucus are higher.

4. Discussion

Fertility is a very complex biological function that depends on several properties of the spermatozoa, including sperm motility. The efficiency of sperm transport in the genital tract is an essential prerequisite for

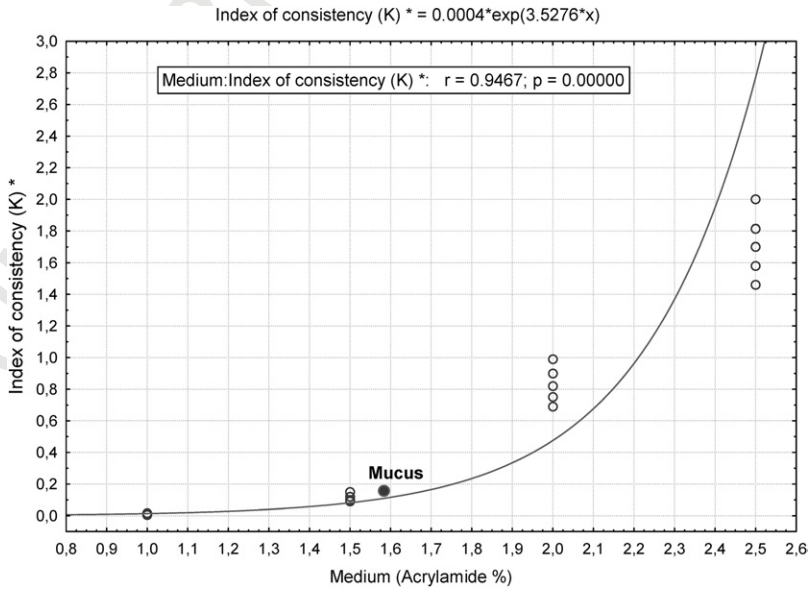

Fig. 1. Rheological properties of acrylamide gel: index of consistency fitted in an exponential manner in relation to proportion of acrylamide in medium (points indicate the values of five assays). Mean values of index for the ovine cervical mucus is shown.

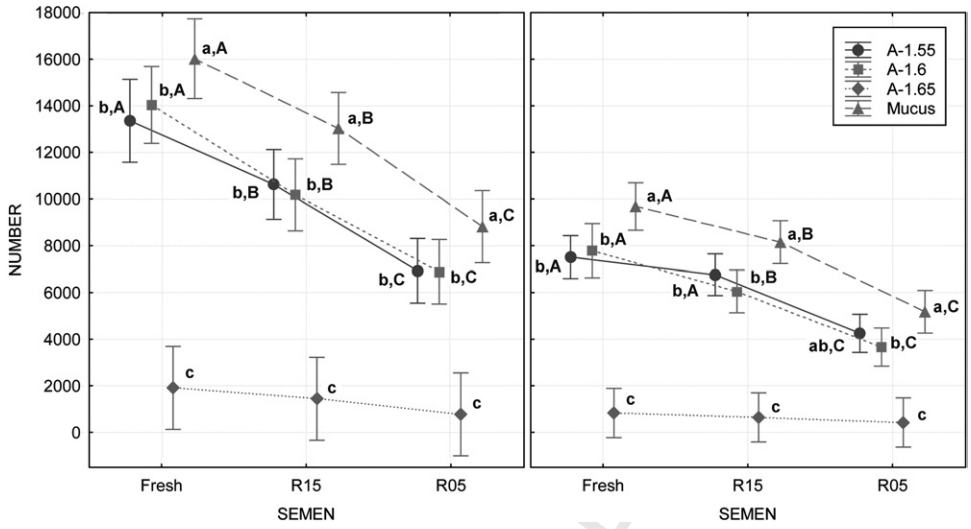

Fig. 2. Number of spermatozoa (LS mean of 12 replicates ± 95% confidence interval) that progresses a distance beyond 20 (D02) or 30 mm (D03) in acrylamide gel or cervical mucus for three semen samples (fresh and refrigerated at 5 °C or 15 °C). Lowercase letters indicate differences between media in each semen sample and progression distance and capital letters indicate differences between semen samples in each media and distance.

the reproduction success [25]. Given this fact, the evaluation of the capacity of spermatozoa to progress through natural mucus (CMPT) or a mucus substitute has been proposed to assess the sperm quality in vitro. The penetration test is one of the methods employed in seminal assessment using the distance traveled by the most advanced spermatozoa in the test device as diagnostic criterion. In this study, we propose an automatic analysis of the sperm numbers that migrates in a column of acrylamide to assess the semen quality in ram.

In our first experiment, a series of acrylamide concentrations were examined. The synthetic mucus have

Table 4
Correlation coefficients (test of significance) between the number of spermatozoa that progress a distance beyond 20 (D02) or 30 mm (D03) in acrylamide gel (A) 1.6% or ovine cervical mucus (Mucus) and the seminal quality parameters.

Parameter	D02				D03			
	Mucus		A 1.6%		Mucus		A 1.6%	
	r	P		P		P		P
TM	0.42	0.093	0.26	0.305	0.43	0.086	0.19	0.461
PM	0.62	0.008	0.55	0.021	0.62	0.006	0.49	0.043
VAP	0.41	0.060	0.40	0.090	0.43	0.068	0.42	0.086
VCL	0.36	0.152	0.21	0.413	0.38	0.137	0.26	0.319
VSL	0.49	0.045	0.48	0.049	0.52	0.034	0.44	0.046
LIN	0.42	0.092	0.59	0.012	0.44	0.080	0.50	0.041
STR	0.59	0.013	0.56	0.019	0.60	0.011	0.43	0.085
WOB	0.24	0.359	0.54	0.024	0.25	0.323	0.49	0.047
ALH	−0.28	0.283	−0.39	0.123	−0.32	0.215	−0.33	0.197
BCF	0.63	0.006	0.40	0.114	0.65	0.004	0.30	0.238
dACR	−0.40	0.002	−0.30	0.018	−0.41	0.001	−0.39	0.049
VIAB	0.45	0.052	0.32	0.062	0.47	0.057	0.35	0.068
MIT	0.37	0.180	0.36	0.187	0.35	0.196	0.32	0.246
APOP	−0.61	0.009	−0.62	0.008	−0.61	0.009	−0.59	0.013

ALH, amplitude of lateral head displacement (μm); APOP, living apoptotic cells, YO-PRO-1+ (%); BCF, head beat-cross frequency (%); dACR, damaged acrosomes, PNA+ (%); LIN, linearity index (%); MIT, active mitochondrial in live spermatozoa (%); PM, progressive motility (%); STR, straightnes. (%); TM, total motility (%); VAP, average path velocity (μm/sec); VCL, curvilinear velocity (μm/sec); VIAB, viability spermatozoa; VSL, straight-line velocity (μm/sec).

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some advantages over natural mucus such as the ability to easily prepare large volumes and the ability to standardize environmental conditions, while the properties of cervical mucus are dependent on the estrus cycle [18] and only a small amount of it is produced by each ewe. Cervical mucus substitutes have been used in a number of studies [11,12,26]. Acrylamide has been previously used as a substitute of cervical mucus in other species (human [19,27]; bull [5,20]). Lorton et al. [27] noted that bull sperm migration in 1.8% acrylamide was similar to sperm migration in bovine cervical mucus. However, bull semen that varied widely in migration distances in bovine cervical mucus maintained similar relative migration distances in this synthetic medium. Eggert-Kruse et al. [19] evaluated polyacrylamide gel as a substitute for human cervical mucus in the sperm penetration test and among other results found that adequate sperm migration in polyacrylamide 1.8% was significantly more frequent in the fertile group.

We have observed a range of acrylamide concentrations (1.5%–1.6%) which allows the greatest number of spermatozoa to penetrate and these sperm counts were close to those observed in the test with cervical mucus. The differences among bull or ram regarding the characteristics of the synthetic medium most suitable for the progression test, can be interpreted according to the different characteristics of the cervical mucus from both species. Our rheological data of acrylamide gel fitted an exponential function, so that the 1.6% (suitable for ram semen) and 1.8% (suitable for bull semen) gels showed a noticeable difference in their consistency index. Moreover, the penetration of spermatozoa in synthetic media is highly dependent on the concentration and viscosity of media, as it has been documented by other authors [12].

In this study, the mean sperm count was always found to be higher in ovine cervical mucus than in any of the tested acrylamide concentrations. This greater difficulty of sperm to progress through the acrylamide has been observed also by Eggert-Kruse et al. [19]. According to these authors, human sperm ability to penetrate the acrylamide medium (concentrations 1.5%–1.8%) correlated significantly with the penetration of human cervical mucus, although polyacrylamide proved to be a stronger barrier so that sperm velocity and duration of progressive motility were markedly reduced in acrylamide.

Cervical mucus has a number of physicochemical properties at the time of ovulation, influenced by sex hormones, that facilitate easy sperm penetration through the uterine cervix [28]. Also, human cervical mucus creates channels in which the spermatozoa become oriented and distributed in a parallel direction to their long axis. Such alignment does not occur into hyaluronate polymer, in which the direction of sperm movement is essentially random [11]. These characteristics of cervical ovulatory mucus, which are not present in the acrylamide medium, might explain the differences observed by us between acrylamide and natural sheep mucus in the ability of progression of ram sperm. We bear in mind that freezing cervical mucus alters its functional properties and such mucus cannot be considered representative of what sperm encounter in in vivo insemination, but given the technical difficulties in handling fresh cervical mucus, we follow the method of Memon and Gustafsson [23] and we believe that the thawed mucus is acceptable control for acrylamide.

To assess the ability of the medium prepared with acrylamide to support sperm motility, we applied the progression test in three models of ovine semen conservation. We must highlight that the cooling-induced damage (cold shock) could explain the behavior of sperm in the test of progression. At present, the most useful method for ovine artificial insemination (vaginal via) is the application of semen cooled at 15 °C, which maintains the fertilizing capacity of sperm stored for 6 to 12 h [29,30], although motility is kept acceptably for up 24 to 48 h after ejaculation [31]. At 4 °C, sperm preservation can be prolonged, but storage time is significantly associated with the deterioration of motility parameters of ram sperm (total progressive motility, VAP, VSL, VCL, ALH, and straightness) [32]. Also, the lambing rates for ram spermatozoa after storage at 5 °C significantly decreased with 0-, 1-, 2-, or 3-day-old semen (60.0%, 34.3%, 33.8%, and 17.1%) [33]. Our results show that the population of sperm that moves beyond the 20 or 30 mm in the progression test is significantly affected by storage temperature. Some in vitro studies report that the spermatozoa stored at 15 °C or 5 °C for 2 days show a high motility (progressive motility and kinetic parameters), so these parameters cannot explain the significant loss of sperm fertilizing capacity in this period. In our study, in which motility is evaluated as a complex variable by this stress test, the results show that the sperm damage is already present at 24 h. These results show the advantages of a functional test, such as the penetration test, which can integrate many cellular characteristics in a single assay.

In the present study, the number of ram spermatozoa that penetrate more than 20 or 30 mm into an acrylamide gel or ovine cervical mucus was significantly and

positively correlated with progressive motility and one kinematics parameter (VSL); while this relationship is negative with acrosomal damage and injuries in the plasma membrane. Other specific correlations of sperm motility parameters, either for the cervical mucus (straightness, BCF) or acrylamide (straightness, LIN, WOB), were also observed. These results are not consistent with those described by Robayo et al. [9], who claim that continuous line velocity (VCL) and average path velocity (VAP) are the only sperm kinematic parameters that presented significant positive correlations with the ability to migrate in sheep cervical mucus. Procedural factors may explain these differences: Robayo et al. [9] analyzed the vanguard sperm distance and we were evaluating the sperm migration efficiency by the number of sperm reaching a specific segment. In this sense, we have to take into account the low or no association observed by Love et al. [34] between all velocity measures and total sperm motility, these authors suggests that the speed with which a sperm moves and how it moves is independent of the total percentage of moving sperm.

The significance of average-path velocity VAP in determining the success of cervical mucus penetration has been supported in several studies [9,11,35] although it has not been associated with fertility. However, differences in sperm migration through cervical mucus in vitro are related to the ability of spermatozoa to colonize the oviduct and to fertilize matured oocytes in vitro [36]. The importance of straight-line velocity VSL for the fertilizing capacity of the spermatozoa has been noted by different authors [8,37] and it has been speculated that a high VSL might be important in sperm transport through the female reproductive tract and penetration of the oocyte vestments [8]. The relationship between the number of cells that had penetrated in the cervical mucus test and fertility has been evaluated in cow [13]. When these authors compared bulls from the low fertility group with those from the high fertility group, the latter showed a higher number of spermatozoa at two defined penetration distances and a significant positive correlation was found between this number of spermatozoa and the nonreturn rates of the bulls.

Apart from findings derived from motility analyses, we found that the occurrence of apoptotic spermatozoa and spermatozoa with damaged acrosome were negatively correlated with the migration capacity of spermatozoa into mucus, while the mitochondrial membrane potential evaluated using a specific fluorophore showed no correlation with this migration. Anilkumar et al. [5] demonstrated that acrosome integrity was significantly and positively correlated with bull sperm penetration in mucus and acrylamide gel. Column filtration techniques have been suggested as useful ways of evaluating acrosome integrity, because they trap membrane-damaged or acrosome-reacted cells but allow motile, membrane-intact sperm to pass through [38]. The acrosome-reacted sperm are especially sticky, and reacted sperm can be seen to stick to glass, even in the presence of albumin [39]. This sticking could explain the negative correlation between acrosomal damage and the reduced ability of sperm to progress in the mucus and acrylamide gel observed in our study.

Garner et al. [40] noted that fluorometric measurement of mitochondrial function was highly correlated with the microscopic estimates of progressive forward motility. However, the importance of mitochondria for sperm motility has recently been reconsidered and it is believed that the mitochondrial activity is also important for maintaining ATP levels in the sperm head and midpiece required for housekeeping processes, such as membrane functionality [41]. As apoptotic spermatozoa, Martínez-Pastor et al. [42] observed that deer spermatozoa with "apoptotic-like" features would not be able to maintain motility for a long time. These authors found that this process was preceded by the loss of mitochondrial membrane potential, but that the loss of mitochondrial activity was not directly related to motility loss. These findings may explain the lack of correlation we have observed between the number of emigrating spermatozoa and mitochondrial status, whereas the proportion of apoptotic cells was negatively correlated with the sperm progression in mucus.

In conclusion, the results of this study suggest that the number of ram spermatozoa capable of going beyond of 20 mm into acrylamide gel, observed by automatic analysis in an optimized sperm migration test, is a useful parameter in the in vitro evaluation of sperm quality and further studies are needed to evaluate its relationship with field fertility.

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