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Sperm subpopulations in ejaculated sperm and spermatozoa recovered from ovine epididymides up to 48 h after death

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ABSTRACT

The objectives of this study were threefold: to identify subpopulations of sperm based on the kinetics of frozen/thawed sheep epididymal spermatozoa or semen collected with an artificial vagina; to evaluate the effects on sperm subpopulations in the thawed samples of post mortem storage at room temperature and the addition of 20% of seminal plasma to the freezing extender and to correlate the percentage of subpopulations with gestation rate following artificial intrauterine insemination. The categorization of the subpopulations was based on sperm kinetic data from Computer Assisted Sperm Analysis (CASA). A hundred ewes were inseminated with thawed spermatozoa and gestation rate was correlated with the proportions of each subpopulation using Pearson correlation matrix and linear regression. Three distinct subpopulations were identified in the thawed samples of either ovine ejaculate collected in artificial vaginas (AV) or ovine spermatozoa retrieved from the cauda epididymis. Subpopulation 1 (SP1) was characterized by spermatozoa with slow and non-linear motion, subpopulation 2 (SP2) was classified as hyperactived spermatozoa and subpopulation 3 (SP3) was composed of spermatozoa with fast, linear motion. The largest subpopulation in all groups was SP1. The semen collected in an artificial vagina had a higher ($P < 0.05$) percentage of SP2 and lower ($P < 0.05$) percentage of SP1 when compared to spermatozoa recovered after death. Increasing time of storage after death had a detrimental effect on sperm samples, increasing ($P < 0.05$) the percentage of SP1 and decreasing ($P < 0.05$) SP2. Length of storage after death was the only variable that influenced, with an inversely proportional relationship, SP3. In samples stored for 48 h after death no SP3 spermatozoa were present. The addition of seminal plasma to the cryopreservative decreased ($P < 0.05$) the subpopulation of hyperactived spermatozoa (SP2). We conclude that, after thawing there are three sperm subpopulations in the spermatozoa obtained from the cauda epididymides and the semen collected in AVs and that the relative proportions of these subpopulations varies with the time of storage post mortem and the presence of 20% of seminal plasma in the extender. However, we conclude that these subpopulations do not correlate with fertility after intrauterine artificial insemination.

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1. Introduction

Recovery of spermatozoa after death may offer the last chance to preserve genetic material from breeding animals which have died unexpectedly (García-Álvarez et al., 2009) and is important in formation of germplasm banks for endangered species (Martinez-Pastor et al., 2005a,b). The collection of viable spermatozoa from the cauda epididymis has been reported in several species (Bertol et al., 2016; Kaabi et al., 2003; Kozioł and Koziorowski, 2015; Weiss et al., 2008); However, factors such as time between death and sperm recovery (Kaabi et al., 2003), storage temperature of the epididymides (Monteiro et al., 2013) and extenders used in cryopreservation (Tamayo-Canul et al., 2011) affect the post-thawing quality and fertility of spermatozoa recovered from post morten cauda epididymides.

Seminal plasma is the secretion from glands associated with the male reproductive tract and mixes with spermatozoa at ejaculation. Sperm recovered from epididymides will not have been in contact with seminal plasma. There are conflicting results regarding i*n vitro* exposure of epididymal spermatozoa to seminal plasma, while some authors report increased viability following cryopreservation (Martínez-Pastor et al., 2006; Rickard et al., 2014), other authors have identified a detrimental effect on sperm survival in dogs, sheep, cattle and buffalo spermatozoa (Dott et al., 1979; Herold et al., 2004).

Computer Assisted Sperm Analysis (CASA) is a computerized and automatic system for capturing and analyzing successive pictures of spermatozoa that, when joined together, form a video documenting the path of each cell (Bergstein et al., 2014). With this system, it is possible to obtain precise and accurate information on the movement of each sperm cell (Amann and Waberski, 2014). The multivariate analysis of the individualized kinetic parameters allows a sample to be analyzed in relation to sperm subpopulations with similar movement patterns. Few studies have used multivariate analysis to identify sperm subpopulations, and relevant information on the identification of fertilization potential is underutilized (Abaigar et al., 1999; Martínez-Pastor et al., 2011).

The objective of this study was to identify sperm subpopulations based on the kinetics of ovine spermatozoa recovered from the cauda epididymidis or ejaculated sperm and to evaluate the effect, on the proportions of sperm subpopulations in thawed samples, of the length of storage post mortem and the addition of 20% seminal plasma to the freezing extender, and to correlate the proportions of these subpopulations with the gestation rate after intrauterine artificial insemination in sheep.

2. Material and methods

2.1. Experiment design

This project was approved by the Ethics Committee on Animal Use (CEUA) of the agricultural sciences sector of the Federal University of Paraná – Brazil, protocol number 52/2016.

Ten cross breed rams, aged between 24 and 48 months, were used. The animals were kept in an intensive system, with access to pasture during the day and were confined overnight. Food supplementation with corn, soybean meal and a mixture of minerals, and water *ad libidum* was provided.

With the use of a sheep as a dummy, semen collection was performed using the artificial vagina (AV) method. The internal temperature of the artificial vagina was 40 °C to 43 °C. AV collections were performed twice a week for two months in the summer (January and February), after collection ejaculated sperm were cryopreserved and analyzed post thaw. The rams were subsequently slaughtered. A section was made in the region of the spermatic funiculum for excision of the scrotum, testis and epididymis. Scrotum, testis and epididymis were transported to the laboratory in a styrofoam box at room temperature (18–25 °C). In the laboratory scrotum, testis and epididymis were removed from the styrofoam box and allocated to empty Becker dishes maintained at room temperature and covered with a paper towel until the end of the period of exposure to room temperature.

Scrotum, testis and epididymis were randomly divided into five groups, corresponding to the period of exposure to room temperature (18–25 °C): zero hours (G0), six hours (G6), twelve hours (G12), twenty-four hours (G24) and forty-eight hours (G48). Each group consisted of four epididymides from four different rams.

2.2. Post mortem spermatozoa recovery

After the period of exposure to room temperature a scalpel blade was used to incise the scrotal skin and extract the testes and epididymides (Abaigar et al., 1999). Testes and epididymides were washed with 0.09% sodium chloride solution warmed to 35 °C. The tail of the epididymis was divided into warmed petri dishes and maintained at 35 °C. Using anatomical tweezers, scissors and a scalpel blade, superficial blood vessels were dissected to minimize blood contamination of semen. The cauda epididymis was sectioned and light pressure was applied to expel semen from the tubules (García-Alvarez et al., 2009). After the first cut all cauda epididymis were sectioned to maximize spermatozoa recovery. Each cauda epididymis was then lavaged with 2 mL of control media (CM) warmed to 35 °C. After five minutes a pipette was used to collect the diluted semen from the Petri dish and the sample was placed in a conical tube (Falcon BD)(Martinez-Pastor et al., 2006). After recovery spermatozoa were cryopreserved and analyzed post thaw.

2.3. Cryopreservation

The control medium (CM) used in the cryopreservation process consisted of 75 mL mother solution (200 mL distilled water, 1.4 g glycine, 2.97 g sodium citrate, 3 g fructose, 0.004 g amikacin), 15 mL skimmed milk, 5 g egg yolk and 4.6 mL distilled water and 4%

glycerol (Vásquez et al., 2013). The SP medium was formed with the addition of 20% seminal plasma to the CM. The seminal plasma was from the same rams from which semen had been collected. The seminal plasma was obtained, and extender with seminal plasma was produced following the methodology of López-Pérez and Pérez-Clariget (López-Pérez and Pérez-Clariget, 2012).

Dose dilution with CM and SP extenders was performed to obtain a concentration of 400 million spermatozoa per mL. Inseminating doses were packed in 0.25 mL French straws with 100 million spermatozoa per dose. The doses were frozen using automated equipment (TK 3000®, TK Congelações, Brazil) with a standard ovine freezing curve "S3P2" that decreased from ambient temperature to 5 °C in steps of 0.5 °C per minute, straws were stabilized at 5 °C for 2 h, after that the temperature decreased 15 °C per minute until −80 °C and in steps of 10 °C per minute until −120 °C then straws were submerged in liquid nitrogen. Samples were thawed in a water bath at 40∘C for 20s. Sperm quality parameters were evaluated immediately after thawing.

2.4. Kinematics analysis

Motility was assessed using a computer-assisted sperm analysis system (CASA) (Hamilton Thorn Motility Analyser – HTMA −IVOS 12–Hamilton Research – Beverly, MA, USA). For evaluation of the sperm kinetics, 30 μL thawed sperm was diluted in 300 μL CM and warmed to 37 °C. Then, 6 μL of diluted semen was deposited into the Makler chamber (Makler Counting Chamber–Selfi-Medical, Haifa, Israel). One frame of the chamber was chosen by the examiner and two other frames were randomly chosen by the equipment and analyzed for total motility (TM,%), progressive motility (PM,%), velocity according to the smoothed path (VAP, μm/s), velocity according to the straight path (VSL, m/s), velocity according to the actual path (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), head beat-cross frequency (BCF, Hz), straightness (STR, %) and linearity index (LIN, %). The setup used was based on the manufacturer's recommendations, (HAMILTON THORNE SETUP FOR IVOS-12.3). The cell size was 5 pixels, cell intensity 55, VAP 75 μ/s, STR 80%, VAP cutoff 21.9 μ/s, VSL cutoff 6.0 μ/s, minimum static intensity gates 0.25, maximum static intensity gates 1.5, minimum static size gates 0.6 and maximum static size gates 8.0, minimum elongation gates 0 and maximum elongation gates 95. Magnification calibration was of 1.95, video source frequency of 60 Hz, 2400 light intensity, low photometer 73 and high photometer 125.

A total of 5560 spermatozoa were analyzed. Individual sperm data values (VAP, VSL, VCL, ALH, BCF, STR and LIN) were saved in an Excel file (Microsoft, Redmon,WA) that was used in the statistical analysis.

2.5. Artificial insemination

Estrus was synchronized in 100 cross breed sheep (74 sheep aged between 9 and 15 months and 26 sheep aged between 15 and 36 months) using intravaginal implants impregnated with 60 mg of medroxyprogesterone acetate (Progespon®, Zoetis, United States) for a period of 14 days at the end of breeding season (July). Five ewes lost their implants and were excluded before artificial insemination. On the day of implant removal 500UI of eCG (Novormon®, Zoetis, United States) was administrated (Anakkul et al., 2014). The ewes were randomly assigned to the groups: AVCM:13 sheep, AVSP:13 sheep, G0CM: 7 sheep, G0SP: 10 sheep, G6CM: 7 sheep, G6SP: 7 sheep, G12CM: 8 sheep, G12SP: 10 sheep, G24CM: 10 sheep and G24SP: 10 sheep. Samples from G48 were not used for artificial insemination because they had very low TM and PM. The inseminating dose was 100 million spermatozoa.

Artificial insemination (AI) was performed between 50 and 56 h after the removal of the intravaginal progesterone implant. After solid and liquid fasting for twelve hours the ewes were sedated with 0.05 mL/Kg of acepromazine then the ewe was placed in the Trendelenburg position. The uterine horns were identified with a 5 mm and 30° angulated laparoscope (Karl Stroz, Tuttlingen, German). The spermatozoa were thawed and immediately deposited into the uterine lumen in the middle of the uterine horn using an Robertson pipet (Minitube Brasil, Porto Alegre, Brazil)(García-Álvarez et al., 2013). Gestation diagnosis was performed by transabdominal ultrasound at 60 days after AI with a 5mHz linear probe (DP2200 VET, Mindray Medical International Limited, Shenzhen, China).

2.6. Statistics

All statistical analyzes were performed in JMP software version 5.0.1 (SAS Institute Inc, Cary, North Carolina, United States of America).

Statistical analysis for identification of sperm subpopulations followed the methodology of Martinez-Pastor et al. (Martinez-Pastor et al., 2005a,b). First, the data of the individual spermatozoa kinetic evaluations were evaluated in a main components procedure in order to reduce the initial variables to the smallest number of principal components, whilst retaining as much information as possible. This analysis allows many variables to be summarized in a smaller number of uncorrelated components. The principal components selected to follow the analysis were those with a variance greater than one (Kaiser criterion). Next, a non-hierarchical (k-means) analysis was performed with 15 clusters, using the principal components identified in the previous analysis as variables. The third step of the cluster analysis was the hierarchical test using the method of average connection to join the clusters obtained in the k-means analysis. To identify the final number of subpopulations, the distance data between clusters were plotted and the abrupt increase of distances was identified in the link for 3 subpopulations (Martinez-Pastor et al., 2005a,b; Martinez-Pastor et al., 2005a,b; Martínez-Pastor et al., 2011).

Chi-square test was used to identify differences in the percentage of each sperm subpopulation in the groups (AV, G0, G6, G12, G24, G48, CM and SP). Pearson correlation matrix and linear regression were used to identify the correlation between fertility and sperm subpopulations in each group. The level of significance was 5% in all statistical tests.

Table 1
Variance, data percentage (%), accumulated data percentage (%) and eigenvectors identified in the principal components analysis of VAP, VSL, VCL, ALH, BCF, STR and LIN.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Variance	3.7	1.9	0.9	0.3	0.2	0.1	0.0
Data percentage	52.9	27.2	12.8	3.9	2.2	0.7	0.3
Accumulated data	52.9	80.1	92.9	96.8	99.0	99.7	100.0
Eigenvectors							
VAP	0.4933	−0.1557	−0.0399	−0.3479	0.1938	0.1819	0.7343
VSL	0.4989	0.0452	0.1256	−0.3349	−0.0614	−0.7345	−0.2792
VCL	0.4631	−0.2946	0.0137	−0.1419	−0.2900	0.5805	−0.5073
ALH	0.3524	−0.4343	−0.0875	0.7818	0.1786	−0.1875	0.0362
BCF	−0.1323	−0.2748	0.9380	−0.0158	0.1575	0.0389	0.0230
STR	0.2725	0.5415	0.2941	0.3499	−0.5954	0.0618	0.2554
LIN	0.2783	0.5741	0.0918	0.1130	0.6807	0.2235	−0.2418

3. Results

Principal components analysis results are described in Table 1. Principal components 1 (PC1) and 2 (PC2) had a variance of 3.7 and 1.9 respectively, and were selected to proceed to the Non-Hierarchical Analysis. PC1 and PC2 correspond to 80.1% of the original information (Table 1). Based on the values of the eigenvectors it can be seen that PC1 is related to high VAP, VSL and VCL, while PC2 is related to STR and LIN.

The dendrogram of the hierarchical analysis is shown in Fig. 1A. The analysis of the distance between clusters, plotted in graph 1B, was used to identify the ideal number of sperm subpopulations, shown in Fig. 1A by dashed line. Three sperm subpopulations were identified in the semen samples collected in the artificial vagina and in the spermatozoa recovered from ovine epididymides kept at room temperature for 48 h after death. The delimitation of the three sperm subpopulations is demonstrated in the multi-dimensional space of PC1 and PC2 in Fig. 1C.

Kinetic parameters of the three sperm subpopulations identified in thawed semen collected in artificial vagina and sperm retrieved from cauda epididymis are shown in Table 2. Kinetic parameters differed ($P < 0.05$) between sperm subpopulations, except for BCF in SP2 and SP3.

Subpopulation 1 (SP1) represents slow spermatozoa (low VAP, VSL and VCL values) with nonlinear motion, but with high flagellar activity (BCF). It is likely that SP1 comprises spermatozoa with circular movement, because they course a short route (VSL). Subpopulation 2 (SP2) is formed by rapidly moving spermatozoa (higher values of VAP, VSL and VCL), high ALH values and average values of STR and LIN. Subpopulation 3 (SP3) is characterized by spermatozoa with high velocities, low ALH and high STR and LIN, being classified as fast spermatozoa with linear motion. The percentage of each subpopulation among the groups studied (AV, G0, G6,

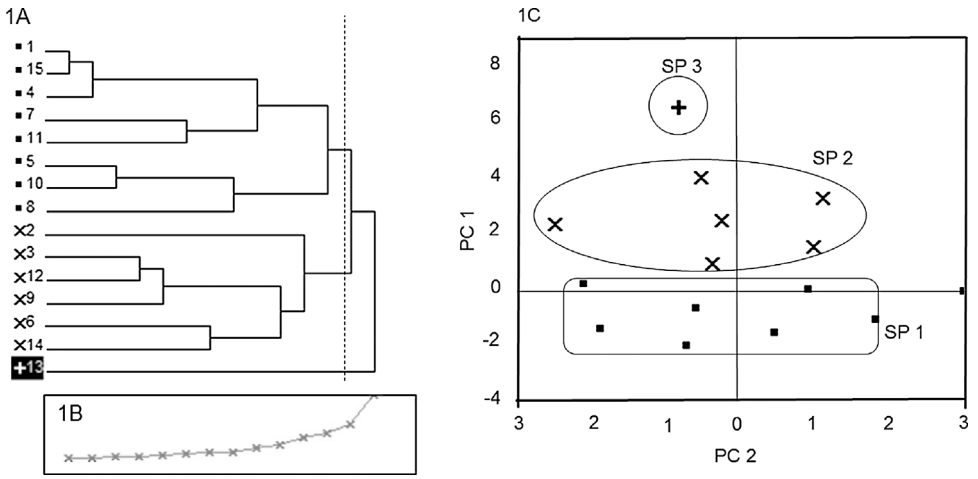

Fig. 1. Hierarchical analysis, graph of distance between clusters and delimitation of subpopulations.
1.A. Dendrogram of hierarchical analysis. The dashed line demarcates the cut position to obtain sperm subpopulations. 1.B. Graph of the distance between cluster. Dendrogram was cut in the region that refers to the exponential increase of the distance between the clusters (dashed line). 1.C. The circle, ellipse and rectangle demarcate the three sperm subpopulations identified after hierarchical analysis in the multidimensional space defined by the two main components extracted from the principal component analysis (PC1 and PC2).
■ SP1: subpopulation 1.
X SP2: subpopulation 2.
+ SP3: subpopulation 3.

Table 2
Mean ± standard deviation of VAP(μm/s), VSL(m/s), VCL(μm/s), ALH(μm), BCF(Hz), STR (%) and LIN (%) of sperm subpopulation 1(SP1), 2(SP2) and 3 (SP3) identified on samples post thaw collected from ten rams in an artificial vagina (AV) and from the twenty cauda epididymides.

	SP1	SP2	SP3
VAP	28.5 ± 16.7 ^{c}	133.3 ± 39.0^{a}	93.5 ± 28.5^{b}
VSL	16.3 ± 11.9 ^{c}	94.1 ± 45.6^{a}	81.5 ± 28.2^{b}
VCL	70.2 ± 40.1 ^{c}	266.0 ± 56.3^{a}	175.8 ± 47.4^{b}
ALH	3.9 ± 2.5 ^{c}	11.1 ± 3.1^{a}	6.8 ± 2.0^{b}
BCF	40.0 ± 17.6 ^{a}	34.5 ± 14.6^{b}	34.1 ± 14.7^{b}
STR	59.2 ± 24.6 ^{c}	69.0 ± 23.1^{b}	86.7 ± 9.6^{a}
LIN	27.3 ± 18.9 ^{c}	34.5 ± 13.6^{b}	47.2 ± 12.1^{a}

Different letters in the same column indicate significant difference ($P < 0.05$) between results.

G12, G24, G48, CM and SP) is described in Table 3.

Comparing semen collected in an artificial vagina and spermatozoa recovered from cauda epididymides immediately after death (G0), it can be seen that SP2 is influenced by spermatozoa origin. The percentage of SP2 in AV groups were higher ($P < 0.05$) than at G0 (CM: 32.3 versus 20.8 and SP: 18.7 versus 12.3). The percentage of SP1 in AVCM was lower ($P < 0.05$) than the percentage of SP1 at G0 (40.8e versus 51.6d).

Increasing post mortem storage time was evaluated by the percentage of subpopulations, and correlated with increasing SP1 to the detriment of subpopulations 2 and 3. SP1 increased ($P < 0.05$) 6 h after death in the CM group and 12 h after death in SP group. SP2 in cryopreserved samples in CM decreased 6 h after death and, in frozen samples SP decreased 12 h post mortem. SP3 decreased after 12 h post mortem in both extenders.

The percentage of sperm subpopulations 1 and 2 was influenced by extenders. SP1 was less frequent in CM at AV and G0 groups when compared to the seminal plasma extender. SP2 followed the inverse pattern of SP1 distribution, with SP2 percentage being higher ($P < 0.05$) in the AVCM and G0CM groups when compared to the cryopreserved samples in the SP extender. SP3 was not influenced by the diluent medium.

Fertility after AI did not differ ($P > 0.05$) between the origin of the spermatozoa (AV or post-mortem), hours of storage after death nor the diluent media used. The gestation rate after AI with semen collected in artificial vaginas and cryopreserved in CM and SP was 15.4%. The gestation rate of the spermatozoa recovered from the epididymis at G0 (CM: 28.6%, SP: 0.0%), G6 (CM and SP: 28.6%), G12 (CM: 12.5% 0%) and G24 (CM: 0%, SP: 30%) did not differ ($P > 0.05$) from one another. There was no significant correlation, in Pearson matrix, between fertility and the percentage of SP1 ($R = 0.091$, $P = 0.80$), SP 2 ($R = -0.162$, $P = 0.65$), SP 3 ($R = 0.03$, $P = 0.93$) and SP2 + SP3 ($R = -0.276$, $P = 0.50$). Nor correlation were identified using linear regression between fertility and SP1 ($R^2 = 0.0082$, $P = 0.80$), SP 2 ($R^2 = -0.0951$, $P = 0.65$), SP 3($R^2 = 0.0009$, $P = 0.93$) and SP2 + SP3 ($R^2 = -0.1156$, $P = 0.80$).

4. Discussion

Ovine ejaculate is composed of a heterogeneous population of sperm cells (Bergstein et al., 2016; García-Álvarez et al., 2010). The

Table 3
Percentage of subpopulation 1 (SP1), 2 (SP2) and 3 (SP3) of post thaw samples cryopreserved with control media (CM) and CM plus 20% of seminal plasma (SP) collected from ten rams in artificial vaginas (AV) and from the cauda epididymis of four epididymis in each group at 0(G0), 6(G6), 12(G12), 24(24) and 48(G48) hours post mortem.

Group	Control media		
	SP1	SP2	SP3
AVCM	40.8 ^{e}	32.3 ^{a}	26.9 ^{ab}
G0CM	51.6 ^{d}	20.8 ^{b}	27.5 ^{a}
G6CM	65.6 ^{c}	10.9 ^{cd}	23.5 ^{ab}
G12CM	75.0 ^{b}	10.4 ^{cd}	14.5 ^{de}
G24CM	74.4 ^{b}	9.8 ^{cd}	15.8 ^{e}
G48CM	87.0 ^{ab}	12.9 ^{c}	0.0 ^{f}
	Seminal plasma		
	SP1	SP2	SP3
AVSP	60.4 ^{c}	18.7 ^{b}	20.9 ^{b}
G0SP	63.2 ^{c}	12.3 ^{c}	24.5 ^{ab}
G6SP	62.0 ^{c}	13.2 ^{c}	24.8 ^{ab}
G12SP	72.1 ^{bc}	8.3 ^{cd}	19.6 ^{bcd}
G24SP	81.8 ^{ab}	6.1 ^{d}	12.1 ^{e}
G48SP	99.5 ^{a}	0.5 e	0.0 ^{f}

Different letters in the same column indicate significant difference ($P < 0.05$) between results.

use of motility mean values simplifies the analysis and considers the sample to be homogeneous (Contri et al., 2012; Martinez-Pastor et al., 2005a,b). For this reason, researchers have used multivariate analysis to identify spermatic subpopulations and the percentage variation of these subpopulations in several stages of sperm processing (Abaigar et al., 1999; Dorado et al., 2011; Gallego et al., 2015).

The present study identified the presence of three subpopulations in ovine semen, similar results were reported by Santolaria et al. (Santolaria et al., 2015). The SP1 identified in this study is characterized by slow spermatozoa with non-linear motion and is related to low quality samples (Martínez-Pastor et al., 2011). SP2 was formed by rapid spermatozoa with average values of STR and LIN, and represents cells that are in the process of hyperactivation because they have high VAP, BCF and ALH concomitantly with low LIN values (Martinez-Pastor et al., 2005a,b). According to Colás et al. (Colás et al., 2010), spermatozoa with LIN $\leq$45% and ALH $\geq$3.5 μm are classified as hyperactivated. SP3 is comprised of fast spermatozoa with linear motion that correspond to good quality seminal samples (Martínez-Pastor et al., 2011). To the author's knowledge this is the first report of identification of subpopulations in ovine sperm recovered from the cauda epididymis.

Spermatozoa recovered from epididymides have the same fertility and viability as ejaculated semen (Álvarez et al., 2012). However, in the present study, it was possible to identify that the semen collected in the artificial vagina had lower percentages of SP1 (slow and nonlinear), and higher SP2 values (fast and average STR and LIN) when compared to the G0 group. According to Martinez-Pastor et al. (Martinez-Pastor et al., 2005a,b) the subpopulation characterized by spermatozoa moving in a slow and nonlinear fashion would be formed mainly by spermatozoa that had not fully matured in the epididymis, explaining the differences between sperm origins (AV and post-mortem). Contri et al. (Contri et al., 2012) identified differences in subpopulational patterns when they compared feline semen collected by electroejaculation and from the epididymis after orchiectomy.

Spermatozoa recovered from cauda epididymides are affected by the time after death, especially when epididymides are kept at room temperature. Under these conditions, total motility and progressive motility decreases up to 24 h post-mortem in sheep (Bergstein-Galan et al., 2017; Kaabi et al., 2003). In the present study, we could identify that the pattern of movement of sperm cells recovered from the cauda epididymides maintained at room temperature is altered by 6 h after death, with increasing SP1 (slow and non-linear) and a corresponding decline in SP2 (fast LIN and STR) in cryopreserved samples with CM. In addition, the subpopulation characterized by spermatozoa with rapid and straight motion (SP3) decreased 12 h after death, coinciding with the onset of degeneration of epididymal tubes reported by Songsasen et al. (Songsasen et al., 1998). In this way, we verified that, although the average of total motility and progressive motility decreases at 24 h after death, there are changes to the sperm movement pattern that occur hours earlier.

SP3 (fast and linear motion) was affected only by the post-mortem storage time. In group G48 this subpopulation disappeared. The decrease in SP3 coincided with the considerable increase in SP1 (slow and non-linear motion). Martinez-Pastor et al. (Martinez-Pastor et al., 2005a,b) also identified an abrupt decrease in the subpopulation characterized by spermatozoa with medium velocities and highly linear motion between 48 and 72 h after death in sperm retrieved from cervid epididymides. The same authors have suggested that this is limit of time in which it would be possible to recover spermatozoa from the epididymis. Our findings corroborate this hypothesis. However, it is true that when spermatozoa are used in reproductive biotechniques (intracytoplasmic injection) their penetration is independent of motility.

Proteins present in seminal plasma exert modulatory functions in motility, viability and sperm function (Mehr et al., 2015; Rodrigues et al., 2013; Soleilhavoup et al., 2014). Authors report that seminal plasma has a decapitating action on sperm cells (Ledesma et al., 2016). Capacitation is a collective term for the changes that spermatozoa suffer when contacting the female reproductive tract, and these include the reorganization of membrane proteins, the cholesterol efflux of the lipid bilayer with consequent increase in membrane fluidity, modulation of intracellular ionic concentration, increased tyrosine kinase activity and the development of hyperactivated motility (Bergstein et al., 2014). The lower incidence of SP2 (hyperactivated spermatozoa) in cryopreserved samples in the presence of seminal plasma may be a result of the decapitating action of plasma proteins. Mortimer et al. (Mortimer et al., 1998) reported that the presence of seminal plasma in the capacitation extender reversibly inhibits the development of hyperactivated motility in human spermatozoa. The decapacitant action of seminal plasma is usually related to cryoinjury reversal and to an increase in the viability period of spermatic samples (López-Pérez and Pérez-Clariget, 2012). However, the higher percentage of SP1 in cryopreserved samples in seminal plasma leads us to believe that seminal plasma had a negative effect on sperm quality, although no effects on fertility were observed. According to Way et al. (Way et al., 2000), when the male ejaculates in the female, cervical mucus acts as a barrier to seminal plasma, so sperm do not naturally remain in contact with seminal plasma for long periods, and such prolonged exposure may promote negative effects on sperm cells.

The gestation rate, after laparoscopic insemination, was lower than that reported by other authors after insemination with ovine sperm recovered from epididymides (Alvarez et al., 2012). We believe that the time (late in the reproductive season) of artificial insemination and the large number of young sheep in the study may have contributed to these results. Sperm subpopulations containing spermatozoa with rapid and linear motion are commonly related to good quality samples (Martínez-Pastor et al., 2011). However, in the present study, we were not able to identify significant correlation between SP3 and fertility after intrauterine AI. Other authors (García-Álvarez et al., 2013) found a significant correlation between the hyperactivated spermatozoa subpopulation and *in vitro* fertility, however, in the present study, the correlation between SP2 and AI was also not significant. Santolaria et al. (Santolaria et al., 2015) also identified three subpopulations in the ovine ejaculate and found no correlation between the subpopulations and fertility after cervical AI in sheep. Some authors report that fertility is more related to sperm viability than to kinetic parameters (Santolaria et al., 2015). This study did not aim to evaluate other spermatozoa variables as morphology, viability and DNA integrity however these parameters may interfere directly in fertility(Gillan et al., 2005; Gosálvez et al., 2009).

In conclusion, we were able to characterize three sperm subpopulations with different sperm kinetic patterns in ovine semen and

in ovine sperm retrieved from the cauda epididymis. Semen collected in an artificial vagina has a higher percentage of cells in a state of hyperactivation when compared to sperm retrieved from the epididymis. Post mortem storage time has a negative effect on sperm subpopulations, increasing the percentage of subpopulations linked to poor quality samples and decreasing desirable subpopulations. The addition of seminal plasma to the cryopreservation extender decreased the subpopulation of hyperactivated spermatozoa and increased the percentage of the subpopulation characterized by spermatozoa with slow and nonlinear motion. It was not possible to correlate subpopulations with fertility after artificial intrauterine insemination.

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References

Álvarez, M., Tamayo-Canul, J., Martínez-Rodríguez, C., López-Urueña, E., Gomes-Alves, S., Anel, L., Martínez-Pastor, F., Paz, P., 2012. Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). Anim. Reprod. Sci. 132, 145–154. http://dx.doi.org/10.1016/j.anireprosci.2012.05.006.

Abaigar, T., Holt, W.V., Harrison, R.a., del Barrio, G., 1999. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol. Reprod. 60, 32–41. http://dx.doi.org/10.1095/biolreprod60.1.32.

Alvarez, M., Tamayo-Canul, J., Anel, E., Boixo, J.C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., de Paz, P., 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. Theriogenology 77, 1111–1118. http://dx.doi.org/10.1016/j.theriogenology.2011.10.013.

Amann, R.P., Waberski, D., 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81, 5–17. http://dx.doi.org/10.1016/j.theriogenology.2013.09.004.

Anakkul, N., Suwimonteerabutr, J., Tharasanit, T., Khunmanee, S., Diloksumpan, P., Berg, D.K., Techakumphu, M., 2014. Sperm distribution and fertilization after unilateral and bilateral laparoscopic artificial insemination with frozen-thawed goat semen. Theriogenology 82, 1137–1144. http://dx.doi.org/10.1016/j.theriogenology.2014.07.032.

Bergstein, T.G., Weiss, R.R., Bicudo, S.D., 2014. Técnicas de análise de sêmen. Rev. Bras. Reprodução Anim. 38, 189–194.

Bergstein, T.G., Bicudo, L.C., Rodello, L., Weiss, R.R., Bicudo, S.D., 2016. Kinematic and spermatic recovery after selection by centrifugation in colloid solutions of ovine cryopreserved semen. Arq. Bras. Med. Vet. e Zootec. 68, 1539–1547.

Bergstein-Galan, T.G., Weiss, R.R., Bertol, M.A.F., Abreu, A.C.M.R., Busato, E., Kozicki, L.E., Bicudo, S.D., 2017. Quality and fertility of frozen ovine spermatozoa from epididymides stored at room temperature (18–25 °C) for up to 48 h post mortem. Theriogenology 96, 69–75. http://dx.doi.org/10.1016/j.theriogenology.2017.04.001.

Bertol, M.A.F., Weiss, R.R., Kozicki, L.E., Abreu de, A.C.M.R., Pereira, J.F.S., Silva, J.J., 2016. In vitro and in vivo fertilization potential of cryopreserved spermatozoa from bull epididymides stored for up to 30 h at ambient temperature (18 (C–20 °C). Theriogenology 86, 1014–1021. http://dx.doi.org/10.1016/j.theriogenology.2016.03.030.

Colás, C., Cebrián-Pérez, J.A., Muiño-Blanco, T., 2010. Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. Int. J. Androl. 33. http://dx.doi.org/10.1111/j.1365-2605.2009.00991.x.

Contri, A., Zambelli, D., Faustini, M., Cunto, M., Gloria, A., Carluccio, A., 2012. Artificial neural networks for the definition of kinetic subpopulations in electro-ejaculated and epididymal spermatozoa in the domestic cat. Reproduction 144, 339–347. http://dx.doi.org/10.1530/REP-12-0125.

Dorado, J., Alcaráz, L., Duarte, N., Portero, J.M., Acha, D., Hidalgo, M., 2011. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient. Anim. Reprod. Sci. 125, 211–218. http://dx.doi.org/10.1016/j.anireprosci.2011.03.013.

Dott, H.M., Harrison, R.a., Foster, G.C., 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. http://dx.doi.org/10.1530/jrf.0.0550113.

Gallego, V., Vílchez, M.C., Peñaranda, D.S., Pérez, L., Herráez, M.P., Asturiano, J.F., Martínez-Pastor, F., 2015. Subpopulation pattern of eel spermatozoa is affected by post-activation time, hormonal treatment and the thermal regimen. Reprod. Fertil. Dev. 27, 529. http://dx.doi.org/10.1071/RD13198.

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., Esteso, M.C., Pérez-Guzmán, M.D., Soler, A.J., 2009. Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: electroejaculation and postmortem collection. Theriogenology 72, 160–168. http://dx.doi.org/10.1016/j.theriogenology.2009.02.002.

García-Álvarez, O., Maroto-Morales, A., Ramón, M., del Olmo, E., Montoro, V., Dominguez-Rebolledo, A.E., Bisbal, A., Jiménez-Rabadán, P., Pérez-Guzmán, M.D., Soler, A.J., 2010. Analysis of selected sperm by density gradient centrifugation might aid in the estimation of in vivo fertility of thawed ram spermatozoa. Theriogenology 74, 979–988. http://dx.doi.org/10.1016/j.theriogenology.2010.04.027.

García-Álvarez, O., Maroto-Morales, A., Ramón, M., Del Olmo, E., Jiménez-Rabadán, P., Fernández-Santos, M.R., Anel-López, L., Garde, J.J., Sole, A.J., 2013. Dynamics of sperm subpopulations based on motility and plasma membrane status in thawed ram spermatozoa incubated under conditions that support in vitro capacitation and fertilization. Reprod. Fertil. Dev. 26.

García-Alvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., Esteso, M.C., Pérez-Guzmán, M.D., Soler, A.J., 2009. Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: electroejaculation and postmortem collection. Theriogenology 72, 160–168. http://dx.doi.org/10.1016/j.theriogenology.2009.02.002.

Gillan, L., Evans, G., Maxwell, W.M.C., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63, 445–457. http://dx.doi.org/10.1016/j.theriogenology.2004.09.024.

Gosálvez, J., Cortés-Gutiérrez, E.I., Nuñez, R., Fermández, J.L., Caballero, P., López-Fernández, C., Holt, W.V., 2009. A dynamic assessment of sperm DNA fragmentation versus sperm viability in proven fertile human donors. Fertil. Steril. 92, 1915–1919.

Herold, F., Aurich, J., Gerber, D., 2004. Epididymal sperm from the African buffalo (Syncerus caffer) can be frozen successfully with AndroMed® and with Triladyl™ but the addition of bovine seminal plasma is detrimental. Theriogenology 61, 715–724. http://dx.doi.org/10.1016/S0093-691X(03)00256-5.

Kaabi, M., Paz, P., Alvarez, M., Anel, E., Boixo, J.C., Rouissi, H., Herraez, P., Anel, L., 2003. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. Theriogenology 60, 1249–1259. http://dx.doi.org/10.3923/ajava.2008.400.408.

Kozioł, K., Koziorowski, M., 2015. Morphological defects of epididymal spermatozoa in male roe deer (Capreolus capreolus) during the reproductive season. Pol. J. Vet. Sci. 18. http://dx.doi.org/10.1515/pjvs-2015-0073.

López-Pérez, A., Pérez-Clariget, R., 2012. Ram seminal plasma improves pregnancy rates in ewes cervically inseminated with ram semen stored at 5 °C for 24 hours. Theriogenology 77, 395–399. http://dx.doi.org/10.1016/j.theriogenology.2011.08.013.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Martínez-Pastor, F., Cesari, A., 2016. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. Anim. Reprod. Sci. 173, 35–41. http://dx.doi.org/10.1016/j.anireprosci.2016.08.007.

Martínez-Pastor, F., Anel, L., Guerra, C., Álvarez, M., Soler, A.J., Garde, J.J., Chamorro, C., de Paz, P., 2006. Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. Theriogenology 66, 1847–1856. http://dx.doi.org/10.1016/j.theriogenology.2006.04.036.

Martínez-Pastor, F., Tizado, E.J., Garde, J.J., Anel, L., de Paz, P., 2011. Statistical Series: opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75, 783–795. http://dx.doi.org/10.1016/j.theriogenology.2010.11.034.

Martinez-Pastor, F., Diaz-Corujo, A.R., Anel, E., Herraez, P., Anel, L., De Paz, P., 2005a. Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. Theriogenology 64, 958–974. http://dx.doi.org/10.1016/j.theriogenology.2005.01.003.

Martinez-Pastor, F., Garcia-Macia, V., Alvarez, M., Herraez, P., Anel, L., Paz, P., 2005b. Sperm subpopulations in iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol. Reprod. 72, 316–327. http://dx.doi.org/10.1095/biolreprod.104.032730.

Martinez-Pastor, F., Garcia-Macias, V., Alvarez, M., Chamorro, C., Herraez, P., Paz, P., De Anel, L., 2006. Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer. Theriogenology 65, 471–485. http://dx.doi.org/10.1016/j.theriogenology.2005.05.045.

Mehr, M.R.-A., Mousavi, M., Ghadamyari, M., 2015. Effect of seminal plasma proteins on membrane cholesterol efflux of ram epididymal spermatozoa. Small Rumin. Res. 129, 88–91. http://dx.doi.org/10.1016/j.smallrumres.2015.06.001.

Monteiro, G.A., Guasti, P.N., Rocha, A.S., Martin, I., Sancler-Silva, Y.F.R., Freitas Dell'Aqua, C.P., Dell'Aqua, J.A., Papa, F.O., 2013. Effect of storage time and temperature of equine epididymis on the viability, motion parameters, and freezability of epididymal sperm. J. Equine Vet. Sci. 33, 169–173. http://dx.doi.org/10.1016/j.jevs.2012.06.010.

Mortimer, S.T., Swan, M.A., Mortimer, D., 1998. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. Hum. Reprod. 13, 2139–2146.

Rickard, J.P., Pini, T., Soleilhavoup, C., Cognie, J., Bathgate, R., Lynch, G.W., Evans, G., Maxwell, W.M.C., Druart, X., De Graaf, S.P., 2014. Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. Reproduction 148, 469–478. http://dx.doi.org/10.1530/REP-14-0285.

Rodrigues, M.A.M., Souza, C.E.A., Martins, J.A.M., Rego, J.P.A., Oliveira, J.T.A., Domont, G., Nogueira, F.C.S., Moura, A.A., 2013. Seminal plasma proteins and their relationship with sperm motility in Santa Ines rams. Small Rumin. Res. 109, 94–100. http://dx.doi.org/10.1016/j.smallrumres.2012.07.032.

Santolaria, P., Vicente-Fiel, S., Palacín, I., Fantova, E., Blasco, M.E., Silvestre, M.A., Yániz, J.L., 2015. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Anim. Reprod. Sci. 163, 82–88. http://dx.doi.org/10.1016/j.anireprosci.2015.10.001.

Soleilhavoup, C., Tsikis, G., Labas, V., Harichaux, G., Kohnke, P.L., Dacheux, J.L., Guérin, Y., Gatti, J.L., de Graaf, S.P., Druart, X., 2014. Ram seminal plasma proteome and its impact on liquid preservation of spermatozoa. J. Proteomics 109, 245–260. http://dx.doi.org/10.1016/j.jprot.2014.07.007.

Songsasen, N., Tong, J., Leibo, S., 1998. Birth of live mice derived by in vitro fertilization with spermatozoa retrieved up to twenty-four hours after death. J. Exp. Zool. 280, 189–196.

Tamayo-Canul, J., Álvarez, M., Mata-Campuzano, M., Álvarez-Rodríguez, M., de Paz, P., Anel, L., Martínez-Pastor, F., 2011. Effect of storage method and extender osmolality in the quality of cryopreserved epididymal ram spermatozoa. Anim. Reprod. Sci. 129, 188–199. http://dx.doi.org/10.1016/j.anireprosci.2011.11.003.

Vásquez, J.H., Núñez, V.H., Florentini, E.a., Gonzales, J.M., Camargo, L.a., Valdivia, M.E., 2013. Effects of five cryoprotective agents on quality of sheep epididymal spermatozoa during pre-freezing. Livest. Sci . 152, 94–99. http://dx.doi.org/10.1016/j.livsci.2012.12.011.

Way, A.M.Y.L., Griel Jr., L.C., Killian, G.J., 2000. Effects of accessory sex gland fluid on viability, capacitation, and acrossome reaction of cauda epididymal bull spermatozoa. J. Androl. 21, 213–219.

Weiss, R.R., Muradas, P.R., Graneman, L.C., Meira, C., 2008. Freezing sperm from cauda epididymis of castrated stallions. Anim. Reprod. Sci. 107, 356. http://dx.doi.org/10.1016/j.anireprosci.2008.05.133.