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Seminal plasma proteins modify the distribution of sperm subpopulations in cryopreserved semen of rams with lesser fertility

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ABSTRACT

Any physiological mechanism involved in sperm selection and semen improvement has effects on heterogeneous sperm populations. This is mainly due to the fact that sperm populations within a single ejaculate have considerable heterogeneity for many

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variables, such as motility which is meaningful in terms of understanding how some sperm cells possess fertility advantages as compared with other cells. In the present research, initially there was a multivariate and clustering analysis used to assess sperm motility data from cryopreserved ram semen to identify subpopulations and compare the distribution of these clusters between rams with lesser and greater fertility. There were four classifications made of sperm subpopulations (clusters): CL1 fast/linear/progressive sperm; CL2 fast/non-linear sperm; CL3 very fast/linear sperm with vigorous beating and CL4 slow/non-linear sperm. Rams with greater fertility had a lesser proportion of sperm considered as "hyperactivated" (CL2) and a greater proportion of slow and non-linear sperm (CL4) than sperm of rams with lesser fertility. In addition, the effects were assessed for the capacity of seminal plasma (SP) and interacting SP proteins (iSPP) that were present during different seasons of the year to improve the distribution of sperm within subpopulations of semen from rams with lesser fertility. The iSPP and SP were obtained by artificial vagina (AV) and electroejaculation (EE) during breeding and non-breeding seasons and added to thawed semen. All the aggregates had a significant effect on the distribution of sperm subpopulations and effects differed among seasons of the year and depending on collection method used. Even though, future studies are needed to assess the contribution of each subpopulation on ram sperm fertility, it is important that a multivariate analysis be used to evaluate the effect of a treatment on sperm quality variables.

Keywords: CASA; Sperm motility subpopulations, Cluster analysis, Ram semen

1. Introduction

Ram sperm cryopreservation induces both a decrease in overall sperm motility and alterations in sperm motility patterns (Watson, 2000; Batellier et al., 2001; Medeiros et al., 2002). This is one of the causes underlying the poor fertilising potential

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of frozen semen when placed in the female reproductive tract by artificial insemination. Seminal plasma (SP) has been evaluated for improving the quality of frozen-thawed semen, however, its effects are inconsistent (Baas et al., 1983; Graham, 1994; Bernardini et al., 2011). Inconsistencies among studies regarding the effects of SP might be related to the variability of its composition due to males from which collections occur, and/or ejaculate variability within the same male (Muiño-Blanco et al., 2008), season of year when ejaculates were collected (Domínguez et al., 2008) and collection method used to procure the semen (Ledesma et al., 2014). In an attempt to address these inconsistencies of findings among studies, a methodology was developed to obtain the fraction of SP proteins that can bind to the sperm membrane, termed interacting SP proteins (iSPP; Bernardini et al., 2011). The addition of iSPP after thawing can improve the quality of frozen semen by reducing some undesirable effects of cryopreservation, such as premature capacitation (Ledesma et al., 2016).

The development of CASA systems (Computer-Assisted Sperm Analysis) have improved the ability to analyse sperm motility, allowing for assessment of individual sperm cells, thus, allowing for the identification and quantification of different sperm subpopulations with specific patterns of movement. In the last 20 years, multivariate statistics have been applied to identify sperm subpopulations, making it possible to determine how specific treatments affect these subpopulations or to evaluate male differences is sperm subpopulations. Different sperm subpopulations have been identified, therefore, in mammalian ejaculates on the basis of the motility characteristics of individual sperm cells (Quintero-Moreno et al., 2003; Martínez-Pastor et al., 2005; Martínez-Pastor et al., 2011).

The purpose of the present study, therefore, was to use the multivariate cluster analysis as a technique to identify sperm subpopulations in frozen ram semen based on

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its motility characteristics to: a) compare the relative sizes of the different sperm populations in rams with greater and lesser fertility and b) evaluate the ability of SP and iSPP collected by different methods and in different seasons of the year, to modify the relative sizes of these sperm populations from rams with lesser fertility.

2. Materials and methods

2.1. Frozen semen of greater and lesser fertility rams

All experiments were performed using frozen sperm from 10 Assaf rams divided in two groups of five rams each, according to their fertility (lesser, 35.3% ± 3.0; greater, 60.4% ± 1.5). Fertility was estimated after routine assessments of the semen doses in the field (obtained by artificial vagina and utilized with standard AI procedures), corrected for the effects of farm, date and inseminator. Frozen semen was supplied by OVIGEN (Centro de Selección y Mejora Genética de Ovino y Caprino de Castilla y León, Toro, Spain).

2.2. Seminal plasma and interacting seminal plasma proteins

Semen samples from another eight Assaf rams were collected either by artificial vagina (AV) or electroejaculation (EE) and were used in this study to obtain seminal plasma (SP) and interacting seminal plasma proteins (iSPP). Collections were conducted during the autumn (breeding season) and spring (non-breeding season). Ejaculates were pooled by collection method and then divided in two aliquots. One aliquot was used to obtain SP and the other was used to obtain the iSPP (Ledesma et al., 2016).

2.3. Experimental design to evaluate the effect of iSSP and SP of sperm of rams with lesser fertility

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Four independent replicates were conducted with frozen sperm from rams with lesser fertility, dividing the samples so as to apply five experimental treatments, adding SP or iSPP obtained by EE or AV collected during breeding or non-breeding season (plus a control). The experimental design was similar to that in a previous study of Ledesma et al. (2016). Briefly, three straws of semen were thawed, layered over Androcoll-O^{TM} and centrifuged to remove dead cells and SP. The use of colloids is very effective to remove SP and allows for obtaining a population of viable sperm (Martínez-Alborcia et al., 2013; Anel-Lopez et al., 2015). The resulting sperm pellet was re-suspended in PBS and an aliquot of sperm suspension (10×10^6 sperm) was added to tubes with five different media and incubated at 37 °C for 1 hour. After this period, samples were analysed by CASA. The incubation media were supplemented with 40 µl of SP or iSSP: 1) SP collected by AV (corresponding to 0.6 mg protein for spring SP or 1.4 mg for autumn SP) in PBS supplemented with 0.5% fructose (PBSF);2) SP collected by EE (corresponding to 0.48 mg protein for spring SP or 1.2 mgfor autumn SP) in PBSF;3) iSPP collected by AV (corresponding to 7 µg for spring collection or 23 µg for autumn collection) in PBSF;4) iSPP collected by EE (corresponding to 3.2 µg for spring collection or 29 µg for autumn collection) in PBSF; and5) Control: PBSF without supplementation.

The amount of iSPP was selected on the basis of the proportional amount of proteins provided by an equivalent volume of complete SP. The concentration of proteins in SP was 35.0 mg/ml for AV and 30.0 mg/ml for EE for the autumn sample and 15.0 mg/ml for AV and 12.0 mg/ml for EE in the spring sample. The concentration of proteins in iSPP in the autumn was 7.8 mg/ml for samples collected with an AV and 5.8 mg/ml for

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samples collected by EE, and in spring it was 0.9 mg/ml for samples collected with an AV and 0.8 mg/ml for samples collected with use of an EE (Ledesma et al., 2016).

2.4. Motility analyses by CASA

To enhance the understanding of the information provided by the CASA and to conduct a more exhaustive analysis of motility, CASA information of the pattern of movement for each sperm cell was obtained from a previous study (Ledesma et al. 2016) and data were re-processed and information is reported that was not reported previously.

The pattern of each sperm cells movements was defined by using a CASA system (ISAS 1.0.4; Proiser SL, Valencia, Spain). A 5 µl drop of semen was assessed in a Makler counting chamber (10 µm depth; Haifa Instruments, Israel) with a phase contrast microscope (Nikon E400 with warmed stage at 37 °C; 10× negative contrast optics). At least four fields and 200 cells were recorded (Basler A312f, Basler AG, Ahrensburg, Germany), in 53 frames/s. Kinematic variables recorded for each sperm cell were: average-path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), linearity (LIN; %), straightness (STR; %), wobble (WOB; %), amplitude of the lateral movement of the head (ALH; µm) and beat-cross frequency (BCF; Hz).

2.5. Statistical analyses

Samples from rams with lesser or greater fertility (without treatment or control samples) were initially analysed to detect the differences in the distribution of sperm subpopulations. The effect of treatment only on sperm from rams with lesser fertility

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(SP or iSPP collected by AV or EE) were subsequently analyzed separately by breeding or non-breeding season.

Data for the CASA information for the pattern of movement of each sperm cell previously reported in Ledesma et al. (2016) was re-assessed to define the sperm subpopulations. Data were processed in the R statistical environment (R Development Core Team, 2011). Subpopulation analysis was performed applying the AGNES clustering algorithm in a two-step procedure (Fernández-Gago et al., 2016).

Data assessing differences between samples from rams with lesser or greater fertility (control samples) were analysed using a general linear model (GLM) without random effects. Information-Theoretic procedures were performed to determine the best fit model, computing AICc (Akaike Information Criterion for small samples; Johnson and Omland, 2004; Symonds and Moussalli, 2011).

To assess the effect of iSPP/SP, AV/EE and breeding/non-breeding season a general linear mixed effects model (GLMM) was performed. This analysis is particularly useful when data are not independent (Pinheiro and Bates, 2000). The use of the GLMM allows for inclusion of data for a sperm sample as a random effect and, thus, accounting for within-sample correlations in all models (Littell et al., 2000).

3. Results

3.1. Analysis of sperm subpopulations

Four sperm subpopulations were defined after multivariate cluster analysis of the 55,225 individual motile sperm cells analysed in this experiment. Data for motility characteristics of these subpopulations are shown in Table 1, and the pattern of movement can be described as follows: subpopulation 1 (CL1) represented those sperm with great velocity (high VCL, VSL and VAP) and the greatest progressive motility, inferred by the greater LIN values. Moreover, the ALH value was the least of all the

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subpopulations, indicating movement with few undulatory characteristics. This population included 68.1% of the total motile sperm.

Subpopulation 2 (CL2) contained active but non-progressive sperm, as indicated by the greater values of VCL and ALH, together with the lesser values of LIN and STR, and moderate BCF. This population might be considered as having a “hyperactivated-like” movement and about 10.9% of the total motile sperm were assigned to this subpopulation.

Subpopulation 3 (CL3) included about 14.3% of the total sperm and was represented by highly motile sperm with the greatest velocity and vigorous beating as indicated by VAP, VCL, VSL and BCF values. This population had great progressive motility, however, less than subpopulation 1 as indicated by LIN and ALH values.

Subpopulation 4 (CL4) contained about 6.7% of the total motile sperm population, and these cells had little motility and motility was non-progressive, as indicated by the least values for VCL, VSL, VAP, ALH and BCF, together with the least LIN, STR and WOB.

3.2 Distribution of sperm within subpopulations between rams with lesser or greater fertility

There were significant differences in the distribution of the subpopulation composed by fast and non-linear sperm movements (CL2) and this subpopulation was composed of slow and non-linear moving sperm (CL4) when rams with lesser or greater fertility were compared (Fig. 1). The CL2 categorization was greater in rams with lesser fertility and the cells with the CL4 categorization was less for semen from these rams (Fig. 1, Supp Table I). There were no significant differences between ram fertility groups for subpopulations composed of fast and linear sperm (CL1) and very fast and linear sperm (CL3).

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2.3. Effect of addition of seminal plasma or interacting seminal plasma proteins

The effect of the different aggregates (SP and iSPP) were analyzed only for samples from lesser fertility rams because the objective of the present research was to evaluate whether the the SP and iSPP additions could improve the semen quality and, consequently, fertilizing capacity. An effect of the interaction between season and treatment (SP or iSPP obtained by AV or EE) was detected for the distribution of the four subpopulations and for this reason treatments with SP and SP fractions during breeding and non-breading seasons were analysed separately.

Examination of the experimental treatment effects on the frequencies of individual sperm in subpopulations CL1, CL2, CL3 or CL4 (Fig. 2) revealed that the proportion of the population with greater velocity and progressiveness (CL1) was enhanced by the addition of SP collected by AV during the breeding and non-breeding season compared with the control (Fig. 2, Supp. Tables II and III).Inconsistent with this finding, addition of iSPP collected with used of EE during both seasons resulted in a decrease in this subpopulation compared with untreated samples. Addition of SP collected with use of EE during the non-breeding season resulted in no differences when treated groups were compared with the control, however, resulted in a decrease in the proportion of this cluster when collected in the breeding season (Fig. 2, Supp. Tables II and III).

Regarding to “hyperactivated-like” sperm (CL2), the least percentage of this subpopulation resulted with the control sperm compared with sperm where there were any additions performed on samples collected during the breeding season (Fig. 2A, Supp. Tables II and III). When samples were collected during the non-breeding season, however, there were no differences among the control and treatment groups (Fig. 2B, Supp. Table II).

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The analysis of the proportion of sperm with greater motility with the greatest vigorous beating (CL3) indicated this fraction was greater in treatments with SP or iSPP added that was obtained by use of EE during both the breeding and non-breeding season compared with the control (Fig. 2, Supp. Tables II and III). There were no differences between the control and iSPP treated group when samples were collected by AV and this subpopulation was absent when treatments included SP collected by use of an AV during both seasons of the year (Fig. 2).

The proportion of sperm with little motility and that had non-progressive motility (CL4) was not different between the control and treatment groups where SP was added that was collected by AV or EE during the breeding season (Fig.2A, Supp. Table II). Samples treated with iSPP that were collected by EE during the non-breeding season and controls had the least proportion of motile sperm in this cluster (Fig. 2B, Supp. Tables II and III).

4. Discussion

The use of multivariate cluster analysis of data from sperm motility patterns in the present study resulted in detection of the presence of four different subpopulations in frozen thawed ram semen, which were differentially distributed when associations with ram fertility were evaluated. It was also observed in the present study that the distribution of sperm subpopulations in frozen semen from rams of lesser fertility were affected by the addition of SP and iSPP and this effect differed depending on season year when collections occurred and semen collection method.

Whereas there has been significant progress made in recent years in the analysis of sperm motility of semen subpopulations that has been facilitated by use

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of the CASA technology in bulls, stallions, gazelle, boars and red deer stags (Abaigar et al., 1999; Abaigar et al., 2001; Quintero-Moreno et al., 2003; Quintero-Moreno et al., 2004; Martínez-Pastor et al., 2005; Muiño et al., 2008; Domínguez-Rebolledo et al., 2009; Mata-Campuzano et al., 2012), fewer studies have focused on cryopreserved ram sperm (Bravo et al., 2011; Mendoza et al., 2012; Luna et al., 2017). The most striking feature of using standard approaches for CASA data analysis is the disappointing results coming from analysis of trough mean values (± standard deviations) of data sets. Examination of typical CASA data shows, that the "normal" statistical distribution is rare, masking treatment effects because the standard deviations are too great to permit the detection when using variance-based statistical tests. This problem can be explained, in part, by the existence of sperm subpopulations, meaning that even if some sperm subpopulations are affected by a treatment, there are other non-responding subpopulations co-existing in the same sample (Holt et al., 1985; Harrison et al., 1996; Martinez-Pastor et al., 2011). The proof-of-concept involves understanding the differences between subpopulations and the biological importance, particularly in cryopreserved samples. These different subpopulations could be cells that respond differently to seminal plasma molecules and to the cryopreservation process and the subpopulations could also be related to differences in fertility.

To understand the relative contribution of each sperm subpopulation to fertility in the present study, samples of frozen semen from rams with different fertility were compared. Cryopreserved sperm from lesser fertility rams had a greater proportion of cells considered to be hyperactivated (CL2) after thawing compared with samples from rams with greater fertility. While it is true that mass sperm motility of ram semen samples is positively correlated with lambing rate (David et al., 2015) it is not known if

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this relationship exists between post thawing motility features and ram fertility when fresh semen is used for insemination. In the present study, assessments occurred as to whether semen from lesser fertility rams contained greater subpopulations of sperm that after thawing are hyperactivated by means of cryocapacitation effects resulting in a lesser half-life of cells. Conversely, cryopreserved sperm from greater-fertility rams had a greater proportion of slow and non-linear moving sperm (CL4) and did have different proportions of any of both high speed clusters after a 1 hour incubation period. This reveals that frozen-thawed semen from greater fertility rams not only have less “cryocapacitated” sperm but also after an incubation period, have a fraction of viable cells that have a decreased swimming speed (changing from C1 to C4 cluster), but maybe still maintain the capacity for fertilization. The key concept behind this observation, according to Martínez-Pastor et al. (2005) is the hypothesis that a highly motile sperm cell would not rapidly lose its motility (unless undergoing extensive damage due to osmotic shock), but rather there would be a progressive decrease in motility. Sperm classified as CL1 and CL3, therefore, would be highly motile and could gradually lose motility and eventually be classified as CL4 sperm and would subsequently become slow enough to be considered immotile. Although the motility post-thawing results of the present study does not allow for conclusive determination if the cells have the capacity for fertilization or not, this information could be a good indicator that the semen sample from males with greater fertility have greater quality characteristics than those from males with lesser fertility.

Considering the decapacitating effect on cryopreserved semen of SP and SP proteins (Barrios et al., 2000; Ledesma et al., 2016) and with the knowledge that protein composition varies with season of year and collection method (Pérez-Pé et al., 2001; Domínguez et al., 2008; Ledesma et al., 2016), the effect of SP and iSPP

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addition to sperm of lesser fertility rams was evaluated. There were resulting differences as a result of season when collections occurred and collection method.

After 1 hour of incubation of sperm from lesser fertility rams with SP collected by use of the AV during the breeding and non-breeding season there was an increase in the population of sperm with the CL1 classification. Similarly, the addition of SP, as well as iSPP obtained by use of EE during both seasons resulted in an increase in the proportion of sperm with the CL3 classification. Sperm with the CL1 together with CL3 classifications could have the greatest fertilising capacity because progressive motility is correlated with the fertilising potential (Donnelly et al, 1998), which is also supported by the distribution of these two subpopulations in the semen of rams with greater fertility in the present study.

Addition of SP and iSPP obtained by EE increased the distribution of sperm in the CL3 classification. After the addition of SP and iSPP obtained with use of an AV, the change if distribution of sperm with the CL3 classification did not occur. This inconsistency of results could be due to the differences in protein concentration and composition of SP obtained with use of EE. In a previous research, sperm collected with use of EE had a greater proportion of those proteins that were cryoprotectives, such as RSVP14 and RSVP20. The use of EE causes a hyper-release of fluids from the seminal vesicles where these proteins originate (Ledesma et al., 2014). None of the treatments evaluated in the present study reduced the population of hyperactivated sperm (CL2) to the extent that the subpopulation distribution of these cells was that of semen from greater fertility rams.

It is interesting to note, that in previous studies (Mortimer and Maxwell 2004; de Graaf et al., 2007; Ledesma et al., 2016) only the average values of the CASA variables were considered instead of assessing sperm subpopulations. In these

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previous studies there was not a significant effect of iSPP collected by AV or EE on sperm motility. In the present study, it could be assessed whether there were effects among treatments on subpopulations of sperm, thus, subtle effects altering the motility pattern of sperm populations could be ascertained. This finding emphasizes the shortfalls of ignoring the variability and multi-modality of semen samples.

In conclusion, the present study provided evidence for the presence of four subpopulations or clusters of sperm cells in frozen ram semen with the predominant one composed of cells with rapid, linear movements and progressively motile sperm. Rams with greater fertility had a lesser proportion of sperm considered as "hyperactivated" and greater proportion of slow and non-linear moving sperm than rams with lesser fertility. Furthermore, the addition of SP and iSPP was found to have very different effects on the distribution of sperm subpopulations depending on the season of the year and semen collection method that was used. Further studies should be aimed at determining the contribution of each sperm subpopulation to fertility of ram semen. With the present research, the importance of the combined use of the CASA system and the multivariate cluster analysis is emphasized to study the effect of a treatment on the sperm motility. The combination of use of these two techniques allow for a greater understanding of the physiology of sperm and allows for improving the quality of cryopreserved semen, especially in males with lesser post-thawing fertility.

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Conflict of interest

The authors have not declared any conflicts of interest.

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Figure legends

Fig 1. Distribution of motile sperm within subpopulations between rams with lesser or greater fertility; Subpopulation 1, black bars; Subpopulation 2, dark grey bars; Subpopulation 3, light grey bars; Subpopulation 4, white bars; Different letters inside or by the side of bars (a–c) indicate differences ($p \leq 0.05$) within subpopulations between rams with greater and lesser fertility.

Fig 2. Distribution of sperm within subpopulations (CLs) among treatments; Subpopulation 1, black bars; Subpopulation 2, dark grey bars; Subpopulation 3, light grey bars; Subpopulation 4, white bars; Different letters (a–c) inside or by the side of bars indicate differences ($p \leq 0.05$) in subpopulations among treatments: SP AV (seminal plasma collected by artificial vagina); iSPP AV (interacting seminal plasma proteins collected by artificial vagina); SP EE (seminal plasma collected by electroejaculation); iSPP EE (interacting seminal plasma proteins collected by electroejaculation), control; During breeding (a) and non-breeding season (b)

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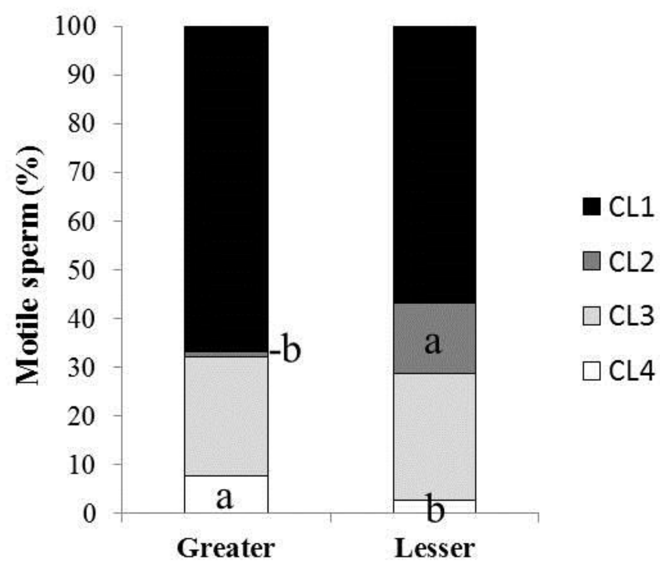

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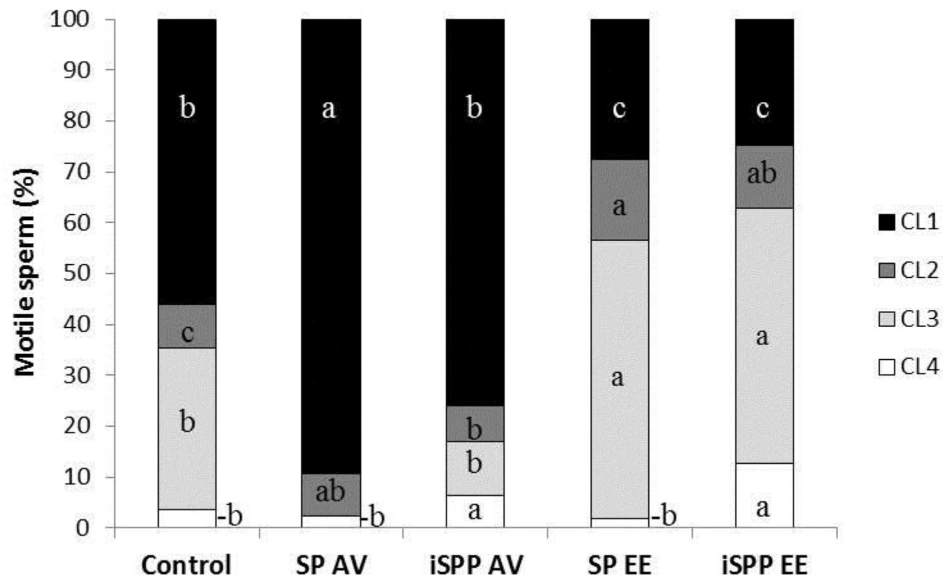

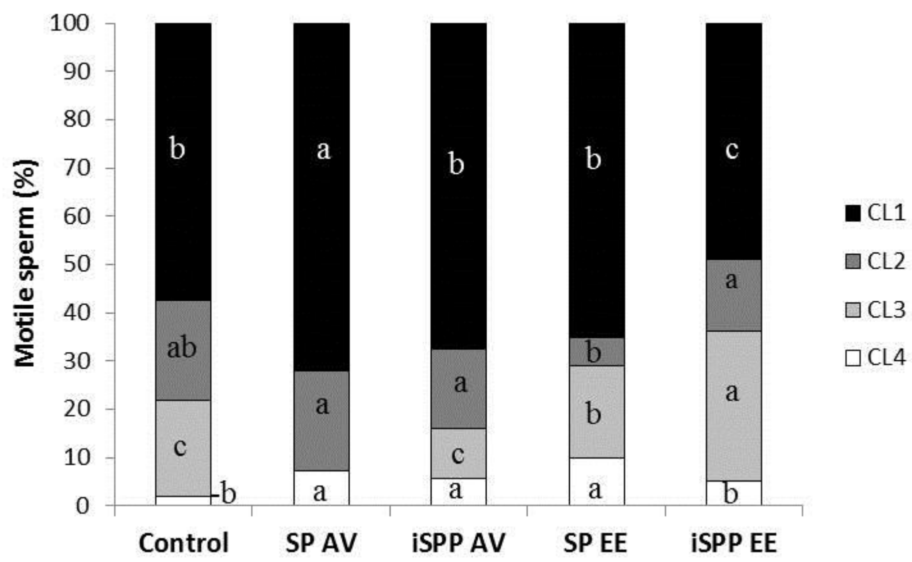

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Table 1

Kinematic variables for the four sperm subpopulations (CL) defined at 60 min post-thawing (mean ± SD)

Variable	CL1	CL2	CL3	CL4
VCL (µm/s)	124.9 ± 45.2	112.6 ± 60.1	189.8 ± 40.7	39.8 ± 21.0
VSL (µm/s)	100.1 ± 42.9	33.4 ± 30.6	129.0 ± 47.8	11.0 ± 7.0
VAP (µm/s)	110 ± 41.1	72.0 ± 54.4	138.8 ± 42.6	23.2 ± 11.5
LIN (%)	82.7 ± 10.2	36.4 ± 22.1	70.7 ± 15.4	30.3 ± 16.6
STR (%)	94.5 ± 4.8	56.9 ± 24.5	94.8 ± 4.4	53.6 ± 21.7
WOB (%)	88.7 ± 7.6	70.9 ± 18.1	75.9 ± 12.5	61.3 ± 17.0
ALH (µm)	1.6 ± 0.5	2.3 ± 0.9	2.8 ± 0.9	1.2 ± 0.4
BCF (Hz)	23.6 ± 6.9	15.0 ± 10.4	27.6 ± 7.2	6.0 ± 4.3

CL1: fast/progressive/linear; CL2: fast/non-linear; CL3: very fast/undulatory/linear; CL4: slow/non-linear; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: lateral head displacement; BCF: beat cross frequency