Title

Clustering of spermatozoa examined through flow cytometry provides more information than the conventional assessment: a resilience to osmotic stress example

Authors

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Abstract

Context: Conventional sperm quality tests may not be sufficient to predict the fertilizing ability of a given ejaculate; thus, rapid, reliable and sensitive tests are necessary to measure sperm function. **Aims:** This study sought to address whether a cluster analysis approach based on flow cytometry variables could provide more information about sperm function. **Methods:** Spermatozoa were exposed to either isotonic (300 mOsm/Kg) or hypotonic (180 mOsm/Kg) media for 5 and 20 min, and were then stained with SYBR14 and propidium iodide (PI). Based on flow cytometry dot-plots, spermatozoa were classified as either viable ($SYBR14^+/PI^-$) or with different degrees of plasma membrane alteration ($SYBR14^+/PI^+$ and $SYBR14^-/PI^+$). Moreover, individual values of EV, SS, green (FL1) and red (FL3) fluorescence were recorded and used to classify sperm cells through cluster analysis. Two strategies of this approach were run. The first one was based on EV and the FL3/FL1 quotient, and the second was based on EV, SS and the FL3/FL1 quotient. **Key results:** The two strategies led to the identification of more than three sperm populations. In the first strategy, EV did not differ between membrane-intact and membrane-damaged sperm, but it was significantly ($P<0.01$) higher in spermatozoa losing membrane integrity. In the second strategy, three out of five subpopulations (SP2, SP3 and SP4) showed some degree of alteration in their plasma membrane with significant ($P<0.01$) differences in EV. In both cluster analysis, SP5 (intact-membrane spermatozoa) presented the lowest EV. Besides, SP3 and SP4 (Strategy 1) and SP5 (Strategy 2) were found to be significantly ($P<0.05$) correlated with sperm functional competence (SFC). **Conclusions:** Cluster analysis based on flow cytometry variables provides more information about sperm function than conventional assessment does. **Implication:** Combining flow cytometry with cluster analysis is a more robust approach for sperm evaluation.

Keywords: cell volume; sperm subpopulations; membrane integrity; osmotic tolerance; flow cytometry

1. Introduction

The journey of spermatozoa from the testis to the site of fertilization involves multiple selection processes that end with a chosen elite subpopulation. Related to this, the concept of ejaculate heterogenicity has been raised (Holt et al., 2004), the elite subpopulation corresponding to those sperm cells that reach the oviduct, form the reservoir and may fertilize the oocyte (Hunter et al., 1980; Rodríguez-Martínez et al., 2005; Bucci et al., 2019). Previous research identified separate motile subpopulations through cluster analysis in several species (pig: Abaigar et al., 1999; Flores et al., 2009; Quintero-Moreno et al., 2004; Fernández-López et al., 2022; dog: Núñez-Martínez et al., 2006; Iberian red deer: Martínez -Pastor et al. , 2005; cattle: Muiño et al., 2008, 2009; horse: A. Quintero-Moreno et al., 2003; donkey: Miró et al., 2005, 2009; human: Davis et al., 1995). All these studies differed in their statistical approach, with disparate complexity in clustering methods (reviewed by Martínez-Pastor et al., 2011). The identification of motile sperm populations allowed determining that exposing cells to stressful conditions, such as anisotonic environment (Quintero-Moreno et al., 2004) or freeze-thawing procedures (Estrada et al., 2017; Flores et al., 2009), leads to changes in the proportions of spermatozoa belonging to each of these subpopulations. In spite of all the aforementioned, not many studies have run cluster analysis using sperm functionality parameters other than motility, nor has consensus yet been reached (Ibanescu et al., 2020).

A source of variation for sperm fertilizing ability and cryotolerance is the ejaculate fraction from which spermatozoa come (Peña et al., 2006). This could be related to the sperm proteome, which differs between fractions (Pérez-Patiño et al., 2019). Moreover, separate sperm subpopulations have been identified in the same ejaculate, with some cells belonging to subpopulations that are deemed to be non-functional (Martinez-Alborcia et al., 2012). On the other hand, spermatozoa are able to adapt to osmotic changes (Chaveiro

et al., 2006; A. M. Petrunkina, Gröpper, et al., 2005). In effect, the male gamete keeps a mechanism of volume regulation, which operates through the activation of membrane channels and allows for compensation of cellular changes resulting from exposure to anisotonic conditions (Barfield et al., 2006; Anna M. Petrunkina et al., 2004). Changes in the osmolality of the environment occur during ejaculation and the transport throughout the female reproductive tract (Cooper & Yeung, 2003; Hunter et al., 1980; Rodríguez-Martínez et al., 2005; C. H. Yeung et al., 2006). The existence of this mechanism could explain why ejaculates with greater proportions of the sperm subpopulation that is more resilient to anisotonic conditions show greater fertilizing ability and cryotolerance (Valencia et al., 2019).

Because conventional sperm quality tests may not be sufficient to predict the fertility and cryotolerance of a given ejaculate, rapid, reliable and sensitive tests are necessary to determine sperm function. Flow cytometry has been used as the leading technique to evaluate sperm functionality, and cell volume has been monitored to investigate the mechanisms underlying volume regulation (Oldenhof et al., 2011; Ching Hei Yeung et al., 2002). It is widely known that volumetric analysis is the most accurate method to measure size changes (A. M. Petrunkina, Gröpper, et al., 2005), and that sperm volume may be evaluated through the Coulter's principle (Ching Hei Yeung et al., 2002). Against this background, the aim of the present study was to determine: a) if the side scatter and electronic volume evaluated through the Coulter principle in sperm populations with distinct degrees of alteration in their plasma membrane differed following exposure to anisotonic conditions; and, b) if the number of sperm subpopulations identified through cluster analyses differed from that found in conventional flow cytometry assessment.

2. Materials and methods

2.1. Seminal samples

Twelve ejaculates coming from different healthy adult boars were involved in this study. All boars were sexually mature, belonged to the Pietrain breed, and were housed in climate-controlled buildings, fed with a standard and balanced diet, and provided with water *ad libitum*. Boars were collected through the gloved-hand method twice a week. After removing the gelatinous fraction, ejaculates were diluted to 30×10^6 sperm/mL in a commercial extender (Vitasem, Magapor, S.L.; Ejea de los Caballeros, Zaragoza, Spain) and transported to the laboratory at 17 °C within two hours after collection. Upon arrival, the sperm quality of each semen dose was evaluated in order to check that minimal quality standards (85% viable sperm, 85% morphologically normal sperm and 80% of total motile sperm) were fulfilled.

As seminal doses were purchased from a local farm (Semen Cardona, S.L.; Cardona; Spain) and the authors of this study did not manipulate any animal, no authorization from an institutional Ethics Committee was required.

2.2. Media

HEPES-buffered saline medium, consisting of 137 mmol/L NaCl, 10 mmol/L glucose, 2.5 mmol/L KOH and 20 mmol/L HEPES, and buffered to pH 7.4 (Harrison et al., 1993), was used as the basis to perform all incubations. This medium was used for isotonic incubations (300 mOsm/Kg). For hypotonic incubation at 180 mOsm/Kg, the concentration of NaCl was 76 mmol/L instead of 137 mmol/L (Anna M. Petrunkina et al., 2004). All media were filtered through a 0.22-µm filter before use.

123 *2.3. Experimental design*

124 Spermatozoa were diluted to a final concentration of 12×10^6 sperm/mL in 1 mL of
125 isotonic medium. After 10 min of incubation at 38 °C, aliquots of 50 µL were mixed with
126 550 µL of isotonic (300 mOsm/Kg) or hypotonic media (180 mOsm/Kg) to reach a final
127 concentration of 1.1×10^6 sperm/mL. Samples were incubated at 38 °C with SYBR14 (100
128 nM) for 10 min, and with PI (12 µM) for further 5 min, in the dark. Volumetric
129 measurements combined with the evaluation of plasma membrane integrity were carried
130 out after incubation at 38 °C for 5 min or 20 min in hypotonic and isotonic conditions,
131 separately.

132

133 *2.4. Flow cytometry analysis*

134 Electronic volume and side scatter of spermatozoa ($SYBR14^+/PI^-$, $SYBR14^+/PI^+$ and
135 $SYBR14^-/PI^+$) were measured using a Cell Laboratory QuantaSC cytometer (Beckman
136 Coulter, Fullerton, California, SA; Serial number AL300087) using the Coulter principle
137 for volume assessment (Ching Hei Yeung et al., 2002). The foundation of this principle
138 is based on measuring changes in the electrical resistance produced by non-conductive
139 particles suspended in an electrolyte solution. This system provides the electronic volume
140 of the particle, based on changes in the electric resistance generated by the particle
141 suspended while passing through a capillary pore. These voltage changes are proportional
142 to the cell volume. It is worth noting that, for this study, the EV channel was calibrated
143 using 10-µm fluorescent beads (EV=200), and the cytometer was not altered from the
144 original configuration provided by the manufacturer. In these conditions, the system
145 presented high accuracy and resolution for volume measurement.

146 Samples were excited with an argon ion laser (488 nm) set at a power of 22 mW.
147 FL1 and FL3 optical filters were original and the optical characteristics for these filters

were: FL1 (green fluorescence): Dichroic/Splitter, dichroic long pass: 550 nm, band pass filter: 525 nm, detection width 505 to 545 nm; and FL3 (red fluorescence): long pass filter: 670/630 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to SYBR14/PI staining. FL1 was used to detect green fluorescence (SYBR14) and FL3 was used to collect red fluorescence (PI). Spill over of SYBR14 into the PI-channel was compensated (2.45%).

2.5. Functional competence test and image analysis

Sperm functional competence (SFCt) was determined as described in Valencia et al. (2019), with some modifications. Briefly, 50 µL of each semen sample - diluted to 12×10^6 sperm/mL as described previously - were mixed with 550 µL of the hypotonic solution (180 mOsm/Kg), containing SYBR14 (100 nM) and propidium iodide (12 mM). Samples were incubated at 38 °C for 5 min, and fixed with 2% glutaraldehyde. Thereafter, spermatozoa were examined under a Laser Scanning Confocal Microscope (Nikon A1R; Nikon, Tokyo, Japan), also equipped with differential interference contrast (DIC), at 400× and 1000× magnification. Acrosome integrity, viability and morphology (including the presence of cytoplasmic droplets) were determined for each spermatozoon, and cells were classified following the criteria established by Jeyendran et al. (1984) for the sHOST. Images obtained under the microscope (fluorescence and DIC) were measured through Image J software, and 200 spermatozoa per sample were examined.

2.6. Statistical analyses

Data were analyzed using a statistical package IBM SPSS for Windows (Ver. 27.0; Chicago, IL, USA) and figures were drawn with GraphPad Prism 8 (Ver. 8.01 GraphPad

172 Software Inc., San Diego, CA, USA). Results are expressed with box-whisker plots and
173 the level of significance was set at $P \leq 0.05$.

174 Data were first tested for normal distribution with Shapiro-Wilk test, and for
175 homogeneity of variances with Levene test, and statistical analyses included two
176 approaches. The first considered the three sperm subpopulations identified by flow
177 cytometry dot-plots ($SYBR14^+/PI^-$, $SYBR14^+/PI^+$ and $SYBR14^-/PI^+$), the sample being
178 the experimental unit. The proportions of each of these three subpopulations were
179 evaluated with a general linear mixed model (intra-subject factor: 5 min and 20 min of
180 incubation; inter-subject factor: hypo and isotonic conditions), followed by post-hoc
181 Sidak test for pair-wise comparisons. EV and SS were evaluated with a general linear
182 mixed model (intra-subject factor: 5 min and 20 min of incubation; inter-subject factor:
183 hypo and isotonic conditions; and sperm subpopulation: $SYBR14^+/PI^-$, $SYBR14^+/PI^+$ and
184 $SYBR14^-/PI^+$), followed by post-hoc Sidak test for pair-wise comparisons.

185 In the second approach, the number of sperm subpopulations was not determined
186 by dot-plots but rather through two separate strategies based on cluster analysis to identify
187 subpopulations, with each sperm cell being the experimental unit. Spermatozoa were
188 classified through two-step cluster analysis based on the log-likelihood distance and the
189 Schwarz's Bayesian Criterion. In the first strategy, cluster analysis was run on the basis
190 of EV and FL3/FL1. In the second strategy, cluster analysis was based on EV, SS and
191 FL3/FL1. The characteristics of the sperm subpopulations resulting from these two cluster
192 analyses are shown in Tables 1 and 2, respectively. The proportions of the obtained sperm
193 subpopulations were evaluated with a general linear mixed model (intra-subject factor: 5
194 min and 20 min after incubation; inter-subject factor: hypo and isotonic conditions;
195 experimental unit: sample), followed by post-hoc Sidak test for pair-wise comparisons.

196 Finally, in order to establish the relationship of the two approaches with sperm
197 functionality, Pearson correlation was calculated between SFCt outcomes and the
198 different sperm subpopulations.
199
200 **3. Results**
201
202 *3.1. Approach 1: Subpopulations identified in flow cytometry dot-plots (conventional*
203 *approach)*
204 As aforementioned, the 12 ejaculates included in this study were exposed to hypo and
205 isotonic conditions for 5 min and 20 min, and the proportions, EV and SS of each
206 subpopulation identified in dot-plots (viable/live: $SYBR14^+/PI^-$, non-viable/moribund:
207 $SYBR14^+/PI^+$ and non-viable/dead: $SYBR14^-/PI^+$) were determined. As shown in Figure
208 1A, the proportions of membrane-intact spermatozoa (i.e. viable/live spermatozoa;
209 $SYBR14^+/PI^-$) after 5 min of incubation in isotonic conditions did not differ from those
210 observed after 20 min of incubation ($P>0.05$). These proportions, however, were greater
211 after 5 min than after 20 min of incubation in anisotonic conditions ($P<0.01$). In addition,
212 and regardless of incubation time, proportions of membrane-intact spermatozoa were
213 greater in isotonic than in hypotonic media, and those of non-viable/dead spermatozoa
214 ($SYBR14^-/PI^+$) were smaller in the former than in the latter. The proportions of
215 spermatozoa losing membrane integrity (i.e., non-viable/moribund spermatozoa;
216 $SYBR14^+/PI^+$) did not show significant differences ($P>0.05$) between 5 and 20 min either
217 in hypotonic or isotonic conditions, although they were significantly ($P<0.05$) higher in
218 the former than in the latter.
219 As shown in Figure 1 (B), EV did not significantly differ ($P>0.05$) between
220 viable/live ($SYBR14^+/PI^-$) and non-viable/dead spermatozoa ($SYBR14^-/PI^+$), either under

isotonic or hypotonic conditions, regardless of incubation time (5 min or 20 min). Yet, EV in spermatozoa losing membrane integrity (non-viable/moribund; $SYBR14^+/PI^+$), was significantly ($P<0.01$) higher than in viable/live and non-viable/dead spermatozoa, in both isotonic and hypotonic conditions. Furthermore, in all sperm subpopulations, EV was significantly ($P<0.01$) higher in hypotonic than in isotonic conditions after both 5 min and 20 min of incubation.

Finally, SS in spermatozoa losing membrane integrity (non-viable/moribund; $SYBR14^+/PI^+$) was significantly ($P<0.01$) higher than in the other two sperm subpopulations (i.e. viable/live, $SYBR14^+/PI^-$; non-viable/dead, $SYBR14^-/PI^+$) after both 5 min and 20 min of incubation (Figure 1C). This parameter, nevertheless, did not differ between 5 min and 20 min of incubation in any of the three subpopulations ($P>0.05$).

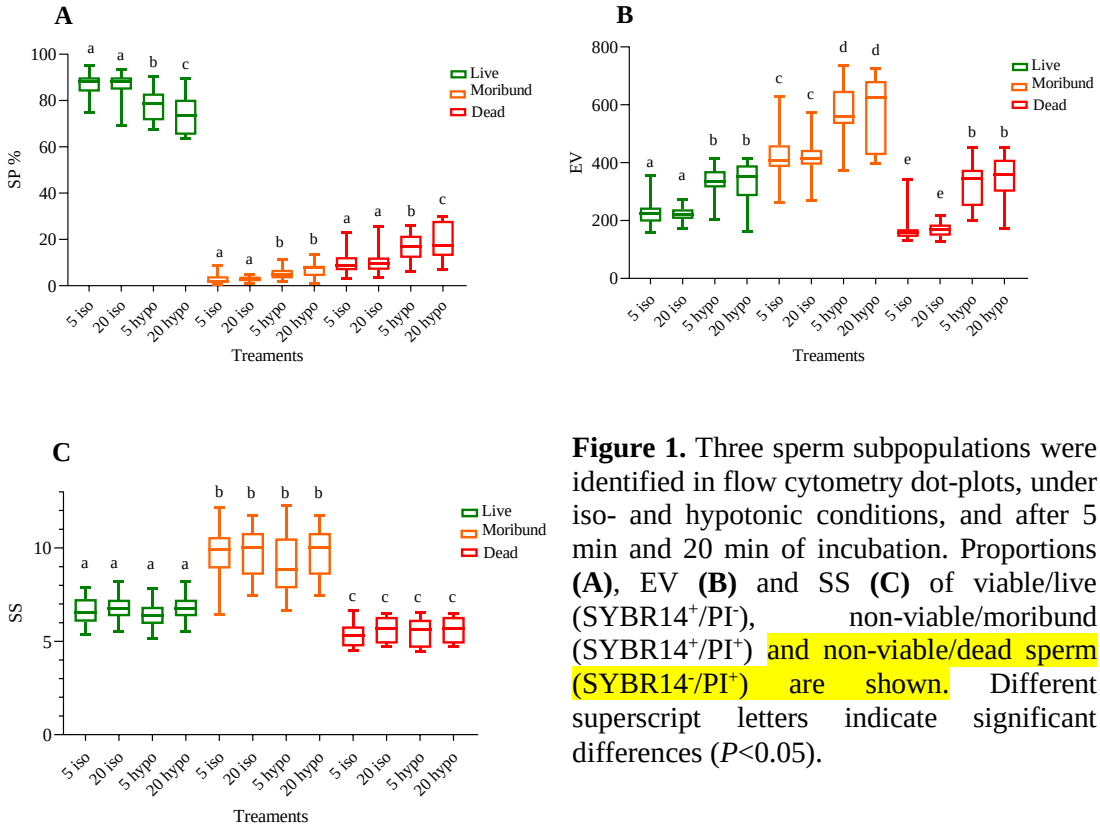

Figure 1. Three sperm subpopulations were identified in flow cytometry dot-plots, under iso- and hypotonic conditions, and after 5 min and 20 min of incubation. Proportions **(A)**, EV **(B)** and SS **(C)** of viable/live ($SYBR14^+/PI^-$), non-viable/moribund ($SYBR14^+/PI^+$) and non-viable/dead sperm ($SYBR14^-/PI^+$) are shown. Different superscript letters indicate significant differences ($P<0.05$).

3.2. Approach 2: Subpopulations identified by cluster analysis

While, as aforementioned, the conventional analysis of dot-plots allowed for the identification of three sperm subpopulations, the first strategy of approach 2 (cluster analysis based on EV and the FL3/FL1 quotient) led to the identification of five subpopulations, three of each (SP2, SP3 and SP4) corresponded to spermatozoa losing plasma membrane integrity (i.e., $SYBR14^+/PI^+$). These three sperm subpopulations significantly ($P<0.01$) differed in their EV and the intensity of FL3 fluorescence (red fluorescence). Remarkably, SP2 showed significantly ($P<0.01$) higher EV and FL3 intensity than SP3 and SP4 did. As depicted in Figure 2A and Table 1, although the maximum values of EV for SP1, SP2, SP3 and SP4 were 1019, 1019, 599 and 326 respectively, SP5, which was considered to be the subpopulation containing membrane-intact spermatozoa ($SYBR14^+/PI^-$) showed the lowest mean of EV (mean ± standard error of the mean: 137.8 ± 0.1) with a maximum of 189, which differed from the other sperm subpopulations ($P<0.01$).

No significant differences between incubation times (5 min vs. 20 min) in spermatozoa exposed to isotonic conditions were observed (except for SP3). Whereas the proportions of SP5 (membrane-intact spermatozoa) and SP3 significantly ($P<0.01$) decreased in hypotonic vs. isotonic conditions after both 5 min and 20 min of incubation, those of SP1, SP2 and SP4 increased significantly ($P<0.01$) (Fig. 2 B). On the other hand, whereas incubation of spermatozoa in hypotonic conditions for 20 min reduced the proportions of spermatozoa belonging to SP5 ($P<0.01$), those of SP4 increased ($P<0.01$) (Fig. 2B).

In the second strategy (cluster analysis based on EV, SS and FL3/FL1), the same subpopulations were identified. SS was significantly ($P<0.05$) greater in SP1 ($SYBR14^-/PI^+$) than in the other subpopulations (Table 2 and Fig. 2C). This could be, as shown in

Figure 3 (A.1, A.2 and A.3), due to the fact that these sperm cells had an exocytosed acrosome, which would change the surface of the acrosome region. In both strategies, SP1 did not show significant differences (P>0.05) between isotonic and hypotonic conditions after 5 min of incubation. As depicted in Figure 3 (C.1 and C.3), sperm cells without an intact membrane and a coiled tail (swollen sperm) were identified; in addition, all spermatozoa with a straight tail did not have their plasma membrane intact. The subpopulation corresponding to non-viable/dead spermatozoa with a coiled tail, however, did not significantly differ from viable/live spermatozoa in head dimensions (Fig. 3B.1). The results of the mixed model (Fig. 2D) showed that, after incubation, the proportions of SP5 ($SYBR14^{+}/PI^{-}$, membrane-intact spermatozoa) were significantly (P<0.01) lower in hypotonic than in isotonic conditions. In contrast, proportions of spermatozoa belonging to SP2 and SP3 were significantly (P<0.01) greater in hypotonic than in isotonic conditions. Except for SP4, exposing spermatozoa to isotonic conditions for a longer period (20 min) did not alter the proportions of subpopulations compared to 5 min (P>0.05). In hypotonic conditions, proportions of SP1, SP2 and SP3 were significantly (P<0.01) greater at 5 min than at 20 min, whereas those of SP4 were significantly (P<0.01) smaller at 5 min than at 20 min. In the case of SP5, no significant differences between 5 min and 20 min were observed when spermatozoa were exposed to isotonic conditions (P>0.05) (Fig. 2D).

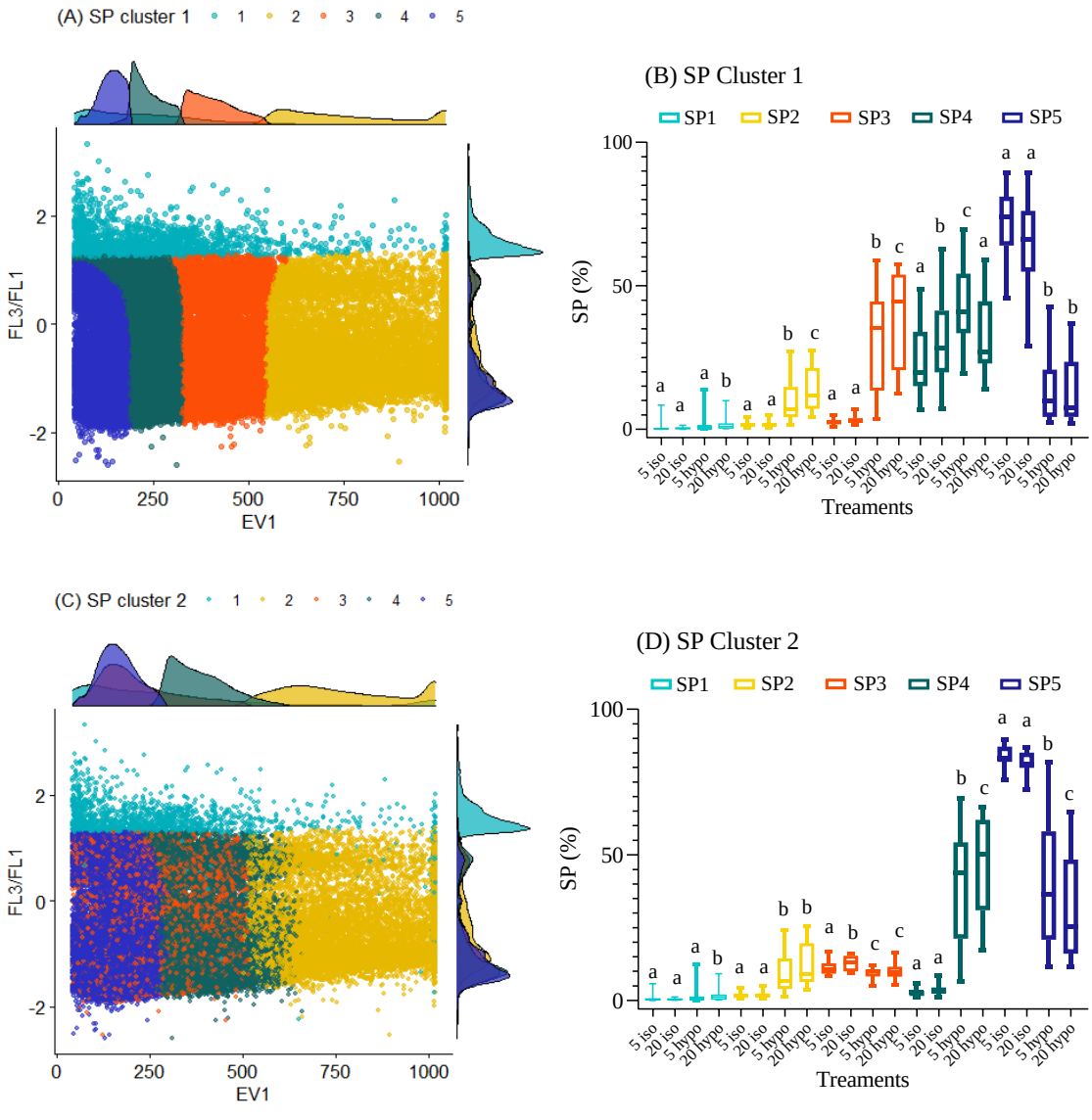

Figure 2. Sperm subpopulations identified through cluster analyses, based on EV and FL3/FL1 (Strategy 1; **A** and **B**), or EV, SS and FL3/FL1 (Strategy 2; **C** and **D**). Box-whisker plots (**B**: strategy 1; **D**: strategy 2) represent the proportions of each SP after incubation in isotonic or hypotonic conditions, for 5 min or 20 min.

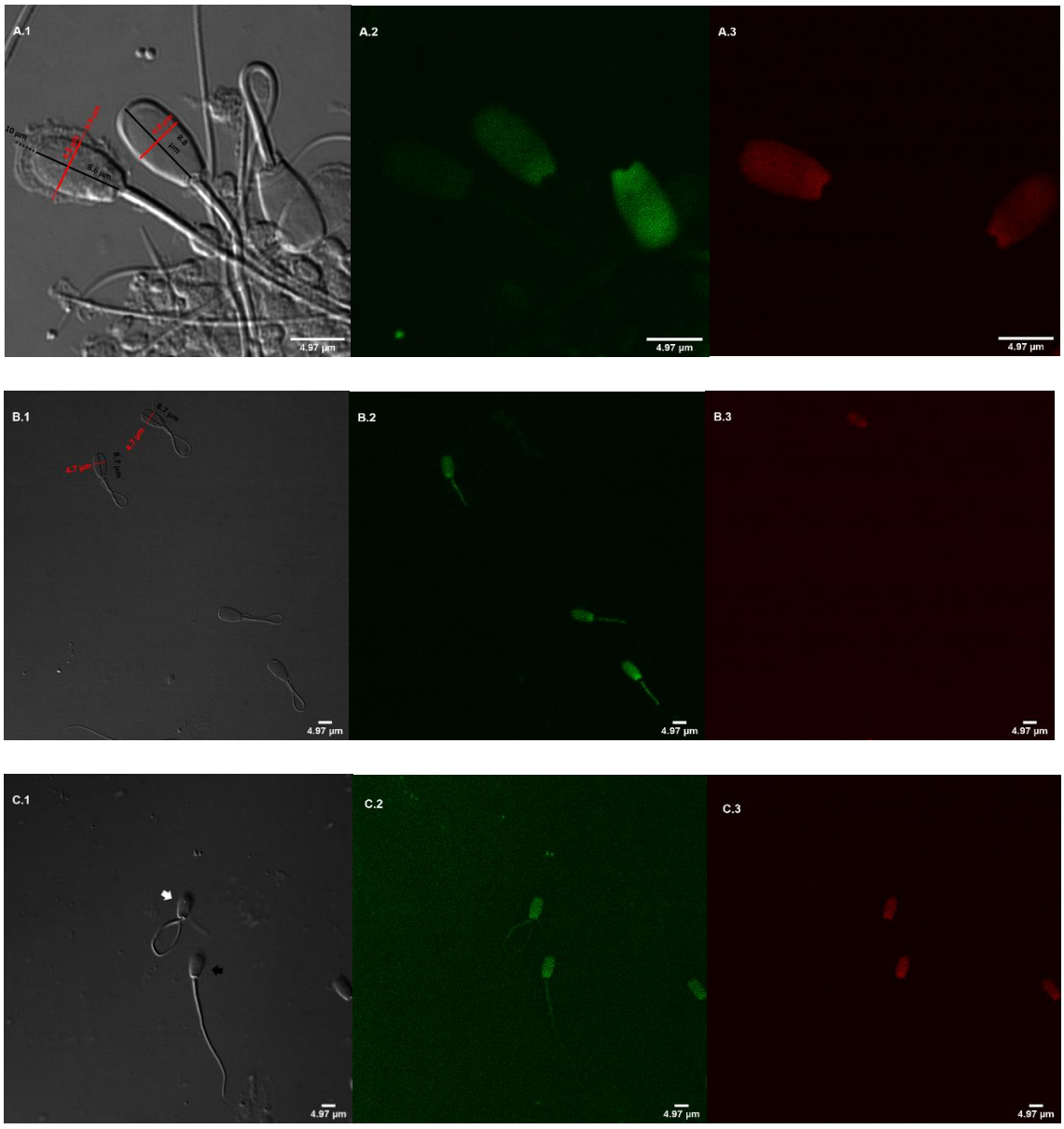

Figure 3. Images captured under a Laser Confocal Scanning Microscope equipped with differential interference contrast showing different sperm subpopulations after exposure to hypotonic conditions for 5 min. (**A.1-A.3**) Different dimensions of sperm head with a damaged or intact acrosome, and with or without an intact membrane (1,000×). (**B.1-B.3**) Different head dimensions in viable/live and non-viable/dead spermatozoa with coiled tails (400×). (**C.1-C.3**) Non-viable/dead spermatozoa with coiled (white arrow) and straight tails (black arrow) (400×).

298 *3.3. Relationship of subpopulations identified by conventional flow cytometry analysis*
299 *(Approach 1) and cluster analysis (Approach 2) with sperm functional competence*
300 Table 3 shows the correlation coefficients between sperm functional competence (SFCt)
301 and the subpopulations identified with flow cytometry dot-plots ($SYBR14^+/PI^-$,
302 $SYBR14^+/PI^+$, and $SYBR14^-/PI^+$), those identified through cluster analyses based on EV
303 and FL3/FL1 (Strategy 1), or those identified through cluster analyses based on EV, SS
304 and FL3/FL1 (Strategy 2), after incubation in hypotonic conditions for 5 min. The
305 proportions of SP3 and SP4 in Strategy 1, and those of SP5 in Strategy 2 were found to
306 be significantly ($P<0.05$) correlated with sperm functional competence. In contrast, the
307 proportions of sperm subpopulations identified in conventional flow cytometry dot-plots
308 were not observed to be correlated to sperm functional competence.
309

4. Discussion

The present work aimed to determine if a cluster analysis approach based on flow cytometry variables (EV, SS and FL3/FL1 quotient) could provide more information on sperm function than the conventional analysis of dot-plots. For this purpose, spermatozoa were exposed to two osmotic conditions (180 mOsm/Kg and 300 mOsm/Kg) for 5 and 20 min, and then evaluated.

Previous studies focusing on the estimation of EV (Oldenhof et al., 2011; Ching Hei Yeung et al., 2002) used flow cytometry to determine membrane integrity with multiple analyses and powerful statistical tools (reviewed by Gil & Fj, 2017). In spite of this, neither flow cytometry variables (EV, SS and fluorescence intensity in different channels) in separate sperm subpopulations were previously evaluated, nor were changes following exposure to hypotonic conditions assessed. In the present study, differences in the EV of spermatozoa from the different subpopulations provided by conventional analysis were found. Under isotonic conditions, spermatozoa losing membrane integrity (i.e., non-viable/moribund sperm; $SYBR14^{+}/PI^{+}$) exhibited the greatest volume followed by viable/live sperm ($SYBR14^{+}/PI^{-}$) and non-viable/dead sperm ($SYBR14^{-}/PI^{+}$). This indicates that over the process of death, the integrity of plasma membrane is lost and sperm cells increase their volume. Related to this, it is worth noting that early necrosis of somatic cells is related to an increase of cell volume because of the accumulation of water in the cytosol and organelles, as well as to changes in the cell surface due to the formation of blebs, which antecedes membrane disruption (reviewed by Pasantes-Morales, 2016). In spermatozoa, the increase of necrotic volume is associated with an uncontrolled opening of quinidine- and tamoxifen-sensitive ion channels, and an accumulation of sodium and chloride ions in the cytoplasm (A. M. Petrunkina, Jebe, et al., 2005). According to Ching Hei Yeung et al. (2002), SS depends on the structural complexity of

spermatozoa and reflects changes in their surface, whereas EV is related to variations of volume when cells experience osmotic stress. In this study, variations in both EV and SS after exposure to hypotonic conditions were observed, particularly in those sperm subpopulations where the increase in the FL3 fluorescence was greater. This indicates that spermatozoa less resilient to osmotic stress and experiencing early necrosis are the ones that exhibit more changes in EV and SS. Interestingly, under hypotonic conditions, EV increased in all sperm subpopulations, notwithstanding viable/live spermatozoa did not differ from non-viable/dead spermatozoa. The increase of EV in viable/live spermatozoa could take place together with coiling of the tail or angulation at the cytoplasmic droplet, which would agree with that reported by Ching Hei Yeung et al. (2002). In contrast, non-viable/dead spermatozoa would also have an increased EV after hypotonic stress but would not show a coiled tail and would have a disrupted membrane (Pérez-Llano et al., 2009).

The increase observed in the SS of SP1, which was the one with the highest FL3/FL1 ratio, could be due to derangements in the plasma membrane due to blebbing after swelling and return to the initial volume (González-Fernández et al., 2012). This coincides with the assumption that PI begins to enter the cell when the volume starts to increase (SP2, SP3 and SP4); when the volume reaches its maximum resistance limit, the cell explodes and the volume returns to a lower EV, showing a greater SS (SP1). Additionally, in the case of SP1, there could be spermatozoa with coiled tails and compromised membrane integrity, and spermatozoa that did not react to incubation in hypotonic medium (straight tail) due to membrane damage prior to incubation, as reported by Pérez-Llano et al. (2009). In this context, one should note that spermatozoa from SP1 presented the highest value of SS and that, in the observations performed herein under the microscope and in agreement with Palacin et al. (I Palacin, P Santolaria & C Soler,

MA Silvestre, 2020), a large number of spermatozoa without an intact membrane and a straight tail also exhibited an exocytosed acrosome, which entailed changes in their surface and head volume. While sperm swelling and coiling of the tail have been used as an indicator of membrane functionality (Jeyendran et al., 1984; Pérez-Llano et al., 2001, 2009), a subpopulation of sperm cells that present a coiled tail after hypotonic stress and have, at the same time, their membrane damaged has also been identified (Pérez-Llano et al., 2009; Valencia et al., 2019). In this work, the EV of spermatozoa with an intact membrane belonging to SP5 (PI^-) was found to increase after incubation in hypotonic conditions. This value reached the maximum value of 189 arbitrary units, from which the PI began to enter, and spermatozoa appeared to switch from SP5 to SP4. The limit of osmotic stress that viable spermatozoa can withstand has been tested in horses (González-Fernández et al., 2012; Oldenhof et al., 2011) and pigs (A. M. Petrunkina & Töpfer-Petersen, 2000) through the Boyle van't Hoff equation. Yet, the maximum EV of a spermatozoon that is losing the integrity of its membrane and thus presents early changes in membrane permeability (i.e., moribund sperm) is not clear. In the present work, the limit of EV in viable spermatozoa could be set at 189, whereas spermatozoa losing the integrity of their plasma membrane and were thus positive for PI staining reached a maximum EV of 661 (SP4 in the second clustering strategy). There was, in addition, another subpopulation (SP1 in the second clustering strategy) with greater PI fluorescence intensity (FL3) and SS but lower EV, which would indicate that, under hypotonic conditions, PI enters as the membrane reaches its maximum resilience to osmotic stress. The plasma membrane then loses its integrity, the cell explodes and loses the acquired volume. It is known that the osmotic resistance of spermatozoa is attributed to volume regulation abilities (Chaveiro et al., 2006; A. M. Petrunkina, Gröpper, et al., 2005), and greater fluidity and flexibility of their plasma membrane (A. M. Petrunkina, Gröpper, et

al., 2005). The plasma membrane of pig spermatozoa is known to contain a high amount of polyunsaturated fatty acids, and a low amount of saturated fatty acids and cholesterol, all with a different point of jellification (Cerolini, 2001; Johnson et al., 2000). Thus, further studies are needed to address if the resilience of an ejaculate to freeze-thawing procedures or a greater fertilizing capacity are related to a higher percentage of spermatozoa with a plasma membrane capable to withstand significant changes in the osmolality of the surrounding milieu. Whether this is related to changes in the proportions of each sperm subpopulation should also be investigated.

As aforementioned, sperm subpopulations with different functional status exist in the ejaculate, which, among other factors, has been related to their response to osmotic changes (Pérez-Llano et al., 2009; A M Petrunkina et al., 2007; Valencia et al., 2019). Because, during thawing, a greater osmotic gradient exists resulting in hypotonic shock and cell death (A M Petrunkina et al., 2007; Yeste, 2016), the sperm resilience to osmotic stress is a determining characteristic to predict the potential damage inflicted by cryopreservation (Chaveiro et al., 2006; A. M. Petrunkina, Gröpper, et al., 2005). Likewise, the sperm ability to fertilize an oocyte, both in vivo and in vitro, has been found to be related to the proportion of spermatozoa capable of withstanding hypotonic stress (Valencia et al., 2019; Yeste et al., 2010). The current research demonstrates that this osmotic resistance may be better evaluated when flow cytometry coupled to clustering analyses is used, as a higher number of sperm subpopulations are identified, which in turn allows identifying, in greater detail, which changes occur and how spermatozoa look like after they are incubated in hypotonic conditions. This is particularly important if one bears in mind that spermatozoa encounter media with different osmolality after deposition in the female reproductive tract, and those sperm cells that show a greater capacity for volume regulation are the ones more able to reach the oviduct (Petrunkina et al., 2007).

Yet, as demonstrated herein, the conventional evaluation with the flow cytometer cannot detect the subtle changes that the different sperm subpopulations identified in the ejaculate experience.

An interesting finding of the present study was that, when using conventional analysis of flow cytometry dot-plots, spermatozoa losing plasma membrane integrity (i.e., moribund sperm) accounted for around 10% of total cells. When sperm subpopulations were set with cluster analysis, those corresponding to spermatozoa losing plasma membrane integrity (SP2, SP3, and SP4) represented up to 50% of total cells. This indicated that this second approach could identify sperm subpopulations with early changes in membrane integrity and volume that could not be detected with conventional analysis. Noticeably, the proportions of SP3 and SP4 showed strong and significant correlations with sperm functional competence, whereas the subpopulations derived from the conventional analysis did not exhibit such correlations. This finding is relevant as, despite the fact that the characteristics related to sperm osmotic resistance and cell volume regulation are not widely studied, they are crucial for the osmotic changes that spermatozoa undergo when they pass from the hypertonic environment in the epididymis tail to the isotonic milieu of seminal plasma and the uterine environment (Cooper &Yeung, 2003; A M Petrunkina et al., 2007). This partly explains why hypoosmotic tests may predict in vitro and in vivo fertility (Jeyendran et al., 1984; Pérez-Llano et al., 2001; Valencia et al., 2019). The osmotic resistance capacity of spermatozoa becomes even more critical in the case of cryopreservation protocols, particularly post-thawing osmolarity changes, transitioning from a hyperosmotic environment aimed at cell dehydration to an isoosmotic environment that aims to restore the cell volume (Oldenhof et al., 2011; Yeste et al., 2016).

Finally, this study creates the need to conduct studies that relate the functioning of membrane channels with the proportions of sperm in each subpopulation, the maximum values of cell volume, and sperm fertility and cryotolerance. Remarkably, separate studies were carried out to test the role of different membrane channels in the regulation of cell volume and during sperm capacitation (Noto et al., 2021; Petrounkina et al., 2004). The current research emphasizes that any evaluation in this realm should consider the possibility of applying clustering strategies with statistical tools using individual data, as this may help understand the relationship between the functioning of plasma membrane channels and volume regulation, and the implications that this has for the resilience to freeze-thawing and fertilizing capacity. Specifically, the research applications of using this cluster analysis approach of sperm subpopulations are focused on studying osmotic changes and cell volume in cryopreservation procedures, as well as identifying these characteristics as markers of freezability. Additionally, it can be applied to studies about sperm capacitation, acrosome exocytosis, and the fertility potential of a given ejaculate.

5. Conclusions

In conclusion, a cluster analysis approach based on flow cytometry variables like EV, SS and FL3/FL1 quotient provides more information on sperm function than the conventional analysis of dot-plots does, as five rather than three sperm subpopulations could be identified. In these five sperm subpopulations, the ones corresponding to spermatozoa losing membrane integrity (i.e., moribund sperm) exhibited the greatest volume followed by viable/live sperm and non-viable/dead sperm, both under hypotonic and isotonic conditions. The EV was seen to increase along with the entry of PI in those sperm cells having a less resilient membrane which, after completely losing plasma

membrane integrity, presented a reduced volume. This study also supports that, in a similar fashion to the assessment of sperm motility by CASA, investigating individual spermatozoa combined with cluster analysis is a more robust approach.

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Data availability

The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest

Marc Yeste is an Editor of Reproduction, Fertility and Development, but was blinded from the peer review process for this paper.

Author contributions

JV and MY designed the study. JV, SB-C, EP and MY ran the experiments. JV wrote the manuscript. EP, SB and MY revised the final version of the manuscript. All authors approved the final version of the Manuscript.

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Tables

Table 1. Sperm subpopulations and different parameters after cluster analysis based on EV and FL3/FL1 quotient (Strategy 1)

Variables	Sperm subpopulations				
	1	2	3	4	5
Number of sperm	1614	12159	33874	73288	117481
FL1	1.0 ± 0.1^{a}	314.9 ± 3.4^{b}	101.7 ± 0.7^{c}	72.4 ± 0.3^{d}	76.0 ± 0.3^{e}
FL3	196.6 ± 8.8^{a}	86.5 ± 2.2^{b}	17.2 ± 0.2^{c}	9.7 ± 0.1^{d}	2.9 ± 0.1^{e}
EV range	44-1019	546-1019	294-599	44-326	44-189
EV (mean)*	247.4 ± 4.8^{a}	762.6 ± 1.4^{b}	409.4 ± 0.3^{c}	236.4 ± 0.2^{d}	137.8 ± 0.1^{e}
SS	9.4 ± 0.1^{a}	11.3 ± 0.1^{b}	6.4 ± 0.0^{c}	5.6 ± 0.0^{d}	5.4 ± 0.0^{e}
FL3/FL1*	46.1 ± 2.0^{a}	1.2 ± 0.0^{b}	1.5 ± 0.0^{c}	1.2 ± 0.0^{b}	0.3 ± 0.0^{d}

Asterisk (*) indicates the sperm parameters included in the cluster analysis. EV: electronic volume; SS: side scattering. Different letters ($^{a, b, c, d}$) mean significant ($P<0.05$) differences between sperm subpopulations.

Table 2. Sperm subpopulations and different parameters after cluster analysis based on EV, SS and FL3/FL1 quotient (Strategy 2)

Variables	Sperm subpopulations				
	1	2	3	4	5
Number of sperm	1703	11133	27261	40609	157710
FL1	134.8 ± 13.2^{a}	319.6 ± 3.4^{b}	96.8 ± 0.9^{c}	92.4 ± 0.5^{d}	71.8 ± 0.2^{e}
FL3	282.1 ± 15.1^{a}	81.5 ± 1.3^{b}	16.2 ± 0.3^{c}	12.9 ± 0.2^{d}	4.1 ± 0.1^{e}
EV range	44-1019	432-1019	44-518	228-661	44-303
EV (mean)*	315.5 ± 6.7^{a}	771.1 ± 1.5^{b}	218.4 ± 0.6^{c}	386.6 ± 0.4^{d}	166.6 ± 0.1^{e}
SS*	14.5 ± 0.3^{a}	11.7 ± 0.0^{b}	11.5 ± 0.0^{c}	5.2 ± 0.0^{d}	4.6 ± 0.0^{e}
FL3/FL1*	41.8 ± 1.9^{a}	1.3 ± 0.0^{b}	1.2 ± 0.0^{b}	1.4 ± 0.0^{b}	0.6 ± 0.0^{c}

Asterisk (*) indicates the sperm parameters included in the cluster analysis. EV: electronic volume; SS: side scattering. Different letters ($^{a, b, c, d}$) mean significant ($P<0.05$) differences between sperm subpopulations.

Table 3. Correlation coefficients between sperm functional competence (SFC) and sperm subpopulations after conventional analysis of dot-plots (*Approach* 1) and cluster analysis (*Approach 2*).

			5 min					20 min				
			SP1	SP2	SP3	SP4	SP5	SP1	SP2	SP3	SP4	SP5
Cluster analysis based on EV and FL3/FL1 quotient (strategy 1)	Hypo	SFC	0,11	0.30	0.59*	-0.63*	-0.46	-0.22	0.21	0.21	-0.14	-0.22
	Iso		0.18	0.08	0.18	0.43	-0.40	-0.12	-0.01	0.39	0.45	-0.47
Cluster analysis based on EV, SS, and FL3/FL1 quotient (strategy 2)	Hypo	SFC	0.13	0.42	0.21	0.50	-0.63*	-0.25	0.28	0.40	0.21	-0.33
	Iso		0.24	0.22	0.56	0.2	0.12	0.13	0.14	0.55	0.35	0.26
			Viable(SYBR14^{+}/PI^{-})	Moribund (SYBR14^{+}/PI^{+})	Non-viable (SYBR14^{-}/PI^{+})			Viable (SYBR14^{+}/PI^{-})	Moribund SYBR14^{+}/PI^{+}	Non-viable (SYBR14^{-}/PI^{+})		
Conventional analysis of dot-plots	Hypo	SFC	0.20	-0.19	-0.14			0.13	-0.07	-0.10		
	Iso		-0.44	0.16	0.38			-0.45	0.15	0.43		

(*) $P<0.05$; EV: electronic volume; SS: side scattering; SP: sperm subpopulation; SFC: sperm functional competence; Hypo: hypotonic incubation (180 mOsm/Kg); Iso: Isotonic incubation (300 mOsm/Kg). Measurements were made after 5 min and 20 min of incubation.