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Sperm survival and heterogeneity are correlated with fertility after intrauterine insemination in superovulated ewes

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Abstract

Efficient animal production involves accurate estimations of fertilizing ability. One key factor is the plasma membrane of the sperm cell, which is actively involved in the cascade of events before oocyte fusion. Many methods are used to analyze the characteristics of this membrane, including partition in aqueous two-phase systems which is an efficient method to analyze sperm surface changes accounting for loss of viability and different functional states. Centrifugal countercurrent distribution (CCCD) analysis can also be used in an aqueous two-phase system to determine the relationship between sperm parameters and in vivo fertility in ewes. In a previous work, we found a significant correlation between two post-CCCD parameters (heterogeneity and recovered viability) and field fertility when the same sample was used after cervical AI. The present study was intended to find out whether the control of several external factors that affect reproductive efficiency is able to increase the correlation coefficient between post-CCCD parameters and fertility. Thus, 90 *Rasa aragonesa* ewes were controlled on the same farm and received intrauterine inseminations using the same technical equipment. The fertilizing ability of the raw semen and sperm samples selected by a dextran/swim-up process was compared using a low number of spermatozoa per insemination (7×10^7) to enhance possible fertility differences. A new post-CCCD parameter was considered; the loss of viability (LV) occurred during the CCCD process. This variable denotes the sperm surviving ability and corresponds to the difference between the total number of viable cells loaded and recovered after the CCCD run. The mean fertility of eight sperm control samples was 60% (range: 25–76%), and there was no significant correlation between standard parameters and in vivo fertility. LV ranged from 2 to 69% (average 27%) and was negatively correlated with fertility ($r = -0.914$,

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$P < 0.01$). Ejaculate heterogeneity (H) ranged from 20 to 47% and was positively, but not significantly, correlated with fertility ($r = 0.391$). A predictive equation for fertility was deduced by multiple analysis with a very high correlation coefficient ($r = 0.967$), and level of significance ($P < 0.005$): predictive fertility PF = 52.546 − 0.594 LV + 0.665 H. The mean fertility of 13 swim-up selected samples was 63% (range: 25–86%). Again, only parameters derived from the CCCD analysis were highly correlated with fertility, especially LV and H ($P < 0.05$).

Keywords: Ram spermatozoa; Intrauterine insemination; Fertility

1. Introduction

One of the main problems in artificial insemination is to develop efficient methods to accurately estimate the fertilizing ability of sires. Several techniques have been developed over the years to correlate different aspects of semen quality with field fertility. They can be divided into basic semen parameter analyses and sperm function assays. The former include assessment of sperm motility [1–3], morphology [4–6], and membrane integrity [7–9]. Other less commonly used techniques involve detecting defective sperm organelles and DNA [10,11] and the analysis of seminal plasma [12,13]. Most parameters are poorly correlated with in vivo fertility when taken individually but results have improved using computer-assisted techniques or new imaging technology [14–17]. Recently, several attempts have been made to analyze how well these simple tests predict fertility [18–21].

Other assays have been developed to evaluate sperm function, such as the sperm penetration assay [22,23], the zona binding assay [24–26] or the hemi-zona assay [27,28], in addition to analyses of the acrosome reaction or capacitation status [29–31]. Although these techniques are still quite time-consuming and technically demanding, they have been used to predict in vivo fertility in several species [32,33].

Despite rapidly developing molecular, genomic and computer techniques, our understanding of fertility is still far from complete. Since fertilization requires several sperm functions, it seems reasonable to use a combination of assays to help predict fertilizing ability more precisely, as suggested by Amann and Hammerstedt [34]. These combinations could include standard semen parameters or others based on sperm function.

The plasma membrane of the sperm cell has several specializations which play unique roles in the cascade of events before fusion with the oocyte. Many methods have been developed to analyze the characteristics of this membrane, including aqueous two-phase systems, which are based on the affinity of the cell surface for immiscible aqueous solutions of polymers, such as dextran (hydrophilic) and polyethylene glycol (PEG, hydrophobic) [35,36]. The extent of partition between the cells in the interface and the PEG-rich upper phase depends on the cell surface properties.

The selectivity and separation resolution can be greatly improved by multistep partitions. Countercurrent distribution (CCD) is a chromatographic process with a stationary (lower) phase and a mobile (upper) phase. The cell sample is partitioned in one system, and the two phases are systematically brought into contact with opposite fresh phases. The loss of viability as a result of dilution [37] and washing during the separation process can be

avoided by including centrifugation (i.e., centrifugal countercurrent distribution, CCCD; [38]).

In previous studies, we have shown that CCCD in an aqueous two-phase system helps to reveal sperm heterogeneity [39–41]. Sperm become more heterogeneous during the maturation process [42], creating sub-populations with different fertilizing abilities. It is especially important to consider heterogeneity in species with variable and unpredictable intervals between mating and ovulation [34], such as sheep. In this sense, ejaculates with heterogeneous sperm would have a higher fertilizing potential than homogeneous ones if there are variable intervals between mating and ovulation. Thus, an index of heterogeneity could provide important information about the fertilizing potential of a given sample. In a previous study [43], we found a significant correlation between heterogeneity and viability after CCCD analysis and the field fertility rate obtained with the same sample after cervical AI. The result was a predictive equation for field fertility (correlation coefficient $r = 0.488$) with a very high level of significance ($P < 0.005$) [43]. The goal of the present study was to analyze whether the correlation coefficient between post-CCCD parameters and fertility could be increased by controlling several external factors that affect reproductive efficiency. Controlled ewes from the same farm received intrauterine inseminations using the same technical equipment. In addition, we compared the fertilizing ability of raw semen with sperm samples selected by a dextran/swim-up process, using a low number of spermatozoa per insemination to enhance possible differences in fertility [44,45].

2. Materials and methods

2.1. Sperm collection

All the experiments were performed with fresh spermatozoa taken from eight mature *Rasa aragonesa* rams using an artificial vagina. This breed corresponds to a local Spanish genotype with a short seasonal anoestrus between May and July. All the rams belonged to the National Association of Rasa Aragonesa Breeding (ANGRA) and were 2–4 years old. They were kept at the Veterinary School under uniform nutritional conditions. Based on the positive results from a previous study, sires underwent an abstinence period of two days, and second ejaculates were pooled and used for each assay, to avoid individual differences [46]. The experiments were performed between October and May at the Veterinary School.

2.2. Preparation of cell samples

The studied samples were: diluted raw semen and plasma-free samples selected by the dextran/swim-up procedure developed by us [47]. The swim-up medium (SM) was based on the formulation of Quinn et al. [48], and devoid of $CaCl_2$ and $NaHCO_3$ to obtain a swim-up sample (SS). Control samples were diluted with SM to the same concentration as selected samples. Sperm samples of approximately 7×10^7 cells were packaged in 0.25-ml straws and kept at 30 °C during the course of the inseminations.

2.3. Assessment of standard semen parameters

Sperm concentration was calculated in duplicate using Neubauer's chamber (Marienfeld, Lauda-Königshofen). Sperm motility was subjectively assessed by visual estimation with a television microscopy system (100×) maintained at 37 °C. Percentage of progressively motile spermatozoa was estimated at intervals of 5%. Semen motility was assessed by the same person throughout the study.

Cell viability (membrane integrity) was assessed by fluorescent staining with carboxyfluorescein diacetate and propidium iodide [7]. The cells were examined under a Nikon fluorescence microscope, and the number of propidium iodide-negative (membrane-intact) spermatozoa and propidium iodide-positive (membrane-damaged) spermatozoa per 100 cells were estimated and recorded. At least 200 cells were counted in duplicates for each sample.

The hypoosmotic-swelling test was performed by diluting 10 μl of the sample in 1 ml of a hypoosmotic solution (7.35 g sodium citrate·$2H_2O$ and 13.51 g fructose in 1 l of distilled H_2O, adjusted to 100 mOsm/l), and then incubated at 37 °C for 30 min. We evaluated 200 cells by counting in at least five different fields under a phase-contrast microscope at magnification 400×.

2.4. CCCD analysis

Preparation of cell samples: After standard sperm evaluation, the sperm sample medium was removed to avoid affecting the CCCD assay. This was done by filtering twice through a 5 μm pore size Millipore disk (Millipore Corp., Bedford, MA) and mixing the sample with 20 volumes of the CCCD polymer-free medium (described below).

Two-phase system: The two-phase system consisted of 5.5% (w/w) dextran T500 (Mr 500.000) from Pharmacia (Uppsala, Sweden), 2% (w/w) polyethylene glycol (PEG; Mr 6000) from Serva Feinbiochemica (New York), 10.5% (w/w) Ficoll 400 (Mr 400.000, Pharmacia), polymer-free medium (0.25 M sucrose, 0.1 mM EGTA, 4 mM sodium phosphate, 5 mM glucose, 10 mM Hepes and 2 mM KOH; final pH 7.5).

Centrifugal countercurrent distribution: The CCCD was carried out using equipment based on Akerlund's apparatus [38]. Sixty chambers are arranged in a circle, allowing transfer of the upper (mobile) phases relative to the lower (stationary) phases. Half of each chamber was in the outer ring and the other half was in the inner ring, and the two rings were able to rotate against each other. Each chamber contained a mixture of the two phases. Centrifugation was used to separate the phases into the denser (bottom) phase in the outer half of each chamber and the lighter (upper) phase in the inner half. Since there was no elution or pumping, the overall process is a circular multistep transfer of 60 upper over 60 bottom batch phases. Each transfer in this centrifugal-enhanced CCD system included shaking the phases at unit gravity to mix them thoroughly and then separation by centrifugation. While the separated phases were still rotating at full speed (1000 × g), each upper (inner) phase was transferred to the next chambers and the next cycle was performed after deceleration [38].

A two-phase system of the above composition was prepared and mixed to perform the CCCD experiments. Batches of 400 g were assembled by weighing out stock solutions to

avoid differences among experiments. In each assay, the volume of the system loaded in chambers 0–59 was the estimated amount to maintain the desired volume of the bottom phase (0.7 ml). Thus, three experiments were performed with the same system under identical conditions to compare the six different samples directly. Two different cell samples obtained from the same semen pool (control and SS, with approximately 1×10^8 cells each) were filtered and loaded simultaneously in chambers 0 and 30, and analyzed under identical conditions by performing 29 transfers (the whole process took approximately 1 h). All operations were carried out at 20 °C. After the run, the solutions were transformed into a single-phase-system by addition of one volume of the polymer-free medium. The fractions were then collected and the cells were counted under a light microscope.

As a consequence of the separation procedure, sperm cell populations with a marked affinity for the lower dextran-rich phase (mainly due to a low hydrophobicity) moved to the left part of the profile. Sperm cells that separated nearly equally in both phases were distributed in the central sector. Finally, sperm populations with a high affinity for the upper PEG-rich phase (mainly due to a high hydrophobicity) moved to the right sector of the profile. An explanatory diagram of the principle of countercurrent distribution is presented

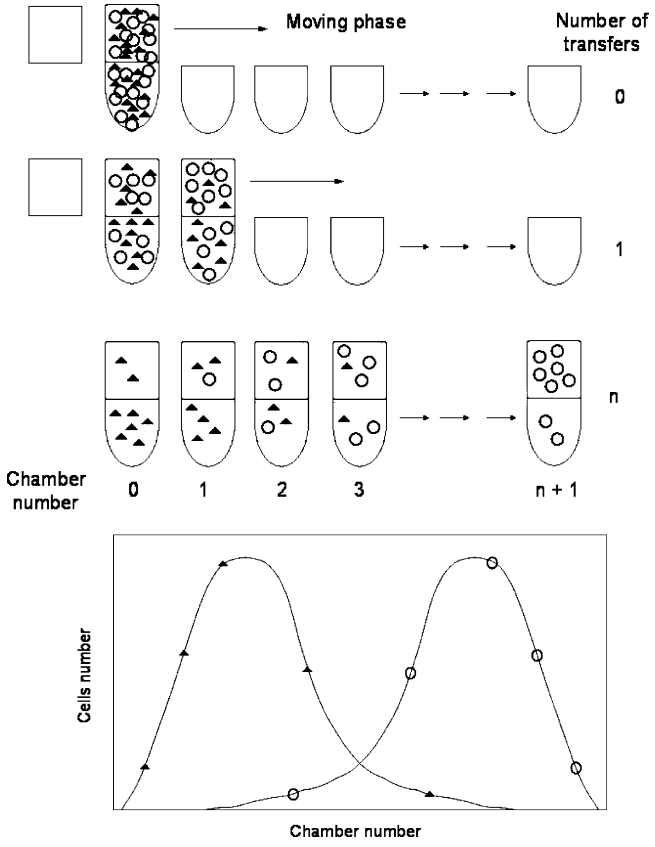

Fig. 1. The principle of countercurrent distribution. For explanation, see text.

in Fig. 1. In order to analyze CCCD results, profiles were divided into three sectors (chambers 0–9, 10–19, and 20–29), corresponding to different cell affinities. Partition results were expressed as the percentage of cells counted in each fraction. For viability assessment, cells from three consecutive chambers throughout the run were pooled and stained as indicated above. Results were expressed as the percentage of viable cells in each sample.

Post-CCCD parameters were derived from the analysis of the obtained partition profile. Total viability (TV) corresponds to the percentage of viable cells with respect to the total cells recovered after the CCCD process (number of cells calculated by Maple V software). The TV was also divided into three fractions (V_1, V_2 and V_3, corresponding to chambers 0–9, 10–19 and 20–29, respectively). This partition is based on previous results in our group [49], where non-viable cells are preferentially located on the left-hand side of the profile, along with acrosome-reacted and immature sperm, whereas viable sperm are mainly partitioned in the central and right sector of the profile. A new parameter was considered called loss of viability (LV) to describe sperm survival (i.e., the difference between the total number of viable cells loaded and recovered after each CCCD run). Finally, profile heterogeneity (H) was defined as the percentage of chambers containing a number of cells = 50% of the cells present in the chamber with the maximum number of cells.

2.5. Hormonal treatment and artificial insemination

A total of 90 *R. aragonesa* ewes were used in the experiment from different farms belonging to ANGRA. All ewes were kept at the Veterinary School and were fed to provide their liveweight maintenance requirements during the experimental work. In each experiment, nine ewes were inseminated comparing three different samples (control and selected obtained from the same semen pool, three ewes per sample/experiment).

2.6. Synchronization of oestrus and superovulation

Oestrus was synchronized using intravaginal sponges containing 30 mg fluorogestone acetate (FGA) (Chrono-gest; Intervet, Salamanca, Spain) inserted for 14 days. Ewes were superovulated with 176 NIH-FSH-S1 units of NIADDK-oFSH-17 (Ovagen ICP-LTD Ltd., New Zealand) in eight doses administered i.m. at 12-h intervals starting 72 h before sponge removal. Each animal received the total dose in 10 ml of solution subdivided into 2 × 2 ml followed by 6 × 1 ml injections. Ewes were checked for oestrus every 8 h using different males.

2.7. Artificial insemination and collection of embryos

Ewes were inseminated in the uterus by laparoscopy using half a straw per uterine horn (0.125 ml sample, 3.5×10^7 sperm), 64 h after sponge removal, as recommended for intrauterine inseminated ewes [50].

Embryos were collected via mid-ventral laparatomy 7 days after the onset of oestrus. Ewes were anesthetized by i.m. injection with 0.4 ml 2% xylazine, and 5 min later, 10 ml of sodium thiopental (20 mg/ml) (Thiobarbital, Braun Medical, Jaen, Spain) was administered by i.v. injection. Both uterine horns were exposed and flushed with pre-warmed

phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) and antibiotics (penicillin and streptomycin). The reproductive tract was flushed with a 2.5% heparin solution in saline before closure in order to minimize post-operative abdominal adhesions.

2.8. Experimental design and presentation of data

A strict criterion for fertility was established as it was assessed as the percentage of embryos recovered from the uterine horns six days after insemination, with respect to the total number of corpora lutea counted in the ovaries. We established a strict criterion of fertility evaluation as only the percentage of recovered embryos was considered, but not the recovered oocytes and embryos. The number of corpora lutea in the ovaries indicates the number of ovulations produced, while recovered embryos and oocytes indicate the number of successful or unsuccessful fertilizations, respectively.

2.9. Statistical analysis

Results are shown as the means (±S.E.M.) of the number of samples indicated in each case. Statistical analyses were carried out using 11.5 SPSS software. Correlations between sperm parameters and in vivo fertility were calculated using Pearson's coefficient.

Stepwise multiple regression analysis was used to determine the variation in fertility and to estimate the regression equations to predict fertility percentage on the basis of sperm parameters. ANOVA was performed to determine whether there were significant differences between both types of samples in some of the above-mentioned parameters.

3. Results

3.1. Semen quality of control samples and in vivo fertility

The mean fertility of the eight control ejaculates was 60% (range: 25–76%). There was no significant correlation between the mean semen quality parameters and in vivo fertility (Table 1).

The parameters derived from the CCCD profiles included viable cells (TV and fractions V_1, V_2, and V_3), LV and H. LV as well as TV showed the highest correlation with fertility. The correlation between LV and fertility was negative, as expected, and very significant ($r = -0.914$, $P < 0.01$). The LV value ranged from 2.18 to 68.57%, with an average value of 26.6%. TV was positively correlated with fertility ($r = 0.803$, $P < 0.05$), mean value 40.06%. Heterogeneity was positively correlated ($r = 0.391$) with fertility, but not significantly (range = 20–47%).

As reported previously, CCCD yields highly reproducible results under identical conditions [38,39]. Four representative CCCD profiles of the eight control samples are shown in Fig. 2. Fertility, TV, LV and H values are shown in Table 2. The correlation between fertility and H was not significant but the latter was included in Table 2, since it can be used to predict fertility, as reported by Pérez-Pé et al. [43]. The LV values of the

Table 1
Mean values of semen quality parameters and correlations with fertility for sperm control samples following IU insemination ($n = 8$)

Parameters	Mean ± S.E.M.	r	P value
Standard semen analyses			
Individual motility	50.00 ± 3.69	0.222	ns
Initial viability	69.54 ± 2.81	0.264	ns
HOS-test	41.93 ± 5.34	0.199	ns
Loaded viability	48.94 ± 2.63	0.420	ns
	118.50 ± 16.22	0.340	ns
Post-CCCD analyses			
TV (%)	40.06 ± 5.57	0.803	<0.05
V_1(%)	21.52 ± 6.57	0.608	ns
V_2(%)	53.79 ± 5.83	0.518	ns
V_3(%)	50.53 ± 7.58	0.551	ns
LV (%)	26.63 ± 8.72	−0.914	<0.01
Heterogeneity	34.88 ± 2.79	0.391	ns

TV: total viability; V_1: viability chambers 0–9; V_2: viability chambers 10–19; V_3: viability chambers 20–29; LV: loss of viability; ns: not significant.

representative samples ranged from 19 to 68.5%, and H from 20 to 40% (Table 2). Samples with the highest fertility had the lowest LV and highest H. Although the sample in Fig. 2c had the highest survival capacity (lowest LV, 19%, Table 2) it did not have the highest fertility rate, possibly due to its relatively low heterogeneity (33%, Table 2). Likewise, the sample in Fig. 2d had the lowest H (20%) and highest LV (68.5%, Table 2), which accounts for the lowest fertility rate. There was a significant correlation with in vivo fertility when LV and H are taken into account, providing a predictive equation for fertility with a very high correlation coefficient ($r = 0.967$) and level of significance ($P < 0.005$):

$$\frac{\mathrm{E}}{\mathrm{CL}} = 52.546 - 0.594\,\mathrm{LV} + 0.665\,\mathrm{H}$$

Each independent variable (LV and H) had a significant effect on fertility ($P < 0.05$).

Table 2
Field fertility (E/CL), recovered total viability (TV), loss of viability (LV) and heterogeneity (H) values corresponding to centrifugal countercurrent distribution profiles for the sperm samples shown in Figs. 1 and 2

Sample	E/CL (%)	TV (%)	LV (%)	H (%)
Control (Fig. 1)				
a	63.22	50.26	22.58	40
b	61.54	31.76	25.40	40
c	57.89	48.92	19.30	33
d	25.00	9.20	68.57	20
Selected (Fig. 2)				
a	77.78	58.47	2.47	47
b	71.67	34.72	17.46	67
c	69.04	35.61	5.20	47
d	39.77	28.28	34.88	27

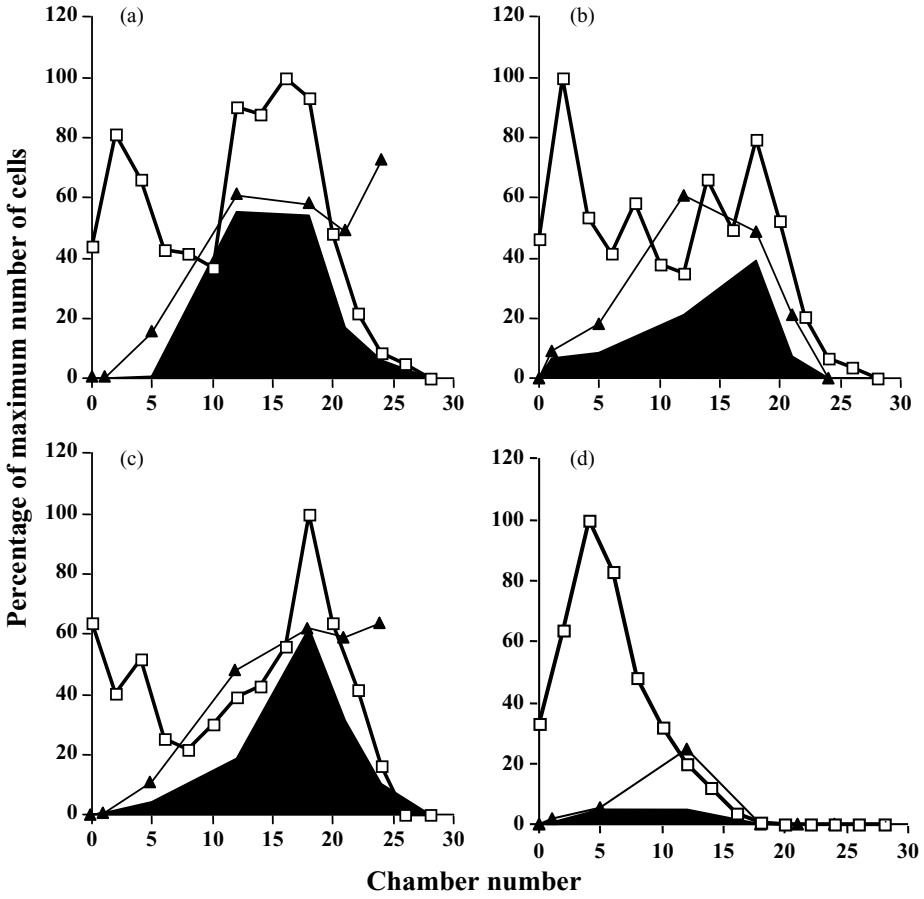

Fig. 2. Representative centrifugal countercurrent distribution (CCCD) profiles of sperm control samples corresponding to (a) 63.2%, (b) 61.5%, (c) 57.8%, and (d) 25% of fertility. (□), Percentage of maximum of cells; (▲), percentage of viable cells; dark area, distribution of total viable cells.

3.2. Semen quality of selected samples and in vivo fertility

Another objective of this work was to investigate the reproductive efficiency of sperm samples selected by a dextran/swim-up procedure [47], and to test whether CCCD can be used to estimate the fertilizing potential of these samples. For this purpose, intrauterine insemination was performed using sperm samples selected by the swim-up procedure (SS), and CCCD analyses were carried down.

The mean fertility value of 13 SS samples obtained from 13 different semen pools) was 63% (range: 25–86%). The mean values of semen quality parameters are shown in Table 3. Again, only the parameters derived from the CCCD profiles were highly correlated with fertility. The viable cells recovered in the right chambers (V_3), LV and H were significantly correlated with fertility ($P < 0.05$).

Table 3
Mean values of semen quality parameters and correlations with fertility for sperm selected samples following IU insemination ($n = 13$)

Parameters	Mean ± S.E.M.	r	P value
Standard semen analyses			
Individual motility	68.21 ± 2.60	0.336	ns
Initial viability	75.64 ± 3.13	0.242	ns
HOS-test	52.00 ± 8.87	0.241	ns
Loaded viability	54.308 ± 2.18	0.147	ns
Post-CCCD analyses			
TV (%)	43.60 ± 2.84	0.361	ns
V_1(%)	27.83 ± 3.60	−0.013	ns
V_2(%)	53.38 ± 3.02	0.270	ns
V_3(%)	46.34 ± 4.72	0.679	<0.05
LV (%)	19.19 ± 5.59	−0.778	<0.05
Heterogeneity	41.15 ± 3.23	0.572	<0.05

TV: total viability; V_1: viability chambers 0–9; V_2: viability chambers 10-19; V_3: viability chambers 20–29; LV: loss of viability; ns: not significant.

Four representative CCCD profiles of the 13 SS are shown (Fig. 3). The combined effect of LV and H on fertility is apparent from the post-CCCD parameters (Table 2). The samples with the highest in vivo fertility (78%, Fig. 3a) had extremely low LV (2.5%) and high H (47%). The sample in Fig. 3b also had high fertility, possibly because the high H (67%, Table 2) could compensate for a relatively high LV (17%, Table 2). The sample in Fig. 3c had a similar fertility rate with lower H (47%), which could be compensated by lower LV (5%). The sample in Fig. 3d had the highest LV and the lowest H, which accounted for the lowest fertility results.

A predictive equation was obtained taking into account LV and H, with a very high correlation coefficient ($r = 0.801$) and level of significance ($P < 0.01$).

$$\frac{\mathrm{E}}{\mathrm{CL}} = 59.344 - 0.647\,\mathrm{LV} + 0.367\,\mathrm{H}$$

3.3. Comparison between control and selected samples

ANOVA was performed to determine whether there were significant differences between samples (control and SS) in some of the above-mentioned parameters.

Table 4
Fertility rate, recovered total viability (TV), loss of viability (LV) and heterogeneity (H) corresponding to CCCD profiles for analyzed samples. Mean values ± S.E.M. of the number of samples indicated in brackets

Sample	Fertility (%)	TV (%)	LV (%)	H (%)
Control (8)	59.89 ± 5.84	40.06 ± 5.57	26.63 ± 8.72	34.88 ± 2.79
Selected (13)	63.02 ± 5.11	43.60 ± 2.84	19.19 ± 5.59	41.15 ± 3.23

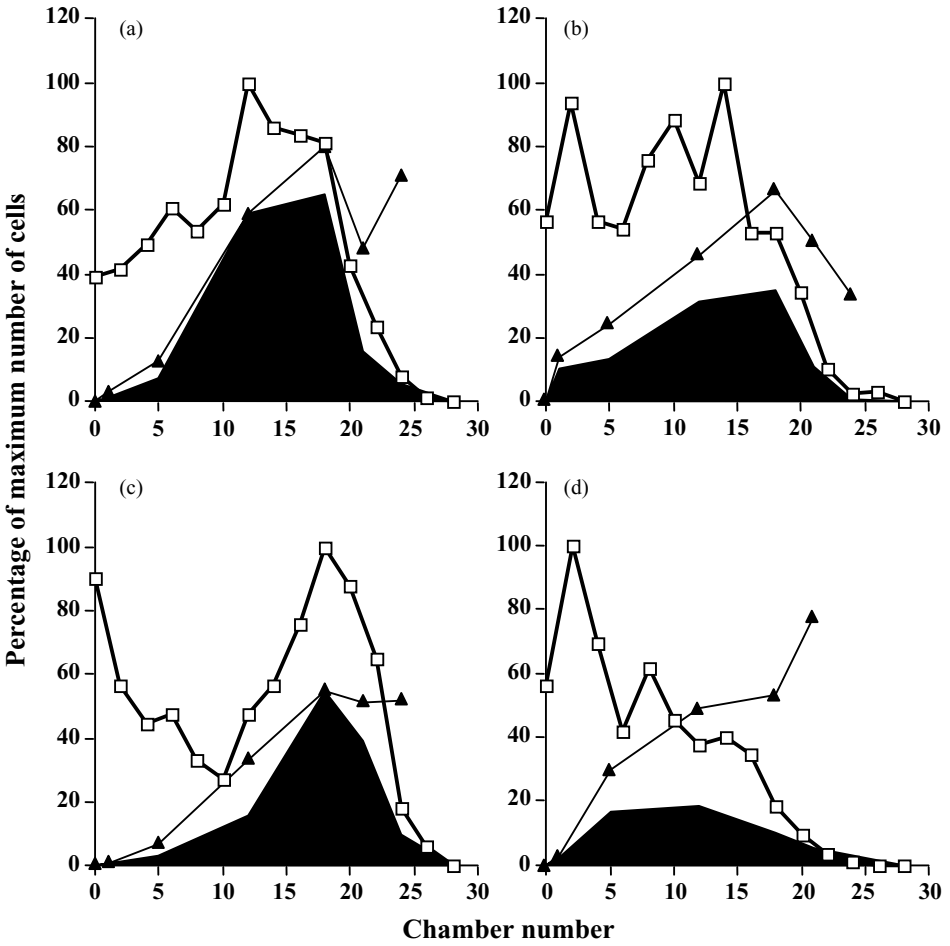

Fig. 3. Representative centrifugal countercurrent distribution (CCCD) profiles of sperm selected samples corresponding to (a) 77.7%, (b) 71.6%, (c) 69.04%, and (d) 39.77% of fertility. (□), Percentage of maximum of cells; (▲), percentage of viable cells; dark area, distribution of total viable cells.

The mean fertility rate was 59.89 ± 5.84 and 63.02 ± 5.11 for control and swim-up selected samples, respectively (Table 4). Similarly, TV and H were higher and LV was lower in swim-up samples than controls, although the differences were not significant. Likewise, no significant differences were found between samples regarding standard or post-CCCD parameters.

4. Discussion

The spermatozoa in an ejaculate are quite heterogeneous, even with regards to fertilizing capacity. Heterogeneity denotes the functional variability in the response of spermatozoa,

which is known to be an inherent characteristic [49,51] and closely related with successful fertilization [34]. Heterogeneity is the result of the coexistence of different sub-populations of sperm in a typical mammalian ejaculate and affects fertilizing ability.

Previous studies have shown that CCCD can be used to assess the intrinsic heterogeneity of a ram ejaculate [49] and changes in heterogeneity after different processes such as freezing–thawing [39], capacitation or induction of the acrosome reaction [31,41], or a selection procedure [52]. The ability of the CCCD to reveal sperm heterogeneity is based on the capacity of this technique to detect subtle changes in the cell surface. Since the fertilization process involves steps with changes of the spermatozoa surface, CCCD could be useful to predict the fertilizing potential of an ejaculate based on heterogeneity and other parameters derived from the profile. In a previous experiment, we analyzed the relationship between heterogeneity and field fertility in ram semen after cervical AI [43]. The predictive equation for field fertility had a highly significant correlation coefficient ($r = 0.488$), demonstrating that this technique is useful to estimate the fertilizing potential of a given semen sample.

The first goal of the present study was to determine whether controlling several external factors that affect fertility could increase the correlation coefficient between post-CCCD parameters and fertility. For this, we performed a similar study with controlled and superovulated ewes located on the same farm and inseminated using the same intrauterine technique. Therefore, comparisons of reproductive results would let us find differences due to the inseminated sample. We obtained a predictive equation for fertility with a significant and higher correlation coefficient between LV and H with fertility. Among all post-CCCD parameters, LV had the highest correlation with fertility. It was negative, as expected, and very significant ($r = -0.914$, $P < 0.01$). The LV could be due to the CCCD process that could cause some sperm damage, specifically in cells more liable to suffer membrane alterations. Thus, LV could reflect the resistance of spermatozoa to stressful events, including insemination and waiting in the female genital tract. Thus, despite the relevance of heterogeneity, sperm resistance capacity should also be considered. Although one ejaculate has many cell populations at different levels of maturity (heterogeneity), the fertilizing ability of the ejaculate will be poor if survival capacity is low. These findings coincide with previous results using fresh ram ejaculates after cervical AI [43]. The regression equation obtained in this work includes sperm resistant capacity and heterogeneity (LV and H), and has a very high and significant correlation coefficient. Therefore, it can be used to predict the fertility rate of an ejaculate inseminated via the intrauterine route.

Several other authors have reported low correlation between standard semen analysis and field fertility [18,53], in addition to our previous study [43]. In our case, sperm concentration was not considered, since the same number of spermatozoa was inseminated in all cases. Moreover, the number of spermatozoa used per dose was very low (7×10^7) in order to enhance possible fertility differences, as reported elsewhere [44,45,54]. Similarly, progressive individual motility and membrane integrity have been considered as compensable seminal traits [44,54]. However, motility does not provide information about the ability to undergo the acrosome reaction or fertilize the oocyte, and only reflects the ability of sperm to reach it. The lack of correlation between initial values of motility or membrane integrity and functionality with fertility also agrees with our previous results on field fertility with fresh ram ejaculates [43]. Thus, these parameters do not appear to provide

information about the resistance of the ejaculate to stressful events, as opposed to LV, which could indicate the sperm surviving capacity.

The second goal of this work was to compare the fertilizing ability of raw semen and sperm selected samples using an intrauterine insemination protocol in controlled ewes. Previous results from our group have shown that the dextran/swim-up procedure selects high-quality ram spermatozoa, as assessed by in vitro parameters [47]. The relationship between higher in vitro sperm quality and the reproductive efficiency of this sample had not been considered yet.

Although the fertility rate, heterogeneity and viability were higher in swim-up selected samples, no significant differences were found between samples. However, the higher fertility rate could be explained by higher total viability and heterogeneity, along with lower loss of viability in selected samples. All these higher parameters would result in higher fertility.

In conclusion, CCCD could be useful to estimate the fertilizing potential of intrauterine injected semen. It would be especially interesting to apply this technique in large breeding centres to discard ejaculates with low reproductive efficiency and to determine the suitability of particular sires in artificial insemination programmers.

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