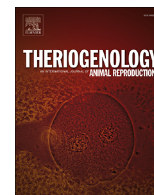
Theriogenology 122 (2018) 1–8

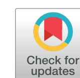
Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com



# Improved bovine *in vitro* embryo production with sexed and unsexed sperm selected by chemotaxis



Esteban Mauricio Dominguez [a, b], Ayelen Moreno-Irusta [a, b], Héctor Alejandro Guidobaldi [a, b], Huberto Tribulo [c], Laura Cecilia Giojalas [a, b, *]

[a] *Centro de Biologia Celular y Molecular, Facultad de Ciencias Exactas, Fisicas y Naturales (FCEFN), Universidad Nacional de Córdoba (UNC), Córdoba, Argentina*

[b] *Instituto de Investigaciones Biologicas y Tecnologicas, Consejo de Investigaciones Científicas y Técnicas (CONICET), UNC-FCEFN, Córdoba, Argentina*

[c] *Instituto de Reproducción Animal Córdoba, Estación Gral. Paz, Paraje Pozo del Tigre, Córdoba, Argentina*

A R T I C L E  I N F O

*Article history:*
Received 21 February 2018
Received in revised form
24 August 2018
Accepted 26 August 2018
Available online 30 August 2018

*Keywords:*
Bovine sperm
Sperm chemotaxis
Sperm capacitation
Sperm DNA fragmentation
*In vitro* fertilization

A B S T R A C T

Assisted reproductive techniques (ART) have been widely used in farm animals in the last decades. Sexed cryopreserved spermatozoa, ovum pick up, *in vitro* embryo production and transfer constitute the ART that have revolutionized the dairy industry. However, the efficiency of some of these techniques is still low due in part to sperm quality, which influences fertilization, embryo development and implantation. The Sperm Selection Assay (SSA), based on sperm chemotaxis towards progesterone, provides a sperm subpopulation enriched with spermatozoa that are capacitated, with intact DNA and low level of oxidative stress. Since the SSA selects a sperm subpopulation at optimum physiological state, the application of the SSA may improve the efficiency of the current ART. The aim of this study was to adapt the SSA for unsexed and sexed bovine frozen-thawed semen samples, and then to test whether sperm selection by the SSA improves the cleavage rate of bovine embryos *in vitro*. The optimal SSA conditions to obtain the higher sperm accumulation percentage given by chemotaxis were the same for both unsexed and sexed semen samples. Thus, sperm accumulation in W2 was significantly higher when: 2 million sperm per mL were placed in W1 (unsexed samples: $12 \pm 1\%$, $p = 0.002$; sexed samples: $14 \pm 3\%$, $p = 0.02$); 1 pM progesterone was placed in W2 (unsexed sample: $9 \pm 1\%$, $p = 0.009$; sexed samples: $11 \pm 2\%$, $p = 0.02$); and to incubate the SSA device for 10 min (unsexed samples: $17 \pm 2\%$, $p = 0.007$; sexed samples: $10 \pm 1\%$, $p = 0.004$). We found that the quality of spermatozoa recovered from W2 in unsexed and sexed semen was enhanced. Thus, the capacitation index was significantly increased (unsexed samples: $1.75 \pm 0.1$, $p = 0.0001$; sexed samples: $1.76 \pm 0.2$, $p = 0.004$), while DNA fragmentation index was significantly decreased (unsexed samples: $0.33 \pm 0.07$, $p = 0.0003$; sexed samples: $0.32 \pm 0.04$, $p = 0.002$). Moreover, the cleavage index of oocytes fertilized with either unsexed or sexed SSA-selected sperm was significantly improved (unsexed samples: $3.2 \pm 0.4$, $p = 0.0001$; sexed samples: $2.3 \pm 0.33$, $p = 0.03$). Thus, we show that the SSA can be used to recruit a bovine sperm subpopulation at optimal functional state regardless of whether the sample is previously sexed, and that this optimal state improves bovine embryo cleavage rate.

© 2018 Elsevier Inc. All rights reserved.

## 1. Introduction

In the last decades, the application of ART has notably improved animal production, but these techniques are still far from being efficient and low cost. The dairy industry is a competitive market where animals with the highest genetic potential are continuously selected for ART. Oocyte recovery is optimized either by ovum pick up [1] from pregnant cows until 3 to 4 gestation months [2], or by laparoscopic ovum pick up from 2 to 3 months old prepubescent calves [3]. These enhanced oocyte technologies are used to produce embryos *in vitro*, which are later transferred to receptive cows. These techniques, combined with sexed spermatozoa samples, are preferred to obtain an offspring of female embryos, which are in

* Corresponding author. Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales, Centro de Biología Celular y Molecular, Av. Velez Sarsfield 1611, X5016GCA - Córdoba, Argentina.

*E-mail address:* lgiojalas@unc.edu.ar (L.C. Giojalas).

https://doi.org/10.1016/j.theriogenology.2018.08.023
0093-691X/© 2018 Elsevier Inc. All rights reserved.

high demand in the dairy industry.

Despite the potential benefits of the application of sexed semen technology in bovine, the percentage of embryos obtained with sexed spermatozoa is lower than the one obtained with unsexed spermatozoa [4]. Moreover, the pregnancy rate of cows artificially inseminated with sexed semen is lower than the rate obtained with unsexed spermatozoa [5–7]. These data suggest that the spermatozoa sexing process may alter sperm quality by inducing DNA damage due to the use of fluorescent dyes and UV light stimulation during sperm sorting [8–10]. Recent literature reported that sexed spermatozoa artificially inseminated lead to a reduction of *in vivo* fertility, but the blastocyst rate is similar to that obtained with unsexed semen under *in vitro* fertilization [11]. In any event, it would be advantageous if optimally functioning spermatozoa could be separated from those with damaged DNA before using them for ART.

Recently, a sperm selection assay (SSA) has been developed in our laboratory. This technique enables the separation of a sperm subpopulation enriched in fertilizing spermatozoa (called 'capacitated sperm'), which present low DNA fragmentation and oxidative stress [12]. Thus, the SSA facilitates the recruitment of physiologically optimal spermatozoa via chemotaxis (a cell chemical guidance) [13,14] towards very low doses of progesterone (P; picomolar range), a hormone secreted by the cumulus cells by the time of ovulation [15–19]. Such selected sperm population at optimum physiological state might support not only fertilization but also early embryo development and implantation [20,21]. Since the spermatozoa selected by SSA present intact DNA, the application of this assay to bovine samples will result in physiologically competent unsexed and sexed samples. The aim of this study was to adapt the SSA, originally developed for human spermatozoa, for unsexed and sexed bovine semen samples, and to test whether by selecting unsexed or sexed spermatozoa by chemotaxis the cleavage rate is improved in bovine embryos *in vitro*.

Here we show that both unsexed and sexed frozen-thawed sperm subpopulations selected by chemotaxis by the SSA are enriched in capacitated spermatozoa which present lower DNA fragmentation, and that the use of these selected spermatozoa in *in vitro* fertilization improves bovine embryo cleavage rate.

## 2. Materials and methods

### 2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Lois, MO, USA), unless otherwise indicated.

### 2.2. Sperm preparation

Experiments were carried out with cryopreserved bovine semen samples. The unsexed samples were provided by Instituto de Reproducción Animal de Córdoba (Córdoba, Argentina), and the sexed samples were purchased from GENPRO (Santa Fe, Argentina). Frozen unsexed samples were obtained from two Holando Argentino and two Braford bulls ($20 \times 10^6$ sperm/mL, 0.5 mL per straw). Frozen sexed samples were obtained from two Holando Argentino bulls ($2 \times 10^6$ sperm/mL, 0.25 mL per straw). Thus, replicates in each set of experiments were performed with different ejaculates from different bulls. On the day of the experiment, semen samples were thawed at 37 °C for 20–30 s. Then, the seminal plasma and cryoprotectant in unsexed samples were removed by the migration-sedimentation technique [22], and by simple centrifugation in sexed samples [23]. Spermatozoa were then diluted in Sp-TALP medium [24], supplemented with 3 mM pentoxifylline [25,26], and further incubated for 1 h at 38.5 °C, in an atmosphere of 5% $CO_2$ in air. The percentage of motile spermatozoa was determined at the end of the incubation time by video microscopy and image analysis [27] with a phase contrast microscope (Nikon Instruments Inc., NY,USA) and NIS elements software (4.30.01 DU1). The movement of spermatozoa was digitally recorded for 10 s at 30 Hz at 10×, and videos were analyzed by FIJI software using the plug-in motility tool. In every experiment, only samples exhibiting ≥90% of motile spermatozoa were used.

### 2.3. Sperm selection assay

The SSA was performed according to Gatica et al. [11]. Briefly, the device used in the SSA consists of two wells; the sperm suspension is placed in one well (W1), and the attractant solution (or culture medium as negative control) is placed in the other (W2). The two wells are connected by a tube, in which a gradient is formed as the attractant solution diffuses from W2 to W1, thus inducing sperm chemotaxis. Either bovine follicular fluid (FF) or P were used as attractant [28,29] as described in the results section. After loading, the device was incubated at 38.5 °C, in an atmosphere of 5% $CO_2$ for different time periods, as specified in the results section. At the end of the assay, the net sperm accumulation in W2 was determined. This parameter was calculated as the difference in the percentage of spermatozoa recovered from W2 with or without attractant solutions.

### 2.4. Sperm capacitation

The ability of spermatozoa to undergo the induced acrosome reaction was determined as an indirect indicator of the level of capacitated spermatozoa [27]. The acrosome status was detected by *in vivo Pisum sativum agglutinin* fluorescein staining (PSA-FITC) [30,31], whereas a similar procedure was also employed in bull spermatozoa samples [32]. Briefly, the sperm suspension was divided into two aliquots, which were incubated with or without 8 μM of the calcium ionophore A23187 and 10 μg/mL PSA-FITC in culture medium, at 38.5 °C for 30 min. Then, the sperm suspensions were fixed in 2% formaldehyde in PBS for 20 min at room temperature, washed by centrifugation in distilled water, and the pellet was let to air dry on a slide. The status of the acrosome was observed at 1,000× under a fluorescence microscope (*Olympus* BX 50, Center Valley, USA). Under the microscope, the acrosome-reacted spermatozoa present a green fluorescent acrosome, while the ones with intact acrosome are unlabeled. The percentage of capacitated spermatozoa was determined as the percentage difference between induced and spontaneous acrosome-reacted spermatozoa (n = 200 cells). A capacitation index was determined as the ratio of the percentage of capacitated spermatozoa obtained before and after the SSA, with or without progesterone, to the percentage of capacitated spermatozoa before the SSA (control without sperm selection).

### 2.5. Sperm DNA fragmentation

The level of DNA fragmentation was evaluated by the Sperm Chromatin Dispersion assay (SCD), as described by Fernandez et al. [33], with minor modifications [34]. The sperm suspension was mixed with 1% low-melting point aqueous agarose at 37 °C, and then 50 μl aliquots were placed on a slide pre-coated with 0.65% standard agarose and dried at 80 °C. The sample was covered with a coverslip and let solidify at 48 °C for 30 min. Then, coverslips were carefully removed, and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) at 22 °C for 7 min, in the dark. The slides were incubated in a neutralizing and lysis solution 1 (0.4 M Tris, 5% 2-

Mercaptoethanol, 1% SDS, 50 mM EDTA, pH 7.5) for 10 min at room temperature, in order to stop DNA denaturation and for protein removal, followed by incubation in neutralizing and lysis solution 2 (0.4 M Tris, 2 M NaCl, 1% SDS, pH 7.5) for 5 min at room temperature. Slides were thoroughly washed in Tris borate-EDTA buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 7.5) for 2 min, and dehydrated in sequential ethanol series. Dried sperm samples were stained with Hoechst 33258 (1 μg/mL). Images of sperm heads from at least 200 cells per treatment were obtained with a fluorescence microscope (Olympus BX 50, Olympus, Center Valley, USA) coupled to a Nikon digital camera (Nikon Instruments Inc., NY, USA). The halo area observed around the head of each spermatozoon was measured using the FIJI program. Sperm heads were classified according to the size of the halo ($\mu m^2$). Four halo patterns were identified: large, $60 \pm 10\ \mu m^2$; medium, $30 \pm 10\ \mu m^2$; small, $10 \pm 10\ \mu m^2$, and without halo. The first two patterns include sperm without DNA fragmentation, while the other two patterns include fragmented DNA cells. The DNA fragmentation index was determined as the ratio of the percentage of fragmented DNA spermatozoa obtained before and after the SSA, with or without P, to the percentage of fragmented DNA spermatozoa before the SSA (control without sperm selection).

### 2.6. Oocyte collection and in vitro maturation

Ovaries from mature cows were obtained from an abattoir (Bustos y Beltran SA, Córdoba, Argentina), preserved at 22–25 °C in a 0.9% NaCl solution supplemented with 100 IU/mL penicillin and 100 mg/mL streptomycin sulfate, and transported to the laboratory within 6 h. The ovaries were cleaned from surrounding tissue, and rinsed in 0.9% NaCl. Follicles under 13 mm size were aspirated with a 19G needle. Grade I, II and III COCs (cumulus-oocyte complexes) [35] were selected under a stereoscopic magnifying glass for *in vitro* maturation, rinsed three times in holding medium consisting of TCM 199 Hank's Salts (Gibco Life Technologies, USA), supplemented with 10% fetal calf serum (FCS), 0.3% faf-BSA, and 0.05 mg/mL gentamycin. Then, the COCs were let matured in maturation medium consisting of TCM 199 Earle's Salts (Gibco Life Technologies, USA), plus 1% FCS, 0.01 UI/mL rhFSH (Gonal F, Merck Laboratory, USA), 0.1 mg/mL glutamine, 2.5 mg/mL sodium pyruvate, and 0.05 mg/mL μg gentamycin. Twenty COCs per 100 μl maturation medium droplets were incubated 22–24 h at 38.5 °C in an atmosphere containing 5% CO2 covered with mineral oil, in 60 × 15 mm Petri dishes.

### 2.7. In vitro fertilization and embryo culture

At the end of the maturation period, COCs were rinsed three times with IVF- SOF medium (315 mg NaCl, 26.8 mg KCl, 2 mg $KH_2PO_4$, 105 mg $NaHCO_3$, 3.25 mg Penicillin, 2.75 mg $C_3H_3O_3Na$, 400 mg faf-BSA, 4.5 mg $C_6H_{12}O_6$, 12.6 mg $CaCl_2H2O$, 23.5 μl $C_3H_5O_3Na$ [60% Syrup], 50 μg/mL heparin, in 50 mL of ultra-pure water). Then, five matured oocytes were placed in a 30 μl IVF-SOF medium droplet and covered with mineral oil, in 60 × 15 mm Petri dishes. Ten microliters of the sperm suspension were added to the droplet containing the COCs to get 1000 spermatozoa per oocyte, according to the rate at which the percentage of cleavage obtained is approximately 20%, as described for unsexed and sexed samples by Trigal et al. [36]. This experimental strategy allows to determine the magnitude of the increment in the embryo cleavage index upon sperm selection by the SSA. The gamete co-incubation time point was considered as day zero of fertilization. Gametes were co-incubated 16–18 h at 39 °C in a high humidity atmosphere enriched with 5% $CO_2$. Presumptive zygotes were vortexed for 2 min to separate cumulus cells, and washed three times in SOF-Citrate medium (314.5 mg NaCl, 26.5 mg KCl, 8.1 mg $KH_2PO_4$, 9.1 mg $MgSO_4 \cdot 7H_2O$, 105 mg $NaHCO_3$, 2.5 mg Gentamicin, 4 mg $C_3H_3O_3Na$, 150 mg faf-BSA, 1.5 mL BME, 0.5 mL MEM, 2.5 mg $C_5H_{10}N_2O_3$, 60 mg acid free HEPES, 5 mg $C_6H_5Na_3O_7$, 25 mg Myo–inositol, 65 mg sodium salt HEPES, 13.1 mg $CaCl_2 \cdot H_2O$, 15 μl $C_3H_5O_3Na$ [60% Syrup] in 50 mL of ultra-pure water). Twenty zygotes per 100 μl SOF-citrate media droplets were incubated covered with mineral oil at 38.5 °C in an atmosphere containing 5% $CO_2$ and 5% $O_2$ in air with high humidity.

### 2.8. Embryo cleavage

At day 2, the occurrence of cleavage, indicated by the presence of two or more cells, was verified under inverted phase contrast microscopy (Olympus CK 2, Japan) at 200× magnification. The percentage of embryo cleavage was calculated as the ratio of the number of zygotes undergoing mitotic division to the total number of mature oocytes incubated with spermatozoa. To know how many times the cleavage rate was modified by fertilization of oocytes with SSA-selected spermatozoa, the cleavage rate index was calculated as the rate of the percentage of cleaved embryos obtained with spermatozoa selected by the SSA (with or without P) to the percentage of embryos obtained with spermatozoa not selected by the SSA. An embryo parthenogenetic control was performed in every experiment, and parthenogenic division was not observed.

### 2.9. Statistical analysis

Differences between treatments were determined by means of a two-way ANOVA, and *a posteriori* Tukey test performed with the Graph Pad Prism 5.01 (La Jolla, CA, USA). The parameters expressed as percentages were previously transformed to the arcsine square root of the proportion. To evaluate the correlation between the percentage of cleaved embryos and the percentage of sperm capacitation or DNA fragmentation, Pearson's correlation coefficient analyses were performed. P values less than 0.05 were considered statistically significant.

## 3. Results

The SSA experimental conditions were adapted for frozen-thawed unsexed and sexed bovine sperm samples. To define the optimum timing and sperm concentration to run the SSA, $10^{-3}$ FF (a known chemoattractant for bovine spermatozoa [29]) was placed in W2, whereas the chemotactic activity of the batch was previously determined (Suppl 1). Firstly, 2 million sperm per mL were placed in W1 and $10^{-3}$ FF in W2, incubating the SSA under different incubation times (5, 10 and 20 min; Fig. 1Ai). A 10 min incubation was enough to get a significant sperm accumulation (unsexed: $p = 0.007$, sexed: $p = 0.004$; Fig. 2A). Then, different sperm concentrations were placed in W1 (1, 2 and 3 million sperm per mL) and $10^{-3}$ FF in W2, incubating the SSA device for 10 min (Fig. 1Aii). When 2 million sperm per mL were placed in W1 a significant sperm accumulation was observed in W2 (unsexed: $p = 0.002$, sexed: $p = 0.02$; Fig. 2B). Under these experimental conditions, we next performed a dose response experiment to verify whether unsexed and sexed bovine spermatozoa also chemotactically orient their movement towards a concentration gradient of P (Fig. 1Aiii). When 1 pM P was placed in W2, a significant increase in the level of sperm accumulation in W2 was observed (unsexed: $p = 0.009$, sexed: $p = 0.02$; Fig. 2C). No statistically significant differences were observed between unsexed and sexed samples for each set of experiments ($p = 0.08$, $p = 0.6$, $p = 0.09$, corresponding to Fig. 2A, B and C, respectively).

Sperm chemotaxis towards 1 pM P was further verified in

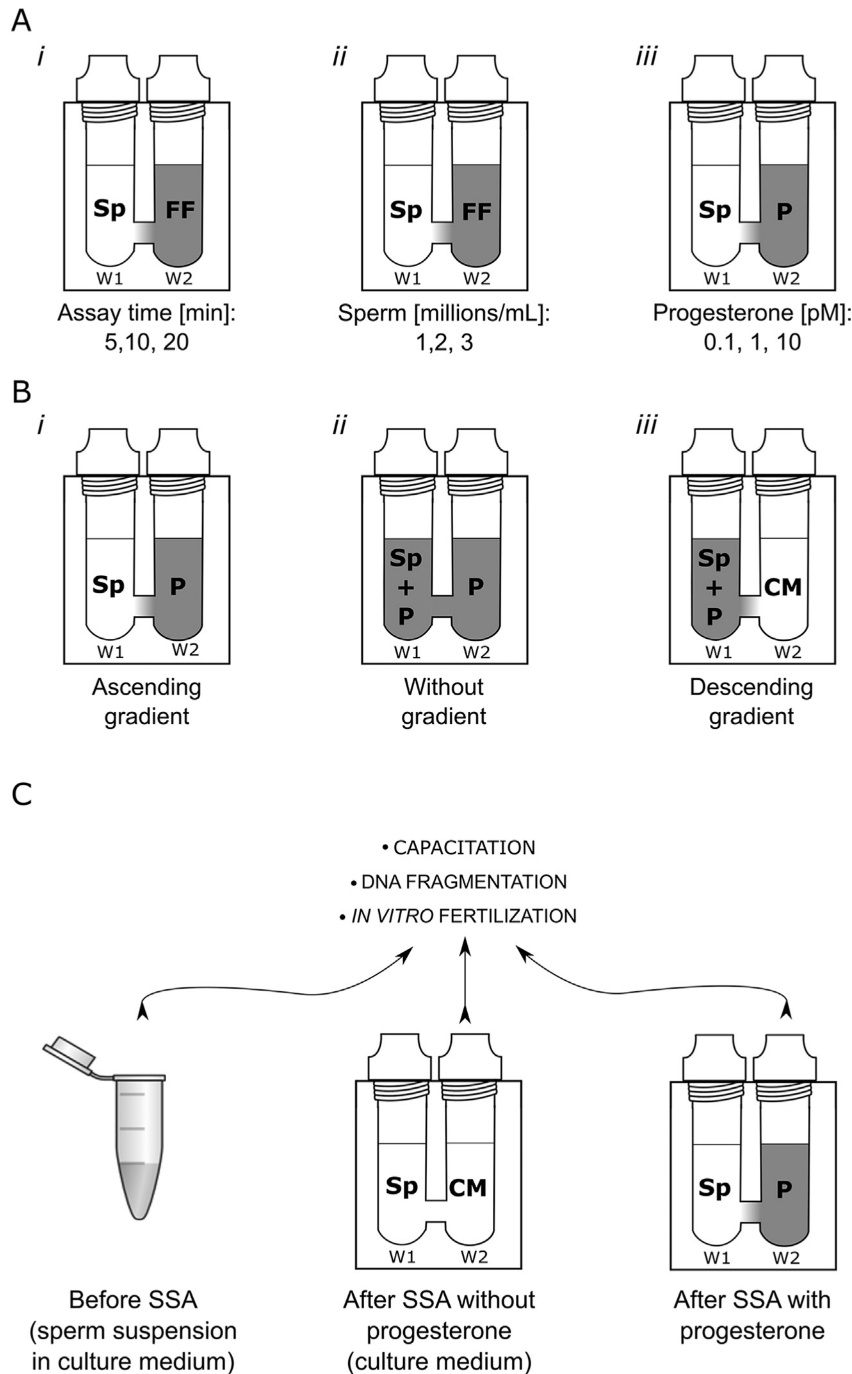



**Fig. 1. Adaptation of the experimental conditions to apply the SSA to frozen-thawed bovine spermatozoa. Ai**, 2 million sperm per mL were loaded in well 1 (W1), and $10^{-3}$ FF were added to well 2 (W2), and the SSA device was incubated for 5, 10, or 20 min; **Aii,** 1, 2, or 3 million spermatozoa per mL were placed in W1, and $10^{-3}$ FF in W2, and the SSA was incubated for 10 min; **Aiii,** 2 million sperm per mL were placed in W1, and 0.1, 1, or 10 pM progesterone (P) was added to W2 for 10 min. **Bi,** 2 million sperm per mL were placed in W1, and 1 pM P in W2, generating an ascending P gradient from W1 to W2. **Bii,** 2 million sperm per mL were loaded to W1, and 1 pM P were added to both wells, and thus it spread to the connecting tube between them, creating no attractant gradient; **Biii,** 2 million sperm per mL and 1 pM P were placed in W1, while culture medium without attractant was placed in W2, generating a descending gradient of P from W1 to W2. **C,** The indexes of sperm capacitation and DNA fragmentation were determined in the sperm population before performing the assay as well as in spermatozoa recovered from W2 after the SSA, with or without P. Embryo cleavage induced in oocytes fertilized with spermatozoa from each of the three groups was also measured. Sp: spermatozoa, FF: follicular fluid, P: progesterone, CM: culture medium.

unsexed and sexed semen samples by performing several controls of the SSA, loading the hormone in different combinations (Fig. 1Bi-iii). Since chemotaxis is dependent on the gradual distribution of the attractant molecule, when P was homogeneously distributed along the SSA device no gradient formed between wells, no sperm accumulation was observed. Conversely, when the attractant was placed together with spermatozoa in W1, the cells sensed a descending gradient of P (from W1 to W2), and consequently, they came back to W1 and accumulate there, causing a significant depletion of spermatozoa in W2 (unsexed: $p = 0.0001$, sexed: $p = 0.0006$; Fig. 2D). A further rationale for these experimental controls can be seen in Giojalas et al. [13]. No statistically significant

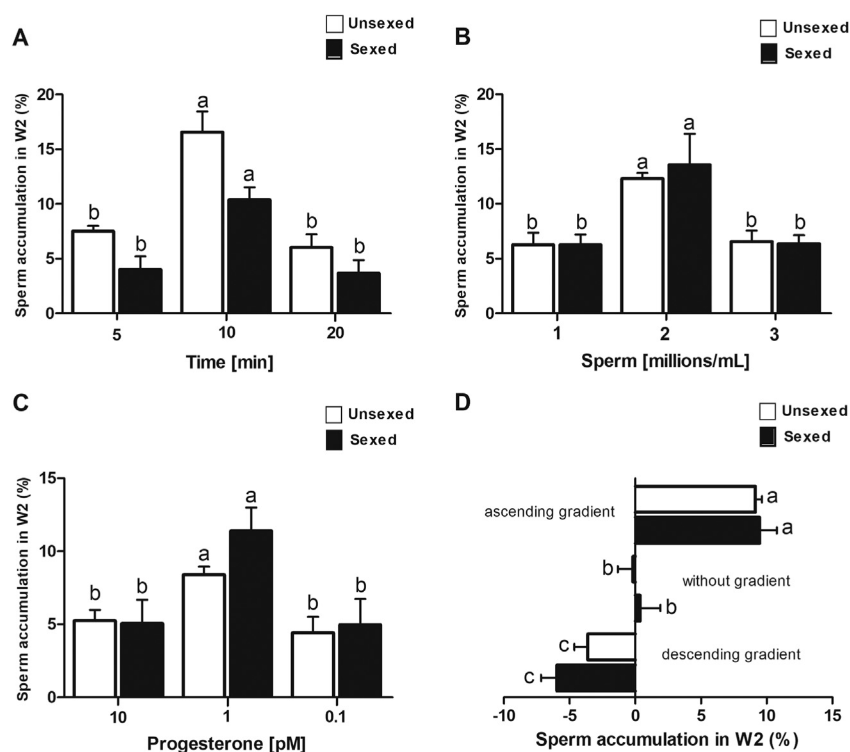



**Fig. 2. Experimental conditions to apply the SSA with frozen-thawed unsexed and sexed bovine semen samples. A**, Percentage of sperm accumulation in W2 as a function of SSA incubation time; **B**, sperm concentration loaded in W1; **C**, progesterone concentration added to W2; and **D**, experimental controls to verify sperm selection mediated by chemotaxis. Follicular fluid was used as the chemoattractant in experiments shown in A and B, while the other experiments were performed with progesterone. White and black bars represent unsexed and sexed sperm samples, respectively. Data are expressed as the mean + SEM of independent experiments performed with different ejaculates from different bulls (three to five experiments were performed with unsexed samples, and four to six with sexed samples). Different letters indicate statistically significant differences between treatments of the same sperm samples (sexed or unsexed).

differences were observed between unsexed and sexed samples ($p = 0.3$). In summary, the optimum conditions to perform the SSA with frozen-thawed unsexed and sexed sperm samples are to place 2 million sperm per mL in W1 and 1 pM P in W2, incubating the SSA device for 10 min, conditions that were applied in the following experiments.

Next, we verified the efficiency of the SSA to recruit the competent unsexed and sexed bovine spermatozoa. After incubating spermatozoa under capacitating conditions, the samples were divided in three groups: sperm not selected by SSA ("Before SSA"), SSA-selected sperm in the presence of P (after SSA + P), and SSA-selected sperm in absence of P (after SSA −P), and the indexes of sperm capacitation and DNA fragmentation were determined (Fig. 1C). We found that the index of capacitated spermatozoa recovered from W2 in the presence of P was significantly higher than the index without P, or before SSA, in both unsexed and sexed samples (unsexed: $p = 0.0001$, sexed $p = 0.004$; Fig. 3A). Moreover, the level of spontaneous acrosome reaction was low enough to visualize the significant increment in induced AR caused by A23187 and did not differ between treatments (Suppl Table 1). In addition, the DNA fragmentation index was significantly lower after the SSA in the presence of P (unsexed: $p = 0.0003$, sexed: $p = 0.002$; Fig. 3 B). No statistically significant differences were observed between unsexed and sexed samples ($p = 0.69$ and $p = 0.76$, respectively).

Then, unsexed and sexed spermatozoa before SSA or recruited with or without P in the SSA device, were used to fertilize oocytes under *in vitro* conditions, determining the cleavage index (Fig. 1C). When the oocytes were fertilized *in vitro* with spermatozoa chemotactically selected by P in the SSA, the cleavage index was significantly improved compared to those oocytes fertilized with spermatozoa before the SSA or those used in the SSA without P (unsexed: $p = 0.0001$, sexed: $p = 0.03$; Fig. 3C). No statistically significant differences were observed between unsexed and sexed samples ($p = 0.49$). The total number of oocytes and cleaved embryos per treatment are shown in Table 1. The percentage of sperm capacitation and DNA fragmentation determined in the population before and after the SSA, with and without P, was correlated with the percentage of cleaved embryos, for unsexed and sexed sperm samples. The level of sperm capacitation was positively and significantly correlated with the embryo cleavage rate, in frozen-thawed unsexed (Fig. 4 A) and sexed (Fig. 4 B) spermatozoa. In other words, the higher the level of capacitated sperm, the higher the embryo cleavage rate. The level of sperm DNA fragmentation was inversely correlated to the embryo cleavage rate in frozen-thawed unsexed (Fig. 4C) and sexed (Fig. 4 D) spermatozoa; thus, the lower the level of fragmented DNA spermatozoa, the higher the embryo cleavage rate. The respective correlation coefficients were very similar between unsexed and sexed samples as shown in the respective figures.

## 4. Discussion

We have previously described the *in vitro* conditions in which the Sperm Selection Assay (SSA) is applied to human sperm samples in order to enrich the capacitated sperm population in a given sample [12]. Here, we show that the SSA can be easily adapted to be used with frozen-thawed, unsexed and sexed bovine spermatozoa, and that the selected spermatozoa present an optimum physiological state that enhances the embryo cleavage rate.

Defining the SSA experimental conditions is critical, since the

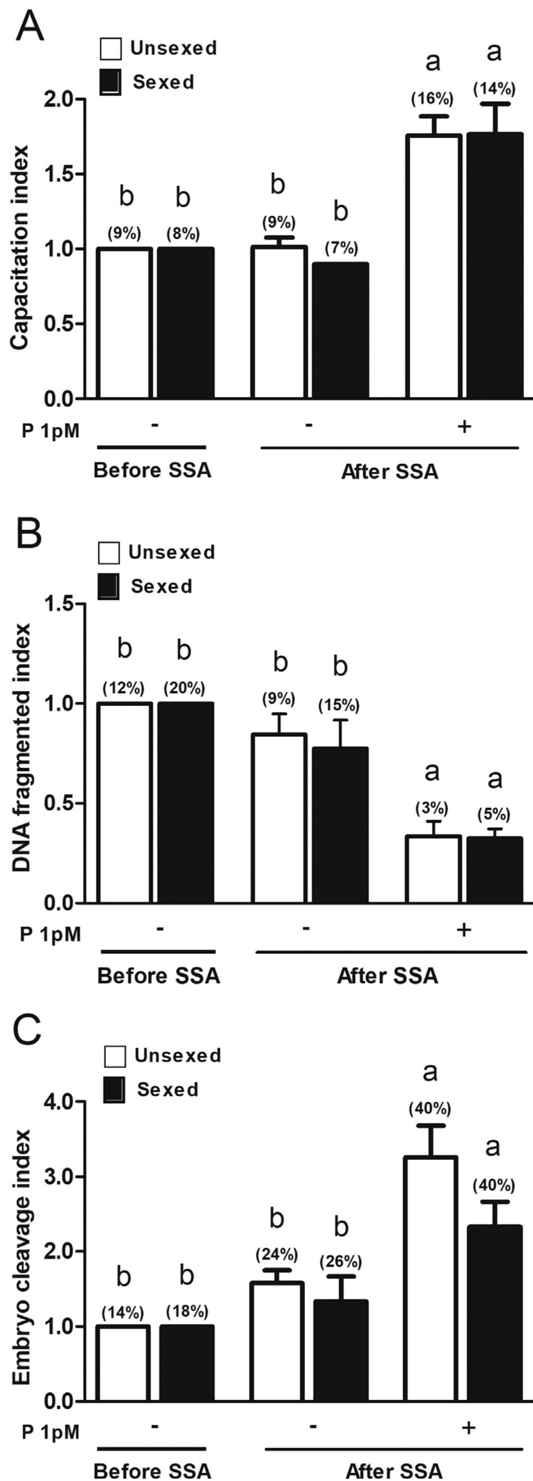



**Fig. 3. Application of the SSA to enhance embryo cleavage. A,** Sperm capacitation index; **B,** DNA fragmentation index; **C,** embryo cleavage index, before and after the SSA performed with or without progesterone. White and black bars represent unsexed and sexed sperm samples, respectively. The corresponding average percentage is shown above each bar. Data are expressed as the mean + SEM of independent experiments performed with different ejaculates from different bulls (seven performed with unsexed samples, and three with sexed samples). Different letters indicate statistically significant differences between treatments of the same sperm samples (sexed or unsexed).

**Table 1**
Number of total oocytes and cleaved embryos obtained per treatment and type of sperm sample.

| Sperm sample | Treatment | N° oocytes | N° cleaved embryos |
|---|---|---|---|
| Unsexed | Before SSA | 155 | 22 |
| | After SSA without P | 167 | 40 |
| | After SSA with P | 176 | 70 |
| Sexed | Before SSA | 112 | 20 |
| | After SSA without P | 164 | 43 |
| | After SSA with P | 167 | 67 |

Embryos were evaluated on day 2 (cleavage). For unsexed samples, one to two ejaculate per bull were used (n = 4) for seven IVF replicates. In the case of sexed samples, one to two ejaculate per bull were used (n = 2) in three IVF replicates.

efficiency of the assay is strictly dependent on assay time and on the sperm and attractant concentrations [12]. When applied to fresh human, rabbit and mouse spermatozoa, the SSA device is incubated for 20 min in the presence of the same picomolar progesterone concentration added to W2, while the sperm concentration loaded in W1 varies according to the species [37]. Here we used frozen-thawed spermatozoa, which may differently respond to the SSA, hence we first adjusted the experimental conditions to this species, which indeed, were slightly different from those observed in others. For instance, the optimum sperm concentration and the duration of the SSA are shorter than those set up for other species spermatozoa. This may be due to the addition of pentoxifylline, which stimulates sperm motility, and so more mobile spermatozoa can rapidly accumulate in the SSA well containing P. At the beginning of the study, we did not know that bovine spermatozoa could also be attracted by progesterone. Therefore, the first setting of the SSA was done with FF, a known attractant for spermatozoa of several species including bulls [29]. Once the basic conditions were set up with FF, we tested the sperm chemotactic attraction towards P. Although the optimum P concentration to perform the SSA is one order of magnitude lower than that concentration necessary to recruit spermatozoa from other species, the chemotactic response is still displayed at the picomolar range [28]. Moreover, according to the SSA controls, the sperm recruited in W2 is only mediated by chemotaxis as also observed in other species [12,38]. This result validates the elemental virtue of chemotaxis, its ability to select capacitated spermatozoa.

Gamete cryopreservation alters some cellular functions, mainly its DNA integrity [38]. Even though the *in vitro* performance of fresh semen samples is higher than that of cryopreserved samples [39], ART procedures are also performed with frozen-thawed sperm samples due to the need for gamete manipulation and transportation [40]. Here we showed that the outcome of the SSA performed with bovine frozen-thawed samples was similar to that obtained with fresh human semen samples (around 10%, expected value of capacitated-chemotactic spermatozoa at any given time) [11]. Therefore, sperm damage caused by cryopreservation procedures may be minimized by performing the SSA after thawing, probably due to its ability to recruit those cells that are in good physiological condition, as observed in frozen-thawed unsexed and sexed semen samples.

For economic reasons, sex-selected spermatozoa combined with ART are applied to spermatozoa from several species; for instance, to replace dairy cattle or to generate polo mare offspring. The most accepted sperm sexing method is cell sorting. Since this technique involves nuclear staining and laser illumination, several undesired effects may be observed in sexed sperm samples, such as reduced sperm viability and DNA damage [41]. In this context, the ability of the SSA to sort out DNA-damaged spermatozoa and to recruit capacitated spermatozoa can be appreciated equally in unsexed and sexed semen samples.

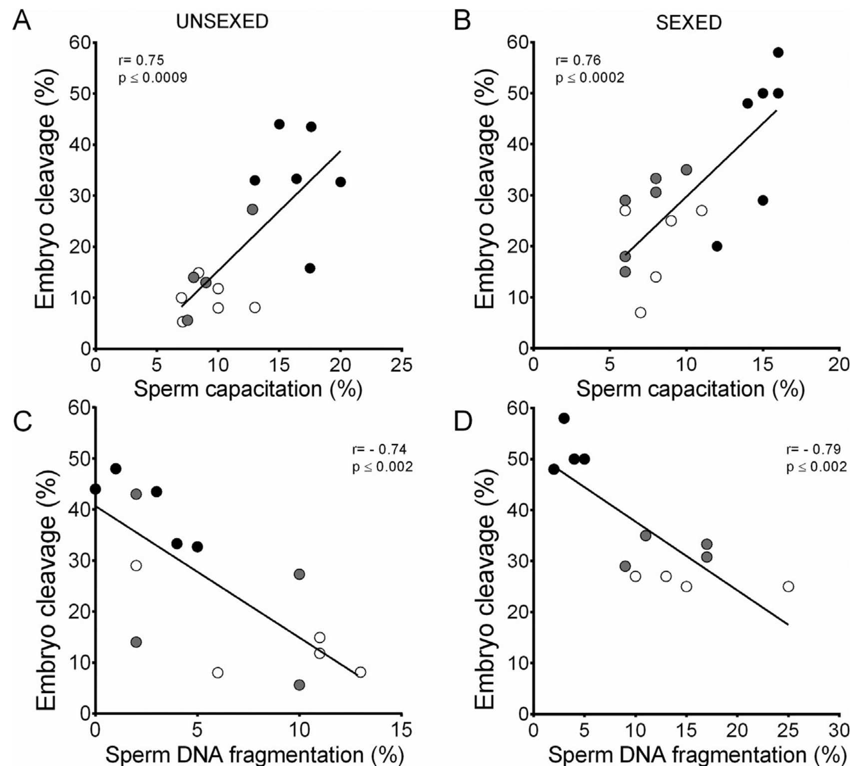



**Fig. 4. Correlation between embryo cleavage and sperm parameters. A** and **B**, percentage of sperm capacitation vs. percentage of embryo cleavage, for unsexed and sexed samples, respectively. **C** and **D, p**ercentage of sperm DNA fragmentation vs. percentage of cleavage, for unsexed and sexed samples, respectively. Spermatozoa before (white dots) and after the SSA, either with the addition of progesterone (black dots) or without it (gray dots). The Pearson correlation values (r) and *p* values are shown in each figure.

Even though differences in number of bulls, straw volume and thawing timing of sexed samples might differentially affect the SSA results as compare to unsexed samples, if this were the case, the expected results would be a poor response in sexed samples due to the mentioned factors. Nevertheless, this was not the case since no significant differences were found between unsexed and sexed samples.

It has been shown that embryos produced by *in vitro* oocyte fertilization with bovine sexed spermatozoa, present reduced expression of genes involved in development [4]. In addition, these authors observed that the embryo cleavage rate was around 30% below those obtained with unsexed spermatozoa. Here we showed that oocytes fertilized *in vitro* with sexed spermatozoa previously selected by the SSA, showed similar rates of embryo cleavage to those fertilized with unsexed spermatozoa. Moreover, the oocytes fertilized *in vitro* with unsexed SSA selected spermatozoa also provided an enhanced cleavage rate. Interestingly, we found that sperm capacitation and low DNA fragmentation are significantly correlated with embryo cleavage rate, which emphasizes the importance of sperm physiological status. Thus, the SSA biotechnology could be beneficial not only for dairy cattle production but also for the reproduction of other species where the female embryos are also preferred.

## 5. Conclusion

Here we show that the use of the SSA with frozen-thawed bovine spermatozoa is as efficient as with human sperm samples. We found that the quality of the sexed sperm population recovered from SSA in the presence of progesterone is similar to the quality of the unsexed population. Finally, we determined that fertilization of oocytes by unsexed or sexed spermatozoa selected by SSA in the presence of progesterone results in a higher embryo cleavage rate.

## Authors' contribution

E.M.D conceived and designed the experiments, acquired, analyzed, and interpreted data; wrote and revised the article. A.M.I acquired, analyzed and interpreted data. H.A.G interpreted data and revised the article. H.T revised the article. L.C.G conceived and designed the experiments; interpreted data, wrote the article, and obtained funding.

## Funding

The study received financial support from Agencia Nacional de Promoción Científica y Tecnológica (PICT Regional "A" - 1995–2011) and Universidad Nacional de Córdoba (203/14; 313/16).

## Conflicts of interest

CONICET and UNC are the owners of the SSA patent. L.C.G and H.A.G are inventors of the SSA device.

## Acknowledgments

L.C.G. and H.A.G. are Principal Investigator and Associate Investigator from CONICET, respectively. E.M.D. and A.M.I are PhD students from Universidad Nacional de Río Cuarto (Argentina) and Universidad Nacional de Córdoba, respectively, and CONICET fellowship holders. The authors thank the advice of Dr. Francisco Ludueña, statistician of our institute.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2018.08.023.

## References


[1] Pieterse MC, Kappen KA, Kruip TAM, Taverne MAM. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology 1988;30:751–62. https://doi.org/10.1016/0093-691X(88)90310-X.

[2] Kruip TAM, Boni R, Wurth YA, Roelofsen MWM, Pieterse MC. Potential use of ovum pick-up for embryo production and breeding in cattle. Theriogenology 1994;42:675–84. https://doi.org/10.1016/0093-691X(94)90384-U.

[3] Majerus V, De Roover R, Etienne D, Kaidi S, Massip A, Dessy F, et al. Embryo production by ovum pick up in unstimulated calves before and after puberty. Theriogenology 1999;52:1169–79. https://doi.org/10.1016/S0093-691X(99)00209-5.

[4] Morton KM, Herrmann D, Sieg B, Struckmann C, Maxwell WMC, Rath D, et al. Altered mRNA expression patterns in bovine blastocysts after fertilisation in vitro using flow-cytometrically sex-sorted sperm. Mol Reprod Dev 2007;74:931–40. https://doi.org/10.1002/mrd.20573.

[5] Seidel GE, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR, et al. Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. Theriogenology 1997;48:1255–64. https://doi.org/10.1016/S0093-691X(97)00368-3.

[6] Seidel GE, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green DG, Cran RD. Insemination of heifers with sexed sperm. Theriogenology 1999;52:1407–20. https://doi.org/10.1016/S0093-691X(99)00226-5.

[7] Underwood SL, Bathgate R, Ebsworth M, Maxwell WMC, Evans G. Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. Anim Reprod Sci 2010;118:7–12. https://doi.org/10.1016/j.anireprosci.2009.06.004.

[8] Sills ES, Kirman I, Thatcher S, Palermo G. Sex-selection of human spermatozoa: evolution of current techniques and applications, vol. 261; 1998. https://doi.org/10.1007/s004040050209.

[9] Seidel GE, Garner DL. Current status of sexing mammalian spermatozoa. Reproduction 2002;124:733–43. https://doi.org/10.1530/reprod/124.6.733.

[10] Enciso M, Cisale H, Johnston SD, Sarasa J, Fernández JL, Gosálvez J. Major morphological sperm abnormalities in the bull are related to sperm DNA damage. Theriogenology 2011;76:23–32. https://doi.org/10.1016/j.theriogenology.2010.12.034.

[11] Vishwanath R, Moreno JF. Review: semen sexing – current state of the art with emphasis on bovine species. Animal 2018:1–12. https://doi.org/10.1017/S1751731118000496.

[12] Gatica LV, Guidobaldi HA, Montesinos MM, Teves ME, Moreno AI, Uñates DR, et al. Picomolar gradients of progesterone select functional human sperm even in subfertile samples. Mol Hum Reprod 2013;19:559–69. https://doi.org/10.1093/molehr/gat037.

[13] Eisenbach M, Giojalas LC. Sperm guidance in mammals [mdash] an unpaved road to the egg. Nat Rev Mol Cell Biol 2006;7:276–85.

[14] Giojalas LC, Guidobaldi HA, Sánchez R. Sperm chemotaxis in mammals. Flagellar Mech Sperm Guid 2015:272–307. https://doi.org/10.2174/9781681081281115010012.

[15] Bar-Ami S, Gitay-Goren H, Brandes JM. Different morphological and steroidogenic patterns in oocyte/cumulus-corona cell complexes aspirated at in vitro fertilization 1. Biol Reprod 1989;41:761–70.

[16] Vanderhyden BC, Tonary AM. Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by a factor(s) secreted by the oocyte. Biol Reprod 1995;53:1243–50. https://doi.org/10.1095/biolreprod53.6.1243.

[17] Chian R-C, Ao A, Clarke HJ, Tulandi T, Tan S-L. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture in vitro. Fertil Steril 1999;71:61–6. https://doi.org/10.1016/S0015-0282(98)00416-6.

[18] Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T. Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes 1. Biol Reprod 2003;68:1193–8.

[19] Guidobaldi HA, Teves ME, Uñates DR, Anastasía A, Giojalas LC. Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. PLoS One 2008;3, e3040. https://doi.org/10.1371/journal.pone.0003040.

[20] Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, Avendaño C, et al. Developmental sperm contributions: fertilization and beyond. Fertil Steril 2009;92:835–48. https://doi.org/10.1016/j.fertnstert.2009.06.030.

[21] Simões R, Feitosa WB, Siqueira AFP, Nichi M, Paula-Lopes FF, Marques MG, et al. Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome. Reproduction 2013;146:433–41. https://doi.org/10.1530/REP-13-0123.

[22] Risopatrón J, Sánchez R, Sepúlveda N, Peña P, Villagran E, Miska W. Migration/sedimentation sperm selection method used in bovine in vitro fertilization: comparison with washing/centrifugation. Theriogenology 1996;46:65–73. https://doi.org/10.1016/0093-691X(96)00142-2.

[23] Correa JR, Zavos PM. Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. Theriogenology 1996;46:1225–32. https://doi.org/10.1016/S0093-691X(96)00293-2.

[24] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988;38:1171–80. https://doi.org/10.1095/biolreprod38.5.1171.

[25] Barakat IAH, Danfour MA, Galewan FAM, Dkhil MA. Effect of various concentrations of caffeine, pentoxifylline, and kallikrein on hyperactivation of Frozen bovine semen. BioMed Res Int 2015;2015:1–7. https://doi.org/10.1155/2015/948575.

[26] Bromfield EG, Aitken RJ, Gibb Z, Lambourne SR, Nixon B. Capacitation in the presence of methyl-β-cyclodextrin results in enhanced zona pellucida-binding ability of stallion spermatozoa. Reproduire 2014;147:153–66. https://doi.org/10.1530/REP-13-0393.

[27] Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan SREM, L.C. G. Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biol Reprod 2002;67:1565–71.

[28] Teves ME, Barbano F, Guidobaldi HA, Sanchez R, Miska W, Giojalas LC. Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. Fertil Steril 2006;86:745–9. https://doi.org/10.1016/j.fertnstert.2006.02.080.

[29] Gil PI, Guidobaldi HA, Teves ME, Uñates DR, Sanchez R, Giojalas LC. Chemotactic response of frozen-thawed bovine spermatozoa towards follicular fluid. Anim Reprod Sci 2008;108:236–46. https://doi.org/10.1016/j.anireprosci.2007.08.006.

[30] Bustos MA, Roggero CM, De la Iglesia PX, Mayorga LS, Tomes CN. GTP-bound Rab3A exhibits consecutive positive and negative roles during human sperm dense-core granule exocytosis. J Mol Cell Biol 2014;6:286–98.

[31] Zoppino FCM, Halón ND, Bustos MA, Pavarotti MA, Mayorga LS. Recording and sorting live human sperm undergoing acrosome reaction. Fertil Steril 2012;97:1309–15. https://doi.org/10.1016/j.fertnstert.2012.03.002.

[32] Kaul G, Sharma GS, Singh B, Gandhi KK. Capacitation and acrosome reaction in buffalo bull spermatozoa assessed by chlortetracycline and Pisum sativum agglutinin fluorescence assay. Theriogenology 2001;55:1457–68. https://doi.org/10.1016/S0093-691X(01)00494-0.

[33] Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66. https://doi.org/10.1002/j.1939-4640.2003.tb02641.x.

[34] Carretero MI, Lombardo D, Arraztoa CC, Giuliano SM, Gambarotta MC, Neild DM. Evaluation of DNA fragmentation in llama (Lama glama) sperm using the sperm chromatin dispersion test. Anim Reprod Sci 2012;131:63–71. https://doi.org/10.1016/j.anireprosci.2012.02.008.

[35] Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, et al. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995;43:141–52. https://doi.org/10.1016/0093-691X(94)00020-U.

[36] Trigal B, Gómez E, Caamaño JN, Muñoz M, Moreno J, Carrocera S. In vitro and in vivo quality of bovine embryos in vitro produced with sex-sorted sperm. Theriogenology 2012;78:1465–75. https://doi.org/10.1016/j.theriogenology.2012.06.018.

[37] Guidobaldi HA, Cubilla M, Moreno A, Molino MV, Bahamondes L, Giojalas LC. Sperm chemorepulsion, a supplementary mechanism to regulate fertilization. Hum Reprod 2017;32:1560–73.

[38] Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Adv Urol 2012;2012:854837. https://doi.org/10.1155/2012/854837.

[39] Richter MA, Haning RV, Shapiro SS. Artificial donor insemination: fresh versus frozen semen; the patient as her own control. Fertil Steril 1984;42:277–80.

[40] Onofre J, Baert Y, Faes K, Goossens E. Cryopreservation of testicular tissue or testicular cell suspensions : a pivotal step in fertility preservation. Hum Reprod Update 2016;22:744–61. https://doi.org/10.1093/humupd/dmw029.

[41] Hamano K. Sex preselection in bovine by separation of X- and Y-Chromosome bearing spermatozoa. J Reprod Dev 2007;53:27–38. https://doi.org/10.1262/jrd.18141.