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# Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function

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### ABSTRACT

In this study, different combinations of 2-step, discontinuous gradient centrifugation were used, consisting of three different combinations of isotonic Percoll (45/60, 60/75 and 45/90%) that allowed us to select different sperm subpopulations from fertile and normozoospermic boars. Our objective in this study is to evaluate the effects of centrifugation through three different discontinuous Percoll gradients on sperm function parameters (motility, viability, morphology, acrosome status, chromatin condensation, DNA fragmentation, ROS generation, tyrosine phosphorylation and intracellular calcium concentration) and the sperm penetrating capacity in an IVF system. All the Percoll treatments evaluated increased the percentage of spermatozoa with normal morphology, the proportion of undamaged DNA, normal chromatin condensation, motion parameters measured by CASA and the percentage of capacitated spermatozoa with tyrosine phosphorylated proteins compared to control group. Finally, the in vitro oocyte penetrating capacity of boar spermatozoa was significantly affected by Percoll centrifugation. All the Percoll treatments increased the penetration rates and mean number of sperm per penetrated oocyte. Despite the efficiency of all three of the sperm treatments tested in selecting spermatozoa with improved sperm parameters and capacity to penetrate oocytes in vitro, the optimum performance of this system was demonstrated after preselecting spermatozoa by centrifugation on a discontinuous 45/90 Percoll gradient. The P45/90 treatment leads to obtain a higher percentage of spermatozoa which develop properly the capacitation process as it was shown measuring tyrosine phosphorylation and intracellular calcium concentration.

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## 1. Introduction

Porcine semen is composed of a heterogeneous population of sperm with varying degrees of structural and functional differentiation and normality (Thurston et al., 2001; Quintero-Moreno et al., 2004; Holt, 2009). In order to enhance the sperm parameters of ejaculates with low sperm quality or to optimize porcine IVF, sperm selection methods have been used to isolate sperm subpopulations

with high fertilizing capacity for use in animal breeding (Rodríguez-Martínez et al., 1997; Morrell, 2006). According to Henkel and Schill (2003), the ideal sperm separation technique should (i) be quick, easy and cost-effective, (ii) isolate as many motile spermatozoa as possible, (iii) not cause sperm damage or non-physiological alterations of the separated sperm cells, (iv) eliminate dead spermatozoa and other cells, including leukocytes and bacteria, (v) eliminate toxic or bioactive substances such as decapacitation factors or reactive oxygen species (ROS), and (vi) allow processing of larger volumes of ejaculates. Among these separation methods in pigs are swim-up (Berger and Parker, 1989), Percoll discontinuous gradient (PDG) (Berger

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and Horton, 1988; Berger and Parker, 1989; Mattioli et al., 1989; Grant et al., 1994; Jeong and Yang, 2001; Matas et al., 2003; Suzuki and Nagai, 2003) and other colloid gradients (Morrell et al., 2009), Sephadex gel filtration (Bussalleu et al., 2009) and glass wool filtration (Berger and Horton, 1988). Gradient density centrifugation is the method most frequently used because it is reported to meet many of these requirements (Morrell, 2006).

Percoll solution consists of silica beads coated with polyvinylpyrrolidone (PVP) to protect the cells from the toxic action of the silica, used for the purification of cells and isolation of subcellular particles (Pertoft, 2000). PDG has been used for separating spermatozoa from different species (Pertoft, 2000) but the technique was withdrawn from human clinical use in 1996, because it could damage spermatozoa (Biotech, 1996).

Since the pioneering studies in the late 1980s in porcine IVF (Berger and Horton, 1988; Berger and Parker, 1989; Mattioli et al., 1989), PDG has been used for the selection of different kinds of boar spermatozoa (frozen–thawed, ejaculated or epididymal) (Grant et al., 1994; Jeong and Yang, 2001; Matas et al., 2003, 2010; Suzuki and Nagai, 2003).

Percoll has been used for the selection of several sperm subpopulations from normozoospermic human seminal samples (Gomez et al., 1996; Buffone et al., 2004). However, a lack of information is related to pig spermatozoa. In the literature different conditions for PDG with different Percoll concentrations, number of layers and centrifugation regimen are described. However, no data exist on the effect of such PDG on sperm functional parameters of fertile, normozoospermic boars.

Our objective in this study was to evaluate the effects of centrifugation through three different discontinuous Percoll gradients on sperm function (morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation and intracellular calcium concentration). Additionally, fertilization in vitro provides information on gamete interaction and the ability of sperm to bind and fertilize homologous oocytes. IVF therefore gives both qualitative and quantitative information on sperm functionality that cannot be tested by these other laboratory tests. Our study therefore aimed at including IVF as an important supplement to those.

## 2. Materials and methods

### 2.1. Ethics

This study was developed following institutional approval from the University of Murcia, and it was performed in accordance with the Animal Welfare regulations of that institution.

### 2.2. Reagents

All chemicals were obtained from Sigma–Aldrich Química, S.A. (Madrid, Spain) unless otherwise indicated.

### 2.3. Sperm collection and semen handling

Semen was collected from mature fertile boars (2–4 years old) from an Artificial Insemination Center using the manual method and a dummy (King and Macpherson, 1973). We evaluated 12 ejaculates from 6 different boars. The sperm-rich fraction was collected in a pre-warmed thermos, while the gel fraction was held on a gauze tissue covering the thermos opening. The semen was then diluted 1:2 with isothermal Beltsville Thawing Solution (BTS) extender (Pursel and Johnson, 1975) and transported to the laboratory. Once in the laboratory, the sample was split in four fractions and added to the different groups (control and 3 Percoll treatments).

The Percoll-group involved layering a 0.5 mL aliquot of spermatozoa on a discontinuous 45 and 90%, 60 and 75%, or 45 and 60% (v/v) Percoll® (Pharmacia, Uppsala, Sweden) gradient (Buffone et al., 2004). For Percoll 45/90 (P45/90), in a 12 mL conic centrifuge tube, 2 mL of 45% Percoll was layered on top of 2 mL of 90% Percoll. Finally, 0.5 mL diluted semen was added, with care taken to avoid mixing the solutions (Matas et al., 2003). The same procedure was used for Percoll 60/75 (P60/75) and Percoll 45/60 (P45/60). The three experimental groups were then centrifuged at  $700 \times g$  for 30 min. The sperm pellet was resuspended in TALP medium (10 mL (Rath et al., 1999)) previously pre-equilibrated at  $38.5^\circ\text{C}$  in 5%  $\text{CO}_2$  in 100% humidified air and washed by centrifugation at  $700 \times g$  for 10 min. For the control group, the spermatozoa were directly diluted in TALP medium and finally sperm concentration adjusted to  $2 \times 10^8$  spermatozoa/mL in all experimental groups.

Seminal parameters were evaluated immediately after sperm treatment: morphology, acrosome status, motility and motion parameters, ROS generation, chromatin condensation, DNA fragmentation, tyrosine phosphorylation, intracellular calcium concentration and in vitro oocyte penetrating capacity.

### 2.4. Morphologic and acrosome sperm evaluation

Wet mounts of semen fixed in buffered 2% glutaraldehyde solution were examined under a phase-contrast microscope (1000 $\times$  magnification, Leica, Wetzlar, Germany) to analyse morphology and acrosomes (Pursel et al., 1972). The proportion of spermatozoa with a normal apical ridge (NAR) was determined on two slides per sample and a total of 200 spermatozoa per slide. Four hundred spermatozoa were categorized according to sperm morphology into those with normal morphology, cells with attached cytoplasmic droplets, tail defects (folded tail, coiled tail) and others (abnormal heads, etc.) (Gadea et al., 2004).

### 2.5. Analysis of motion parameters

Motion parameters were determined using a CASA system (ISAS®, Proiser, Valencia, Spain). The CASA-derived motility characteristics studied were total motility (%), progressive motility (%), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), straight-line velocity (VSL,  $\mu\text{m/s}$ ), average path velocity (VAP,  $\mu\text{m/s}$ ), linearity of the curvilinear trajectory (LIN,

ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ) and beat cross-frequency (BCF, Hz). A 7  $\mu\text{L}$  drop of the sample was placed on a warmed (37 °C) slide and covered with a 24 mm  $\times$  24 mm cover slip.

The setting parameters were: 100 frames in which spermatozoa had to be present in at least 15 in order to be counted, 50 images per second. Images were obtained at 200 $\times$  magnification using a negative contrast phase objective (Nikon, Tokyo, Japan). Spermatozoa with a VAP < 10  $\mu\text{m}/\text{s}$  were considered immotile. A minimum of five fields per sample was evaluated, counting a minimum of 200 spermatozoa per sub-sample. For statistical analysis the mean measurements of CASA for each field were entered into the ANOVA model.

## 2.6. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10,000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analysed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

### 2.6.1. Production of reactive oxygen species

Production of ROS was measured by incubating the spermatozoa in TALP medium in the presence of 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (0.5  $\mu\text{M}$ ) for 60 min at 37 °C (Gadea et al., 2005a). This dye is a fluorogenic probe commonly used to detect cellular ROS production. H<sub>2</sub>DCFDA is a stable cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter. Measurements were expressed as mean green intensity fluorescence units (mean channel in the FL1) and this was used as an index of ROS generation.

### 2.6.2. Determination of chromatin condensation

Sperm chromatin was stained with propidium iodide (PI) for the determination of sperm chromatin condensation (Gadea et al., 2005b). Sperm samples were centrifuged (1200  $\times$  g, 3 min) and the pellet was resuspended in a solution of ethanol and phosphate-buffered saline (PBS) (70/30, v/v) for 30 min for sperm membrane permeabilization and stored at –20 °C until analysis. After thawing, the samples were centrifuged, the supernatant was discarded and the pellet was resuspended in a PI solution (10 mg/mL) in PBS. Samples were maintained in darkness for 1 h before the flow cytometric analysis. Red PI fluorescence was collected with a FL3 sensor using a 650 nm band-pass filter. Measurements were expressed as mean red intensity fluorescence units (mean channel in the FL3) and this was used as an

index of the state of the chromatin condensation, as this is directly related to the PI uptake by DNA.

### 2.6.3. Evaluation of sperm DNA fragmentation by TUNEL

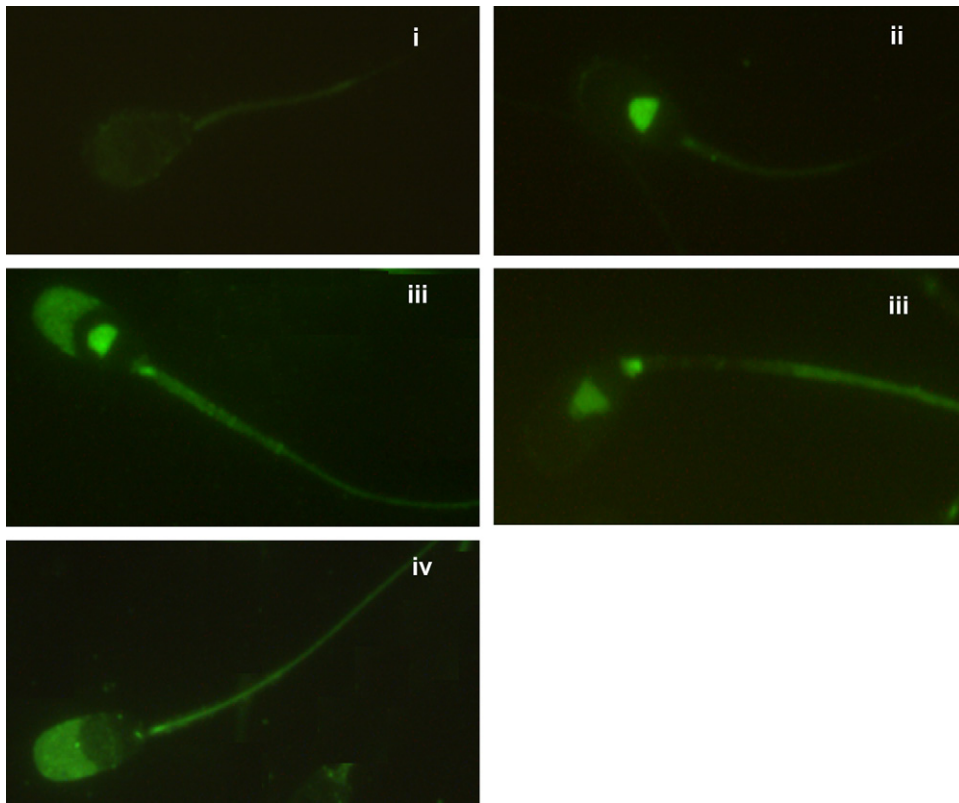
Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) staining was used to determine sperm DNA fragmentation according to the method described previously (Gadea et al., 2008). In brief, the cells were concentrated by centrifugation, fixed in a solution of ethanol and PBS (70/30, v/v) for 30 min for sperm membrane permeabilization, and stored at –20 °C. Cells (approx. 10<sup>6</sup>) were washed twice with PBS and resuspended in 50  $\mu\text{L}$  terminal deoxynucleotidyltransferase (TdT) reaction buffer containing: 10  $\mu\text{L}$  5 $\times$  concentrated buffer solution, 1  $\mu\text{L}$  (15 units) TdT, 0.25 nmol fluorescein-dUTP (BODIPY@-FL-X-14-dUTP, Invitrogen SA, Barcelona, Spain) (0.25  $\mu\text{L}$ ) and 39  $\mu\text{L}$  distilled water.

Negative controls were incubated in fluorescein-dUTP in the absence of enzyme terminal transferase. The cells were incubated with the reaction buffer for 60 min at 37 °C, then rinsed twice and measured by flow cytometry. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter and two populations were determined. The cells with fragmented DNA presented an intense green nuclear fluorescence, in comparison with spermatozoa with non-fragmented DNA which presented low green fluorescence. Measurements were expressed as the percentage of cells with high green intensity fluorescence and this was used as an index of the DNA fragmentation, as it is directly related to the dUTP uptake by DNA.

## 2.7. Localization of proteins phosphorylated in tyrosine residues

Immunofluorescence was employed to determine the localization of proteins phosphorylated in tyrosine residues (Tardif et al., 2001). Sperm from the different Percoll and control groups were washed with PBS and centrifuged at 270  $\times$  g for 10 min. Spermatozoa were fixed in 2% formaldehyde solution for 60 min at 4 °C. Spermatozoa were washed once in PBS and blocked with 2% (w/v) BSA–H<sub>2</sub>O and incubated overnight at 4 °C before addition of primary antibody. The sperm were washed and resuspended in PBS, smeared onto a microscope slide, and allowed to air dry. Slides were then incubated for 1 h with anti-phosphotyrosine monoclonal antibody at 4 °C (clone 4G10, 1:200, Millipore, Madrid, Spain) rinsed with PBS, and incubated for an additional 1 h with fluorescein-conjugated goat anti-mouse antibodies (1:400, Bio-Rad Laboratories, Madrid, Spain). After rinsing with PBS, samples were mounted on the slides with 90% glycerol/PBS (v/v). Sperm were observed with a Nikon microscope equipped with fluorescent optics (excitation 450–490 nm: B2-A filter, 4003) for anti-phosphotyrosine antibody labelling.

Two hundred spermatozoa per slide and two 2 slides per sample were classified into four groups according to the localization of the anti-phosphotyrosine monoclonal antibody signal: (i) no signal of fluorescence; (ii) fluorescent signal only presents in the equatorial subsegment, triangular in appearance (Jones et al., 2008), (iii) signal in



**Fig. 1.** Boar spermatozoa classified into four groups according to the localization of the anti-phosphotyrosine monoclonal antibody signal: (i) no Ab signal, (ii) signal only in the equatorial subsegment, triangular in appearance, (iii) signal in equatorial segment and in acrosome area and/or tail and (iv) signal only in acrosome area and/or tail.

equatorial segment and in acrosome area or/and tail and (iv) signal only in acrosome or/and tail (Fig. 1).

### 2.8. Determination of intracellular calcium concentration

Calcium concentration was measured according to the method reported previously (Tardif et al., 2003; Matas et al., 2010). To measure intracellular free  $\text{Ca}^{2+}$  spermatozoa were incubated with  $2.5 \mu\text{M}$  fura-2/AM in a buffer medium consisting of  $2.7 \text{ mM}$  KCl,  $1.5 \text{ mM}$   $\text{KH}_2\text{PO}_4$ ,  $8.1 \text{ mM}$   $\text{Na}_2\text{HPO}_4$ ,  $137 \text{ mM}$  NaCl,  $5.55 \text{ mM}$  glucose, and  $1 \text{ mM}$  pyruvate for 45 min at  $37^\circ\text{C}$ . The extracellular unloaded fura-2 was removed by centrifugation ( $700 \times g$ , 5 min). Washed sperm were resuspended in the same buffer to a concentration of  $3 \times 10^8$  cells/mL and incubated at  $37^\circ\text{C}$  for 15 min in dark. Then, spermatozoa were centrifuged ( $700 \times g$ , 5 min) and resuspended in TALP medium. As a negative control, spermatozoa were also resuspended in PBS media without calcium (control PBS). Fluorescence was monitored using Jasco FP-6300 spectrofluorometer (Jasco, Madrid, Spain) for a further 30 min. Excitation wavelengths alternated between 340 and 380 nm with emission held at 510 nm. At the end of the experiments, sperm were lysed with Triton X-100 (0.5%), and then calcium was depleted by addition of  $25 \text{ mM}$  EGTA. Intracellular  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_i$  was calculated as previously described (Gryniewicz et al., 1985).

The equation used for calculation was as follows:  $[\text{Ca}^{2+}]_i = K_d \times (R - R_{\min}/R_{\max} - R) \times S_f/S_b$  where  $R$  indicates the fluorescence ratio  $340/380 \text{ nm}$ ;  $R_{\min}$  indicates the minimum values of fluorescence;  $R_{\max}$  indicates the maximal fluorescence values;  $S_f$  indicates the fluorescence intensity in the  $\text{Ca}^{2+}$ -free medium (excitation  $380 \text{ nm}$ );  $S_b$  indicates the fluorescence intensity under  $\text{Ca}^{2+}$  saturation conditions and  $K_d$  ( $224 \text{ nM}$ ) is the  $\text{Ca}^{2+}$  constant dissociation for Fura-2AM. For the statistical analysis the calcium concentration (nM/L) was recorded  $t$  from 0 to 1800 s and every 30 s for every experimental group and replicate. Finally the mean value during the incubation period was calculated.

### 2.9. In vitro maturation and fertilization

#### 2.9.1. Oocyte collection and in vitro maturation

Within 30 min of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline ( $0.9\%$  (w/v) NaCl) containing  $100 \mu\text{g}$  kanamycin sulphate  $\text{mL}^{-1}$  at  $37^\circ\text{C}$ , washed once in  $0.04\%$  (w/v) cetrimide solution and then twice in saline. Oocyte-cumulus cell complexes were collected from non-atretic follicles ( $3\text{--}6 \text{ mm}$  diameter), washed twice in DPBS supplemented with  $4 \text{ mg}$  polyvinyl alcohol (PVA)  $\text{mL}^{-1}$ , and twice more in maturation medium previously equilibrated for at least 3 h at  $38.5^\circ\text{C}$  under  $5\% \text{ CO}_2$  in  $100\%$  humidified air. Only oocytes



**Table 1**

Percentages of morphologically normal sperm, sperm with abnormal morphology and normal apical ridge (NAR) recovered from different Percoll discontinuous gradient systems (mean values  $\pm$  sem).

Treatment	Normal (%)	Cytoplasmic droplets (%)	Altered tail (%)	Other abnormalities (%)	NAR (%)
Control	82.35 $\pm$ 2.79 <sup>a</sup>	11.25 $\pm$ 1.96 <sup>a</sup>	6.30 $\pm$ 2.09 <sup>a</sup>	0.10 $\pm$ 0.07	88.80 $\pm$ 2.85
P45/60	80.75 $\pm$ 2.97 <sup>a</sup>	13.50 $\pm$ 2.33 <sup>a</sup>	5.60 $\pm$ 1.77 <sup>ab</sup>	0.15 $\pm$ 0.11	84.00 $\pm$ 2.92
P60/75	87.35 $\pm$ 2.34 <sup>a</sup>	8.60 $\pm$ 1.81 <sup>ab</sup>	3.70 $\pm$ 1.37 <sup>ab</sup>	0.35 $\pm$ 0.26	87.40 $\pm$ 4.46
P45/90	96.25 $\pm$ 0.89 <sup>b</sup>	3.30 $\pm$ 0.80 <sup>b</sup>	0.45 $\pm$ 0.17 <sup>b</sup>	0.00 $\pm$ 0.00	88.80 $\pm$ 3.22
<i>p</i> -Value	<0.01	<0.01	0.04	0.40	0.73

Different letters (a,b) in the same column indicate significant differences ( $p < 0.05$ ).

harvested within 2 h of slaughter with a complete, dense cumulus oophorus were matured (Matas et al., 1996). The medium used for oocyte maturation was NCSU-37 (Petters and Wells, 1993); supplemented with 0.57 mmol cysteine L<sup>-1</sup>, 1 mmol dibutyryl cAMP L<sup>-1</sup>, 5  $\mu$ g insulin mL<sup>-1</sup>, 50  $\mu$ mol  $\beta$ -mercaptoethanol L<sup>-1</sup>, 10 iu equine chorionic gonadotrophin mL<sup>-1</sup> (Foligon, Intervet International BV, Boxmeer), 10 iu human chorionic gonadotrophin mL<sup>-1</sup> (Veterin Corion, Divasa Farmavic, Barcelona, Spain), and 10% (v/v) pig follicular fluid. Groups of 50 oocytes were cultured in 500  $\mu$ L maturation medium for 20–22 h at 38.5 °C and 5% CO<sub>2</sub> in air. Once cultured, the oocytes were washed twice, transferred to fresh maturation medium without hormones or dibutyryl cAMP, and cultured for a further 20–22 h. Under these conditions the efficiency of oocyte maturation is higher than 90% (Coy et al., 1999; Coy and Romar, 2002).

### 2.9.2. *In vitro* fertilization

After maturation oocytes were mechanically stripped of cumulus by gentle aspiration with a pipette. Denuded oocytes were washed three times in TALP medium and groups of 25–30 oocytes transferred to each well of four-well Nunc multidishes (Nunc, Roskilde, Denmark) containing 250  $\mu$ L TALP medium previously equilibrated at 38.5 °C under 5% CO<sub>2</sub> (Matas et al., 2003, 2010). Sperm suspensions (250  $\mu$ L) from each treatment group were added to the fertilization wells to obtain a final concentration of  $1 \times 10^5$  cells mL<sup>-1</sup>. At 18–20 h post-insemination, putative zygotes were fixed for 30 min (0.5% glutaraldehyde in PBS), stained for 15 min (1% Hoechst 33342 in PBS), washed in PBS containing 1 mg/mL polyvinylpyrrolidone, mounted on glass slides and examined under an epifluorescence microscope at 400 $\times$  magnification for evidence of sperm penetration. Penetration rate and mean number of spermatozoa per penetrated oocyte were assessed for each group (Gadea et al., 1998; Matas et al., 2007).

### 2.10. Statistical analysis

Data are expressed as the mean  $\pm$  SEM and analysed by ANOVA, considering the specific sperm treatment as the main variable and ejaculate as covariable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at  $p < 0.05$ . *In vitro* penetration values (categorical data) were modeled according to the binomial model of parameters by arcsin transformation of the data and were analysed by ANOVA. Pearson correlation index

was calculated for chromatin condensation and TUNEL assay values.

## 3. Results

### 3.1. Sperm morphology and acrosome status

Centrifugation in a discontinuous Percoll gradient 45/90 increased the percentage of spermatozoa with normal morphology to values higher than 95% from the 82% in the control group ( $p < 0.01$ , Table 1). The reduction in morphological abnormalities achieved by density centrifugation was based on a reduction in the percentage of spermatozoa with cytoplasmic droplets and tail defects. However, the use of P45/60 and P60/75 was not efficient enough to increase the percentage of spermatozoa with normal morphology. On the other hand, the acrosome status evaluated as NAR was not affected by the Percoll treatment (ranged from 84 to 88.80%,  $p = 0.73$ , Table 1).

### 3.2. Motility and motion parameters

The percentage of total sperm motility was not affected by the selection by Percoll density gradient (ranged from 60.62 to 67.53%,  $p = 0.06$ , Table 2). However, progressive motility was higher for the spermatozoa selected by P60/75 than control (62.71% vs 53.45%,  $p = 0.03$ , Table 2), while progressive motility for P45/60 and P45/90 offered intermediate values. All the motion parameters analysed (velocity, straightness, linearity, etc.) were increased after Percoll treatment compared to the control group ( $p < 0.01$ , Table 2) without differences between the Percoll groups, except for VSL which was higher for P60/75 than P45/60 and P45/90 ( $p < 0.05$ , Table 2).

### 3.3. ROS generation, chromatin condensation and DNA fragmentation

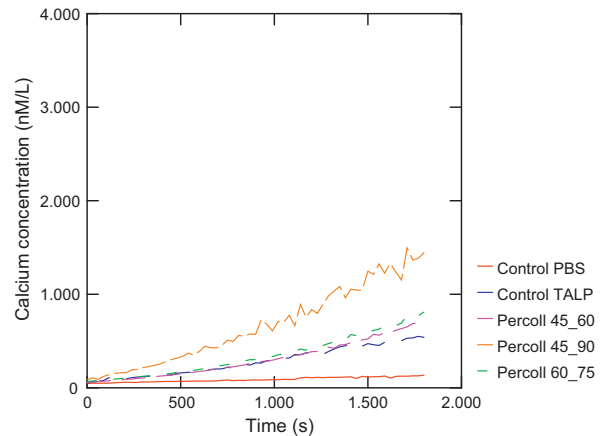
Measurements of the ROS generated revealed higher values for sperm treated by Percoll 45/60 than control ( $p < 0.01$ , Table 3) while no differences were found for the other Percoll groups (P60/75 and P45/90) compared to control ( $p > 0.05$ , Table 3).

Sperm chromatin condensation was affected by the Percoll treatments; the chromatin after Percoll treatments was less condensed than control, with higher values of fluorescence units that correspond with higher IP uptake measured by flow cytometry ( $p < 0.01$ , Table 3). At the same time, the Percoll treatment reduced the percentage of spermatozoa with fragmented DNA ( $p < 0.01$ , Table 3). When

**Table 2**  
Motility parameters measured by CASA obtained from sperm recovered from different Percoll discontinuous gradient systems (mean  $\pm$  sem).

Treatment	Motility (%)	Progressive motility (%)	VCL ( $\mu\text{m/s}$ )	VSL ( $\mu\text{m/s}$ )	VAP ( $\mu\text{m/s}$ )	LIN (%)	STR (%)	WOB (%)	ALH ( $\mu\text{m}$ )	BCF (Hz)
Control	65.65 $\pm$ 1.86	53.45 $\pm$ 2.36 <sup>a</sup>	59.66 $\pm$ 2.73 <sup>a</sup>	33.85 $\pm$ 2.71 <sup>a</sup>	43.57 $\pm$ 2.64 <sup>a</sup>	50.79 $\pm$ 2.21 <sup>a</sup>	68.08 $\pm$ 1.84 <sup>a</sup>	69.36 $\pm$ 1.59 <sup>a</sup>	1.32 $\pm$ 0.04 <sup>a</sup>	11.41 $\pm$ 0.39 <sup>a</sup>
P45/60	62.50 $\pm$ 2.04	58.86 $\pm$ 2.59 <sup>ab</sup>	86.79 $\pm$ 2.70 <sup>b</sup>	61.42 $\pm$ 2.89 <sup>bc</sup>	71.26 $\pm$ 2.74 <sup>b</sup>	67.00 $\pm$ 2.11 <sup>b</sup>	79.57 $\pm$ 1.62 <sup>b</sup>	80.00 $\pm$ 1.54 <sup>b</sup>	1.48 $\pm$ 0.05 <sup>b</sup>	15.23 $\pm$ 0.41 <sup>b</sup>
P60/75	67.53 $\pm$ 1.75	62.71 $\pm$ 2.23 <sup>b</sup>	89.39 $\pm$ 2.45 <sup>b</sup>	67.51 $\pm$ 2.47 <sup>c</sup>	76.05 $\pm$ 2.44 <sup>b</sup>	71.01 $\pm$ 1.27 <sup>b</sup>	82.68 $\pm$ 0.81 <sup>b</sup>	82.37 $\pm$ 0.93 <sup>b</sup>	1.45 $\pm$ 0.03 <sup>b</sup>	14.76 $\pm$ 0.26 <sup>b</sup>
P45/90	60.62 $\pm$ 2.10	56.98 $\pm$ 2.67 <sup>ab</sup>	82.97 $\pm$ 3.15 <sup>b</sup>	57.98 $\pm$ 2.82 <sup>b</sup>	67.18 $\pm$ 2.80 <sup>b</sup>	65.99 $\pm$ 1.84 <sup>b</sup>	80.01 $\pm$ 1.26 <sup>b</sup>	78.51 $\pm$ 1.29 <sup>b</sup>	1.51 $\pm$ 0.05 <sup>b</sup>	14.59 $\pm$ 0.47 <sup>b</sup>
p-Value	0.06	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01

VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity of the curvilinear trajectory (VSL/VCL), STR: straightness (VSL/VAP), WOB: Wobble (VAP/VCL), ALH: amplitude of lateral head displacement, BCF: beat cross-frequency.  
Numbers within columns with different superscripts (a,b,c) differ ( $p < 0.05$ ).



**Fig. 2.** Calcium concentration (nM) in boar spermatozoa recovered from different Percoll discontinuous gradient systems and diluted in TALP medium. Fluorescence was measured by Fura-2 and monitored using spectrofluorimeter for 60 min.

we studied the relation between DNA fragmentation and chromatin condensation we observed a significant relation between these parameters ( $r = -0.63$ ,  $p < 0.01$ ), the samples with highest chromatin condensation showed the highest DNA fragmentation.

### 3.4. Tyrosine protein phosphorylation

Indirect phosphotyrosine immunolocalization showed that the treatment by Percoll increased the percentage of cells with phosphorylation of tyrosine, used as an index of capacitation ( $p < 0.01$ , Table 4). So, while in the control group most of the spermatozoa (84.35%) have not undergone the tyrosine phosphorylation, the P45/60 and the P60/75 groups displayed phosphorylation in 80–90% of the spermatozoa and finally in P45/90 group more than 95% of the spermatozoa showed phosphotyrosine signal (Table 4). The pattern of staining is also different in the P45/90 group with a higher proportion of cells with signal in equatorial region, acrosome and tail compared to the P45/60 and P60/75 groups which show a similar proportion of only equatorial segment staining (Table 4).

### 3.5. Intracellular calcium concentration

Since intracellular  $\text{Ca}^{2+}$  influx is one of the crucial biochemical events occurring during capacitation, this experiment was performed to study changes in  $\text{Ca}^{2+}$  uptake between sperm groups. Calcium intake by the sperm increased through the incubation time (30 min) in the presence of TALP medium, while incubation in a calcium-free medium (PBS), used as control, resulted in a constant calcium concentration at a basal level during the time of study (mean value =  $84.26 \pm 7.52$  nM/L; Fig. 2). Treatment with Percoll affected the calcium concentration ( $p < 0.01$ ) (Fig. 2). P45/90 induced the highest values in calcium concentration (mean value =  $658.72 \pm 65.27$  nM/L), values significantly higher ( $p < 0.01$ ) than in the control group (mean value =  $276.14 \pm 16.68$  nM/L), P45/60 group

**Table 3**

ROS generation, chromatin decondensation and DNA fragmentation (TUNEL) obtained from sperm recovered from different Percoll discontinuous gradient systems (9 replicates).

Treatment	ROS generation (UF)	Chromatin decondensation (UF)	DNA fragmentation (%)
Control	21.28 ± 0.85 <sup>a</sup>	13.83 ± 0.64 <sup>a</sup>	5.26 ± 0.31 <sup>a</sup>
P45/60	27.63 ± 1.86 <sup>b</sup>	22.82 ± 0.84 <sup>b</sup>	2.97 ± 0.11 <sup>b</sup>
P60/75	25.09 ± 1.71 <sup>ab</sup>	21.66 ± 0.75 <sup>b</sup>	3.08 ± 0.25 <sup>b</sup>
P45/90	21.98 ± 1.27 <sup>a</sup>	21.57 ± 0.79 <sup>b</sup>	2.44 ± 0.31 <sup>b</sup>
<i>p</i> -Value	<0.01	<0.01	<0.01

Different letters (a,b) in the same column indicate significant differences ( $p < 0.05$ ).

UF: arbitrary units of fluorescence.

**Table 4**

Percentage of sperm with phosphotyrosine immunoreactive signal obtained from sperm recovered from different Percoll discontinuous gradient systems.

Treatment	No signal (%)	Only equatorial segment (%)	Equatorial segment and acrosome and/or tail (%)	Acrosome or/and tail (%)
Control	85.50 ± 2.66 <sup>a</sup>	7.25 ± 2.28 <sup>a</sup>	2.75 ± 1.06 <sup>a</sup>	4.50 ± 1.95 <sup>a</sup>
P45/60	19.50 ± 5.01 <sup>b</sup>	46.80 ± 3.0 <sup>b</sup>	32.90 ± 5.63 <sup>b</sup>	0.80 ± 0.21 <sup>b</sup>
P60/75	7.70 ± 3.43 <sup>bc</sup>	51.75 ± 5.95 <sup>b</sup>	40.00 ± 6.65 <sup>b</sup>	0.55 ± 0.19 <sup>b</sup>
P45/90	3.15 ± 1.15 <sup>c</sup>	20.05 ± 4.99 <sup>a</sup>	75.40 ± 6.05 <sup>c</sup>	1.40 ± 0.44 <sup>b</sup>
<i>p</i> -Value	<0.01	<0.01	<0.01	0.01

Different letters (a,b,c) in the same column indicate significant differences ( $p < 0.05$ ).

**Table 5**

IVF results obtained from sperm recovered from different Percoll discontinuous gradient systems.

Treatment	<i>N</i>	PEN (%)	SPZ/O <sup>a</sup>
Control	205	49.76 ± 3.50 <sup>a</sup>	1.78 ± 0.11 <sup>a</sup>
P45/60	246	87.40 ± 2.12 <sup>b</sup>	3.20 ± 0.16 <sup>b</sup>
P60/75	158	87.34 ± 2.65 <sup>b</sup>	4.17 ± 0.20 <sup>c</sup>
P45/90	204	98.04 ± 0.97 <sup>c</sup>	4.31 ± 0.20 <sup>c</sup>
<i>p</i> -Value		<0.01	<0.01

*N*: number of inseminated oocytes; PEN: penetration percentage from *N*; SPZ/O: mean number of penetrated sperm per oocyte.

Different letters (a,b,c) in the same column indicate significant differences ( $p < 0.05$ ).

<sup>a</sup> Based on penetrated oocytes.

(299.26 ± 21.20 nM/L) and P60/75 group (341.96 ± 23.53) without differences between these last three groups.

### 3.6. In vitro oocyte penetrating capacity

The in vitro fertilizing capacity of boar sperm was significantly affected by Percoll centrifugation. All the Percoll treatments increased the penetration rates and mean number of sperm per penetrated oocyte ( $p < 0.01$ , Table 5). The penetration rate increased from 50% in control group to 87% for P45/90 and P60/75 groups, and the highest value in penetration rate was for P45/90 group, at close to 100% (Table 5). The mean number of penetrated sperm per oocyte was affected by Percoll treatment in a similar way to penetration rate (Table 5). However, in all the groups male pronuclear formation was near to 100% without differences between groups.

## 4. Discussion

Under in vivo conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus. During this process, not

only are progressively motile spermatozoa selected but male germ cells also undergo physiological changes in a process called capacitation, which is a fundamental prerequisite for the sperm's functional competence with regard to acrosome reaction (Bedford, 1983; Yanagimachi, 1998).

Under in vitro conditions, fractionation of sperm by density gradient centrifugation can separate these subpopulations, resulting in a considerable improvement in the quality of sperm recovered in the pellet. Several reports indicate that higher percentages of motile and morphologically normal human sperm can be recovered from the pellet, in comparison with lower density fractions (Menkveld et al., 1990; Mortimer and Mortimer, 1992; Chen and Bongso, 1999; Ollero et al., 2000). Furthermore, sperm collected from the pellet exhibit less DNA damage (Larson et al., 1999; Sakkas et al., 1999) and produce less reactive oxygen species (ROS) than those recovered from the lower density layers, where abnormal sperm are predominantly found (Ollero et al., 2001). However in boar spermatozoa there is a lack of information about these sperm parameters after Percoll centrifugation and how the Percoll gradient conditions could affect the sperm parameters. In this study, we used different combinations of isotonic Percoll (P45/60, 60/75 and 45/90%) from fertile and normozoospermic boars that led us to select different sperm subpopulations that could be characterized by different sperm parameters.

In relation to sperm morphology, in human spermatozoa, Percoll gradient centrifugation reduces the percentage of morphological alterations, particularly when teratospermia is not severe (Hall et al., 1995). In boar sperm, Percoll density gradient has been used to isolate sperm subpopulations with cytoplasmic droplet (low-density) from spermatozoa without this droplet (higher density) (de Vries and Colenbrander, 1990). On the contrary, a reduction in sperm abnormalities other than cytoplasmic droplets, such as tail loops, after Percoll 35/70 has been previously reported (Waberski et al., 2006). Under our experimental

conditions only Percoll 45/90 is able to induce a significant reduction in both kinds of morphoanomalies, results that are in agreement with those reported in humans (Buffone et al., 2004). Analysis of the NAR showed that just after Percoll centrifugation no changes were detected in acrosome status, in agreement with data obtained previously (Grant et al., 1994), although when frozen–thawed spermatozoa were used and showed severe acrosome damage the use of Percoll centrifugation improved the NAR status compared to swim-up selection (Zheng et al., 1992).

The effect of Percoll in selecting porcine sperm with higher motility has been well documented (Berger and Parker, 1989; Horan et al., 1991; Grant et al., 1994; Waberski et al., 2006). However, other studies have not detected significant differences. This is probably related to the high variability that could be present in some boars, differences in the Percoll density procedures (Berger and Horton, 1988; Suzuki and Nagai, 2003) or differences in the system of measurement. In this study, we used a high speed camera (50 images/s, Basler AG, Ahrensburg, Germany) and a high number of images (100 images) per analysis, to more extensively and accurately follow the sperm motility pattern unlike previous studies (camera 25 pictures/s, 25 pictures analysed). As a consequence of this setting, the ALH values are lower and BCF higher than in previous studies (Gadea et al., 2005b). On the other hand, the use of a capacitating media as TALP medium (Rath et al., 1999), with a high level of bicarbonate and calcium in the composition, induced an early capacitation process that implies the presence of aggregates or pairs of spermatozoa. These sperm aggregates are not well recognized by the software because the total area of the particles is higher than setup. In consequence, the percentage of total and progressive motility could be underestimated, but it did not affect the motion parameters.

Only the gradient 60/75 selected higher percentage of progressive motility spermatozoa than control and it could be related to a selective effect on motility produced by the top layer of Percoll 60% that is not done by Percoll 45%. However, the differences in the pattern of movement reported in this study showed that the Percoll selection is important to optimize recovery of a higher percentage of spermatozoa with a fast-linear movement which could be related to the capacitation process as a significant increase of tyrosine phosphorylation and calcium intake was observed. This sperm subpopulation with fast motion characteristics could be related to the fertilizing capacity (Davis et al., 1995; Quintero-Moreno et al., 2004; Jędrzejczak et al., 2005).

The effect of Percoll on ROS generation could be complex. Centrifugation and resuspension of the resulting pellet subject cells to mechanical forces due to close packing (Abidor et al., 1994), and centrifugation could lead to sublethal damage (Alvarez et al., 1993). Centrifugation can also be detrimental to the spermatozoa because of the production of a burst of reactive oxygen species by a discrete subpopulation of cells characterized by significantly diminished motility and fertilizing capacity (Aitken and Clarkson, 1988) and the elimination of the antioxidant compounds that are present in the seminal plasma. On the other hand, Percoll separation could compensate by eliminating dead

cells, cells with cytoplasmic droplets and debris that generate a high proportion of ROS. These data overall suggest that the best balance is for P45/90 and the worse results are seen for P45/60, probably because it is not the most effective procedure for eliminating the damaged cells or because the packing is closer and the subsequent mechanical forces higher. This is in concordance with studies in humans that show that these fractions display higher ROS production (Aitken and Clarkson, 1988; Ollero et al., 2001).

Another interesting point is the fact that capacitating spermatozoa produce controlled amounts of ROS that regulate downstream events such as an increase in cAMP, protein kinase A (PKA) activation and phosphorylation of PKA substrates (de Lamirande and O'Flaherty, 2008). It is possible that ROS generation in spermatozoa after Percoll 45/90 could be related to the capacitation process while in the other experimental groups ROS generation could be related to the presence of dead cells and cytoplasmic droplets. However this hypothesis must be confirmed in further studies.

The subpopulations of spermatozoa selected by Percoll gradient differ not only in terms of conventional criteria such as motility and morphology, but also in terms of their DNA integrity as measured in human studies by different techniques such as the SCSA (Ollero et al., 2001), Comet assay (Van Kooij et al., 2004) and TUNEL (Mitchell et al., 2011). In humans the selection of spermatozoa by density gradient centrifugation also increases the percentage of mature spermatozoa with normal chromatin condensation compared to control (Le Lannou and Blanchard, 1988; Hammadeh et al., 2001; Mitchell et al., 2011). We observed that chromatin condensation and DNA fragmentation were similar among Percoll separated samples but better than in the control group, which suggests that when Percoll density is used to select motile and viable spermatozoa it indirectly selects spermatozoa with a nuclear normal structure and DNA (Mitchell et al., 2011). We also reported in this study a significant relationship between chromatin condensation and DNA fragmentation, as previously studied in different species (Fraser and Strzezek, 2007; Gadea et al., 2008; Mitchell et al., 2011) that suggests that abnormally condensed spermatozoa chromatin is also related with higher DNA fragmentation.

Spermatozoa washed on a Percoll gradient are partly capacitated (Berger and Parker, 1989) or have undergone the early stages of the acrosome reaction (De Maistre et al., 1996). Protein tyrosine phosphorylation has been associated with the ability of normal sperm to undergo capacitation and fertilize an egg (Urner and Sakkas, 2003). The pattern of tyrosine phosphorylation in boar is different from other species where the flagellum appears to be the principal sperm compartment presenting tyrosine-phosphorylated proteins. In boar the flagellum is less important (Petrunkina et al., 2001; Tardif et al., 2001) and the most important area is the equatorial area, which shows a specific triangular pattern of tyrosine phosphorylated proteins (Jones et al., 2008). Fluorescence is also present over the anterior acrosome, partly due to nonspecific binding of the second layer antibody and partly due to phosphoproteins within the acrosomal matrix (Tardif et al., 2001; Jones et al., 2008). In this study we confirmed that



Percoll increases the percentage of cells with phosphorylated tyrosine and that the pattern of staining is different in the different Percoll procedures, which may play a key role in sperm–zona pellucida interaction (Flesch et al., 2001). In Percoll 45/90 the percentage of cells with any kind of tyrosine phosphorylation is near to 100% and the signal extends to most of the spermatozoon (acrosome, equatorial segment and tail). In other Percoll groups (45/60 and 60/75), although the percentage of phosphorylated cells is higher than 75%, the extension of the signal is lower than P45/90. In accordance with other authors (Petrunkina et al., 2001; Urner and Sakkas, 2003), we suggest that the phosphorylation-positive spermatozoa could have a competitive advantage for penetrating the oocytes. However, this fact must be confirmed in further studies, including, for example, in vitro production of embryos.

Changes in intracellular calcium concentrations are associated with different aspects of sperm function such as capacitation, acrosome reaction, sperm chemotaxis and hyperactivation (Bedu-Addo et al., 2008; Costello et al., 2009). In this study we measured calcium in spermatozoa treated by Percoll density gradient and obtained results that follow the same pattern as those obtained in the study of tyrosine-phosphorylated proteins. The higher values obtained in P45/90 group suggests that the spermatozoa completed the capacitation process properly and are more capable of proceeding with the fertilization process.

The Percoll procedure yields pure fractions of spermatozoa, whereas the washing technique produces cell pellets that contain a heterogeneous population of spermatozoa along with amorphous material and seminal plasma (Grant et al., 1994). Percoll centrifugation not only selected a sperm subpopulation but also allowed some “decapacitation” factors to be removed from the sperm membrane and/or seminal plasma (Furimsky et al., 2005), which could facilitate subsequent sperm capacitation and acrosome reaction (Nolan and Hammerstedt, 1997).

Several studies have previously demonstrated that Percoll centrifugation leads to selection of a subpopulation of sperm with a higher ability to penetrate oocytes in vitro (Berger and Horton, 1988; Berger and Parker, 1989; Grant et al., 1994; Jeong and Yang, 2001; Suzuki and Nagai, 2003). Previously, we have shown that fresh spermatozoa treated with Percoll showed faster oocyte penetration and higher penetrability than control; the time of female and male pronucleus formation was affected by the sperm treatment and was faster for the Percoll group, while cleavage rate and rate of blastocyst formation were also higher after Percoll treatment (Matas et al., 2003). These differences are related to changes in some important spermatozoa parameters such as motility parameters, calcium intake, ROS generation, lipid membrane disorder and acrosome reaction (Matas et al., 2010). However, Percoll gradient did not improve embryo development after ICSI injection (García-Rosello et al., 2006).

Among all the sperm tests that have been developed, IVF tests are the most suitable for assessing overall sperm function during fertilization. The binding and penetration of the zona pellucida is one of the most important barriers the spermatozoa must overcome in the fertilization process. Also, the interaction with the oocyte plasma

membrane appears to explain much of the variability in sperm fertilizing potential (Gadea et al., 2005b). We used the IVF system to evaluate the penetration capacity and assumption that an elevated number of sperm could penetrate the oocytes. As we expected, the highest values for penetration were obtained in Percoll 45/90 because all the sperm parameters were better than or equal to control. However for Percoll 45/60, where similar values for morphology, worse values for in ROS generation and better motility, chromatin condensation, DNA and capacitation than control were observed, there was a higher penetration rate than control.

In the present study, three combinations of Percoll gradient density were tested and as expected, the highest penetration rates were observed after pretreatment with Percoll 45/90. Firstly, this procedure selected the highest number of morphologically normal spermatozoa with less alteration in the chromatin structure and less DNA damage; the motion characteristics are also improved and the percentage of capacitated spermatozoa steadily increases throughout incubation. All these characteristics lead to the highest proportion of penetration of oocytes in vitro.

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