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Obtainment of Enriched Capacitated Sperm in Pure Breed Dog Ejaculates Through Swim Up in an in Vitro Capacitation Medium

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Citation

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Abstract

The aim of this work was to identify capacitated dog sperm after a swim up procedure in vitro capacitation conditions (swim up/IVC). For this, freshly obtained sperm-rich fractions of ejaculates from 18 pure breed individuals were subjected to a swim up procedure in 10 mL of a defined IVC medium. Afterwards, samples were fractionated in 5 sections correlatively named from F0 to F4. The identification of capacitated sperm was conducted through determination of sperm motion characteristics, chlortetracycline (CTC) assay and phosphorylated DARPP-32 protein/total protein kinase A ratio. According to this, the greatest percentages of capacitated sperm were in fractions F2 (viable CTC+ spermatozoa= 91.8±0.8%) and F3. Results suggest that the application of swim up/IVC could be a useful tool to optimize the collection of capacitated sperm from pure breed canine ejaculates.

Key words: Dog Sperm; Pure Breed; Capacitation; Swim up

Introduction

Artificial insemination is common in canine pure breeds (Caturla-Sánchez et al.,2018; Mason, 2018). However, the utilization of more sophisticated techniques such as ICSI are much less commonly used (Van Soom et al., 2014; Nagashima et al., 2015, 2019; Hollinshead and Hanlon, 2017; Mason, 2018; Nagashima et al., 2019). This discrepancy is due to that studs with high genetic/economic value usually suffer a significant drop in their reproductive performance linked with the presence of poor-quality ejaculates (Borge et al., 2011; Marelli et al., 2020). Thus, the development of feasible techniques to improve reproductive performance is an increasing demand in pure breed dogs. One of the easiest techniques that can be utilized to enrich a poor-quality ejaculate is the swim up procedure. (García-López et al., 1996). This procedure has been successfully utilized in species like bovine (Ricci et al., 2009). However, its success in dog has been low (Dorado et al., 2016). This could be due to a lack of knowledge regarding the exact location of high-quality sperm after the swim up. This work has been centred in identifying this placement. For this, freshly collected sperm-rich semen fractions were subjected to a standard swim up procedure concomitantly with a IVC procedure (swim up/IVC; Rota et al., 1999; Albarracín et al., 2004a). Afterwards, separate fractions of the sample were carefully taken, and sperm were evaluated to detect of capacitated sperm in each fraction. Capacitation was evaluated by analyzing motion characteristics, chlortetracycline (CTC) assay (Albarracín et al., 2004a) and protein kinase A (PKA) activity by analyzing the pDARPP-32/PKA ratio (Hemmings et al., 1984; Córdova et al., 2012; Resende Chaves et al., 2021). The information obtained would be a first step to optimize in vitro reproductive techniques in dog.

Material and Methods

Animals and samples collection

All procedures. Including the written consent of dog owners, were approved by the Autonomous University of Barcelona Animal Care and Use Committee (procedure number 3692) and performed following the Animal Welfare Law issued by the Regional Catalan Government (Generalitat de Catalunya). Semen was from 18 dogs of previously unknown fertility, aged 3-9 years, belonging to different pure breeds (Table 1). Dogs were housed at a commercial kennel centre (Mascotes Costabrava, Les Oliveres Mas Gros, 56, E-17473 Ventalló, Girona, Spain). Animals were housed in huts with outdoor kennels, exercised once daily and fed a balanced diet with water ad libitum. Semen was collected by manual stimulation without the presence of an oestrous female. A sperm-rich fraction of the ejaculate from each dog was utilised. An initial sperm analysis (cell membrane integrity through eosin-nigrosine staining and subjective motility) was performed immediately after collection. Samples that showed percentages of cell membrane integrity and/or subjective total motility below 50% were discarded. Subsequently, another more complete analysis of selected samples was performed before starting the swim up/IVC procedure (Table 1).

Table 1: Description of individuals utilized in the study and their ejaculates

Dog	Breed	Age (years old)	Concentration (10^6 spz/ml)	Cell membrane integrity (%)	Altered acrosomes (%)	Total motility (%)	Progressive motility (%)	Total morphological abnormalities (%)	Head abnormalities (%)	Neck and midpiece abnormalities (%)
1	Beagle	5	420	97.2	0.3	93.5	63.1	75.4	15.1	34.7
2	Beagle	6	305	67.6	9.9	61.6	45.8	11.7	2.4	3.6
3	Beagle	6	670	99	0.1	96.7	77.1	54.6	17.3	21.8
4	Beagle	7	455	91.3	0.8	90.2	69	81	26.1	45.4
5	Beagle	6	221	84.2	2.7	85.8	75.8	12.6	3.5	3
6	GR	3	476	75.1	4.7	70.9	49.4	5.1	0	1.7
7	LR	6	390	60.8	6.9	62.6	37	54.9	7.6	29.8
8	LR	5	291	56.5	9.7	59.9	52.1	21.7	6.1	6.1
9	Beagle		684	93.1	0.7	95.1	78.9	6	0	1.2
10	Shiva Inu	7.5	330	52.4	6.5	47.8	32	82.5	45.7	22.7

11	LR	3	119	98.6	0.6	99.1	85.1	66.6	21.1	26.1
12	WHWT	5	467	94.4	1	97.9	77.3	21.7	2.6	12.5
13	Beagle	2	480	61	6.9	58.9	61	11.1	0	4.7
14	BM	3	990	54.3	8.1	54.5	43.2	79.4	38.2	22.4
15	BM	6	1115	81.7	1.7	79.8	65.7	3.8	0	2
16	CS	3	372	38.4	7.4	41.2	29.9	82	36	31.1
17	WHWT	5	805	92.5	0.6	91	85.1	35.7	19.1	7.8
18	CS	7	591	66.7	6.5	64	37.6	21.2	7	11.9

GR: Golden Retriever; LR: Labrador Retriever; WHWT: West Highland White Terrier; BM. Bernese Mountain; CS: Cocker Spaniel

Swim up/IVC procedure

Swim up/IVC was performed through incubation of sperm in 10mL of a specifically designed medium (SPZ; Albarracín et al., 2004 a, b 250x106 sperm were gently placed at the bottom of a tube that previously contained 10 mL of SPZ medium. Sperm were incubated in this medium for 4 hours at 38.5ºC in a 5% (v:v) CO2 atmosphere accurately avoiding any movement. Tubes were kept vertical during all the incubation time. Afterwards, samples were separated in four fractions by gentle aspiration. The uppermost 4 mL comprised the F4 fraction, whereas the following 4 mL was the F3 one. The next fraction (F2) was the next 1mL of the tube. The F1 was the cloudy 0.5 mL medium below the F2. The remaining volume was then last fraction (F0; Fig. 1). Fractions were separated by estimating both and macroscopical appearance. At the end of the incubation, a 3µL aliquot of each fraction was taken to analyse motility patterns. Afterwards, fractions F2, F3, F4 were centrifuged at 5000 g for 30 s. The obtained pellet was re-suspended in 250µL of fresh ZP and then, together with F0 and F1, were subjected to the evaluation of concentration, percentages of cell membrane integrity and morphological abnormalities, the chlortetracycline (CTC) test and analysis of the tyrosine phosphorylation levels of DARPP-32 and total amount of PKA ratio (pDARPP-32/PKA ratio), as described below.

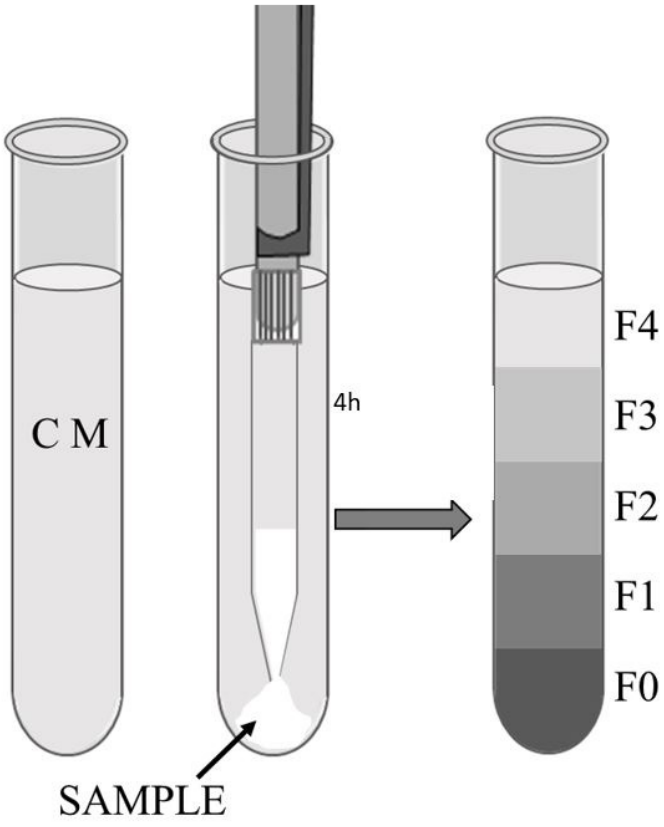

Figure 1: Swim up/IVC procedure scheme. CM: capacitation medium. F0-F4: fractions obtained after the application of the swim up/IVC procedure.

Percentages of cell membrane integrity, acrosome integrity, morphological abnormalities, motility and concentration

Percentages of both cell membrane integrity and acrosome integrity were determined by flow cytometry, following the recommendations and settings established by Lee et al. (2008). Samples were examined using a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA). The sheath flow rate was of 4.17 µL/min. electronic volume (EV) and side scatter (SS) were recorded as EV/SS dot plots for 10,000 events per replicate. The analyzer threshold was adjusted to exclude subcellular debris and cell aggregates. Compensation was used to minimize fluorescence spill-over. Information on all events was collected in List-mode Data files and processed using the Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter). Data were corrected following Petronian et al. (2010). Percentages of membrane-intact spermatozoa were determined using the LIVE/DEAD® Sperm Cell membrane integrity Kit (SYBR14/PI), following Garner & Johnson (1995). Acrosome integrity was determined through the fluorescein isothiocyanate-conjugated Arachis hypogaeaagglutinin (FITC-PNA)/ethidium homodimer (EthD-1) procedure, following Cooper & Yeung (1998). Total morphological abnormalities were determined in samples fixed in a 0.3% (w: v) glutaraldehyde solution in PBS. Fixed samples were then observed through a phase-contrast microscope at 1000X augmentations. A minimum of 200 sperm per sample were analyzed Motility parameters and sperm concentration of samples were determined by means of a computerized system analysis (CASA; Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain) as described in Albarracín et al. (2004a, b), and utilizing a Leja® 4 chamber slide of 20 µm (SC 20-0104-b; Leja Products; Nieuw Vennep, The Netherlands). The analysed motility parameters were curvilinear velocity (VCL), linear velocity (VSL), mean velocity (VAP), linear coefficient (LIN), straightness coefficient (STR), wobble coefficient (WOB), mean lateral head displacement (mALH), frequency of head displacement (BCF), dance (DNC), absolute angular mean displacement (absMAD) and algebraic angular mean displacement (algMAD) as defined in Quintero- Moreno et al. (2003). Total motility was defined as the percentage of motile sperm with VAP10µm/s. Sperm concentration of samples was analysed through the CASA system.

Chlortetracycline test

For this, 100µL-aliquots of each fraction were centrifuged during 10 min at 600g at 16ºC. CTC was determined after incubation of cells with 45µL of a 180 mM CTC solution as described in Albarracín et al. (2004a).

Analysis of tyrosine phosphorylation levels of the DARPP-32 protein

For this analysis, samples were centrifuged at 10.000g during 30s at 15ºC and the resultant pellet was immediately frozen in liquid N2 and further stored at -80ºC until analysis. When stated, sperm pellets were homogenised by sonication in 400 µL of ice-cold 50-mM Tris-HCl buffer (pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA), 10mM ethylene glycol tetraacetic acid (EGTA), 25mM 1,4-dithiothreitol (DTT), 1.5% (w/v) Triton X-100, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamidine, 10 µg/mL leupeptin and 1mM Na2VO4, (Homogenization Buffer). Samples were then centrifuged at 13.000g during 15 min at 4ºC. Resultant supernatants were subsequently diluted to a final concentration of 1.2µg/µL in Homogenization Buffer. Then, 40µL of each sample were processed through polyacrylamide gel electrophoresis in the presence of SDS and subsequent Western blot analysis as in Albarracín et al. (2004a). The pDARPP-32 expression was detected by using the anti- pDARPP-32 Thr 75 primary antibody (Cell Signal Technology, catalogue number #2301; Leiden, the Netherlands) and peroxidase-conjugated anti-rabbit secondary antibody (donkey anti-rabbit Santa Cruz technology, Santa Cruz, California, USA). Nitrocellulose membranes were then subjected to stripping. Afterwards, stripped membranes samples were tested for PKA detection by applying an anti-PKA antibody (catalogue number PA5-70360; Thermo Fisher Scientific, USA). Finally, bands intensity was measured with the programme Image J Fiji (Schindelin et al., 2012) and the pDARPP-32/PKA ratios for each point were calculated taking those of whole ejaculates as reference with the arbitrary value of 100. Final dilution for both primary antibodies was 1:1000 (v/v), while dilution for secondary ones was 1:5000 (v/v).

Statistical methods

All parameters were analysed by using the SAS® statistical package v9.0 (SAS Institute Inc., Cary, NC, USA). Statistical differences in values of all analysed parameters were evaluated by applying the GLM procedure after assessing normality with a Shapiro-Wilk test. Linear correlations between CTC and pDARPP32 data were determined by using the "Linear Regression" procedure. Motile sperm subpopulation structures were analysed through the FASTCLUS procedure by counting 5757 spermatozoa and then determined as Luna et al. (2017). Percentages of spermatozoa belonging to each subpopulation were calculated and compared through a GLM. In all analyses, the level of significance was set at $P<0.05$. Data are shown as mean±standard error of the mean (S.E.M.).

Results

Concentration, cell membrane integrity, altered acrosomes and morphological abnormalities

Mean concentration of sperm-rich fractions was of 510.1x106±61.4x106 sperm/mL (Table 2). Concentrations in the separate fractions were significantly (P<0.05) lower, with minimal levels in fractions F3 and F4 (1.1x106±0.1x106 sperm/mL and 0.9x106±0.1 sperm/mL respectively; see Table 2). Cell membrane integrity of the initial ejaculate (75.8±1.5%) were significantly (P<0.05) higher than those from the obtained fractions, where no significant differences were detected among them (Fig. 2A). Likewise, no significant differences were observed in the percentage of altered acrosomes among experimental points (Fig. 2B). Mean percentage of total morphological abnormalities in the initial ejaculate was of 40.4±7.3% (Fig. 3A). No significant differences were detected among all experimental groups (Fig. 3A). However, percentages of both head abnormalities and distal cytoplasmic droplets were significantly (P<0.05) higher, whereas tailabnormalities were significantly (P<0.05) lower, in the whole ejaculate when compared with all obtained fractions. (Table 2).

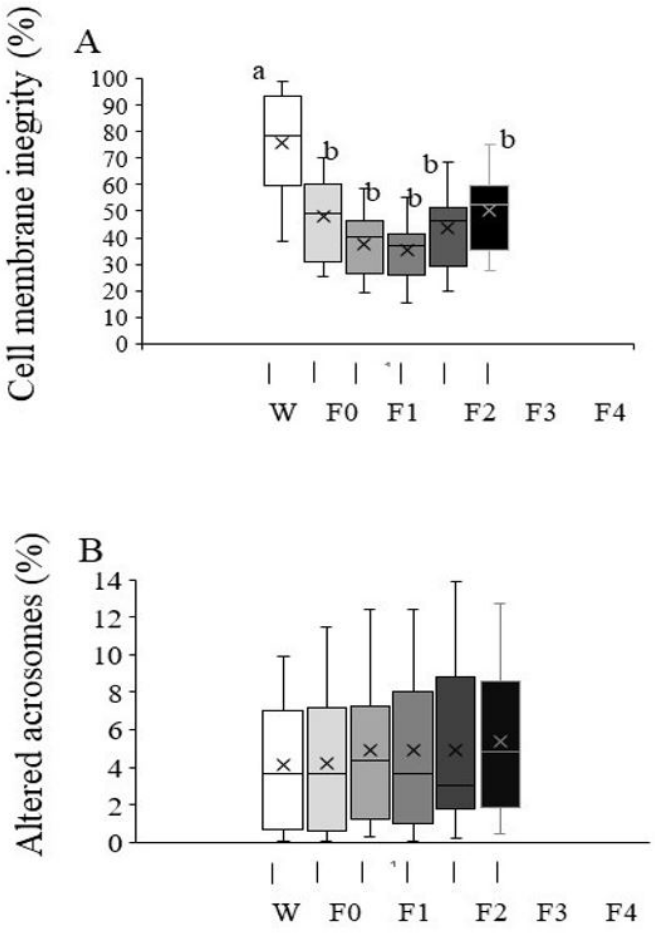

Figure 2: Effect of swim up/IVC procedure on percentages of cell membrane integrity (2A) and structurally altered acrosomes (2B). Whole (white bars): initial samples. F0-F:4 fractions obtained after the application of the swim up/IVC procedure. F0, descending grated bars. F1: ascending grated bars. F2: plain light grey bars. F3: plain dark grey bars. F4: plain black bars. Different letters indicate significant (P<0.05) differences among groups. Results are expressed as mean±S.E.M. for 18 separate ejaculates, one from each stud shown in Table 1.

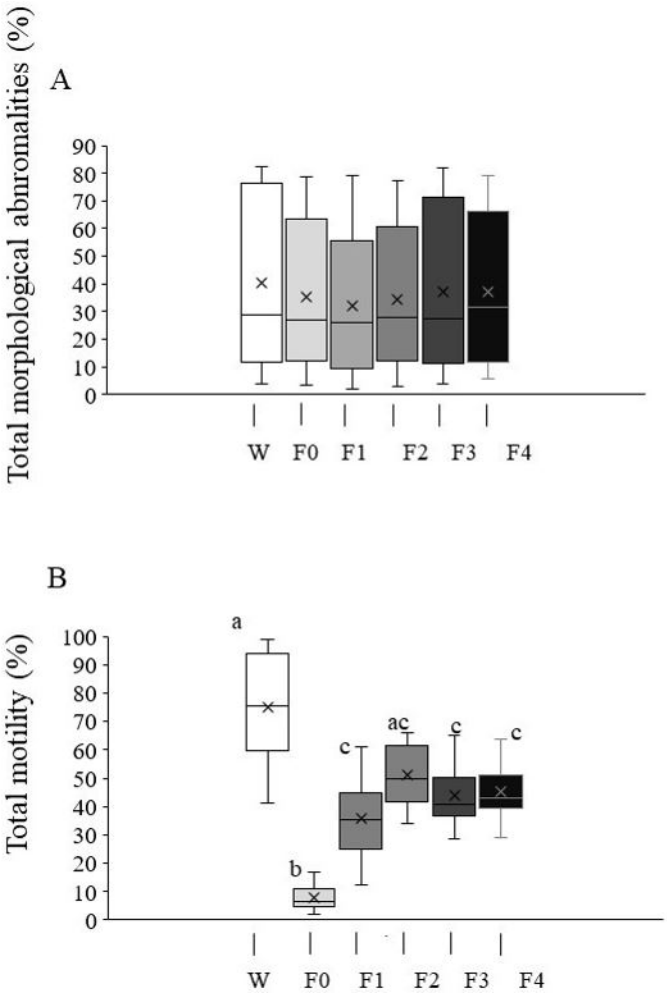

Figure 3: Effects of swim up/IVC on sperm total morphological abnormalities (3A) and total motility (3B). Whole (white bars): initial samples. F0-F:4 fractions obtained after the application of the swim up/IVC procedure. F0, descending grated bars. F1: ascending grated bars. F2: plain light grey bars. F3: plain dark grey bars. F4: plain black bars. Different letters indicate significant ($P<0.05$) differences among groups. Results are expressed as mean±S.E.M. for 18 separate ejaculates, one from each stud shown in Table 1.

Table 2: Percentages of specific morphological abnormalities and sperm concentration in initial ejaculates and sperm fractions obtained after the application of the swim up plus in vitro capacitation procedure.

	Whole ejaculate	F 0	F 1	F 2	F 3	F 4
Head abnormalities (%)	13.8±3.4^{a}	0.7±0.1^{b}	0.9±0.1^{b}	0.3±0.1^{b}	0.5±0.1^{b}	2.5±0.3^{c}
Neck and midpiece abnormalities (%)	16.0±3.2^{a}	14.1±1.9^{a}	11.9±1.6^{a}	14.2±2.2^{a}	16.0±2.5^{a}	12.8±1.9^{a}
Tail abnormalities (%)	7.0±1.5^{a}	19.5±2.7^{b}	17.1±2.0^{b}	19.0±2.2^{b}	20.5±2.5^{b}	23.7±2.6^{b}
Proximal cytoplasmic droplet (%)	2.5±0.2^{a}	1.9±0.2^{a}	2.8±0.2^{a}	2.4±0.2^{a}	2.9±0.3^{a}	3.2±0.3^{a}
Distal cytoplasmic droplets (%)	1.1±0.2^{a}	0.1±0.1^{b}	0.0±0.0^{b}	0.0±0.0^{a}	0.1±0.1^{b}	0.2±0.1^{b}
Concentration (10^{6} sperm/mL)	510.1±61.4^{a}	417.6±31.4^{a}	3.0±0.7^{b}	2.1±0.3^{bc}	1.1±0.1^{c}	0.9±0.1^{c}

Motion characteristics

The swim up/IVC procedure induced an overall decrease of the percentage of total motility in all fractions (Fig. 3B), accompanied with a significant (P<0.05) decrease of VSL, VAP, LIN and WOB values in F1, F2, F3 and F4. Furthermore, VAP values in F1 were significantly lower (P<0.05) than that of F3 (54.0±1.4 μm/sg in F3 vs. 60.2±1.4 μm/sg in F4; Table 3). These decreases were concomitant with a significant (P<0.05) increase of mALH, BCF, DNC and absMAD (Table 3). Regarding F0, there was a significant (P<0.05) increase of STR and BCF, together with significant (P<0.05) decreases of VCL, VAP and WOB (Table 3).

Table 3: Mean motion parameters of initial ejaculates and sperm fractions obtained after the application of the swim up plus in vitro capacitation procedure.

	Whole ejaculate	F0	F1	F2	F3	F4
VCL (μm/sg)	108.7±0.3^{a}	104.8±0.4^{b}	103.9±0.7^{b}	107.1±1.3^{ab}	108.9±1.6^{ab}	107.3±1.5^{ab}
VSL (μm/sg)	56.4±0.9^{a}	57.9±1.6^{a}	42.4±1.1^{b}	46.7±1.8^{b}	47.0±2.1^{b}	44.9±2.3^{b}
VAP (μm/sg)	76.7±0.7^{a}	66.5±1.1^{b}	54.0±1.4^{c}	57.9±2.3^{cd}	60.2±1.4^{d}	57.6±1.6^{cd}
LIN (%)	52.1±1.3^{a}	55.2±2.8^{a}	41.1±2.0^{b}	43.6±2.3^{b}	43.3±2.5^{b}	42.4±2.0^{b}
STR (%)	73.9±3.8^{a}	87.3±4.2^{b}	79.0±4.1^{ab}	80.7±4.0^{ab}	79.2±4.3^{ab}	78.2±4.6^{ab}
WOB (%)	71.7±4.7^{a}	63.2±4.1^{b}	52.1±3.7^{c}	53.8±3.9^{c}	54.9±4.2^{c}	54.0±4.6^{c}
mALH (μm)	3.89±0.05^{a}	4.01±0.06^{a}	4.57±0.05^{b}	4.42±0.06^{b}	4.50±0.06^{b}	4.47±0.07^{b}
BCF (Hz)	8.47±0.08^{a}	8.98±0.11^{b}	9.49±0.15^{c}	10.09±0.17^{d}	9.05±0.12^{b}	9.07±0.12^{b}
DNC (μm^{2}/sg)	424.3±7.6^{a}	421.0±6.8^{a}	475.2±7.7^{b}	473.9±7.2^{b}	488.9±8.0^{b}	479.0±7.5^{b}
absMAD (º)	100.8±1.6^{a}	109.6±2.4^{ab}	115.3±3.1^{b}	115.7±3.4^{b}	117.0±3.9^{b}	114.6±2.7^{b}
algMAD (º)	-0.12±0.25^{a}	-0.57±0.89^{a}	-0.23±0.41 ^{a}	0.21±0.44^{a}	0.25±0.67	-0.34±0.66^{a}

Both motility parameters and sperm fractions have been described in the Material and Methods section. Different superscripts in a row indicates significant (P<0.05) differences when compared with the respective value of the whole ejaculate. Results are expressed as means±S.E.M. for 18 separate ejaculates, one from each stud shown in Table 1.

Four sperm subpopulations were defined in the initial samples (Table 4). These subpopulations were classified following their velocity characteristics in a decreasing manner, being Subpopulation 1 (SP1) the fastest group and Subpopulation 4 (SP4) the slowest (Table 4). Otherwise, SP3 would correspond to that in which most of capacitated sperm are included (Table 4). Data indicated that the most abundant subpopulation in the initial samples, was SP2 (35.1±5.2%; Fig. 4). The percentage of sperm included in the SP3 significantly (P<0.05) increased in F1, F2, F3 and F4, when compared with whole samples, reaching maximal values in F2 (57.6%±6.2%; see Fig. 4). This increase was accompanied with a concomitant decrease in the percentages of both SP1 and SP2 in both F1 and F2, whereas in both F3 and F4 there was only a significant (P<0.05) decrease of the SP2 (Fig. 4).

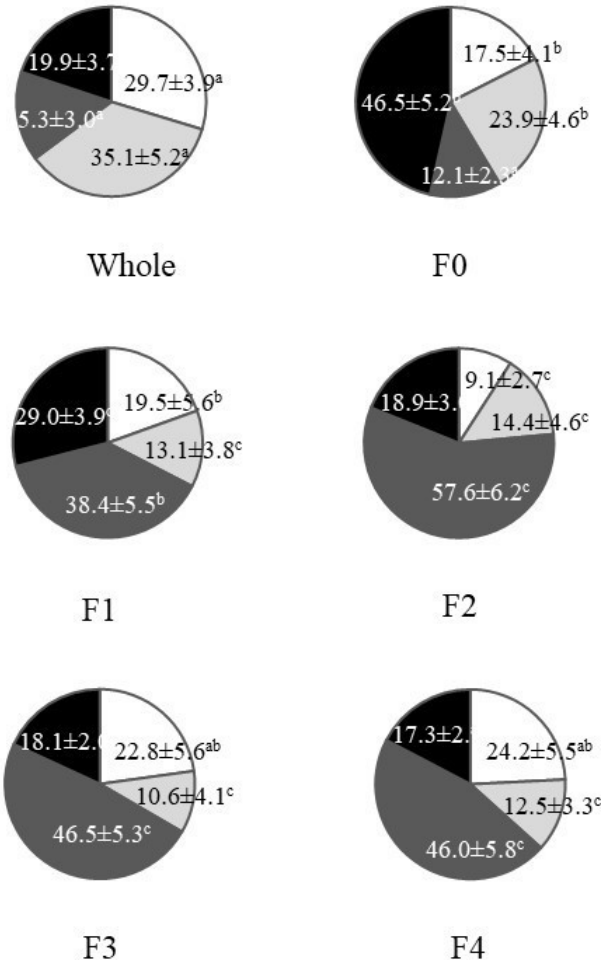

Figure 4: Sperm subpopulations structure of experimental groups. White: Subpopulation 1. Light grey: Subpopulation 2. Dark grey: Subpopulation 3. Black: Subpopulation 4. Whole: initial samples. F0-F:4 fractions obtained after the application of the swim up/IVC procedure. Different letters indicate significant ($P<0.05$) differences among groups. Results are expressed as mean±S.E.M. from 5757 sperm belonging to 18 separate ejaculates, one from each stud shown in Table 1.

Table 4: Kinematic parameters of the separate motile sperm subpopulations analysed in samples without the application of the swim up/IVC procedure.

	Subpopulation 1	Subpopulation 2	Subpopulation 3	Subpopulation 4
VCL (μm/s)	189.3±5.3	121.0±3.6	145.7±4.9	24.6±2.2
VSL (μm/s)	171.4±3.8	97.6±3.5	41.4±2.7	4.1±0.9
VAP (μm/s)	175.8±3.9	108.2±4.1	68.4±1.9	18.0±1.1
LIN (%)	91.2±4.0	80.8±3.6	29.5±2.3	16.9±1.1
STR (%)	98.2±5.5	91.4±5.0	62.0±4.8	23.3±2.7
WOB (%)	94.0±5.9	90.7±6.6	47.1±3.8	72.6±5.2
mALH (μm)	3.15±0.07	2.59±0.05	7.18±0.09	1.16±0.02
BCF (Hz)	9.47±0.10	21.37±0.14	13.51±0.13	2.98±0.08
DNC ($\mu m^2/s$)	600.0±25.6	312.6±14.9	1044.6±31.9	29.7±7.3
absMAD (°)	27.4±1.3	299.6±4.9	195.0±2.9	21.4±1.1
algMAD (°)	-0.34 ±0.28	-27.89±4.33	4.01±0.35	0.17±0.29

Motility parameters and sperm fractions have been described in the Material and Methods section. Data are expressed as mean±S.E.M. from 18 separate ejaculates, one from each stud shown in Table1.

Percentage of CTC+ sperm

Percentage of both total sperm with a capacitation-like CTC pattern (CTC+sperm) and viable, CTC+ sperm were significantly ($P<0.05$) higher in F2 and F3 (Figure 5: A, B).

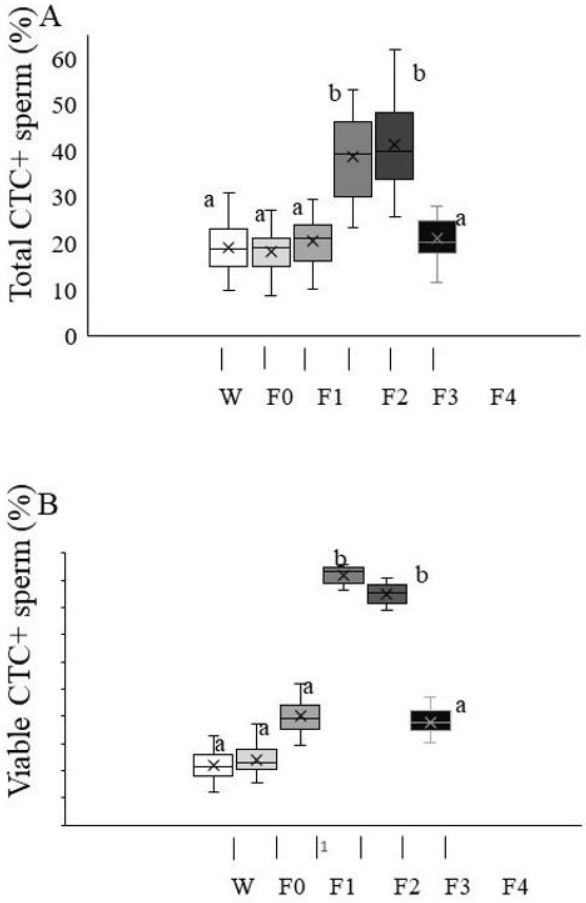

Figure 5: Effects of swim up/IVC on CTC Test. A). Percentages of total CTC+ sperm. B). Percentages of viable CTC+ sperm. Different letters indicate significant ($P<0.05$) differences among groups. Results are expressed as mean±S.E.M. for 18 separate ejaculates. Whole (white bars): initial samples. F0-F:4 fractions obtained after the application of the swim up/IVC procedure. F0, descending grated bars. F1: ascending grated bars. F2: plain light grey bars. F3: plain dark grey bars. F4: plain black bars. Results are expressed as mean±S.E.M. for 18 separate ejaculates, one from each stud shown in Table 1.

pDARPP-32/PKA ratio

Both initial samples (100.0±0.0 arbitrary units) and F0 (101.1±1.8 arbitrary units) presented the lowest pDARPP-32/PKA ratios. Maximal values were observed in both F2 (198.0±2.8 arbitrary units) and F3 (205.3±1.3 arbitrary units; Fig. 6B).

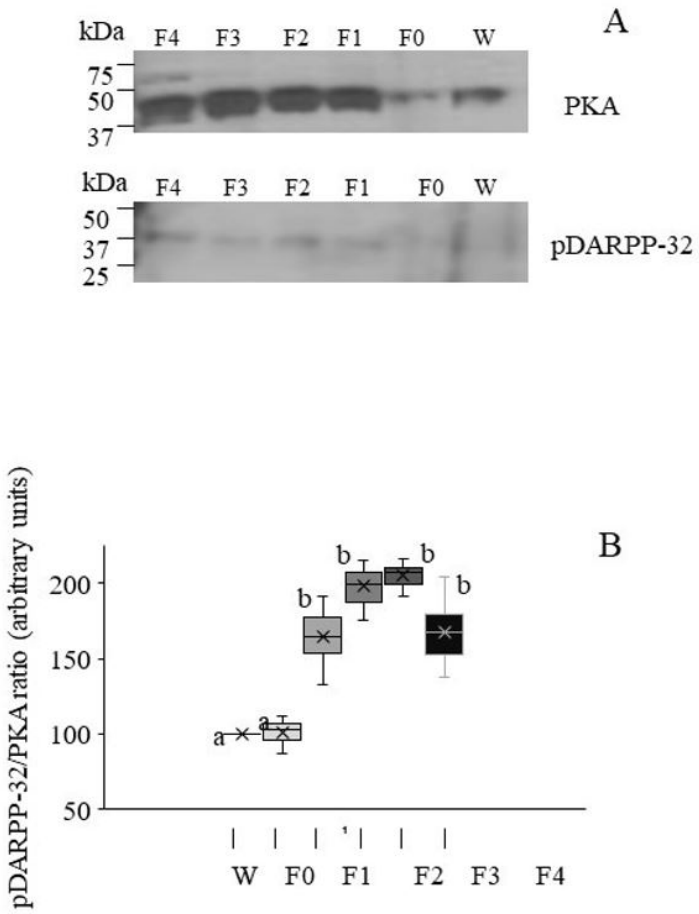

Figure 6: Effects of swim up/IVC on the pDARPP-32 PKA ratio. A). Representative figure for a Western blot showing the presence of PKA (PKA) and pDARPP32 (pDARPP32). Molecular weights are placed at the left side of each blot. B): pDARPP32/PKA ratios. W: initial samples F0-F:4 fractions obtained after the application of the swim up/IVC procedure. F0, descending grated bars. F1: ascending grated bars. F2: plain light grey bars. F3: plain dark grey bars. F4: plain black bars. Different letters indicate significant ($P<0.05$) differences among groups. Results are expressed as mean±S.E.M. for 7 separate ejaculates.

Linear correlations between chlortetracycline assay data and pDARPP-32/PKA ratio ones

There were significant ($P<0.05$) linear correlations between CTC+ sperm and pDARPP- 32/PKA ($r=0.7083$; Fig. 7A) and between viable CTC+ sperm and pDARPP-32/PKA ratio ($r=0.8486$; Figure 7B).

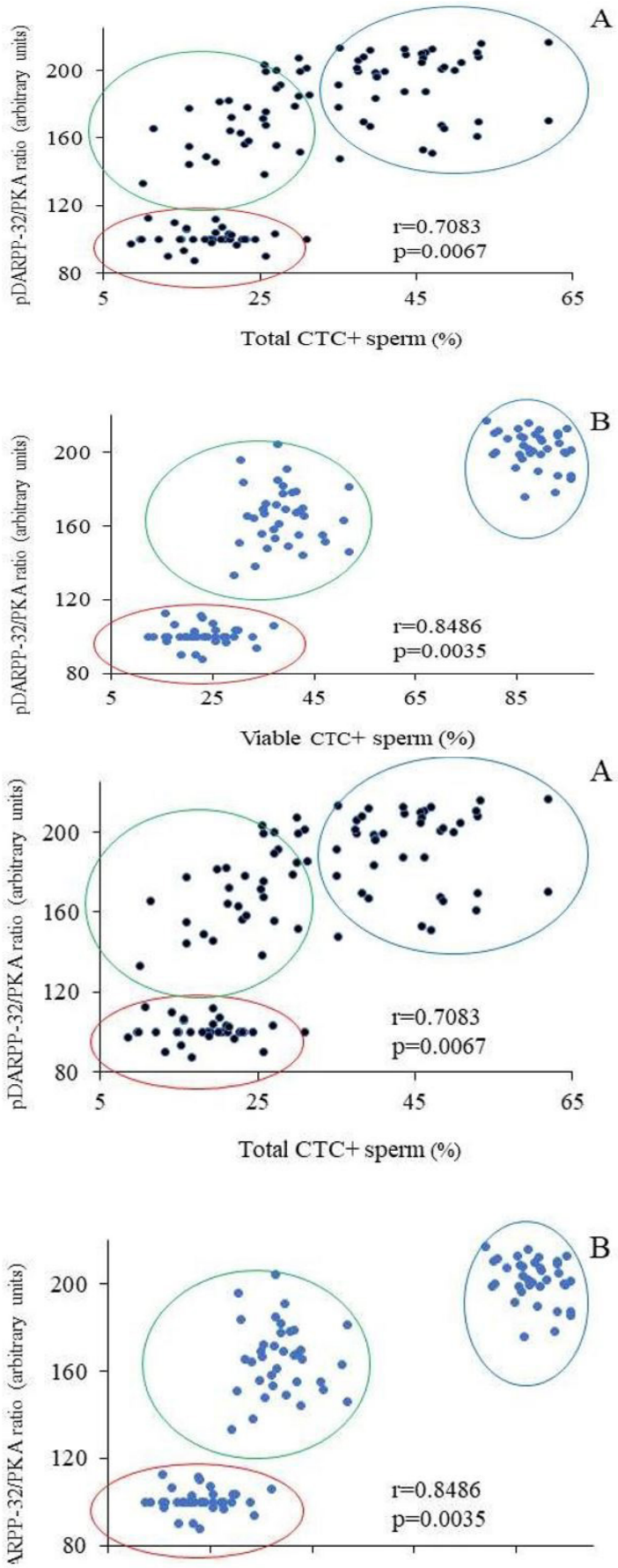

Figure 7: Distribution and linear correlation values between pDARPP-32/PKA ratios and percentages of either total, CTC+ sperm and viable, CTC+ cells. A: distribution points of pDARPP-32/PKA vs. percentages of total, CTC+ sperm. B: distribution points of pDARPP-32/PKA ratio vs. viable CTC+ sperm. P and r values are shown for each plot. Red ellipses: values of whole ejaculates and F0 fractions samples. Green ellipses: values of F1 and F4 fractions. Blue ellipses: values of F2 and F3 fractions.

Discussion

Results indicate that most of capacitated dog sperm obtained through swim up/IVC are accumulated at fraction volumes placed below the upper one. This is different to that observed in other species such as bovine, in which those sperm are accumulated uppermost (Holt et al., 2010). Species-specific sperm motility characteristics can be an explanation for this discrepancy. Thus, canine spermatozoa show faster and more linear motion characteristics than those observed in species like human (Nascimento et al.,2008). These species-specific differences could be related to the different timing that each species need to achieve the full sperm capacitation status, which in turn will be closely related with the time between ejaculation and ovulation (Petrunkina et al., 2003; Plant and Zeleznik, 2014). In this way, the precise motion characteristics of each species would be an important factor in explaining these differences. Our results indicate the existence of a low percentage of sperm that underwent capacitation under our experimental conditions. This result could be linked to that the final yielding of IVC-capacitated sperm widely varied among studies (Rota et al., 1999; Petrunkina et al., 2003; Albarracín et al., 2004a). Moreover, dog semen quality tends to be low in pure breed individuals (Soler et al., 2017). Thus, a poor-quality ejaculate would yield few capacitated sperm. Poor semen quality of these pure breed individuals might be linked with consanguinity linked to inbreeding (Van Eldik et al., 2006; Fitzpatrick and Evans, 2009; Inhorn et al., 2009; Bateson and Sargan, 2012; Ferenčaković et al., 2017). Thus, an optimization of dog IVC protocols would be very useful to increase the yielding of sperm with full fertilizing abilities in pure breeds with poor-quality ejaculates.

Results suggest that the utilization of the pDARPP-32/PKA ratio could be a useful tool to determine capacitation in dog sperm. The only species in which this ratio has been described are horse (Córdova et al., 2012) and boar (Resende Chaves et al.,2021).

The DARPP-32 is a regulatory subunit of the protein phosphatase 1 (Svenningsson et al., 2004). DARPP32 is a specific substrate for PKA (Svenningsson et al., 2004). Thus, capacitation-linked PKA activation leads to an increase in pDARPP-32 levels (Córdova et al., 2012; Resende Chaves et al., 2021). In this way, we postulate the determination of the pDARPP-32/PKA ratio as a useful marker for capacitation in mammalian sperm. In summary, results suggest that swim up/IVC could be a useful tool to separate significant amounts of capacitated sperm from pure breed dog ejaculates.

Conflict of Interest

There is no conflict of interest to declare.

Author Contributions

L.T.-R., O. B.-P.: main experimental analyses and semen collection from studs.

E. P.: main responsible for housing and keeping dogs

A. P.: collaborated in performing all experiments

T. R.: data analysis and obtaining funding.

M.M. R. Á.: data analysis and interpretation and manuscript writing.

J.E. R.-G.: experimental design, data interpretation, writing of manuscript and obtaining funding.

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