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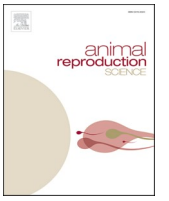

# BoviPure® density-gradient centrifugation procedure enhances the quality of fresh and cryopreserved dog epididymal spermatozoa

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ABSTRACT

BoviPure® is a salt solution containing colloidal silica particles coated with silane used to select sperm (e.g., ruminants) by density-gradient centrifugation (DGC). This research assessed the suitability of the BoviPure-DGC and swim-up methods for selecting dog epididymal sperm in fresh, chilled and frozen-thawed samples on post-treatment sperm quality. Sperm samples ($n = 60$ epididymides) were recovered by retrograde flushing from thirty orchiectomized adult dogs. Thereafter, 20 sperm pools, containing sperm aliquots of three randomly selected animals, were used for chilling (at 5 °C for 24 h) and freezing (in liquid nitrogen vapors). Sperm selection by BoviPure-DCG and swim-up was performed in both individual and pooled samples, including non-selected samples as controls. Overall, after BoviPure-DGC selection a higher sperm retrieval rate was obtained than the swim-up selection in both individual ($P < 0.05$) and pooled ($P < 0.01$) samples. BoviPure-DGC improved ($P < 0.05$) the total (TM) and progressive (PSM) sperm motilities, curvilinear (VCL) and straight-line (VSL) velocities, linearity (LIN), wobble (WOB), beat-cross frequency (BCF), and integrity of plasmatic (IPM) and acrosomal (IAM) membranes of individual samples in comparison with non-selected samples. In pooled samples, however, the BoviPure-DGC improved ($P < 0.05$) the PSM, VCL, WOB, and IPM of chilled and frozen-thawed samples. The swim-up method improved ($P < 0.05$) only some kinematic variables of the individual (VCL, WOB and BCF) and cryopreserved pooled samples (VCL and ALH) in comparison with non-selected samples. In conclusion, BoviPure-DGC was more effective for recovering and selecting both fresh and cryopreserved dog epididymal sperm than the swim-up procedure improving the kinematic variables, and membranes intactness.

## 1. Introduction

The recovery and cryopreservation of dog epidydimal sperm from genetically important individuals, or wild canids that have died unexpectedly, enables the application of assisted reproductive techniques (ART) (Hewitt et al., 2001). The successful recovery of these epididymal spermatozoa, however, depends on several factors that limit cell survival. The management techniques of testes and

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epididymis such as the time and temperature of storage, recovery method, or extender type, may influence sperm survival (Hewitt et al., 2001; Martins et al., 2012; Mota Filho et al., 2014). It has been previously reported that dog epididymal sperm may be successfully recovered when they are stored at low temperatures for a long time (i.e., 4–5 °C for up to 8 days) (Chima et al., 2017; Yu and Leibo, 2002). Under these conditions, it has been possible to obtain desirable results of motility, plasma membrane integrity, and fertilizing ability of dog epidydimal spermatozoa for use in ARTs ( Marks et al., 1994; Hewitt et al., 2001; Hori et al., 2005).

The major issue found after epididymal sperm retrieval is sample contamination. Epididymal spermatozoa commonly become contaminated with environmental microorganisms (Santiago-Moreno et al., 2009), interstitial fluid, epididymal cells, and blood (Martinez-Pastor et al., 2006), which may interfere with their optimal cryopreservation. Epididymal samples may contain normal and viable spermatozoa and other undesirable elements such as non-functional or abnormal sperm, blood, debris cell, dead cells, etc., as has been demonstrated by Muñoz-Fuentes et al. (2014) in wild canids epididymal spermatozoa. These unwanted elements may affect live sperm, probably due to the production of reactive oxygen species (ROS) that accumulate in deleterious concentrations, which impair fertilization (Nichi et al., 2007). This highlights the need to apply dog epididymal sperm purification techniques such as density gradient centrifugation (DGC) (Hishinuma and Sekine, 2004) or swim-up procedures to remove undesirable elements or cells as has been done in other domestic or non-domestic species (Santiago-Moreno et al., 2009; Chatdarong et al., 2010; Muñoz-Fuentes et al., 2014; Pradieé et al., 2018).

In the last decade, a variety of methods have been applied for selecting canine ejaculated sperm such as swim-up (Bukowska et al., 2011; Dorado et al., 2016), Sephadex filtration columns (Mogas et al., 1998), glass wool (Kim et al., 2010), and DGC using colloidal commercial solutions such as Percoll® (45/90%), ISolate® (50/90%), PureCeption® (40/80%), PureSperm® (40/80%), and Androcoll-C® (Dorado et al., 2016, 2013, 2011a, 2011b; Phillips et al., 2012). Few works have been carried out to purify canine epididymal spermatozoa (Hishinuma and Sekine, 2004; Muñoz-Fuentes et al., 2014). All aforementioned sperm selection methods were suitable for purifying canine spermatozoa. However, these results have demonstrated great variability in terms of motility and kinematics variables. Swim-up methods are based on the innate tendency of sperm cells to migrate through a medium in contact with the semen. This method allows the selection of highly-motile dog spermatozoa to be used in vitrification techniques (Sánchez et al., 2011). Some modification of the swim-up technique with Androcoll-C is also an effective method to select spermatozoa in canine semen preservation protocols (Dorado et al., 2016). DGC methods separate cells according to sperm density (Sharma and Agarwal, 2020; Beydola et al., 2013) which could be related to sperm head dimension. BoviPure®, a selective washing method based on DGC, is an iso-osmotic salt solution containing colloidal silica particles coated with silane used to select ejaculated ruminant (bull and ram) sperm for further application in ARTs (Arias et al., 2017; Galarza et al., 2018a, 2018b, 2018c; Samardzija et al., 2006). Silane-coated silica media have also been proposed to be used in single or two layer centrifugation for selecting ejaculated canine spermatozoa (Dorado et al., 2013; Gálvez et al., 2015; Phillips et al., 2012). To the best of our knowledge, there is no evidence of using BoviPure® to select epididymal dog sperm, either fresh nor cryopreserved. Hence, this research was aimed to assess the suitability of BoviPure-DGC and swim-up methods for selecting dog epidydimal sperm in fresh, chilled, and frozen-thawed samples. In addition, the influence of sperm selection methods on morphometric characteristics of the sperm head was also evaluated.

## 2. Material and methods

BoviPure® (BP-100) and BoviDilute® (BD-100) were obtained from Nicadon Laboratory (Nidacon, Mölndal, Sweden). All diluents and media were prepared in the Animal Reproduction Biotechnology Laboratory, University of Cuenca, Ecuador, using reagent-grade chemicals purchased from Sigma Chemical Co., (St. Louis, Missouri, USA).

### *2.1. Sperm recovery and initial evaluation*

All dogs were handled according to procedures approved by the Honorable Board of Directors of the Faculty of Agricultural Sciences from the University of Cuenca, and this research was performed in accordance with the chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (07/8/2019), regarding the protection of animals used in scientific experiments.

Testes and their epididymides were obtained from thirty healthy adult dogs of various breeds aged from 1 to 6 years after routine bilateral orchiectomy during a sterilization campaign of dogs undertaken by the Faculty of Agricultural Sciences of the University of Cuenca, Cuenca, Ecuador. The testes and their intact epididymis were placed into sterile Ziploc® bags (properly labeled: left or right). Samples were kept in ringer lactate solution at room temperature and transported to the Animal Reproduction Biotechnology Research Laboratory housed in 'Irquis farm' owned by University of Cuenca (3°04'48.1"S 79°04'31.0"W) within 2 h of collection. All tissues were stored at 5 °C for 72 – 96 h.

Cauda epididymis and vas deferens were dissected from each testis and placed in a dry pre-cooled petri dish. Epididymal sperm samples were collected by retrograde flushing administering 1 ml of cooled (5 °C) TCG-EY medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 0.54 mM Streptomycin, 2.14 mM Penicillin plus 20% egg yolk [v/v]; pH: 7.16 and osmolality: 354 mOsm/kg; Santiago-Moreno et al., 2009) through vas deferens and then the cauda epididymides were sliced. The content was recovered by pipetting and then placed into a 1.5 ml Eppendorf tube. This procedure was performed within a cold-chamber (5 °C). The percentage of motile sperm and the quality of sperm movement (scored on a scale from 0 *[lowest]* to 5 *[highest]*) were initially evaluated via phase contrast microscope (Nikon Eclipse, Nikon Instruments Inc., New York, USA). .

### 2.2. Experimental design

This experimental work was carried out in two studies. A total of sixty sperm samples were recovered by retrograde flushing from sixty canine epididymis and stored at 5 °C until processing. The total volume (approximately 1000 μl) of each sample was split into two aliquots of 400 μl and 600 μl used for study 1 and 2, respectively (Fig. 1).

#### 2.2.1. Study 1: sperm selection in fresh individual sperm samples

The study 1 was designed to evaluate the effect of sperm selection methods in sixty individual samples immediately after recovery process. Two 200-μl aliquots were taken from each sperm sample and then were selected by BoviPure-DGC and swim-up methods, respectively. Before sperm selection, a small aliquot (10 μl) was taken to measure sperm variables (motility and membranes integrity); this aliquot was considered as the control (non-treated semen samples).

#### 2.2.2. Study 2: sperm selection in pooled sperm samples

The study 2 was designed to evaluate the effect of sperm selection methods in pooled samples, either fresh or cryopreserved (chilled and frozen-thawed). A total of twenty pooled samples were made mixing three 600-μl aliquots (remaining) from individual samples randomly selected. Sperm concentration of each pooled sample was measured using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) and adjusted to a volume of 3 ml at $100 \times 10^6$ spermatozoa/ml using the same pre-cooled TCG-EY medium. Total volume of each pooled sperm sample was divided into three 1000-μl sub-aliquots. These aliquots were then maintained as fresh sample, chilled, or frozen-thawed. Each of these samples was purified with BoviPure-DGC and swim-up treatments with non-treated samples considered as controls.

*Sperm selection in fresh samples*. The first 1000-μl sub-aliquot of each pooled sample was subjected to the BoviPure-DGC and swim-up purified procedures using 200-μl of that sample for each method.

*Sperm selection in cryopreserved samples*. The second and third 1000-μl sub-aliquots were cooled and frozen, respectively, and then purified with both sperm selection methods. The cold-stored (at 5 °C for 24 h) sperm samples were warmed at room temperature and, subsequently purified with BoviPure-DGC and swim-up methods, taking two aliquots of 200-μl respectively, for each procedure. The last sample was frozen in liquid nitrogen ($LN_2$) vapors in two steps as described by Galarza et al. (2021). The first step consisted in equilibrating these 1000-μl (at $100 \times 10^6$ sperm/ml) for one h at 5 °C. The second step consisted in added another 1000-μl of a second cooled extender made of TCG-EY + 10% glycerol (v/v) (Sigma G9012, St. Louis, MO, USA) to the first aliquot sample (volume equal, 1:1) and equilibrated for one h more. Thereby, the final volume (2 ml) of these samples prior to conventional freezing reached a final concentration of $50 \times 10^6$ sperm/ml and 5% glycerol. Sperm samples were manually loaded into 0.25 ml IVM French straws (L'Aigle Cedex, France) and sealed with polyvinyl-alcohol (Sigma P8136, St. Lois, MO, USA). Straws were frozen using two ramps placed inside a Styrofoam cryo-box of 30 × 29 × 31 cm of length, width and height respectively, that contained 1.7 liters of $LN_2$ (up to 2 cm of height). The $LN_2$ was filled into the box 30 min before freezing process to equilibrate the $LN_2$ vapors inside the cryo-box. Straws were placed in the first ramp at 24 cm above the $LN_2$ surface and exposed to $LN_2$ vapors for 1 min, and then placed in a second lower ramp at 10 cm for 1 min more above the $LN_2$. Finally, straws were plunged in $LN_2$ to cool to − 196 °C and kept for three months.

Frozen straws ($n = 60$) were thawed by placing them in a water bath at 37 °C for 30 s. The contents of every two thawed straws

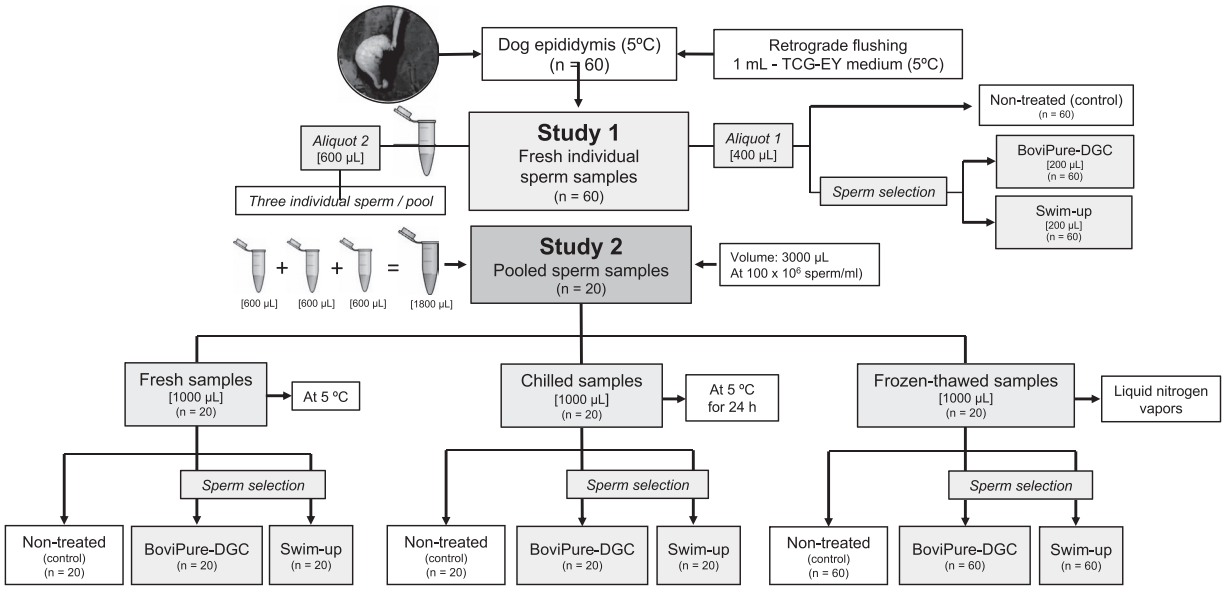


**Fig. 1.** Experimental design for assess the suitability of BoviPure-DGC and swim-up methods for selecting dog epididymal sperm in fresh, chilled, and frozen-thawed samples.

were poured into a dry 1.5 ml Eppendorf tube obtaining a total volume > 400 μl and then purified with both sperm selection methods (200-μl for each method).

### 2.3. Sperm selection procedures

Sperm selection was performed in individual samples and fresh, chilled and frozen-thawed pooled samples. The entire sperm selection process for both BoviPure-DGC and swim-up methods were performed at room temperature (i.e. cold stored sperm samples were allowed to warm to room temperature prior to being used).

#### 2.3.1. Swim-up

This procedure was performed as described by Bukowska et al. (2011) with slight modifications. Briefly, a volume of 500-μl of sperm TALP medium (NaCl 114 mM; KCl 3.2 mM; $NaH_2PO_4$ $H_2O$ 0.3 mM; Na Lactate 10 mM; $CaCl_2H_2O$ 2 mM; MgCl 6 $H_2O$ 0.5 mM; HEPES 10 mM; $NaHCO_3$ 25 mM; 6 mg/ml BSA; 0.11 mg/ml Na Pyruvate; and 5 μl/ml gentamycin; 348 mOsm/kg; pH: 7.2), previously warmed to 38.5 ºC, was placed in a 1.5 ml Eppendorf tube, and 200 μl of each sperm sample (individual or pooled) warmed to room temperature was placed under the layer of TALP medium. Samples were kept in the incubator at 38.5 °C for 30 min. Thereafter, the 200 μl upper layer (supernatant) was recovered and placed in a 1.5 ml Eppendorf tube for subsequent analysis of sperm quality.

#### 2.3.2. BoviPure-DGC

Following the protocol described by Galarza et al. (2018c), BoviPure® solution was diluted with BoviDilute® solution to obtain BoviPure Bottom layer medium and BoviPure Top layer medium, at 80% and 40% concentrations, respectively. The BoviPure-DGC columns were prepared in 1.5 ml conical Eppendorf tubes: equal volumes (200 μl) of BoviPure Bottom layer and Top layer medium were successively layered in the tubes. The individual or pooled sperm samples adjusted at room temperature were gently placed on top of the BoviPure Top medium. The columns were centrifuged at 300 *g* for 20 min. After centrifugation, the fluid above the sperm pellet was carefully removed. Each final pellet was then re-suspended in 100 μl TCG-EY medium (at room temperature), and sperm quality variables were evaluated.

### 2.4. Sperm analysis

The sperm kinematics analysis was objectively assessed using the 'Motility and Concentration' module of a CASA system (Sperm Class Analyzer, SCA Evolution 6.4.0.99 - © 2018 - software, Microptic S.L., Barcelona, Spain), coupled to a phase contrast microscope (Nikon Eclipse Ci-E; Nikon Instruments Inc., New York, USA; negative phase contrast [Ph1] with green filter). Individual or pooled sperm samples used to evaluate the kinematic variables were adjusted to a concentration of $20 \times 10^6$ sperm/ml with the TCG-EY medium. Briefly, the 5-μm aliquot of sperm samples was loaded into a warmed (37 °C) slide and covered with a coverslip. At least three fields and at least 200 sperm tracks per field (average: 600 spermatozoa per sample evaluated) were evaluated at 100 X for each sample slide (image acquisition rate 25 frames/s). The following sperm kinematic variables were assessed, as previously described by Galarza et al. (2021): total motility (TM, %), progressive sperm motility (PSM, %), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), oscillation (WOB), beat-cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, μm).

Plasma and acrosome membrane status were assessed using a double association of fluorescent probes – propidium iodide (PI, Sigma P4170) and fluorescein isothiocyanate conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381) - according to Galarza et al. (2018a). A total of 200 sperm cells per slide were examined using a Nikon Eclipse Ci-E epi-fluorescence light microscope (Nikon Instruments Inc., New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450 – 490 nm, and emission: 520 nm) and four subpopulations of cells were quantified, i.e., those showing in proportions: (1) intact plasma membrane/intact acrosome (IPIA); (2) intact plasma membrane/damaged acrosome (IPDA); (3) damaged plasma membrane/intact acrosome (DPIA); and (4) damaged plasma membrane/damaged acrosome (DPDA). In addition, the total proportion of cells presenting an intact plasma membrane equivalent to live sperm (IPM: IPIA + IPDA) and intact acrosomal membrane (IAM: IPIA + DPIA) were calculated.

Sperm head morphometry analysis was performed automatically using the ASMA (*Automated Sperm Morphology Analysis*) module of the same CASA system (SCA®) in fresh (non-frozen), non-selected frozen-thawed and selected BoviPure-DGC and swim-up samples. Microscope slides were prepared for each sample, placing 5 μl of sperm pool sample at the end of the slide and dragging the drop across with another. Smears were air-dried and stained using a SpermBlue® staining technique as previously described by Galarza et al. (2021). 100 images of heads were acquired to measure the morphometric dimensions of the sperm sample: length (L, μm), width (W, μm), area (A, $\mu m^2$), and perimeter (P, μm).

### 2.5. Statistical analysis

The results are presented as means ± SEM. All statistical analyses were performed using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by Shapiro-Wilk test, were transformed to *arcsine* (percentages values) or *log10* (numeric values) before analysis.

In *individual sperm samples* analysis, a general linear model (GLM) with a one-way ANOVA and Tukey post hoc multiple comparison tests were used to examine the effect of sperm selection methods on sperm kinetic variables and plasma membrane and acrosome

status. In *pooled sperm samples* analysis, a factorial ANOVA and Tukey post hoc multiple comparison tests were used to examine the effects of the interactions between *type of sperm sample* (fresh, chilled and frozen-thawed) and *sperm selection method* (non-treated, BoviPure-DGC and swim-up) on sperm kinetic variables and plasma membrane and acrosome status. In addition, in the analysis of the frozen-thawed samples, a one-way ANOVA was used to determine the effect of sperm selection on the morphometry of the sperm head in the fresh (non-frozen) and frozen-thawed samples of either non-selected or BoviPure-DGC and swim-up selected sperm.

## 3. Results

Mean values of the kinematics parameters and status of plasma and acrosome membranes in individual or pooled sperm samples before (non-selected) and after BoviPure-DGC and swim-up treatments are shown in Tables 1 and 2, respectively. Sperm head dimensions analysis of fresh and frozen-thawed pooled samples are included in Table 3.

### 3.1. Study 1. Sperm selection in individual sperm samples

Sperm concentration (x $10^6$ sperm/ml) in individual non-selected sperm samples was 311.1 ± 39. The sperm retrieval rate was greater ($P < 0.05$) after BoviPure-DGC selection than after swim-up selection (42.5 ± 3.4% vs. 31.3 ± 2.7%, respectively).

The values of TM, PSM and VSL after BoviPure-DCG treatment were greater ($P < 0.001$) than in the non-treated samples and after swim-up treatment. The VCL ($P < 0.001$), VAP ($P < 0.0001$), WOB ($P < 0.01$), and BCF ($P < 0.0001$) values were greater after BoviPure-DCG and swim-up treatments than in the non-selected sperm samples. Moreover, the proportion of LIN after BoviPure-DGC treatment was greater ($P < 0.05$) than in the non-selected sperm samples (Table 1).

The proportion of sperm with intact plasma and intact acrosomal membrane (IPIA), intact plasma membrane or viability (IPM), and intact acrosomal (IAM) were greater ($P < 0.05$) after BoviPure-DCG treatment than in the non-treated sperm samples. The undesirable sperm subpopulation with damaged plasma membrane and damaged acrosome was reduced ($P < 0.01$) after BoviPure-DGC treatment (Table 1).

### 3.2. Study 2. Sperm selection in pooled sperm samples

Similar to individual samples, sperm retrieval rate in pooled samples were greater ($P < 0.01$) after BoviPure-DGC treatment than after swim-up treatment whether in fresh (56.2 ± 2.1% vs. 45.5 ± 3.3%), chilled (58.4 ± 3.6% vs. 32.7 ± 2.8%), or frozen-thawed samples (46.2 ± 2.1% vs. 28.7 ± 3.4%), respectively. The interaction '*sperm selection methods* x *type of sperm samples*' had no significant effect ($P > 0.05$) on the sperm variables examined. Overall, the sperm kinematics variables and integrity of sperm membranes were lower in the cryopreserved samples (frozen-thawed and chilled-warmed) than in the fresh samples counterpart.

The TM proportion was greater ($P < 0.05$) after BoviPure-DGC treatment than after swim-up treatment in chilled and frozen-thawed sperm samples. The proportion of PSM of chilled samples, however, was greater ($P < 0.05$) after BoviPure-DGC treatment than in the non-treated sperm samples and after swim-up treatment. Moreover, the proportion of PSM of chilled sperm selected with

**Table 1**
Kinematics variables and status of plasma and acrosome membranes (mean ± SEM) for individual samples of dog epididymal sperm before and after subjection to BoviPure-DCG and swim-up procedures.

| Variables | Fresh individual samples ($n = 60$) | Sperm selection methods | | *P-value* |
|---|---|---|---|---|
| | | BoviPure-DCG ($n = 60$) | Swim-up ($n = 60$) | |
| TM (%) | 72.2 ± 2.31$^{b}$ | 81.0 ± 1.58$^{a}$ | 68.4 ± 2.71$^{b}$ | 0.0005 |
| PSM (%) | 19.0 ± 1.56$^{b}$ | 31.5 ± 2.07$^{a}$ | 24.2 ± 2.06$^{b}$ | 0.0000 |
| VCL (μm/s) | 58.3 ± 1.99$^{b}$ | 72.6 ± 2.83$^{a}$ | 67.8 ± 2.70$^{a}$ | 0.0003 |
| VAP (μm/s) | 32.3 ± 1.46$^{b}$ | 45.3 ± 1.64$^{a}$ | 41.3 ± 1.88$^{a}$ | 0.0000 |
| VSL (μm/s) | 20.5 ± 0.91$^{b}$ | 30.1 ± 1.36$^{a}$ | 26.6 ± 1.53$^{b}$ | 0.0000 |
| STR (%) | 58.7 ± 0.98 | 61.5 ± 0.93 | 59.5 ± 1.50 | 0.2141 |
| LIN (%) | 35.9 ± 1.12$^{b}$ | 40.3 ± 0.89$^{a}$ | 38.2 ± 1.44$^{ab}$ | 0.0308 |
| WOB (%) | 55.8 ± 1.09$^{b}$ | 61.4 ± 0.88$^{a}$ | 60.2 ± 1.37$^{a}$ | 0.0011 |
| ALH (μm) | 2.7 ± 0.08 | 3.1 ± 0.13 | 2.9 ± 0.11 | 0.1322 |
| BCF (Hz) | 5.3 ± 0.13$^{b}$ | 6.2 ± 0.13$^{a}$ | 6.2 ± 0.21$^{a}$ | 0.0000 |
| IPIA (%) | 80.1 ± 1.89$^{b}$ | 87.4 ± 1.55$^{a}$ | 83.5 ± 1.58$^{ab}$ | 0.0124 |
| IPDA (%) | 4.4 ± 0.81 | 2.4 ± 0.66 | 3.3 ± 0.72 | 0.1731 |
| DPIA (%) | 8.8 ± 0.75 | 6.9 ± 0.51 | 8.0 ± 0.56 | 0.1268 |
| DPDA (%) | 6.7 ± 0.87$^{a}$ | 3.2 ± 0.62$^{b}$ | 5.1 ± 0.72$^{ab}$ | 0.0095 |
| IPM - viability (%) | 84.6 ± 1.46$^{b}$ | 89.8 ± 1.00$^{a}$ | 86.9 ± 1.04$^{ab}$ | 0.0215 |
| IAM (%) | 89.0 ± 1.42$^{b}$ | 94.3 ± 1.21$^{a}$ | 91.6 ± 1.29$^{ab}$ | 0.0092 |

$^{a–c}$ Different superscripts within a same row differ significantly ($^{a–b–c}$ $P < 0.05$ and $^{a–c}$ $P < 0.01$). TM: total motility; PSM: progressive sperm motility; VCL: curvilinear velocity; VAP: average path velocity; VSL: straight line velocity; STR: straightness; LIN: linearity; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; IPIA: intact plasma membrane/intact acrosome; IPDA: intact plasma membrane/damaged acrosome; DPIA: damaged plasma membrane/intact acrosome; DPDA: damaged plasma membrane/damaged acrosome; IPM: intact plasma membrane (or viability); and IAM: intact acrosomal membrane.

**Table 2**
Kinematics variables and status of plasma and acrosome membranes (mean ± SEM) for pooled samples either fresh, chilled or frozen-thawed dog epididymal sperm before and after subjection to BoviPure-DCG and swim-up procedures.

| Variables | Fresh samples | | | Chilled samples | | | Frozen-thawed samples | | |
|---|---|---|---|---|---|---|---|---|---|
| | Non-treated ($n = 20$) | BoviPure-DCG ($n = 20$) | Swim-up ($n = 20$) | Non- treated ($n = 20$) | BoviPure-DCG ($n = 20$) | Swim-up ($n = 20$) | Non-treated ($n = 60$) | BoviPure-DCG ($n = 60$) | Swim-up ($n = 60$) |
| MP (%)*Ø | 75.5 ± 3.63$^{ab}$ | 84.3 ± 2.31$^{a}$ | 81.1 ± 4.26$^{a}$ | 60.7 ± 4.26$^{bc}$ | 72.5 ± 4.19$^{ab}$ | 53.3 ± 3.83$^{c}$ | 37.7 ± 4.43 $^{cd}$ | 53.4 ± 3.35$^{c}$ | 31.1 ± 3.64$^{d}$ |
| MP (%) *Ø | 24.1 ± 3.51$^{ab}$ | 35.5 ± 3.72$^{a}$ | 31.7 ± 4.17$^{a}$ | 15.1 ± 2.97$^{bc}$ | 30.9 ± 3.11$^{a}$ | 16.6 ± 2.50$^{bc}$ | 8.3 ± 1.54$^{c}$ | 18.5 ± 2.32$^{bc}$ | 10.8 ± 1.83$^{c}$ |
| VCL (μm/s) *Ø | 63.5 ± 3.84$^{abc}$ | 88.2 ± 9.72$^{a}$ | 90.4 ± 10.75$^{a}$ | 54.7 ± 2.94$^{bc}$ | 75.1 ± 3.22$^{ab}$ | 56.7 ± 3.62$^{bc}$ | 38.5 ± 2.56$^{e}$ | 52.8 ± 2.29 $^{cd}$ | 59.0 ± 5.45$^{bc}$ |
| VAP (μm/s) *Ø | 38.3 ± 2.61$^{ab}$ | 56.9 ± 6.03$^{a}$ | 54.9 ± 7.03$^{ab}$ | 29.9 ± 2.69 $^{cd}$ | 48.7 ± 2.47$^{ab}$ | 38.2 ± 2.82$^{abc}$ | 22.5 ± 1.60$^{d}$ | 34.2 ± 1.85$^{bc}$ | 38.8 ± 3.28$^{abc}$ |
| VSL (μm/s) *Ø | 26.4 ± 2.48$^{abc}$ | 44.2 ± 6.36$^{a}$ | 41.4 ± 6.94$^{a}$ | 22.5 ± 2.65$^{b}$ | 36.9 ± 2.54$^{a}$ | 29.1 ± 2.67$^{ab}$ | 16.5 ± 1.32$^{c}$ | 26.8 ± 1.99$^{ab}$ | 25.3 ± 2.55$^{abc}$ |
| STR (%) | 62.5 ± 2.55 | 70.5 ± 3.22 | 67.2 ± 3.38 | 62.8 ± 3.09 | 67.4 ± 1.75 | 68.8 ± 2.15 | 67.1 ± 1.85 | 70.7 ± 2.24 | 64.6 ± 3.17 |
| LIN (%) | 41.1 ± 2.35 | 47.0 ± 2.16 | 42.3 ± 2.45 | 37.9 ± 3.70 | 45.9 ± 2.24 | 48.5 ± 2.61 | 42.7 ± 3.00 | 48.9 ± 2.90 | 45.6 ± 3.36 |
| WOB (%)Ø | 59.8 ± 1.58$^{ab}$ | 63.6 ± 1.38$^{a}$ | 60.2 ± 1.43$^{ab}$ | 53.7 ± 3.31$^{b}$ | 63.4 ± 1.84$^{a}$ | 66.5 ± 2.04$^{a}$ | 58.8 ± 2.51$^{ab}$ | 64.2 ± 2.36$^{a}$ | 65.9 ± 2.53$^{a}$ |
| ALH, μm)*Ø | 2.8 ± 0.16$^{ab}$ | 3.0 ± 0.20$^{ab}$ | 3.1 ± 0.21$^{ab}$ | 2.4 ± 0.11$^{abc}$ | 3.1 ± 0.15$^{a}$ | 2.4 ± 0.13$^{abc}$ | 1.9 ± 0.13$^{c}$ | 2.2 ± 0.11$^{bc}$ | 2.4 ± 0.21$^{b}$ |
| BCF (Hz) | 6.39 ± 0.26 | 6.4 ± 0.23 | 5.7 ± 0.28 | 6.1 ± 0.45 | 6.4 ± 0.34 | 5.8 ± 0.44 | 5.2 ± 0.32 | 6.0 ± 0.28 | 6.2 ± 0.52 |
| IPIA (%)*Ø | 80.9 ± 2.80$^{a}$ | 86.2 ± 2.77$^{a}$ | 83.6 ± 3.10$^{a}$ | 56.8 ± 3.39$^{bc}$ | 69.1 ± 2.96$^{b}$ | 59.3 ± 3.31$^{bc}$ | 39.7 ± 1.88$^{d}$ | 56.4 ± 2.04$^{c}$ | 41.6 ± 1.78$^{d}$ |
| IPDA (%)* | 5.7 ± 1.82$^{ab}$ | 4.1 ± 1.68$^{ab}$ | 5.9 ± 2.30$^{ab}$ | 10.5 ± 2.43$^{a}$ | 6.2 ± 1.15$^{ab}$ | 7.7 ± 2.02$^{ab}$ | 2.7 ± 0.62$^{b}$ | 2.4 ± 0.55$^{b}$ | 4.3 ± 0.96$^{ab}$ |
| DPIA (%)*Ø | 8.3 ± 0.91 $^{cd}$ | 6.5 ± 0.52$^{d}$ | 6.3 ± 0.79$^{d}$ | 16.5 ± 1.90$^{ab}$ | 12.6 ± 1.32$^{bcd}$ | 13.2 ± 1.28$^{bc}$ | 21.7 ± 2.35$^{a}$ | 15.7 ± 1.07$^{ab}$ | 20.5 ± 1.23$^{a}$ |
| DPDA (%)*Ø | 5.2 ± 0.97$^{e}$ | 3.2 ± 1.03$^{e}$ | 4.1 ± 1.03$^{e}$ | 15.8 ± 2.33 $^{cd}$ | 12.1 ± 1.96$^{de}$ | 18.0 ± 2.46$^{bcd}$ | 35.9 ± 2.98$^{a}$ | 25.5 ± 1.78$^{a}$ | 33.6 ± 1.63$^{a}$ |
| IMP (%)*Ø | 86.5 ± 1.67$^{a}$ | 90.3 ± 1.39$^{a}$ | 89.6 ± 1.43$^{a}$ | 67.3 ± 2.73$^{bc}$ | 75,3 ± 2.87$^{b}$ | 67.0 ± 2.43$^{bc}$ | 42.3 ± 1.94$^{d}$ | 58.8 ± 2.22$^{c}$ | 45.9 ± 1.95$^{d}$ |
| IMA (%)*Ø | 89.1 ± 2.40$^{ab}$ | 92.8 ± 2.49$^{a}$ | 89.9 ± 2.88$^{a}$ | 73.4 ± 3.07 $^{cd}$ | 81.7 ± 2.20$^{bc}$ | 72.5 ± 3.09 $^{cd}$ | 61.4 ± 3.03$^{d}$ | 72.1 ± 1.65 $^{cd}$ | 62.1 ± 1.44$^{d}$ |

$^{a–e}$ Different superscripts within a same row differ significantly ($^{a-b-c-d-e}$ $P < 0.05$; $^{a-c,\ b-d,\ c-e}$ $P < 0.01$; and $^{a-d,\ b-e}$ $P < 0.001$). * Asterisk indicates significant differences between *types of sperm samples* (fresh, chilled, and frozen-thawed) ($P < 0.001$). Ø Symbol indicates significant differences between *sperm selection method* (non-treated, BoviPure-DGC, and swim-up) ($P < 0.01$). TM: total motility; PSM: progressive sperm motility; VCL: curvilinear velocity; VAP: average path velocity; VSL: straight line velocity; STR: straightness; LIN: linearity; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; IPIA: intact plasma membrane/intact acrosome; IPDA: intact plasma membrane/damaged acrosome; DPIA: damaged plasma membrane/intact acrosome; DPDA: damaged plasma membrane/damaged acrosome; IPM: intact plasma membrane (or viability); and IAM: intact acrosomal membrane.

**Table 3**
Mean sperm head measurements (mean ± SEM) of length (L), width (W), area (A), and perimeter (P) for fresh (non-frozen) and frozen-thawed dog epididymal sperm samples and selected by BoviPure-DGC and swim-up procedures.

| Variables | Fresh (non-frozen) ($n = 20$) | Frozen-thawed sperm | | | *P*-value |
|---|---|---|---|---|---|
| | | Non-treated ($n = 20$) | Sperm selection method | | |
| | | | BoviPure-DGC ($n = 20$) | Swim-up ($n = 20$) | |
| L (μm) | 5.7 ± 0.11 | 5.9 ± 0.05 | 5.8 ± 0.05 | 5.7 ± 0.08 | 0.2213 |
| W (μm) | 3.4 ± 0.06$^{ab}$ | 3.5 ± 0.02$^{a}$ | 3.4 ± 0.04$^{ab}$ | 3.3 ± 0.02$^{b}$ | 0.0086 |
| A (μm$^2$) | 19.6 ± 0.39$^{a}$ | 20.7 ± 0.26$^{a}$ | 20.2 ± 0.41$^{a}$ | 17.6 ± 1.08$^{b}$ | 0.0005 |
| P (μm) | 13.1 ± 0.24 | 13.7 ± 0.10 | 13.3 ± 0.10 | 13.0 ± 0.12 | 0.0570 |

$^{a–b}$ Different superscripts within the same row differ significantly ($P < 0.05$).

BoviPure-DGC reached similar values to those of fresh samples, irrespective of the sperm selection method. In chilled sperm samples, the VAP and VSL values were increased ($P < 0.05$) after BoviPure-DGC treatment compared with the values of non-treated samples. However, in frozen-thawed sperm samples, the VCL and VAP values were improved ($P < 0.05$) after both BoviPure-DGC and swim-up treatments compared with the values of non-treated samples. Moreover, the VSL value was greater ($P < 0.05$) after the BoviPure-DGC selection than in the non-treated sperm sample. The proportion of WOB after BoviPure-DGC and swim-up treatments were greater ($P < 0.05$) than in non-treated samples, however, only in chilled sperm (Table 2).

Regarding the status of sperm membranes, the proportion of sperm with intact plasma membrane and intact acrosome (IPIA) and

the overall proportion of sperm with intact plasma membrane (IPM) after BoviPure-DCG treatment were greater ($P < 0.05$) than after swim-up treatment and in non-treated samples, in frozen-thawed sperm (Table 2).

Morphometric analysis showed that fresh sperm head dimensions were not altered ($P > 0.05$) after the freezing-thawing process. Also, after BoviPure-DGC treatment, no sperm head dimensions were altered. After the freezing-thawing process, however, the head width was lower ($P < 0.05$) after swim-up treatment than in non-treated sperm. Curiously, the head area after swim-up treatment was smaller ($P < 0.05$) than in all other sperm samples (fresh and frozen-thawed either non-treated or selected with BoviPure-DCG) (Table 3).

## 4. Discussion

This research evaluated the efficacy of the BoviPure-DGC and swim-up procedures for selecting dog epididymal sperm from either individual or cryopreserved pooled samples. Our findings revealed that the BoviPure-DGC method was more suitable for purifying spermatozoa than the swim-up method based on a higher sperm recovery rate with improved kinematics and membranes integrity.

Canine ejaculate sperm selection by swim-up, either in fresh samples (Bukowska et al., 2011) or chilled (Dorado et al., 2016) has improved sperm quality increasing both motility and viability compared to its control counterpart. However, the most important drawback of the use of this method has been attributed to low sperm recovery rates, which limits its use to in-vitro conditions in ARTs (Beydola et al., 2013). We assume that the effectiveness of the swim-up method is mainly due to the sperm-TALP nutrient medium. Thus, the spermatozoa with better kinematic (compared with non-selected spermatozoa) from individual samples or chilled-warmed and frozen-thawed pooled samples floated towards the upper part of the tube with the sperm-TALP suspension as detailed by Holt et al. (2010). Nevertheless, the results of this work showed also that the swim-up method did not improve the integrity of either the plasma or acrosomal membranes. Therefore, the effectiveness of the swim-up method in the selection of dog epididymal sperm is limited only to the improvement of some sperm kinetic variables. The results of our study developed in dog epididymal sperm are consistent with those obtained in dog ejaculated sperm (Bukowska et al., 2011; Dorado et al., 2016) and other species (Santiago-Moreno et al., 2014; Chatdarong et al., 2010) after swim-up selection.

There are several studies that compare the effectiveness of commercial solutions based on colloidal silica particles, coated or not with silane (e.g., Percoll®, ISolate®, PureCeption®, PureSperm® or Androcoll-C®) compared to conventional techniques such as swim-up or filtration in the selection of canine sperm from ejaculated semen (Dorado et al., 2016, 2013, 2011; Phillips et al., 2012) or epidydimal samples (Muñoz-Fuentes et al., 2014). All these commercial products have been demonstrated to be useful for selecting dog sperm, enhancing the sperm quality of fresh or cryopreserved samples, especially motility and viability. To the best of our knowledge, there are no reports on the use of BoviPure-DGC on the selection of dog epidydimal sperm, thus this research constitutes the first report of the efficacy of BoviPure-DGC in canines.

BoviPure-DGC has proven effective to select the ejaculated spermatozoa with high motility and viability from different species (e.g., bull, goat or ram) (Arias et al., 2017; Galarza et al., 2018a; Samardzija et al., 2006), and thus it is a recommended technique to select ram sperm prior to the IVF process (Galarza et al., 2018b, 2019, 2018c). Galarza et al. (2018a), (2018c) suggest that kinematic activity of ram semen cold-stored for 48 h and then selected by BoviPure-DGC is maintained, which led to improved viability and membrane integrity in comparison with non-treated samples. The results of this work indicate that the dog epididymal sperm selected by BoviPure-DGC had greater velocities and progression ratio values than swim-up selected or non-selection samples, as was previously demonstrated by Oliveira et al. (2006) and Dorado et al. (2011a) in dog ejaculated sperm. The biological relevance of these findings in dogs has been established in fresh or frozen-thawed sperm either from ejaculated (Silva et al., 2006) or epididymal sperm (Varesi et al., 2014; Mota Filho et al., 2014), since high motilities, velocities and DNA integrity are benchmarks for greater in vivo and in vitro fertility.

Consistent with previous studies developed in semen ejaculates (Hernández-López et al., 2005; Maxwell et al., 2007), the BoviPure-DGC procedure allowed purification of dog sperm with greater integrity of plasma (viability) and acrosome membranes. Moreover, our results demonstrated that BoviPure-DGC was more effective than swim-up for selecting dog epididymal spermatozoa with intact plasmalemma and acrosome membranes. The sperm population with both plasma and acrosome membranes intact will be able to reach the site of fertilization after artificial insemination with the capacity to penetrate the oocyte, as the acrosome membrane is intact and prepared to undergo acrosome reaction (Dorado et al., 2011a; Santiago-Moreno et al., 2020).

Furthermore, the findings of this study showed that the BoviPure-DGC procedure did not alter the sperm head dimensions, while the swim-up method reduced the width and area of the head of those frozen-thawed samples. It is known that the freezing-thawing process provokes disruption of sperm membranes and microtubules influenced by osmotic stress. Consequently, the osmotic stress determines changes in morphometric head dimensions of epididymal or ejaculated sperm after the freezing-thawing process (Watson, 2000; O'Brien et al., 2019). Previous studies have determined that cryopreserved epididymal samples (Esteso et al., 2006) or ejaculated sperm (Bóveda et al., 2020; Hidalgo et al., 2007) suffer a reduction of the size of the head compared to pre-freeze values. It has been suggested that over-condensation of sperm chromatin, plasma and acrosome membrane injuries, as well as acrosome loss and damage in the cell cytoskeleton could cause a reduction in the head size (Arruda et al., 2002; Santiago-Moreno et al., 2016). Cerdeira et al. (2020) determined that conventional freezing tended to decrease the head dimensions of ejaculated dog sperm. However, another study determined a correlation between the cat epididymal sperm head area and chromatin condensation (Alves et al., 2018). The authors suggest that sperm head size decreases due to increases in DNA compaction during epididymal transit, but there is less chance of DNA damage during cryopreservation. The results of the present study showed that the freezing-thawing protocol used did not affect the sperm head dimensions. Unlike the BoviPure-DGC procedure, the frozen-thawed sperm samples selected by swim-up had decreased width and area of the head. This reduction of head dimensions may be due to the hyperosmolarity (> 345 mOsm/kg) of the

Sperm-TALP medium used with the swim-up method, which may cause sperm dehydration. These results also suggest that the swim-up purification procedure selects frozen-thawed dog epididymal sperm with a smaller head but with higher velocities and seemingly, DNA more compacted and less damaged.

## 5. Conclusions

In conclusion, the BoviPure-DGC procedure enhanced the motilities, velocities, movement trajectory, and integrity of plasma and acrosome membranes of fresh, chilled, and frozen-thawed dog epididymal sperm. In addition, the BoviPure-DCG treatment was more effective for selecting fresh or cryopreserved epididymal sperm than the swim-up treatment concerning sperm retrieval rate, kinematic parameters as well as membranes intactness, and head morphometry of cryopreserved spermatozoa. This new procedure in dogs could improve in vitro fertilization results; however, this has to be proven yet. This is the first attempt to use the BoviPure-DGC to select canine epididymal sperm and it is recommended for use in cryopreservation procedures and other reproductive technologies.

## CRediT authorship contribution statement

**D.A. Galarza:** Conceptualization, Methodology, Formal analysis, Validation, Writing – original draft, Visualization. **D.I. Jara, E.B. Paredes and J.X. Samaniego:** Methodology, Formal analysis and Resources. **M.S. Méndez, M.E. Soria, F. Perea and E. Muñoz-León:** Formal analysis and Resources. **J. Santiago-Moreno:** Conceptualization, Writing – review & editing, Supervision.

## Conflict of interest

The authors have no conflicts of interest to declare.

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