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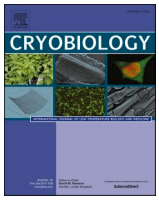

Epididymal bull sperm selection by Percoll® density-gradient centrifugation prior to conventional or ultra-rapid freezing enhances post-thaw sperm quality

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ABSTRACT

This study evaluated the effectiveness of Percoll® density gradient centrifugation (Percoll-DGC) for selecting bull epididymal sperm prior to conventional slow (CS) or ultra-rapid (UR) freezing and its effects on sperm quality. Fifteen pooled samples from 30 epididymides (2 different samples/pool) of 15 bulls were split into two aliquots assigned to either CS or UR freezing. Samples were either selected using Percoll-DGC (40/80 %) or left non-selected (control), resulting in four pre-freezing treatments: Percoll-CS, Control-CS, Percoll-UR, and Control-UR. The CS freezing used 5 % glycerol, exposing sperm straws to liquid nitrogen (LN_2) vapors, while UR freezing used 100 mM sucrose with direct submersion of 30 μL samples into LN_2. Overall, sperm quality was higher in CS treatments than in UR treatments. Pre-freezing, Percoll-CS improved straight-line velocity (VSL), linearity (LIN), and beat-cross frequency (BCF) compared to Control-CS ($P < 0.05$). Similarly, Percoll-UR treatment enhanced progressive motility (PSM), velocities, straightness (STR), amplitude of lateral head displacement (ALH), and BCF compared to Control-UR ($P < 0.05$). Post-thaw, Percoll-CS demonstrated higher kinematic parameters, viability, and acrosome integrity compared to Control-CS ($P < 0.05$). Meanwhile, Percoll-UR improved viability and acrosome integrity relative to Control-UR ($P < 0.05$). Notably, both Percoll-UR and Control-UR resulted in lower DNA fragmentation compared to Percoll-CS. In conclusion, Percoll-DGC selection prior to CS freezing significantly improved post-thaw sperm quality, including kinematics, viability, and acrosome integrity. For UR freezing, Percoll-DGC primarily enhanced post-thaw viability and acrosome integrity.

1. Introduction

Sperm cryopreservation significantly extends the storage time of mammalian spermatozoa by completely inhibiting metabolic activity. It has a fundamental impact on livestock production, as its application in artificial insemination (AI) is essential for cross-breeding and genetic improvement programs [49]. For decades, the conventional slow (CS) freezing technique, which employs penetrating cryoprotectant agents, has been widely used for storing bovine semen [54]. Typically, the CS freezing technique requires a lengthy equilibration period prior to freezing, followed by exposure of sperm samples to static liquid nitrogen (LN_2) vapors. This exposure generates initial cooling rates ranging from 40 to 60 °C/min [18]. However, the cooling rates applied during the CS freezing process can cause cryoinjuries and structural damage to bovine sperm. Such damage is primarily due to intracellular ice crystal formation [25], which is further exacerbated by cold shock, oxidative stress, and osmotic stress [19].

Ultra-rapid (UR) freezing serves as an alternative to CS freezing for cryopreserving bull sperm. The UR freezing technique is performed by directly immersing microdroplets (30–50 μL) of sperm samples, extended with high concentrations of non-penetrating cryoprotective agents (e.g., sucrose or trehalose) into LN_2 [20,35]. This direct contact

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with LN_2 generates exceptionally high cooling rates ranging from 2000 to 10,0000 °C/min [22]. Sucrose, the most commonly used non-penetrating cryoprotective agent in UR freezing of ruminant sperm [35], increases the osmolarity of the freezing medium, leading to cellular dehydration [42,45] However, a recent study reported low cryosurvival rates of ultra-rapidly frozen bull sperm [13]. In contrast, some species of wild ruminants have demonstrated high cryoresistance, particularly when epididymal sperm is used [35].

Epididymal sperm have been effectively utilized for cryopreservation and subsequent application in AI [30] The use of epididymal sperm is particularly justified in cases where bulls of high genetic value die unexpectedly or experience reproductive failures, among other scenarios. Cryopreserved bull epididymal sperm have been successfully employed in AI, resulting in successful births [9], as well as in other reproductive biotechnologies, such as *in vitro* embryo production (IVP) and intracytoplasmic sperm injection (ICSI). Evidence suggests that bull epididymal sperm are more cryoresistant than ejaculated sperm [10]. However, sperm cryosurvival is influenced by the initial quality after recovery and prior to the freezing process [8]. The presence of dead and non-functional spermatozoa, blood cells, cellular debris, and other dead cells in epididymal sperm samples can compromise viable spermatozoa [15]. These undesirable elements may lead to uncontrolled production of reactive oxygen species (ROS), triggering apoptosis and DNA fragmentation [53]. Therefore, implementing sperm purification procedures before freezing could enhance sperm quality both prior to and after cryopreservation.

Sperm selection by density gradient centrifugation (DGC) is a procedure that isolates sperm cells based on their density, enabling the selection of motile and morphologically normal sperm. Live and motile spermatozoa with intact membranes are denser than degenerated or dead spermatozoa, as well as other cellular debris and bacteria [3] Consequently, during centrifugation at 300×*g*, they settle in the denser gradient (pellet). Percoll®, a commercial colloidal silica medium with particles (15–30 nm in diameter) coated with non-dialyzable polyvinylpyrrolidone, has been effectively used to select bull spermatozoa [11]. Studies have demonstrated that Percoll-DGC (40/80 %) efficiently selects highly motile frozen-thawed bull spermatozoa for use in procedures such as *in vitro* fertilization [31,41,52], without affecting gene expression [3]. Most research, including those aforementioned studies, has successfully employed Percoll-DGC to select bull sperm after cryopreservation. However, the use of Percoll-DGC for the selection of bull epididymal sperm prior to freezing has not yet been reported.

It is well established that the cryopreservation process affects the dimensions of the sperm head, whether of ejaculated or epididymal origin. This variation in head dimensions is primarily attributed to osmotic stress experienced by sperm during the freezing stage [14,35]. During supercooling, sperm are exposed to hypertonic solutions formed by solutes, which force the cells to undergo significant dehydration prior to freezing. This severe dehydration can also cause chromatin damage, compromising its integrity and condensation. Reductions in sperm head dimensions have been reported following conventional slow freezing [17]. However, the use of ultra-rapid freezing could help minimize these detrimental effects caused by osmotic stress.

The present study hypothesized that selecting epididymal bull sperm through Percoll-DGC before either conventional slow or ultra-rapid freezing could enhance kinematic parameters (such as motility, velocity, progression ratio parameters) and maintain membrane integrity by mitigating the harmful effects of high ROS production, such as DNA fragmentation. Therefore, the objective of this work was to evaluate the impact of sperm selection and cryopreservation method on the quality and morphometry of bull epididymal spermatozoa.

2. Materials and methods

2.1. Chemicals

All diluents and media were prepared in the Animal Reproduction Biotechnology Research Laboratory using reagent-grade chemicals. Percoll® (Sigma P1644) and other reagents used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany).

2.2. Sperm collection and initial evaluation

This research was conducted in compliance with the guidelines provided in chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (August 07, 2019), which addresses the protection of animals used in scientific experiments. The research project was approved by the Honorable Board of Directors of the Faculty of Agricultural Sciences from the University of Cuenca.

Thirty bovine testicles (15 left and 15 right) with intact epididymides were collected from fifteen adults, sexually matured, and healthy bulls routinely slaughtered at a local abattoir. The testicles were transported in a Styrofoam box at room temperature (22 °C) to Animal Reproduction Biotechnology Research Laboratory within 2 h. The cauda epididymis and vas deferens were carefully dissected and isolated from each testicle and placed in a dry Petri dish. Epididymal sperm samples were obtained by the retrograde flushing method [14], were 1 mL of TCG-EY medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 0.54 mM Streptomycin, 2.14 mM Penicillin, +6 % egg yolk [v/v]; pH: 7.16 and osmolality: 341 mOsm/kg) was administered through vas deferens, followed by slicing the cauda epididymis with a scalpel. The recovered fluid was placed into a 1.5 mL Eppendorf tube, and its volume was measured. This procedure was performed at room temperature (22 °C).

The epididymal sperm samples were then subjected to an initial evaluation. The percentage of motile sperm and the motility quality were assessed using a phase contrast microscope (Nikon Eclipse, Nikon Instruments Inc., New York, USA) at 100× magnification. Only sperm samples with a motility percentage greater than 70 % and a motility score above 2 on a scale from 0 (lowest) to 5 (highest) were included in the subsequent experimental work.

2.3. Experimental design

Two epididymal sperm samples (regardless of bull, or left or right epididymis) from fifteen bulls were randomly allocated to create *'epididymal pooled sperm samples'* (n = 15). The volume of each pooled sample was measured using a graduate micropipette (Boeco®, Germany). Sperm concentration was determined using a photometer (SDM 1; Minitube®, Germany).

Each pooled sample was initially divided into two equal-volume aliquots, which were then diluted using conventional slow (CS) and ultra-rapid (UR) freezing media, respectively. Subsequently, each aliquot from CS and UR freezing media was further divided into two sub-aliquots: one subjected to sperm selection by Percoll® density gradients centrifugation (Percoll-DCG) and another left without sperm selection (non-selected, considered as the control). Prior to freezing, four treatments were established based on the combination of *'sperm selection'* and *'freezing medium'* and their kinematic parameters were evaluated: 1) Percoll-CS, 2) Control-CS; 3) Percoll-UR; and 4) Control-UR.

Consequently, sperm samples from these treatments were frozen according to the freezing method (see below). After three months, samples from all treatments were thawed and then the kinematic parameters, viability, acrosomal integrity, DNA fragmentation, and morphometric head dimensions were evaluated (Fig. 1).

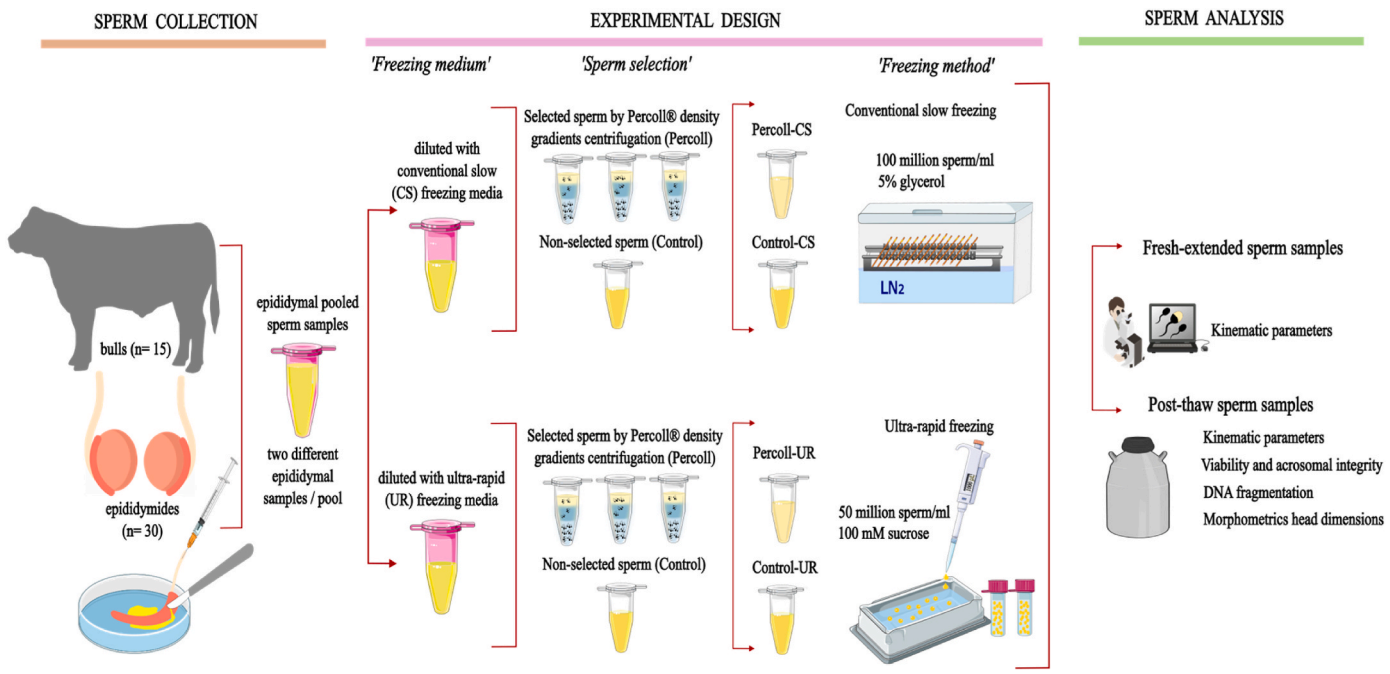

Fig. 1. Experimental design scheme for the collection, dilution, selection, cryopreservation, and analysis of bull epididymal spermatozoa.

2.4. Sperm selection

The aliquots from Percoll-CS and Percoll-UR treatments were subjected to sperm selection using Percoll-DGC. For this, the Percoll® solution was diluted with BoviDilute® solution (BD-100; Nidacon, Mölndal, Sweden) to obtain the Percoll Bottom layer medium and the Percoll Top layer medium, at concentrations of 80 % and 40 %, respectively. The Percoll-DGC columns were prepared in 1.5 mL conical Eppendorf tubes: equal volumes (200 μL) of Percoll Bottom layer and Top layer medium were successively layered into the tubes. The pooled sperm samples (200 μL) from the Percoll-CS and Percoll-UR treatments, adjusted at room temperature (22 °C), were gently placed on top of the Percoll Top medium. Three Eppendorf tubes of Percoll columns were prepared to select sperm samples from each of the Percoll-CS and Percoll-UR treatments. The columns were centrifuged at 300×*g* for 20 min. After centrifugation, the fluid above the sperm pellet was carefully removed. Three pellets were then resuspended in 600 μL of TCG-EY medium (at room temperature), and the kinematic parameters were evaluated. After selection, the sperm concentration was determined using a photometer (SDM 1; Minitube, Germany). The selected sperm samples were adjusted to a final volume of 1 mL and a concentration of 200 × 10^6 sperm/mL.

2.5. Conventional slow (CS) freezing

The sperm samples from the Percoll-CS and Control-CS treatments of each pooled sample were adjusted to an initial volume of 1 mL and a concentration of 200 × 10^6 sperm/mL using TCG-EY medium. Subsequently, an equal volume (1 mL) of TCG-EY medium containing 10 % glycerol (v/v) (Sigma G9012, St. Louis, MO, USA) was added to these samples. As a result, the final volume of the samples prior to CS freezing was 2 mL, with a final concentration of 100 × 10^6 sperm/mL and 5 % glycerol (final osmolarity of CS freezing medium: 1637 mOsm/kg). The samples were then loaded into 0.25 mL IVM French straws (L'Aigle Cedex, France), sealed with polyvinyl alcohol (Sigma P8136, St. Louis, MO, USA), and equilibrated for 2 h at 5 °C.

Conventional slow freezing was performed using a Cryogenic Freezing Unit® (Minitube 15043/0736, Germany; dimensions of 64 × 41 × 26 cm of length, width, and height, respectively), which contained 5.6 L of LN_2. The straws were placed in a floating rack 5 cm above the LN_2 surface and exposed to static LN_2 vapors for 10 min. Finally, the straws were plunged into LN_2 at −196 °C.

All frozen straws from Percoll-CS (n = 75) and Control-CS (n = 75) treatments were thawed by submerging them in a water bath (Memmert, WNB22 10L, Germany) at 37 °C for 30 s. The contents were then poured into 1.5 mL Eppendorf tubes and incubated for 5 min at 37 °C. Sperm quality parameters were subsequently evaluated (see below).

2.6. Ultra-rapid freezing (UR)

Similar to the CS freezing treatments, the sperm samples from the Percoll-UR and Control-UR treatments were adjusted to an initial volume of 1 mL and a concentration of 100 × 10^6 sperm/mL using TCG-EY medium. Subsequently, an equal volume (1 mL) of TCG-EY medium containing 200 mM sucrose (w/v) (Sigma S1888, St. Louis, MO, USA) was added to these samples. Consequently, the final volume of the samples prior to UR freezing was 2 mL, with a final concentration of 50 × 10^6 sperm/mL and 100 mM sucrose (final osmolarity of UR freezing media: 473 mOsm/kg). The samples were equilibrated for 30 min at 5 °C. Ultra-rapid freezing was performed by pipetting and plunging 30 μL drops directly into LN_2 from a height of 15 cm. The formed spheres were stored in 2 mL cryogenic vials (Wuxi NEST Biotechnology, Jiangsu, China) in a cryotank at −196 °C [13].

Spheres from both the Percoll-UR (n = 30 cryotubes) and Control-UR (n = 30 cryotubes) treatments were thawed using a handmade warming device (STC-3008) [13]. The spheres were placed on plates warmed at 65 °C, and the thawed contents were immediately recovered into a 10 mL glass beaker; this warming process lasted 3 s. The contents were then transferred to 1.5 mL Eppendorf tubes and centrifuged at 300×*g* for 5 min at room temperature. The supernatant was removed, and 100 μL of TCG-EY medium was added to the pellets. The samples were then incubated for 5 min at 37 °C before sperm quality analysis.

2.7. Sperm analysis

The sperm kinematic parameters from each treatment, whether fresh (after sperm selection and prior to cryopreservation) or frozen-thawed epididymal sperm samples, were objectively assessed using a CASA

system (Sperm Class Analyzer, SCA-Evolution® 2018, v.6.4.0.99 software. Microptic S.L., Barcelona, Spain), coupled to a phase contrast microscope (Nikon Eclipse model 50i; with negative contrast capability [Ph1] with green filter) with the following settings: 25 frames/s, head area 5–70 μm^2, velocity limit for slow sperm 10 μm/s, velocity limit for medium sperm 25 μm/s, velocity limit for fast sperm 70 μm/s, and minimal straightness for progressive spermatozoa 70 % [13]. A minimum of three fields and 600 sperm tracks were evaluated at 100× magnification. The following parameters were analyzed: total sperm motility (TM, %), progressive sperm motility (PSM, %), curvilinear velocity (VCL, μm/s), average-path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz).

Sperm viability (plasma membrane integrity, %) and acrosomal integrity (%) were determined using a combination of fluorescent probes – propidium iodide (PI, Sigma P4170) and fluorescein isothiocyanate conjugated peanut (*Arachis hypogaea)* agglutinin (PNA-FITC, Sigma L7381) [13,24]. A total of 200 sperm cells per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (400× magnification with an excitation: 330–380 nm, and emission: 420 nm). Spermatozoa with intact plasma membrane (live) and intact acrosomes did not emit fluorescence, whereas spermatozoa with damaged plasma membranes (dead) and damaged acrosomes were stained red and green, respectively.

The DNA fragmentation (indicative of condensed or unstable chromatin) was evaluated using the acridine orange staining [29]. Post-thawed sperm samples from each experimental treatment were spread on a slide, air-dried, and fixed overnight in Carnoy solution (methanol and acetic acid in 3:1 ratio). The smears were air-dried and incubated in buffer solution (80 mM, citric acid and 15 mM Na_2HPO_4; pH 2.5) at 75 °C for 5 min to test chromatin stability. Subsequently, the slides were stained with acridine orange hydrochloride solution (A8097, Sigma Aldrich Co., 0.2 mg/mL) for 5 min and washed with water to remove background staining. Immediately, while still wet, evaluation was performed under epifiuorecense microscopy (600× magnification, excitation: 510–560 nm, and emission 590 nm). At least 300 spermatozoa from at least ten random fields were examined per slide. Two fluorescence categories were identified: 1) spermatozoa stained green were considered to have normal chromatin; and 2) spermatozoa stained yellow-green to red considered to have damaged chromatin.

The morphometric dimensions of the spermatozoa head and midpiece were assessed using the ASMA module *(Automated Sperm Morphology Analysis)* from CASA system, coupled to a Nikon Eclipse 50i microscope (600× magnification). For this analysis, 14 μL of post-thaw sperm samples from each treatment was mixed with 7 μL of SpermBlue® stain, and a smear was prepared. The slides were dried at room temperature, mounted with synthetic resin (Eukitt® mounting medium UN1866; PanReac AppliChem), and covered with a 22 × 44 mm cover slip. The morphometric dimensions of the sperm head (including length, width, perimeter, area, elongation, ellipticity, roughness and regularity) and midpiece (width and area) were evaluated on 100 cells per slide.

2.8. Statistical analysis

Values for sperm variables that showed non-normal distributions, as determined by the Shapiro-Wilk test, were transformed to *arcsine* (for percentages values) or *Log$_{10}$* (for numeric values) prior to analysis. Before freezing, the effects and interactions of *sperm selection* and *freezing media* were analyzed using a factorial ANOVA under the general linear model (GLM) procedure. Differences between means were determined using the least squares test. After thawing, the effects and interactions of *sperm selection* and *freezing method* were assessed using the same factorial ANOVA, and differences between means were analyzed with the least squares test. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$. All statistical analyses were performed using SAS (Statistical Analysis System) software for Windows, version 9.3; (SAS Institute, Inc., Cary, NC, USA). Data are expressed as means ± S.E.M (standard error of the mean).

3. Results

3.1. Pre-freezing analysis: sperm selection and freezing media

The kinematic parameters of epididymal sperm samples, whether selected or non-selected (control) with Percoll and diluted with CS or UR freezing media, are included in Table 1. Significant interactions ($P < 0.05$) between *freezing media* × *sperm selection* were for the PSM, WOB, and ALH variables in sperm samples diluted with CS and UR freezing media before cryopreservation (Table 1).

Overall, sperm samples from both CS treatments showed higher values ($P < 0.05$) than those from both UR treatments in the following kinematic parameters: PSM, VSL, STR, and BCF. In fact, the Control-CS treatment resulted in higher values for all kinematic parameters compared to the Control-UR treatment ($P < 0.05$). After the Percoll-CS treatment, VSL, LIN, and BCF values increased significantly compared to their Control-CS counterparts ($P < 0.05$). Similarly, after the UR-Percoll treatment, the values of PSM, VCL, VAP, VSL, STR, ALH, and BCF significantly increased compared to the Control-UR counterpart ($P < 0.05$). Notably, the Percoll-UR treatment presented a lower percentage of WOB compared to the Control-UR treatment ($P < 0.05$) (Table 1).

3.2. Post-thawing analysis: sperm selection and freezing method

The kinematic parameters of epididymal bull sperm samples, whether selected or non-selected (control) with Percoll and frozen using the CS or UR method, are shown in Table 2. Post-thaw viability, acrosomal integrity, and DNA fragmentation of sperm samples from different treatments are depicted in Fig. 2. Significant interactions ($P < 0.05$) between *sperm selection* and *freezing method* were for all kinematic variables, except for the progression ratio parameters (i.e., STR, LIN and WOB) (Table 2).

Post-thaw TM and PSM values were significantly higher ($P < 0.05$) in sperm samples from both CS freezing treatments compared to both UR freezing treatments. The Percoll-CS treatment produced higher post-thaw values of TM, PSM, VCL, VAP, VSL, ALH, and BCF compared to the Control-CS treatment ($P < 0.05$). However, no significant differences

Table 1
Pre-freezing kinematic values (mean ± SEM) of bull epididymal sperm selected and non-selected (control) with Percoll, and then diluted with conventional slow (CS), or ultra-rapid (UR) freezing media. Total sperm samples analyzed, n = 15 pools per treatment.

Kinematic parameters	Conventional slow freezing treatments		Ultra-rapid freezing treatments	
	Percoll-CS	Control-CS	Percoll-UR	Control-UR
TM (%)	93.2 ± 3.4^{ab}	94.5 ± 3.4^{ab}	86.0 ± 3.4^{bc}	77.5 ± 3.4^{c}
PSM (%) *	51.7 ± 4.0^{a}	53.4 ± 4.0^{a}	37.8 ± 4.0^{b}	21.6 ± 4.0^{c}
VCL (μm/s)	83.7 ± 3.5^{a}	79.6 ± 3.5 ^{ab}	75.1 ± 3.5^{b}	59.8 ± 3.5^{c}
VAP (μm/s)	45.1 ± 1.9^{ab}	40.3 ± 1.9^{bc}	39.4 ± 1.9^{c}	32.9 ± 1.9^{d}
VSL (μm/s)	32.0 ± 1.6^{a}	27.5 ± 1.6^{bc}	24.8 ± 1.6^{c}	18.1 ± 1.6^{d}
STR (%)	67.8 ± 1.3^{a}	65.6 ± 1.3^{a}	59.8 ± 1.3^{b}	54.1 ± 1.3^{c}
LIN (%)	38.7 ± 1.0^{a}	35.4 ± 1.0 ^{bc}	33.6 ± 1.0 ^{cd}	32.4 ± 1.0^{d}
WOB (%) **	55.2 ± 0.9 ^{bc}	52.4 ± 0.9 ^{cd}	54.7 ± 0.9^{b}	59.2 ± 0.9^{a}
ALH (μm) *	3.6 ± 0.1^{a}	3.8 ± 0.1^{a}	3.6 ± 0.1^{a}	3.2 ± 0.1^{b}
BCF (Hz).	9.2 ± 0.3^{a}	8.2 ± 0.3^{b}	6.7 ± 0.3^{c}	4.6 ± 0.3^{d}

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 (ALH); and BCF beat-cross frequency. $^{a–d}$ Different superscripts within a same row differ significantly between treatments ($P < 0.05$). * Significant interaction between sperm selection and freezing media (*$P < 0.05$, **$P < 0.01$).

Table 2
Post-thaw kinematic values (mean ± SEM) of bull epididymal spermatozoa selected and non-selected (control) with Percoll, and then cryopreserved by conventional slow (CS), or ultra-rapid (UR) freezing. Total sperm samples analyzed, n = 45 straws per treatment.

Kinematic parameters	CS freezing treatments		UR freezing treatments	
	Percoll-CS	Control-CS	Percoll-UR	Control-UR
TM (%) *	68.8 ± 2.0^{a}	55.1 ± 2.0^{b}	26.0 ± 2.0 ^{cd}	23.1 ± 2.0^{d}
PSM (%) *	37.8 ± 1.1^{a}	28.2 ± 1.1^{b}	9.7 ± 1.1 ^{cd}	9.1 ± 1.1^{d}
VCL (μm/s) **	60.0 ± 1.6^{a}	52.1 ± 1.6^{b}	57.4 ± 1.6^{a}	59.4 ± 1.6^{a}
VAP (μm/s) **	31.9 ± 0.8^{a}	26.6 ± 0.8^{b}	30.1 ± 0.8^{a}	30.5 ± 0.8^{a}
VSL (μm/s) **	22.8 ± 0.7^{a}	18.3 ± 0.7^{b}	21.3 ± 0.7^{a}	21.3 ± 0.7^{a}
STR (%)	65.3 ± 0.7	63.9 ± 0.7	65.2 ± 0.7	64.2 ± 0.7
LIN (%)	37.2 ± 0.9	34.9 ± 0.9	37.7 ± 0.9	36.0 ± 0.9
WOB (%)	54.1 ± 0.8	52.2 ± 0.8	55.1 ± 0.8	53.6 ± 0.8
ALH (μm) *	2.7 ± 0.6^{a}	2.5 ± 0.6^{b}	2.7 ± 0.6^{a}	2.8 ± 0.6^{a}
BCF (Hz) *	7.3 ± 0.2^{a}	6.2 ± 0.2 ^{bc}	6.1 ± 0.2^{c}	5.7 ± 0.2^{c}

TM, total motility; PSM, progressive motility; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head (ALH); and BCF beat-cross frequency. $^{a–d}$ Different superscripts within a same row differ significantly between treatments $P < 0.05$. Asterisks expresses significant interaction between *sperm selection* and *freezing method*, *$P < 0.05$, **$P < 0.01$.

were observed between UR freezing treatments (i.e., Percoll-UR and Control-UR, $P > 0.05$) for any kinematic parameters. Notably, the post-thaw speeds and ALH values of the Percoll-UR and Control-UR treatments were similar to those of the Percoll-CS treatment and significantly higher ($P < 0.05$) than the Control-CS treatment (Table 2).

Sperm samples from Percoll-CS treatment exhibited higher percentages of post-thaw viability and acrosomal integrity compared to samples from the Control-CS ($P < 0.05$), Percoll-UR ($P < 0.01$) and Control-UR ($P < 0.001$) treatments. After the Percoll-UR treatment, post-thaw viability and acrosomal integrity increased significantly ($P < 0.05$) compared to its Control-UR counterpart. Additionally, both Percoll-UR and Control-UR treatments resulted in significantly lower percentage of DNA fragmentation in frozen-thawed sperm samples compared to the Percoll-CS treatment ($P < 0.05$) (Fig. 2).

In morphometric analysis, it was observed that after both CS freezing treatments, the sperm head area and regularity, as well as the midpiece area, were greater than those observed after both UR freezing treatments ($P < 0.05$). Sperm samples from the Percoll-CS treatment showed significantly higher post-thaw values ($P < 0.05$) for head roughness, head regularity, and midpiece area compared to samples from their Control-CS counterpart (Table 3).

4. Discussion

The findings from this study demonstrate that bull epididymal spermatozoa selected using Percoll-DCG and subsequently cryopreserved via CS freezing in LN_2 vapors significantly improved post-thaw kinematic parameters, viability, and acrosomal integrity. In contrast, sperm selection with Percoll followed by ultra-rapid freezing improved only post-thaw viability and acrosomal integrity, despite enhancing some sperm kinematic parameters prior to cryopreservation. These results suggest that sperm selection with Percoll prior to conventional slow freezing substantially enhances the cryosurvival of bovine epididymal spermatozoa. This study constitutes the first report on the effect of sperm selection on epididymal bull sperm quality both before and after conventional and ultra-rapid freezing.

Sperm selection by DGC separates motile sperm from non-motile,

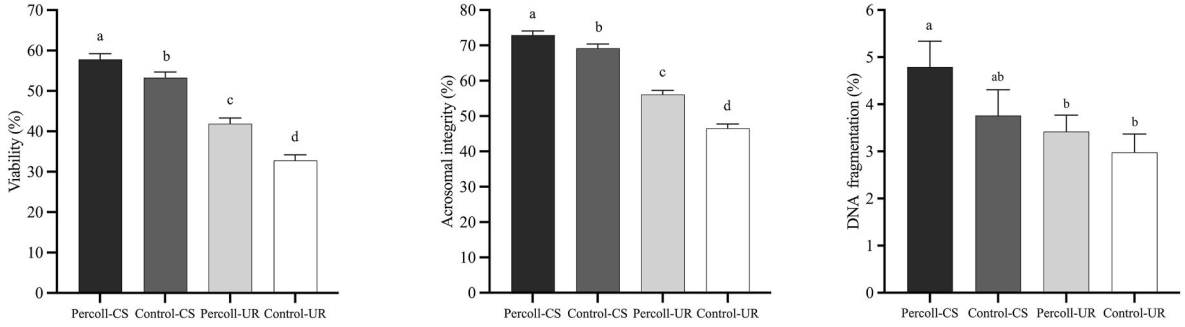

Fig. 2. Viability, acrosomal integrity, and DNA fragmentation of bull epididymal spermatozoa selected and non-selected (control) with Percoll, and then cryopreserved by conventional slow (CS), or ultra-rapid (UR) freezing. $^{a–d}$ Different superscripts in each bar express significant difference between treatments, $P < 0.05$.

Table 3
Measurements of head and midpiece (mean ± SEM) of bull epididymal sperm selected and non-selected (control) with Percoll, and then cryopreserved by conventional slow (CS), or ultra-rapid (UR) freezing.

Morphometric variables	CS freezing treatments		UR freezing treatments	
	Percoll-CS	Control-CS	Percoll-UR	Control-UR
Head				
Length (μm)	9.2 ± 0.05	9.2 ± 0.03	9.3 ± 0.06	9.3 ± 0.07
Width (μm)	4.5 ± 0.03	4.5 ± 0.02	4.5 ± 0.04	4.6 ± 0.02
Area (μm^2)	38.2 ± 0.5^{b}	38.2 ± 0.2^{b}	39.3 ± 0.4^{a}	39.5 ± 0.4^{a}
Perimeter (μm)	20.1 ± 0.11	20.2 ± 0.04	21.0 ± 0.84	20.1 ± 0.17
Ellipticity	2.1 ± 0.01	2.0 ± 0.01	2.0 ± 0.02	2.0 ± 0.02
Elongation	0.3 ± 0.002	0.3 ± 0.003	0.3 ± 0.004	0.3 ± 0.004
Roughness	1.20 ± 0.008^{a}	1.18 ± 0.005^{b}	1.22 ± 0.006^{a}	1.21 ± 0.007^{a}
Regularity	0.85 ± 0.002^{a}	0.86 ± 0.002^{b}	0.84 ± 0.002^{c}	0.84 ± 0.002^{c}
Midpiece				
Width (μm)	1.00 ± 0.1^{b}	1.05 ± 0.1^{a}	0.95 ± 0.1^{b}	0.98 ± 0.1^{b}
Area (μm^2)	7.7 ± 0.2^{b}	7.1 ± 0.2^{b}	8.9 ± 0.2^{a}	8.5 ± 0.2^{a}

$^{a–d}$ Different superscripts within a same row differ significantly between treatments $P < 0.05$.

dead sperm and with fragmented DNA, sperm with morphological abnormalities, seminal plasma, blood cells, bacteria, cell debris, and other elements that negatively affect sperm kinematic, viability, and fertilizing capacity after cryopreservation [15,31,32,36]. Previous evidence indicates that sperm selection by DGC improves the motility and function of frozen-thawed bull sperm from ejaculated semen [41,46], even without affecting gene expression [3]. It is most common to separate sperm after thawing, for example, during semen preparation for IVF. As a result, several studies have been conducted to evaluate the effectiveness of sperm selection by DGC in enhancing the quality of fresh-extended, chilled, and frozen-thawed sperm in various species, including bulls [3,16], rams [15,51], horse [27], wisent (*Bison bonasus*) [11], and wild mountain ruminants [43]. The results of the present study are consistent with these previous works, considering that the bull sperm used were of epididymal origin and the selection was performed before cryopreservation.

There are few reports on the use of bull sperm selection prior to freezing to remove abnormal and non-motile sperm, with the aim of enhancing post-thaw semen quality and fertility [16]. Alternative sperm selection methods to DGC, such as rheotaxis and thermotaxis, have been successfully employed prior to freezing, leading to improved motility, structural integrity, and fertilizing ability in bulls [33].

Although kinematic parameters and sperm membrane integrity decreased after both freezing methods, the results of the present study demonstrate that ultra-rapid freezing significantly impacted the motility and membrane integrity of bull epididymal spermatozoa. Following the UR freezing method, TM, PSM, viability, and acrosome integrity values were lower than those obtained with CS freezing. These findings are consistent with previous studies on ultra-rapid freezing (with 100 mM sucrose) of spermatozoa from wild ungulates [6]. The notable differences observed may be attributed to variations in cooling and thawing rates. Ultra-rapid cooling rates during the freezing process can induce cold shock damage, leading to modifications in the plasma and acrosomal membranes, increasing their permeability, and reducing sperm motility and metabolic activity [7,37]. Moreover, cryoinjury to the plasma and mitochondrial membranes can result in changes to mitochondrial membrane potential, which is one of the main causes of reduced motility following the ultra-rapid freezing process [22].

High concentrations of disaccharides in the ultra-rapid freezing medium (100 mM) could be another factor contributing to the reduction in post-thaw sperm quality. Similarly, studies have shown that increasing concentration of sucrose or trehalose correlate with a decrease in bovine sperm motility and viability [1]. In agreement with these findings, the addition of higher sucrose concentrations in ultra-rapid freezing or vitrification media [2,13,24] has been associated with reduced kinematic parameters following thawing or warming, respectively. While Percoll-DGC selection improved sperm kinematics prior to ultra-rapid freezing, it only enhanced viability and acrosomal integrity after thawing.

The cryopreservation process induces high levels of apoptosis and damage to DNA and chromatin in bull sperm, whether derived from ejaculates [12] or epididymal samples [30]. Chromatin integrity is critical for successful fertilization, as abnormalities can adversely affect sperm quality (e.g., motility), fertility potential [5,26,39], and early embryo development [50]. The precise mechanism of chromatin damage during cryopreservation remains unclear. It may result from the mechanical effects of ice crystallization or chemical damage induced by cryoprotectants. However, it is primarily attributed to oxidative stress [47] and the activation of caspases [34].

Another key factor contributing to DNA damage in bull spermatozoa is the freezing and thawing rates [44]. Exposure to LN_2 vapors and the cooling rates achieved through conventional slow freezing (initially rapid: 40–60 °C/min) can destabilize chromatin and trigger DNA damage, as described in the pathways above. This likely explains why conventional freezing causes greater DNA damage compared to ultra-rapid freezing methods.

The findings of this study demonstrate that the selection of epididymal bull sperm with Percoll-DGC prior to CS and UR freezing did not reduce DNA fragmentation. Similar results were reported by Eberhardt et al. [11] in wisent epididymal sperm (*Bison bonasus*) selected with Percoll-DCG (45/90 %) before conventional freezing. In contrast, DNA fragmentation after thawing decreased when bull ejaculated semen was selected with colloidal solutions (e.g., Bovipure®) prior to conventional freezing [40]. Apparently, the origin of bull sperm (ejaculate or epididymal) and the sperm selection method influence DNA fragmentation in bull sperm.

In addition, this study demonstrated that ultra-rapid freezing rates have a significant more positive effect on DNA integrity compared to slow freezing rates. Earlier studies suggested that ultra-rapid freezing produced either lower [23] or similar [21] DNA integrity relative to conventional slow freezing. However, recent research has shown that sperm subjected to ultra-rapid freezing exhibits higher DNA integrity than those frozen by conventional methods [38,48]. The effectiveness of ultra-rapid freezing, particularly when using non-permeable cryoprotectants (e.g., 100 mM sucrose), is attributed to its ability to preserve critical physiological parameters of sperm, such as DNA integrity [22]. This evidence suggests that ultra-rapid freezing of bull epididymal sperm could be used successfully in *in vitro* fertilization trials, where DNA integrity plays a crucial role in early embryonic development.

Some evidence suggests that the dimensions of the bull sperm head may serve as an indicator of sperm cryosurvival [17]. Previous studies indicate that sperm with smaller head areas are associated with better quality and fertility [28]. Variations in sperm head shape and volume/area may influence the rate of water exchange across the plasma membrane during the freeze-thaw process [55]. It has been proposed that over-condensation of sperm chromatin, plasma membrane damage, acrosome loss, and cytoskeletal damage could lead to a reduction in sperm head size [4]. In this study, sperm selection did not significantly affect head dimensions, except for roughness and regularity. Furthermore, regardless of sperm selection, CS freezing resulted in smaller head and midpiece areas compared to UR treatments.

In conclusion, this study demonstrated that CS freezing resulted in better cryosurvival of bull epididymal sperm compared to UR freezing; however, UR freezing led to lower DNA fragmentation. Additionally, Percoll density gradient centrifugation prior to conventional slow freezing enhanced the post-thaw kinematic parameters, viability, acrosomal integrity of bull epididymal spermatozoa. In contrast, following UR freezing, Percoll-DGC improved only post-thaw viability and acrosomal integrity. Based on these findings, sperm selection prior to conventional slow freezing is a highly effective strategy for cryopreserving bull epididymal sperm.

CRediT authorship contribution statement

Mauricio Duma: Writing – original draft, Methodology, Investigation. **Diego A. Galarza:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Kelly Delgado:** Methodology, Investigation. **Angie Morocho:** Methodology, Investigation. **Guido Bermúdez:** Methodology. **Manuel E. Soria:** Supervision, Investigation. **María S. Méndez:** Supervision, Investigation. **Esteban Muñoz-León:** Methodology. **Fernando P. Perea:** Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Declaration of competing interest

None of the authors have any conflict of interest to declare.

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