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Original Research Article

Cushioned and high-speed centrifugation improve sperm recovery rate but affect the quality of fresh and cryopreserved feline spermatozoa

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ABSTRACT

The development of endoscopic transcervical catheterization (ETC) in the queen increases the interest in handling fresh and cryopreserved feline semen. The ETC requires a small volume of the insemination dose with a high concentration, not easily reached with the actual frozen technique in this species. Centrifugation is widely used to concentrate spermatozoa for several purposes, but this process is detrimental to spermatozoa. This study verified the effects of conventional and cushioned centrifugation on fresh and cryopreserved feline spermatozoa. To this, semen was collected from 20 toms, grouped in seven pools and diluted. After dilution, the pools were divided into two aliquots, the first used for centrifugation on fresh semen, and the second, after freezing, on cryopreserved semen. Centrifugation regimens were: conventional at 500×*g*, conventional at 1000×*g*, and cushioned (iodixanol) at 1000×*g*. The sperm recovery rate was calculated for the three centrifugation regimens, and sperm kinematics, membrane and acrosome integrity, and plasma membrane stability on viable spermatozoa were assessed as endpoints.

The data reported in this study showed that the centrifugation at 500×*g* resulted in negligible effects on both fresh and cryopreserved spermatozoa, but the lower recovery rate (62.4 ± 3.1 % and 60.2 ± 1.6 %, respectively) underlines the loss of a large proportion of spermatozoa, unfavourable in a species with small total sperm ejaculated. On the other hand, the centrifugation at 1000×*g* improved the recovery rate (86.9 ± 4.3 % and 89.8 ± 2.4 % in fresh and cryopreserved samples, respectively), but was more deleterious for feline spermatozoa, especially in cryopreserved samples (i.e. total motility of 40.7 ± 5.4 % compared with 57.2 ± 9.8 % in cryopreserved uncentrifuged samples, $P < 0.05$), resulting in artificial insemination doses of lower quality. The recovery rate in cushioned centrifugation appeared less efficient, likely due to the small volume of feline samples, which makes difficult the separation of sperm pellet and cushioned fluid. Interestingly, in cryopreserved samples centrifuged at 1000×*g* the number of viable spermatozoa with membrane destabilization (31.3 ± 3.2 %) was greater than uncentrifuged (4.1 ± 0.7 %, $P < 0.05$) and those centrifuged at 500×*g* (9.8 ± 1.3 %, $P < 0.05$), suggesting modifications induced by the cryopreservation amplifies centrifugation sublethal damage on feline spermatozoa. Cushioned centrifugation on cryopreserved samples showed kinematics similar to uncentrifuged samples, but higher viable spermatozoa with membrane destabilization (37.4 ± 3.4 % vs 4.1 ± 0.7 %; $P < 0.05$). In felines, g-force is crucial for sperm recovery rate during centrifugation, with better results at 1000×*g*; on the other hand, greater g-forces could have a significant impact on the quality of feline insemination dose, especially in cryopreserved samples.

1. Introduction

In the cat artificial insemination, especially with cryopreserved semen, has limited diffusion [1] despite cryopreserved spermatozoa being able to fertilize successfully the oocyte in this species [2–4]. As a result, the number of studies on feline ejaculated sperm cryopreservation is very limited in the literature [1,5].

The artificial insemination techniques traditionally described for the queen are surgical insemination, via laparotomic [3,6,7] or laparoscopic [8] approach, which poses ethical issues, or blind transcervical

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insemination [9], with an acceptable pregnancy rate but requiring long training and technical ability. Recently, a technique of endoscopic transcervical catheterization (ETC) in the queen was described [10], with the birth of live kittens. The procedures were performed by a semirigid sialendoscope using a specifically designed catheter [11]. The authors reported a successful pregnancy rate of 100 % even though the results were obtained on a small number of animals [11]. Nevertheless, the possibility of obtaining pregnancy by ETC increases the interest in the preservation of semen in this species but requires the development of efficient procedures to prepare fresh or frozen artificial insemination doses. Pregnancy with ETC was obtained using fresh semen collected by urethral catheterization after pharmacological induction (UrCaPI), which provides a lower volume (mean of about 10 μL) and higher concentration (mean about 1800 × 10^6 sperm/mL) compared with other collection techniques [2,12], suitable conditions for intrauterine insemination in the queen with fresh semen. On the other hand, in several cases the UrCaPI does not allow the collection of enough spermatozoa to perform effectively artificial insemination [13], making electroejaculation a preferable alternative to collecting useable spermatozoa. Furthermore, the liquid (cooling) or solid (freezing) storage of spermatozoa requires the dilution of semen with specific extenders, increasing the volume. Thus, centrifugation is an unavoidable procedure aimed to reach an insemination volume dose compatible with the ETC.

Centrifugation is a procedure extensively used in semen manipulation, to wash spermatozoa for assisted reproductive technology [14], concentrate spermatozoa [15], remove seminal plasma [16–18], remove cryoprotectants [19], or remove other detrimental or undesired substances [15]. Centrifugation is not an innocuous procedure for mammalian spermatozoa. In literature, the data reported by numerous studies that conventional centrifugation allows sperm pellet formation but with a negative effect on cells [20]. Evidence has suggested that the recovery rate of sperm cells after centrifugation is related to the g-force applied, however an excessive centrifugation force reduced the viability of spermatozoa, probably because of the physical pressure against the wall of the tube [20,21]. The data reported in the literature showed that a centrifugation speed exceeding 1800×*g* could be deleterious for equine sperm motility [22], while centrifugation at 600×*g* [23], 900×*g* [22], and 1000×*g* [24] had a negligible effect on equine sperm characteristics. In a study conducted in the bull, single (SLC) and double layer centrifugation (DLC) were performed effectively to improve the quality of the semen samples. The authors showed that the bull samples treated by SLC and DLC had a similar sperm quality after cryopreservation compared with the untreated and cushioned centrifugation (CC) samples [25]. In recent years, a cushioned centrifugation was proposed to minimize the impact of the g-force on equine spermatozoa. In the bottom of the centrifugation tube, a dense fluid (i.e. iodixanol) creates a layer, preventing spermatozoa from being mechanically pushed down against the wall of the tube [26].

This study aimed to verify the impact of centrifugation, as a procedure to reduce the insemination dose volume, on feline spermatozoa. Furthermore, the possible mitigation role of cushioned centrifugation on the adverse effects related to higher centrifugation forces was also considered in fresh and cryopreserved feline semen.

2. Materials and methods

The experimental protocol for semen collection was approved by the Ethics Committee of the Department of Veterinary Medicine of Teramo (Protocol number 30023, 25 October 2022). The experiments were performed after obtaining explicit owner consent.

2.1. Animals, semen collection, and preliminary evaluation

This study was conducted on 21 pure breed toms (8 Maine Coon; 5 British shorthair; 5 Ragdoll; 3 Scottish) presented for semen evaluation or long-term preservation. The toms were aged 13–32 months and weighed 4.8–8.3 kg. All the males were maintained under natural photoperiod conditions and provided with cat commercial dry food (Feline Adult, Hill's Science Plan) and water ad libitum.

Semen was collected by electroejaculation, as previously described [27]. Pharmacological restriction and analgesia were achieved by the combination of medetomidine (80 mg/kg intramuscularly; Domitor, Vetoquinol Italia, Forlì-Cesena, Italy) and ketamine (5 mg/kg intramuscularly; Ketavet 100, MSD Animal Health, Milan, Italy) [28]. If the tom showed an increased respiratory or cardiac frequency of more than 20 % or the presence of voluntary movements, interpreted as sensitivity to the procedures, 1–4 mg of propofol (Propovet, Zoetis Italia S.r.l., Rome, Italy) were administered intravenously. After the achievement of the analgesic effect, electroejaculation was performed following the previously described schedule of stimulation [28]. Pre-warmed (37 °C) sterile 1.5-mL Eppendorf tubes were used to collect semen during the procedure. After collection, each sample was transferred within 5 min to the laboratory for evaluation and manipulation.

The semen samples were evaluated for volume and concentration. Sperm volume was determined using a validated variable volume micropipette (Acura 825; 10–100 μL, Socorex ISBSA Lausanne, Switzerland) [29]. Sperm concentration was estimated using a Burker counting chamber (Merck, Leuven, Belgium), after dilution of the raw semen at 1:100 with formol (1 %) isotonic saline solution. The total sperm in the sample (TSS, x 10^6 sperm/ejaculate) was also calculated as follows: volume x concentration. A 5-μL drop of each sample was evaluated for subjective motility, under a phase-contrast microscope Olympus BX-51 equipped with a 38°C-warmed plate (Olympus Europe, Hamburg, Germany) at 200× magnification. Sperm morphology was evaluated using a phase-contrast microscope (Olympus BX-51, Milan, Italy) at magnification: ×1000, after dilution (1:10 in saline solution with 3 % glutaraldehyde) (Hancock, 1957), and normal sperm morphology was estimated as the proportion of normal shape spermatozoa on the total spermatozoa (at least 200 cells).

Only ejaculates with subjective motility >70 % and normal morphology >70 % were included in the experimental design.

2.2. Experimental design

In the present study, 21 tomcats were collected by electroejaculation, but only 20 samples met the criteria of inclusion and were used for the experiments. To manage a sufficient volume of semen and to reduce the inter-individual effect, the samples were pooled randomly in seven pools. After preliminary evaluation, each pool was diluted at 40 × 10^6 sperm/mL with a TRIS extender (TRIS) supplemented with 20 % egg yolk (TEY) [1]. Then, the pool was split into two aliquots, for the first (trial on fresh spermatozoa) and the second (trial on cryopreserved spermatozoa) experiment.

The first aliquot (fresh sample) was subdivided into three subsamples and centrifuged conventionally at 500×*g* for 10 min (CE500), at 1000×*g* for 10 min (CE1000) and cushioned centrifugated at 1000×*g* for 10 min (CU1000). Similarly, the second aliquot, after freezing/thawing (cryopreserved sample) was subdivided into three subsamples and centrifuged conventionally at 500×*g* for 10 min (CE500), at 1000×*g* for 10 min (CE1000) and cushioned centrifugated at 1000×*g* for 10 min (CU1000). Fresh and cryopreserved samples were evaluated for concentration by Burker chamber; objective motility by CASA system; membrane and acrosome integrity, and lipid membrane disorder on viable spermatozoa by flow cytometry before (control - CTR) and after the different centrifugation regimens.

2.2.1. Effect of centrifugation on fresh feline sperm

The first aliquot of the pools was used for the evaluation of the effects of centrifugation regimens on fresh semen. In brief, each aliquot was diluted 1:1 with TEY to reach the concentration of 20 × 10^6 sperm/mL and split into four sub-samples in 0.5 mL plastic and sterile vials (Biosphere SafeSeal tube, Sarstedt, Numbrecht, Germany). The first was

not centrifuged and served as control, the second was centrifuged at 500×*g* for 10 min (CE500); the third was centrifuged at 1000×*g* for 10 min (CE1000); the fourth was centrifuged at 1000×*g* for 10 min with cushion medium (CU1000). Cushioned centrifugation was performed using the non-ionic iodixanol (Optiprep™, Axis-Shield PoC AS, Olso, Norway), deposited at the bottom of the centrifugation tube, using a non-toxic intravenous cannula needle (24 gauge, Delta Ven® T, Delta Medspa, Mantova, Italy) connected with a 2 piece 1-mL syringe (Injekt®-F, B. Braun, Melsungen, Germany). After centrifugation, the supernatant was removed using the cannula, as above described, and transferred to a different tube (supernatant aliquot). For the cushion centrifugation sample, after the supernatant removal, the cushioned medium was removed using the intravenous cannula needle, as above described. For all the samples, the pellet was resuspended with the same volume removed using TEY, to restore the original conditions (bottom aliquot).

Semen handling was performed, in all the cases, at room temperature (21 °C), and the centrifugations were performed with a MySPIN 12 (Heathrow Scientific, Vernon Hills, IL, USA).

Before (control CTR) and after centrifugation, bottom and supernatant aliquots were evaluated for sperm concentration, objective sperm motility by computer-assisted sperm analyzer (CASA); membrane and acrosome integrity, and lipid membrane disorder on viable spermatozoa by flow cytometry. Objective motility, membrane and acrosome integrity, and lipid membrane disorder on viable spermatozoa were also evaluated after 60 min post-centrifugation.

2.2.2. Effect of centrifugation on frozen-thawed feline sperm

The second aliquot from each pool was cryopreserved as follows. After the first dilution, the samples were diluted 1:1 with TEY containing 8 % glycerol to allow a final concentration of 20 × 10^6 sperm/mL. After equilibration at room temperature for 20 min, the samples were loaded in 0.25 plastic French straws (IMV Technologies, L'Aigle, France). The straws were equilibrated at 5 °C for 25 min with a rate of 0.2 °C/s and then cryopreserved with the technique previously reported by Zambelli, with a constant cooling rate of 3.85 °C/min [5]. The straws were plugged in liquid nitrogen and stored at the same temperature for one week.

After storage, the samples were thawed in a waterbath at 37 °C for 30 s and the resulting semen was centrifuged with the same procedure described for the trial on fresh samples. Seminal endpoints were: concentration, recovery rate, objective motility, membrane, and acrosome integrity, and lipid membrane disorder on viable spermatozoa. Sperm-hyaluronan binding assay was also tested on cryopreserved feline spermatozoa centrifuged at different regimens. Objective motility, membrane and acrosome integrity, and lipid membrane disorder on viable spermatozoa were evaluated also after 60 min post centrifugation.

2.3. Semen evaluation

2.3.1. Sperm concentration and recovery rate

Sperm concentration was measured soon before and after the different centrifugation regimens, using the Burker chamber, as above described. Both the bottom and the supernatant aliquot were evaluated. The sperm recovery rate (SRR, %) was calculated only on the bottom aliquot of centrifuged samples as [concentration after centrifugation/concentration before centrifugation] x 100.

2.3.2. Sperm kinematics

Objective sperm kinematics were evaluated by an IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA) as previously reported [30]. An aliquot of diluted semen was warmed in a waterbath at 37 °C for 5 min, then a 5 μL drop was loaded in a pre-warmed Makler chamber (Sefi Medical Instruments, Haifa, Israel), and 12 non-consecutive fields were analyzed. Total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), the amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, as VSL/VAP, %), and linearity (LIN, as VSL/VCL, %) were recorded by the system and used for the statistical analysis. Spermatozoa with a VAP ≥80 μm/s and STR ≥75 % were classified as progressive.

2.3.3. Membrane and acrosome integrity

Membrane and acrosomal integrity were evaluated flow-cytometrically using propidium iodide and Alexa-Fluo-PNA double stain. In brief, the sample was incubated with 2.4 μM of propidium iodide (PI) and 5 μg/mL of Alexa-Fluo-conjugated agglutinin derived from peanuts (Alexa-Fluo-PNA) for 10 min at darkroom temperature. The analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter, San Jose, CA, USA) equipped with a 488-nm wavelength laser, and the data were analyzed using the software CytoExpert (Beckman Coulter, USA). A gate was based on forward scatter (FSC, area) and side scatter (SSC, area) to consider only events with dimensions compatible with spermatozoa. On this gate, the Alexa-Fluo-PNA fluorescence was detected using the 525/40 nm band-pass filter (area), while the PI fluorescence was detected at a 610/20 nm long-pass filter (area). The flow rate was between 280 and 420 events/s and the acquisitions were stopped at 20,000 morphologically gated events. This stain association resulted in four different subpopulations: sperm with membrane integrity and acrosome integrity showing no fluorescence (PI-/PNA-); sperm with membrane integrity and an acrosome reaction (PI-/PNA+); sperm with a damaged membrane and acrosome integrity (PI+/PNA-); and sperm with a damaged membrane and a reacted acrosome (PI+/PNA+).

2.3.4. Lipid membrane disorder on viable spermatozoa

Lipid membrane disorder in viable spermatozoa was estimated using a combination of calcein-AM and merocyanine 540. Viable cells were identified using calcein-AM (CAM - Molecular Probes Inc., Eugene, OR, USA) [31]. In this subpopulation, the lipid disorder was studied with merocyanine 540 (M540 - Molecular Probes Inc., Eugene, OR, USA), a molecule able to intercalate between plasma membrane lipids when reduced packaging was present [32]. In brief, the M540 stock solution (54 mM in DMSO) was diluted at 1:39 in TRIS to obtain a 1.35 mM working solution. After an incubation time of 30 min with calcein-AM and a further 10 min with M540, the samples were evaluated using the CytoFLEX flow cytometer (Beckman Coulter). For all evaluations, a gate was created with the side scatter and forward scatter plot to consider only sperm cells. Viable spermatozoa, positive to CAM, were detected with a 525/40 nm band-pass filter (area), while spermatozoa stained or not with M540 were collected with the 585/42 nm band-pass filter (area). The flow rate was between 280 and 420 events/s, and the acquisition was stopped at 20,000 events within the morphological gate. This stain association resulted in four different subpopulations, but only viable sperm with no lipid disorder (CAM+/M540-) and viable sperm with lipid disorder (CAM+/M540+) were considered.

2.3.5. Feline sperm hyaluronan binding assay

To test the functionally feline cryopreserved spermatozoa treated with different centrifugation regimens, the hyaluronan binding assay (HBA - CooperSurgical, Trumbull, CT, USA) was performed, according to the manufacturer's instructions. In brief, a 7-μL aliquot of different treatments was loaded on a hyaluronan-coated glass chamber and covered with a Cell Vu coverslip featuring a 0.1 × 0.1 mm grid (Fig. 1). After 10 min of incubation at room temperature, the chamber was examined under a phase-contrast microscope Olympus BX51 at 100× magnification. The hyaluronan-bound (HB) motile sperm percentage was calculated with the following formula:

% HB sperm = (bound motile sperm / (bound motile sperm + unbound motile sperm))*100

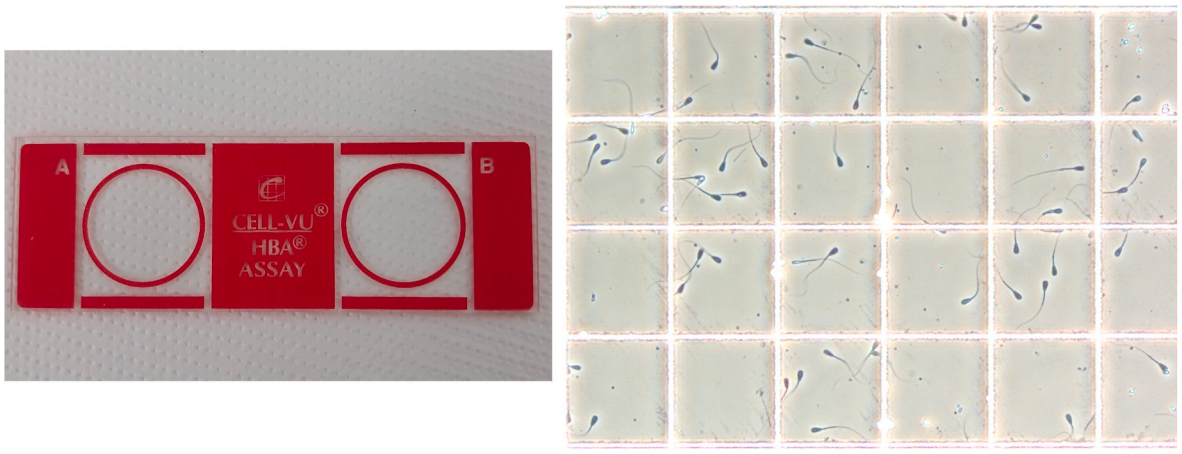

Fig. 1. Image of the slide used for the Feline sperm hyaluronan binding assay (A) and a representative microscopic image (phase-contrast microscope, 100× magnification) of a field with the 0.1× 0.1 mm grid used to quantify motile bound spermatozoa (B).

2.4. Statistical analysis

The values reported in this study were reported as mean ± standard error of the mean (SEM). The normal distribution of the data was tested using the Shapiro-Wilks test, and the homoscedasticity was checked with Levene's test. Where the data were not normally distributed, a log transformation was performed before the statistical analysis. In the tables, untransformed data were reported.

The sperm recovery rate in samples submitted to different centrifugation regimens was compared with a general linear model (GLM) based on ANOVA. Similarly, the effect of the centrifugation regimen was tested using the GLM, in which the centrifugation regimen (CTR, CE500, CE1000, and CU1000), the nature of the sample (fresh vs cryopreserved), and the time of analysis (soon after resuspension for fresh samples, after thawing for cryopreserved sample – T0; 60 min after resuspension or thawing – T60) were used as fixed factors, followed by the Scheffè test for the *post-hoc* evaluation. Hyaluronan bounded spermatozoa in cryopreserved centrifuged samples were compared using the ANOVA, followed by the Scheffè test for the *post-hoc* evaluation. Sperm attributes in the bottom and supernatant aliquots were also compared with the GLM.

Agreement between the PI negative population considered spermatozoa with membrane integrity, and CAM positive population, as spermatozoa viable due to intracellular metabolism, was tested by calculating the Pearson's correlation coefficient.

In all the cases, the significance was considered with $P < 0.05$. The statistical analyses were performed using the SPSS 15.0 software package (SPSS Inc. Chicago, IL, USA).

3. Results

One ejaculate (4.8 %) was not included in the trials, due to the yellowish colour of the sample and the low quality of spermatozoa present (subjective motility <30 %), suggesting urine contamination. The mean seminal volume collected in the animals included in this study was 68.6 ± 18.2 μL, with a mean concentration of 351.7 ± 31.9 × 10^6 sperm/mL. Subjective motility was 75.3 ± 3.3 % and the proportion of spermatozoa with normal morphology was 77.1 ± 2.9 %.

3.1. Effect of centrifugation on fresh feline sperm

The seminal characteristics in fresh cat spermatozoa before (CTR) and after different centrifugation regimens are reported in Table 1 and Table 2. Sperm concentration in the bottom samples was similar between the control and CE1000 ($P > 0.05$), while a reduced concentration was found in CE500 ($P < 0.05$). Concentration in CU1000 showed intermediate values. Conversely, a greater concentration of spermatozoa in the CE500 supernatant was found compared with conventional and cushioned centrifugation at 1000×*g*. As a result, the SRR of the CE500 was lower compared with CE1000 ($P < 0.05$), while in CU1000 SRR had intermediate values (Fig. 2). Sperm kinematics were modified depending on the centrifugation regimen. Compared with the uncentrifugated

Table 1
Kinematics in fresh feline pools (single ejaculates from 20 toms, 2–3 ejaculates/pool) prepared without centrifugation (CTR), after conventional centrifugation at 500×*g* (CE500) and 1000×*g* (CE1000), and cushioned centrifugation (CU1000).

	CTR	CE500		CE1000		CU1000	
	Whole	Bottom	Supernatant	Bottom	Supernatant	Bottom	Supernatant
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Concentration (x 10^6/mL)	42.3 ± 2.4^{a}	26.2 ± 2.1^{b}	13.2 ± 3.2^{c}	36.9 ± 1.4^{a}	0.76 ± 0.3^{d}	32.3 ± 2.6^{ab}	3.3 ± 1.3^{cd}
SRR (%)	–	62.4 ± 3.1^{a}	–	86.9 ± 4.3^{b}	–	76.2 ± 2.9^{ab}	–
Total motility (%)	68.5 ± 5.5^{ab}	73.8 ± 6.5^{a}	74.5 ± 3.9^{a}	73.2 ± 6.5^{a}	73.5 ± 3.4^{a}	61.8 ± 4.9^{b}	75.9 ± 2.1^{a}
Progressive motility (%)	49.1 ± 5.2^{a}	51.4 ± 5.8^{a}	64.1 ± 5.3^{b}	60.2 ± 8.4^{b}	61.2 ± 4.5^{b}	39.1 ± 5.3^{c}	55.5 ± 4.1^{ab}
VAP (μm/s)	107.3 ± 9.5^{a}	114.1 ± 6.2^{ab}	127.5 ± 5.8^{b}	129.3 ± 11.3^{b}	133.6 ± 9.2^{b}	109.6 ± 4.1^{a}	119.9 ± 3.9^{ab}
VSL (μm/s)	94.4 ± 8.8^{a}	93.8 ± 6.9^{a}	116.8 ± 5.1^{b}	114.5 ± 10.8^{b}	116.2 ± 11.2^{b}	85.8 ± 4.3^{ac}	92.3 ± 4.8^{c}
VCL (μm/s)	164.6 ± 6.9^{a}	182.3 ± 9.4^{b}	180.5 ± 8.2^{b}	176.3 ± 11.9^{b}	181.8 ± 12.1^{b}	163.7 ± 6.9^{a}	173.4 ± 7.1^{b}
ALH (μm)	4.2 ± 0.7^{a}	6.4 ± 0.2^{b}	5.5 ± 0.7^{ab}	6.9 ± 0.5^{b}	4.9 ± 0.7^{ab}	7.1 ± 0.3^{c}	7.3 ± 0.2^{c}
BCF (Hz)	36.7 ± 1.1^{ab}	36.8 ± 1.7^{ab}	40.1 ± 1.8^{ab}	41.4 ± 1.9^{b}	42.6 ± 1.9^{b}	36.1 ± 1.8^{a}	37.6 ± 2.8^{ab}
STR (%)	82.7 ± 2.3^{a}	93.4 ± 2.7^{b}	91.4 ± 2.6^{b}	84.7 ± 1.6^{a}	92.3 ± 3.5^{b}	87.2 ± 1.7^{a}	86.2 ± 2.8^{ab}
LIN (%)	53.1 ± 3.1^{a}	50 ± 1.7^{a}	53.2 ± 2.1^{a}	54.3 ± 3.8^{a}	53.8 ± 3.3^{a}	47.7 ± 2.5^{a}	49.1 ± 1.5^{a}

Sperm recovery rate (SRR), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN). Different letters in superscript (a-d) referred to significant differences ($P < 0.05$).

Table 2
Plasma membrane and acrosomal integrity subpopulations and plasma membrane destabilized subpopulations in fresh feline pools (single ejaculates from 20 toms, 2–3 ejaculates/pool) prepared without centrifugation (CTR), after conventional centrifugation at 500×*g* (CE500) and 1000×*g* (CE1000), and cushioned centrifugation (CU1000).

	CTR	CE500		CE1000		CU1000	
	Whole	Bottom	Supernatant	Bottom	Supernatant	Bottom	Supernatant
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
PI-/PNA- (%)	70.1 ± 3.5^{a}	71.6 ± 3.1^{a}	72.4 ± 2.9^{a}	73.3 ± 2.3^{a}	72.5 ± 3.1^{a}	61.2 ± 2.9^{b}	70 ± 2.7^{a}
PI-/PNA+ (%)	0.7 ± 0.4^{a}	1.2 ± 0.5^{a}	0.9 ± 0.4^{a}	0.81 ± 0.5^{a}	1.5 ± 0.2^{a}	0.9 ± 0.7^{a}	0.8 ± 0.5^{a}
PI+/PNA- (%)	26.1 ± 2.7^{ab}	23.2 ± 2.1^{ac}	22.5 ± 2.2^{ac}	21.7 ± 2.1^{a}	23.1 ± 1.6^{ac}	32.1 ± 1.2^{b}	23.1 ± 1.9^{c}
PI+/PNA+ (%)	3.8 ± 0.9^{a}	3.9 ± 1.5^{ab}	3.5 ± 1.2^{a}	3.6 ± 1.6^{a}	3.1 ± 1.8^{a}	5.9 ± 1.5^{b}	5.3 ± 1.7^{b}
CAM+/M540- (%)	66.1 ± 4.9^{ab}	67.4 ± 2.4^{ab}	72.2 ± 2.9^{b}	63.7 ± 2.8^{a}	71.8 ± 2.6^{b}	53.1 ± 3.2^{c}	67.5 ± 2.4^{ab}
CAM+/M540+ (%)	2.2 ± 0.5^{a}	1.6 ± 0.2^{a}	1.4 ± 0.5^{a}	8.2 ± 2.2^{b}	1.3 ± 1.5^{a}	13.1 ± 2.9^{b}	2.7 ± 0.6^{a}

Sperm with membrane integrity and acrosome integrity (PI-/PNA-), sperm with membrane integrity and acrosome damage (PI-/PNA+), sperm with membrane damage and acrosome integrity (PI+/PNA-), sperm with membrane damage and acrosome damage (PI+/PNA+); live sperm with membrane stability (CAM+/M540-), live sperm with membrane destabilization (CAM+/M540+). Different letters in superscript (a/b/c) referred to significant differences ($P < 0.05$).

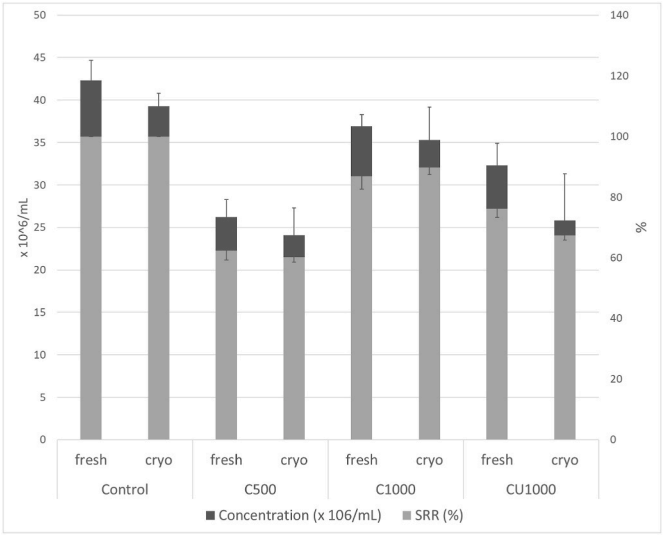

Fig. 2. Histograms reporting the concentration (dark grey) and sperm recovery rate (SRR; pale grey) in feline fresh and cryopreserved samples without centrifugation (control) or centrifugated at 500×*g* (CE500), 1000×*g* (CE1000), and cushioned at 1000×*g* (CU1000).

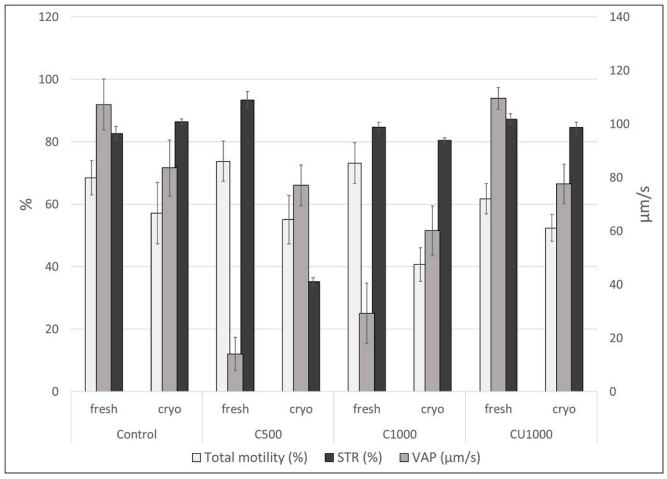

Fig. 3. Histograms reporting total motility ™, straightness (STR), and average path velocity (VAP) in feline fresh and cryopreserved samples without centrifugation (control) or centrifugated at 500×*g* (CE500), 1000×*g* (CE1000), and cushioned at 1000×*g* (CU1000).

control, CE500 bottom spermatozoa showed similar values except for VCL ($P < 0.05$), ALH ($P < 0.05$), and STR ($P < 0.05$). In CE1000 bottom samples, similar total motility was reported, with an increase in the progressive motility ($P < 0.05$), as a result of increased sperm velocities ($P < 0.05$). Spermatozoa in CU1000 showed total motility comparable with that in the CTR, but significantly lower ($P < 0.05$) compared to that recorded in CE500 and CE1000 (Fig. 3). Progressive motility was significantly lower than the control and the other centrifugation regimens ($P < 0.05$), possibly due to the wider ALH recorded in this sample (Table 1).

Spermatozoa with membrane and acrosome integrity were lower in CU1000 bottom samples compared with the other samples, with a contextual increase in sperm with membrane and acrosome damage (Table 2). Sperm membrane integrity, defined as unstained spermatozoa using propidium iodide, and viability, as the population of spermatozoa with positivity to CAM, were consistent ($r = 0.923$; $P < 0.001$). A significant increase in the proportion of viable sperm with plasma membrane destabilization was found in CE1000 ($P < 0.05$) and CU1000 ($P < 0.05$) (Fig. 4).

Samples evaluated after 60 min showed similar values for all sperm attributes (data not shown).

3.2. *Effect of centrifugation on frozen-thawed feline sperm*

The concentration and SRR in frozen-thawed centrifuged samples reflect the findings of the corresponding treatment on fresh semen (Fig. 2).

As expected, cryopreservation dramatically impacted sperm characteristics, with lower values for total (Fig. 3) and progressive motility (for both $P < 0.05$) and sperm velocities (VAP, VSL, and VCL; $P < 0.05$) in CTR samples. On the other hand, the other kinematics (ALH, BCF, STR, and LIN) were similar to the values recorded in fresh feline semen. The conventional centrifugation at 1000×*g* negatively affected sperm total and progressive motility, which were lower among the treatments (Table 3). These values appeared to be the result of reduced velocities (VAP, VSL, and VCL; $P < 0.05$) and progressiveness (STR and LIN; $P < 0.05$) of spermatozoa in this treatment. Interestingly, the supernatant samples of all the centrifugation treatments showed greater values for total and progressive motility, VCL, and BCF ($P < 0.05$). Furthermore, in CE1000 and CU1000, the supernatant values for VAP, VSL, STR, and LIN were greater compared with the correspondent pellet sample (Table 3; $P < 0.05$).

A general agreement between propidium iodide-negative spermatozoa and spermatozoa positive to CAM was found in frozen-thawed samples, according to the findings of fresh semen ($r = 0.928$; $P < 0.001$). After cryopreservation, an increase in spermatozoa with membrane damage was found in all the samples (Table 4). A great proportion

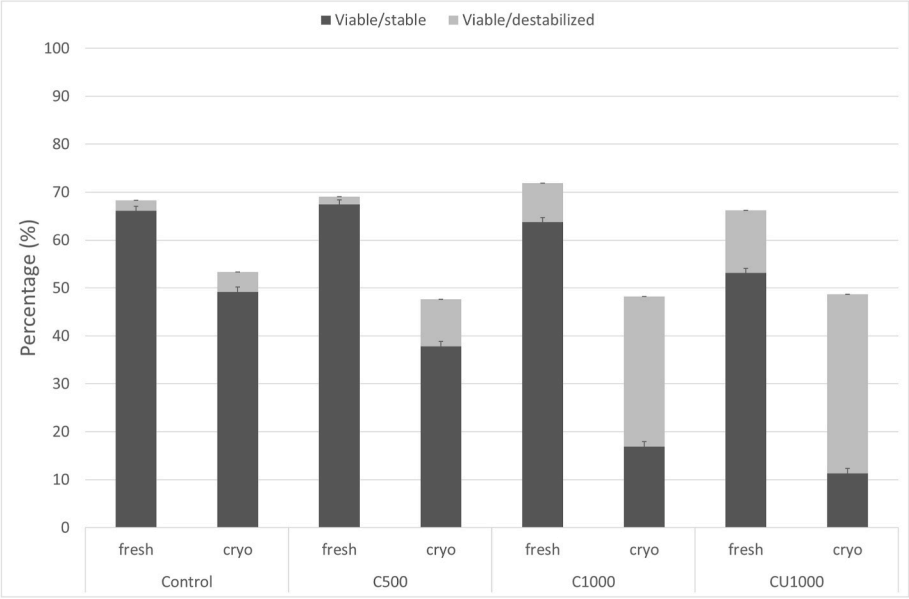

Fig. 4. Cumulative histograms reporting viable spermatozoa with the stable plasma membrane (calcein positive and merocyanine negative – dark grey) and viable spermatozoa with membrane destabilization (calcein positive and merocyanine positive – pale grey) in feline fresh and cryopreserved samples without centrifugation (control) or centrifugated at 500×*g* (CE500), 1000×*g* (CE1000), and cushioned at 1000×*g* (CU1000).

Table 3
Kinematics parameters in cryopreserved feline pools (single ejaculates from 20 toms, 2–3 ejaculates/pool) prepared without centrifugation (CTR), after conventional centrifugation at 500×*g* (CE500) and 1000×*g* (CE1000), and cushioned centrifugation (CU1000).

	CTR	CE500		CE1000		CU1000	
	Whole	Bottom	Supernatant	Bottom	Supernatant	Bottom	Supernatant
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Concentration (x 10^6/mL)	39.3 ± 1.5^{a}	24.1 ± 3.2^{b}	12.1 ± 4.7^{c}	35.3 ± 3.9^{a}	1.5 ± 0.3^{d}	25.8 ± 5.5^{ab}	3.2 ± 0.4^{d}
SRR (%)	–	60.2 ± 1.6^{a}	–	89.8 ± 2.4^{b}	–	67.3 ± 1.4^{a}	–
Total motility (%)	57.2 ± 9.8^{a}	55.1 ± 7.8^{a}	75.4 ± 9.1^{b}	40.7 ± 5.4^{c}	81.1 ± 9.7^{b}	52.4 ± 4.3^{a}	72.7 ± 8.5^{b}
Progressive motility (%)	48.1 ± 9.1^{a}	43.7 ± 8.5^{a}	66.5 ± 6.7^{b}	35.3 ± 5^{ac}	74.8 ± 8.9^{b}	43.3 ± 4.7^{a}	65.1 ± 9.2^{ab}
VAP (μm/s)	83.6 ± 10.5^{a}	77.1 ± 7.6^{a}	85.4 ± 9.8^{a}	60.2 ± 9.2^{b}	87.7 ± 10.4^{a}	77.6 ± 7.4^{a}	83.7 ± 11.4^{a}
VSL (μm/s)	75.5 ± 8.6^{ab}	69.5 ± 6.7^{b}	78.2 ± 8.6^{a}	48.5 ± 7.4^{c}	79.9 ± 9.8^{a}	67.4 ± 8.3^{b}	79.4 ± 12.6^{a}
VCL (μm/s)	128.1 ± 11.5^{a}	128.3 ± 8.3^{a}	139.8 ± 13.6^{b}	120.6 ± 9.9^{a}	142.8 ± 12.9^{b}	129.3 ± 7^{ab}	135.7 ± 10.2^{ab}
ALH (μm)	4.6 ± 0.3^{ab}	4.9 ± 0.4^{b}	4.2 ± 0.4^{a}	5.1 ± 0.5^{b}	4.9 ± 0.3^{b}	5.2 ± 0.4^{b}	4.3 ± 0.4^{a}
BCF (Hz)	37.4 ± 1.7^{a}	35.7 ± 1.4^{a}	41.7 ± 1.7^{b}	35.4 ± 1.5^{a}	40.8 ± 1.6^{b}	35.2 ± 1.8^{a}	42.1 ± 1.6^{b}
STR (%)	86.4 ± 0.9^{a}	85.2 ± 1.3^{ab}	91.9 ± 1.4^{a}	80.3 ± 0.8^{b}	89.6 ± 1.5^{a}	84.6 ± 1.7^{ab}	91 ± 1.9^{a}
LIN (%)	54.4 ± 2.8^{a}	53.5 ± 1.9^{a}	56.8 ± 3.7^{a}	40.3 ± 3.4^{b}	55.4 ± 3.2^{a}	49.2 ± 3.1^{ab}	56 ± 4.2^{a}

Sperm recovery rate (SRR), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN). Different letters in superscript (a-d) referred to significant differences ($P < 0.05$).

Table 4
Plasma membrane and acrosomal integrity subpopulations, plasma membrane destabilized, and hyaluronan-binded (HB) sperm subpopulations, in cryopreserved feline pools (single ejaculates from 20 toms, 2–3 ejaculates/pool) prepared without centrifugation (CTR), after conventional centrifugation at 500×*g* (CE500) and 1000×*g* (CE1000), and cushioned centrifugation (CU1000).

	CTR	CE500		CE1000		CU1000	
	Whole	Bottom	Supernatant	Bottom	Supernatant	Bottom	Supernatant
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
PI-/PNA- (%)	54.3 ± 3.8^{ab}	49.3 ± 4.4^{a}	53.9 ± 2.7^{ab}	48 ± 3.2^{a}	57.2 ± 2.9^{b}	47.6 ± 4.6^{a}	57.1 ± 2.1^{b}
PI-/PNA+ (%)	1.1 ± 0.3^{a}	0.9 ± 0.4^{a}	1.1 ± 0.3^{a}	1.2 ± 0.4^{a}	1.3 ± 0.3^{a}	1 ± 0.3^{a}	0.9 ± 0.2^{a}
PI+/PNA- (%)	22.1 ± 2.6^{a}	26 ± 3.1^{ab}	28.3 ± 1.4^{b}	24.6 ± 2.7^{ab}	29.2 ± 1.2^{b}	22.5 ± 2.8^{a}	30.1 ± 1.3^{b}
PI+/PNA+ (%)	23.4 ± 1.9^{a}	23.8 ± 2.2^{a}	16.1 ± 1.3^{b}	25.7 ± 2.2^{a}	13.1 ± 1.6^{b}	28.7 ± 1.8^{a}	11.7 ± 1.1^{b}
CAM+/M540- (%)	49.2 ± 3.7^{a}	37.8 ± 3.2^{b}	52.7 ± 2.8^{a}	16.9 ± 2.6^{c}	54.2 ± 1.9^{a}	11.3 ± 2.7^{c}	50.4 ± 2.6^{a}
CAM+/M540+ (%)	4.1 ± 0.7^{a}	9.8 ± 1.3^{b}	1.1 ± 0.4^{a}	31.3 ± 3.2^{c}	1.9 ± 0.7^{a}	37.4 ± 3.4^{c}	5.8 ± 1.2^{ab}
HB sperm (%)	32.8 ± 6.2^{a}	28.6 ± 8.4^{a}	–	20.7 ± 7.2^{a}	–	19.9 ± 8.6^{a}	–

Sperm with membrane integrity and acrosome integrity (PI-/PNA-), sperm with membrane integrity and acrosome damage (PI-/PNA+), sperm with membrane damage and acrosome integrity (PI+/PNA-), sperm with membrane damage and acrosome damage (PI+/PNA+), live sperm with membrane stability (CAM+/M540-), live sperm with membrane destabilization (CAM+/M540+), motile sperm bounded to hyaluronic acid (HB sperm). Different letters in superscript (a/b/c) referred to significant differences ($P < 0.05$).

of spermatozoa with membrane damage showed acrosome reaction in all the pellets after centrifugation, independently of the technique and regimen. Consistently with kinematic data, spermatozoa in the supernatant showed a greater proportion of spermatozoa with membrane integrity and acrosome integrity and a lower proportion of spermatozoa with membrane and acrosome damage compared to the correspondent pellet sample ($P < 0.05$). Differences were found in the proportion of live spermatozoa with a destabilized membrane in the different treatments. A significant increase in membrane destabilization was found in CE500 compared with the CTR samples ($P < 0.05$), and a further increase was found in both CE1000 ($P < 0.05$; Fig. 5) and CU1000 ($P < 0.05$). In all the supernatant samples, spermatozoa with membrane stability appeared predominant (Table 4). In samples analyzed 60 min after centrifugation, a significant reduction in sperm total and progressive motility ($P < 0.05$), spermatozoa with plasma membrane integrity ($P < 0.05$), and live sperm with plasma membrane destabilization ($P < 0.05$) were found in all the treatments, while a significant increase in sperm with plasma membrane damage ($P < 0.05$) was detected, suggesting the shift of destabilized spermatozoa within sperm with membrane damage (Fig. 6). After centrifugation, part of the spermatozoa appeared able to bind hyaluronic acid. The proportion of HB sperm in cryopreserved feline samples, summarized in Table 4, showed significantly higher percentages for CTR and CE500 compared with CE1000 ($P < 0.05$) and CU1000 ($P < 0.05$).

4. Discussion

In this study, spermatozoa collected by electroejaculation from different males resulted in samples with lower volume and larger concentration compared with other studies performed with a similar technique [33,34]. Previous studies performed by our group, however, showed consistent results [29,30,35]. The data were also consistent with the results of other recent studies using electroejaculation [36,37]. These findings suggest that differences in the sedation protocol, probe conformation, localization, or amount of retrograde flow [38] during collection could explain the dissimilarities. Seminal characteristics were comparable with other studies performed with the same technique [35,37,39], and with semen collected by artificial vagina [33,34,40]. These findings confirmed that electroejaculation is a suitable technique for collecting good-quality spermatozoa [13].

4.1. Role of centrifugation and cushioned centrifugation on fresh spermatozoa and comparison with the different species

Previous studies showed that centrifugation, however, resulted in mechanical stress related to the compression of spermatozoa against the plastic wall of the tube [23,26]. In feline reproduction, centrifugation was performed in most studies on feline semen cryopreservation as a step of the procedure [5,9,41–43]; unfortunately, comparative research for the optimization of this procedure was never reported.

The findings presented in the present study suggested that fresh spermatozoa withstood both conventional (500×*g*) and high centrifugation (1000×*g*) forces since most parameters were not affected by the procedure. The difference was present, however, in the recovery rate of spermatozoa, as the number of spermatozoa recovered after centrifugation corresponded to the number of spermatozoa lost in the supernatant. In the horse, it was estimated that the centrifugation at 400 to 600×*g* reduces the total amount of spermatozoa by about 20–40 %, likely due to the supernatant aspiration [44,45]. In our study, the centrifugation at 500×*g* resulted in the loss of nearly 40 % of spermatozoa, consistent with previous studies.

Interestingly, the centrifugation appeared selective for spermatozoa since spermatozoa in the supernatant of the CE500, CU1000, and more evident CE1000 showed superior properties compared with the correspondent bottom aliquot. Most studies on the sperm centrifugation effect focused on characteristics of sperm in the pellet, reasonably because of the useable aliquot of the cell. Although studies in humans and horses showed that spermatozoa in the supernatant were present in most samples also at a higher centrifugation rate [21,46], little attention was devoided to characterize sperm quality in the supernatant, representing the aliquot lost. The data presented in this study suggested that spermatozoa removed in the supernatant have better attributes compared with the pellet one, possibly because of their increased ability to remain in suspension. Reasonably, the spermatozoa lost in the supernatant are not only part of the semen, but also a better proportion of the sperm population, thus particular attention should be paid to limiting as much as possible sperm loss via the supernatant. Few studies were performed to verify the impact of centrifugation on feline semen. In a study on epididymal cat semen, spermatozoa of high kinematic quality were recovered after Percoll centrifugation, however with a low recovery number of cells [47]. Similar results were recorded in wild felines, since an increasing proportion of motile sperm, with normal tail and intact acrosome, was recovered using the SLC procedure in leopards [48]. The authors concluded that SLC may be useful to breed effectively wild felid

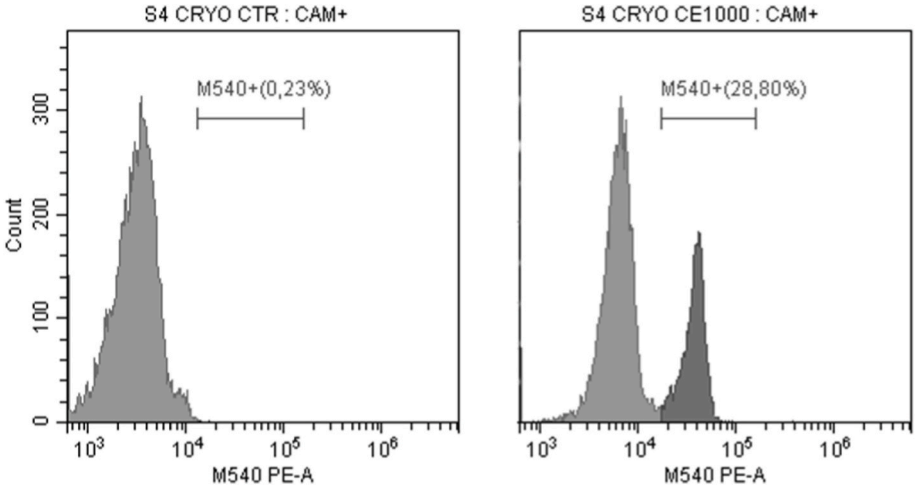

Fig. 5. Representative flow cytometric histograms of feline viable cryopreserved spermatozoa (gate on Calcein AM positive cells) with membrane stability (left peak in the histograms) and with membrane destabilization (right peak in the histograms) in the same sample without centrifugation (left graph) or after conventional centrifugation at 1000×*g* (CE1000; right graph).

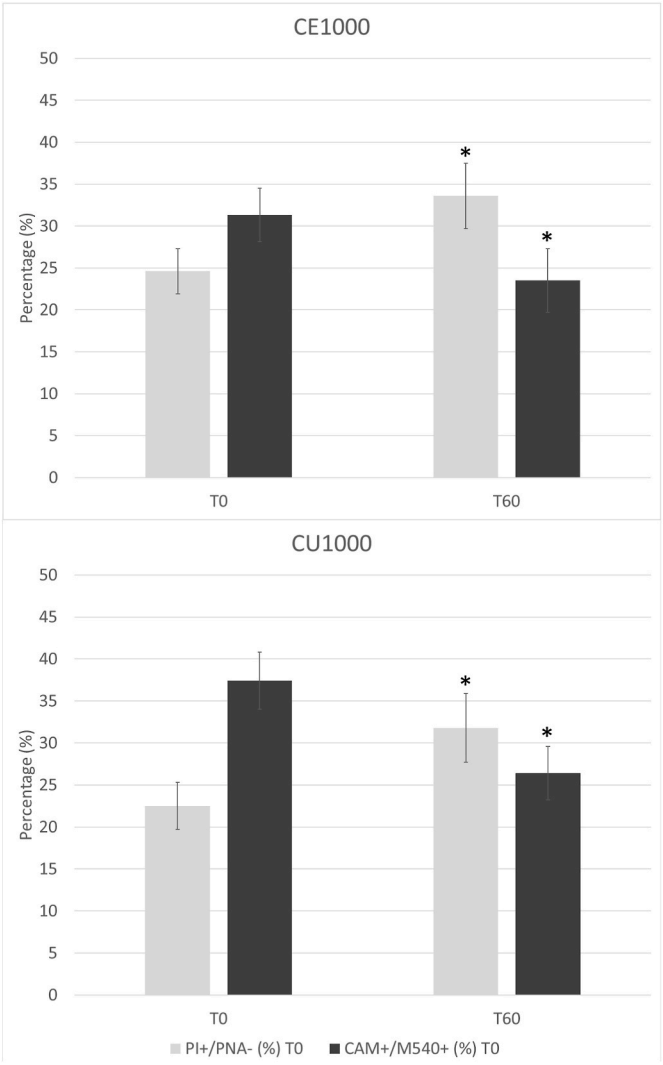

Fig. 6. Histograms reporting the percentage of cryopreserved feline spermatozoa with plasma membrane damage and acrosome integrity (PI+/PNA-) and of viable spermatozoa with plasma membrane destabilization (CAM+/M540+) after thawing (T0) and after 60 min of incubation (T60) in samples treated with conventional centrifugation at 1000×*g* (CE1000) and with cushioned centrifugation at 1000×*g* (CU1000). Values at T60 designed with asterisk (*) differ significantly from T0 ($P < 0.05$).

males with poor sperm quality. In a study conducted on cat epididymal semen, SLC and swim-up were compared for their efficiency in providing spermatozoa with improved characteristics compared to the starting sample. The authors reported the improvement of sperm quality using both the techniques of semen separation but found decreased motility and acrosome integrity in cryopreserved epididymal cat sperm treated with SLC compared to simple washing [49]. Moreover, the sperm yield after SLC was lower in felines [49] compared to other species such as boar [50] and stallion [51].

To improve the recovery of spermatozoa after centrifugation, cushioned centrifugation was proposed, especially in some species with large ejaculates, such as horses [22–24,52] and pigs [53]. This technique uses a nonionic iodinated high-density molecule (iodixanol 60 %) to create a fluid cushion at the bottom of the tube, avoiding contact between spermatozoa and the wall of the tube [15]. The inclusion of this compound allows the increase in the centrifugation force reducing the impact on spermatozoa in the horse [23,24]. Differently from the findings shown in the stallion, in our study the recovery rate was not greatly efficient, since part of the spermatozoa were almost present in the supernatant after the centrifugation, although the centrifugation force was slightly lower than previously reported. It should be remarked that the centrifugation force and time were assumed from studies in other species, thus specific aspects such as sperm head surface and density [54] or extender composition, were not considered or optimized in the study, suggesting that specific studies on feline sperm cushioned centrifugation are needed to reach conclusions on this matter. On the other hand, the presence of spermatozoa in the supernatant could only partially explain the low recovery rate detected in the present study since the data suggested that part of the spermatozoa was lost in other manners. Part of the spermatozoa likely was lost during the procedure of removal of iodixanol at the end of centrifugation, as suggested in a study evaluating this procedure on equine spermatozoa [22]. This hypothesis could explain the results reported in the present study, considering that the small volume of semen and the cushioned solution used, make problematic the accurate removal of the bottom fluid in the cat.

Otherwise studies in the stallion [22,23,52], cushioned centrifugation seemed to have an impact also on feline sperm characteristics, such as the reduction in total motility, the increase in spermatozoa with plasma membrane damage, and the number of viable spermatozoa with destabilized membrane compared with the other treatments. Ioxidanol is non-toxic for spermatozoa since several studies showed comparable sperm characteristics in samples with and without cushioned centrifugation in different species [23,53,55,56]. Similarly, in a study performed on in-vitro embryo culture, the inclusion of 25 %, 40 %, or 100 % iodixanol demonstrated limited toxicity for embryo development since a percentage of 86 %, 85 %, and 82 % of embryos grow to blastocysts after 72 h of culture, respectively, was recorded [55]. No specific studies, however, were carried out to demonstrate the toxicity of the molecule at increasing concentrations during co-incubation with spermatozoa.

Interestingly, an increase in membrane destabilization in viable spermatozoa was detected in both samples centrifuged with or without cushioned medium at a greater centrifugation rate. Previous studies suggested that membrane destabilization, detected by merocyanine, could be associated with the early stage of capacitation in different species [32,57,58]. Merocyanine was able to detect membrane disorder occurring during apoptosis in several somatic cells [59], or apoptotic bodies observed in human semen [60]. The findings of this study suggested that higher gravitational forces could induce destabilization of feline spermatozoa compatible with early apoptosis, but these modifications could be considered negligible for sperm viability and plasma membrane integrity since no differences were present after 1 h of incubation. The effect of centrifugation was negligible on acrosomal structure and integrity in feline spermatozoa at different centrifugation rates.

4.2. Impact of centrifugation and cushioned centrifugation on cryopreserved feline spermatozoa

Consistently with fresh semen, also in cryopreserved feline spermatozoa, the percentage of viable sperm with plasma membrane destabilization was significantly higher in the faster centrifugation rate and cushioned pellet samples. This data confirmed a higher susceptibility of cell membranes after the cryopreservation process to centrifugation. Cryopreservation induces several damaging stresses including osmotic, oxidative, toxic, and mechanic stress. As the temperature decreases to the liquid nitrogen temperature or increases to restore the physiologic temperature after thawing, the formation and dissolution of ice in the extracellular environment result in osmotic stress [61]. Freeze fracture studies of membranes before, during, and after cooling show clear evidence of phase separation events, which are only partially reversed after rewarming [62,63], making the plasma membrane pivotal in sperm cryodamage.

The detrimental semen characteristics after cryopreservation in feline spermatozoa were reported also in the present study, confirming previous findings, in which cooling and storage at liquid nitrogen

temperature caused motility and vigour loss in feline spermatozoa [1]. Similar results were recorded in wild felids since leopard spermatozoa are susceptible to cold damage, with sperm with lower total and progressive motility after chilling and freezing [48]. Moreover, data reported in the present study also suggested that modification induced by cryopreservation could make feline spermatozoa more sensitive to centrifugation damage, amplifying the effect noted in fresh semen. A proportion of cryopreserved feline spermatozoa were able to bind hyaluronic acid, as demonstrated in humans [64,65]. On the other hand, the proportion appeared dependent on the centrifugation regimen with samples uncentrifuged or centrifuged at 500×*g* with greater ability to interact with hyaluronic acid compared with samples centrifugated at 1000×*g* or prepared with cushioned centrifugation. The ability to interact with the hyaluronic acid could be the expression of the hyaluronic acid receptor on the sperm surface [64], and it was related to certain sperm attributes. In humans, non-motile, immature, and acrosome-damaged spermatozoa are not able to interact with hyaluronic acid [64]. Although the results reported in the present study should be considered preliminary, it appears that spermatozoa with different membrane statuses had a different ability to bind hyaluronic acid. The major difference between samples with higher or lower HB ability was the level of plasma membrane destabilization in viable spermatozoa, suggesting this structural modification could be implied in the reduced ability of sperm hyaluronan binding ability. Specific studies are necessary, however, to confirm more specifically this aspect in feline spermatozoa.

5. Conclusion

The data presented in this study showed that centrifugation has an impact on fresh and, more importantly, on cryopreserved spermatozoa. This effect appeared related to the centrifugation force and was not mitigated by the use of cushioned centrifugation. Even though centrifugation appeared an unavoidable procedure to prepare insemination doses from semen collected by electroejaculation, especially when cryopreservation semen was used for intrauterine insemination in the queen, the question related to what regimen produces better results remained unresolved. Centrifugation at 500×*g* showed a lower impact on sperm quality but resulted in the worst sperm recovery, the use of a faster centrifugation rate, with or without cushioned solutions, resulted in larger spermatozoa recovered, but of lower quality. Due to increasing interest in the practical use of cryopreserved semen in feline ETC, more studies are required to define efficient strategies for cryopreserved sperm manipulation in this species.

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CRediT authorship contribution statement

Alessia Gloria: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Marco Cunto:** Writing – review & editing, Resources, Investigation. **Daniele Zambelli:** Writing – review & editing, Investigation. **Claudia Bracco:** Resources, Investigation. **Giulia Ballotta:** Data curation. **Alberto Contri:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization.

Declarations of competing interest

None.

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