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Seminal quality comparison of first and second cryopreserved ejaculates of Alpine kid goats by flow cytometry

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Research Article

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Summary

Discarding the first ejaculate is recommended as an alternative for improving seminal quality after long sexual resting, especially when semen should be used for cryopreservation. However, when the males are not in sexual resting the necessity to discarding the first ejaculate is still unknown. Therefore, this study aimed to compare by flow cytometry the quality of the first and second ejaculates. Ten kids and uniform goats between 5 and 6 months of age were used in a completely randomized design. Semen collection was carried out every 4 days, until a total of five ejaculates per animal in each treatment was completed. The fresh and frozen semen collected were processed and analyzed using macroscopic and microscopic parameters, resistance test, hypo-osmotic medium test, and flow cytometry (FC). The FC parameters were production of reactive oxygen species, plasma and acrosomal membrane integrity, and lipid peroxidation of the plasma membrane. The ejaculates did not differ for the resistance test, the reactivity in the hypo-osmotic medium and for the macroscopic and microscopic seminal parameters, except for sperm volume and concentration. The first ejaculate had a higher percentage of minor and total defects. None of the FC parameters analyzed differed between the first and second ejaculates. The first and second ejaculates demonstrated similar seminal qualities, so for Alpine kid goats without a sexual resting period, discarding the first ejaculate it is not recommended.

Introduction

In the natural male physiology, the non-ejaculated sperm stored in the tail of the epididymidis and the ampulla of the ductus deferens is gradually eliminated in urine or undergoes cell degeneration (Hafez, 1995). Consequently, after long periods of sexual rest, it is possible that there is a reduction in seminal viability due to a high percentage of degenerated cells in the ejaculate (Firman *et al.*, 2015), reduced sperm motility, and low fertilizing ability (Kastelic and Thundathil, 2008). However, when males are not sexually resting the necessity to discarding the first ejaculate it is still unknown.

Although discarding the first ejaculate is a common practice, studies point to a decrease in sperm quality after the first (Martins *et al.*, 2006) or fourth and fifth ejaculates (Shamsuddin *et al.*, 2000), demonstrating the inconclusiveness of this recommendation. Furthermore, some studies using adult goats observed inconsistent results regarding seminal characteristics such as mass activity and sperm concentration, volume and motility (Shamsuddin *et al.*, 2000; Barkawi *et al.*, 2006; Gopinathan *et al.*, 2021; Morrell *et al.*, 2022).

For that reason, there is a need to use accurate analysis to precisely verify the recommendation of discarding or not the first ejaculate. In this context, flow cytometry (FC) analysis is an excellent tool to evaluate seminal quality due to its capacity to acquire data on thousands of spermatozoa within a sample of semen, making measurements on single cells in a few minutes (Gillan *et al.*, 2005). The FC uses fluorescent probes to evaluate several semen characteristics such as the integrity and peroxidation of plasma and acrosomal membranes, mitochondrial potential, and the production of reactive oxygen species (ROS) (Baumber *et al.*, 2000; Hossain et al., 2011), with high reproducibility and the capacity to analyze a large number of samples (Basiji *et al.*, 2007). This allows the correlation between cell parameters and potential fertilizer capacity more accurately (Hossain *et al.* (2011)).

Therefore, this study aimed to compare the seminal quality of the first and second cryopreserved ejaculates of the Alpine kid goats using FC.

Table 1. Composition of experimental diet on natural matter basis

Component	(%)	(% total diet)
Forage		60.00
Tifton hay	100.0	60.00
Concentrate		40.00
Corn meal	75.54	30.22
Soybean meal	23.96	9.58
Calcareous	0.50	0.20
Chemical characteristics	Nutritional composition (% DM)	
CP	14.37	
NDF_{ap}	44.31	
EE	0.74	
MM	4.71	

CP, crude protein; EE, ether extract; MM, mineral matter; NDF_{ap}, neutral detergent fibre (ashes and protein free).

Materials and methods

Local, animals and diet

The animals were housed in Viçosa, Minas Gerais, Brazil, in the Caprine Sector of the Universidade Federal de Viçosa. Ten healthy Alpine kid goats were used, with initial ages between 5 and 6 months, and with a body condition score of 2.5–3.0, which was scored by lumbar palpation, always by the same handler, based on scale of 0 (very thin) to 5 (obese), as described by Hervieu *et al.* (1991). The kids were housed in individual stalls and had free access to mineral salt and water. Before starting the experiment, for 30 days the animals were conditioned to detect females in oestrus and to ejaculate in the controlled experimental regimen with collections occurring until the time viable spermatozoa were detected in the ejaculate. There was control of endoparasites and ectoparasites using the treatment regimen routinely used for goats at the location this experiment was conducted. The total diet was formulated according to the Agricultural and Food Research Council (AFRC) (1998), and composed of Tifton hay and concentrate based on a corn and soybean meal diet (Table 1).

Semen collection, treatments, and fresh semen analysis

Before the experimental period, the animals were submitted to andrological examinations according to the Andrological Examination and Evaluation of Animal Semen Manual of the Brazilian College of Animal Reproduction (CBRA, 2013). The animals were assigned to one of the treatments in a completely randomized design (CRD). The treatments were: (i) first ejaculate and (ii) second ejaculate. Semen collection was carried out every 4 days until a total of five ejaculates per animal in each treatment was completed. The semen samples were collected in the morning using an estrous female restrained in a squeeze chute for goat species, as described by Dias *et al.* (2017). The first and second ejaculates were collected sequentially. Progressive sperm motility and vigour were evaluated as recommended by the Brazilian College of Animal Reproduction (CBRA, 2013).

The hypo-osmotic test was conducted using fresh semen samples to determine the functional integrity of the sperm plasma membrane. Spermatozoa that responded to the test (reactive) with coiling or tail folding were considered to be normal after the addition of hypo-osmotic solution and by subtracting from this value the percentage of spermatozoa that had a folded tail when there was morphological evaluation. The methodology used was that described by Bittencourt *et al.* (2005).

For sperm concentration, a 10-µl aliquot of each semen sample was collected and diluted in 1.99 ml of saline formaldehyde solution, resulting in a dilution factor of 1:200 (semen:solution). The number of spermatozoa was quantified using a Neubauer chamber and the total sperm concentration in the ejaculate was determined. The value was determined in cm^3 (sperm/cm^3) by conversion to ml considering there was a ratio of 1:1 (sperm/ml).

The longevity of the viability of spermatozoa in fresh semen was evaluated using a thermoresistance test that consisted of placing 0.5 ml of diluted semen in a preheated microtube at 37°C and evaluating the motility and vigour of the sperm in a 37°C bath every 30 min during a 3 h period.

The sperm morphology was determined by placing 0.5 ml of diluted semen in 1 ml of PBS in microtubes and then evaluating 400 spermatozoa in each ejaculate using a phase-contrast optical microscope (×1000) using criteria described by Blom (1973) and preconized by CBRA (2013) for determining major, minor and total abnormalities.

Semen cooling and freezing

Ejaculates were diluted in commercial medium (Botubov®) and were subsequently evaluated for osmolarity. The osmotic pressure of the medium used in the experiment was always as similar as possible to that of the osmotic pressure of the seminal plasma (282 mOsm/kg). After obtaining the final dilution, the ejaculates were placed in a 0.25-ml plastic tube (IMV®) at room temperature and frozen in liquid nitrogen at −196°C, at a concentration of 25×10^9 sperm per straw. Ten straws were stored per animal from each ejaculate.

Plastic straws were placed in a glass test tube (15 ml), which was hermetically sealed. Subsequently, this tube was placed inside a plastic tube (240 ml) containing 120 ml of absolute alcohol, and both were stored at room temperature (24°C) and then placed in a refrigerator at 5°C for 3 h, using a cooling curve (45 min) and equilibrium time of 2 h and 15 min.

Freezing was conducted in liquid nitrogen vapour (−179°C), placing the straws that were in equilibrium at 5°C, on a platform screened at a height of 5 cm from liquid nitrogen for 15 min. After this period, the straws were immersed in liquid nitrogen. Straws were subsequently conditioned in an appropriate canister and stored in a cryogenic container for further analysis.

Thawing and semen analysis

Straws were thawed in a 37°C water bath for 30 s, placed in 1.5-ml plastic tubes (Eppendorf®) and homogenized for immediate analysis of progressive motility and sperm vigour using a phase-contrast microscope at ×200 (CBRA, 2013). A semen sample from each ejaculate, after thawing, was submitted to the thermoresistance test to evaluate sperm motility after thermal stress. Sperm progressive motility and vigour were evaluated for 3 h every 30 min in a 37°C water bath, as described by Penitente-Filho *et al.* (2017). The hypo-osmotic swelling test and sperm morphology evaluation were performed as previously described in this manuscript for fresh semen.

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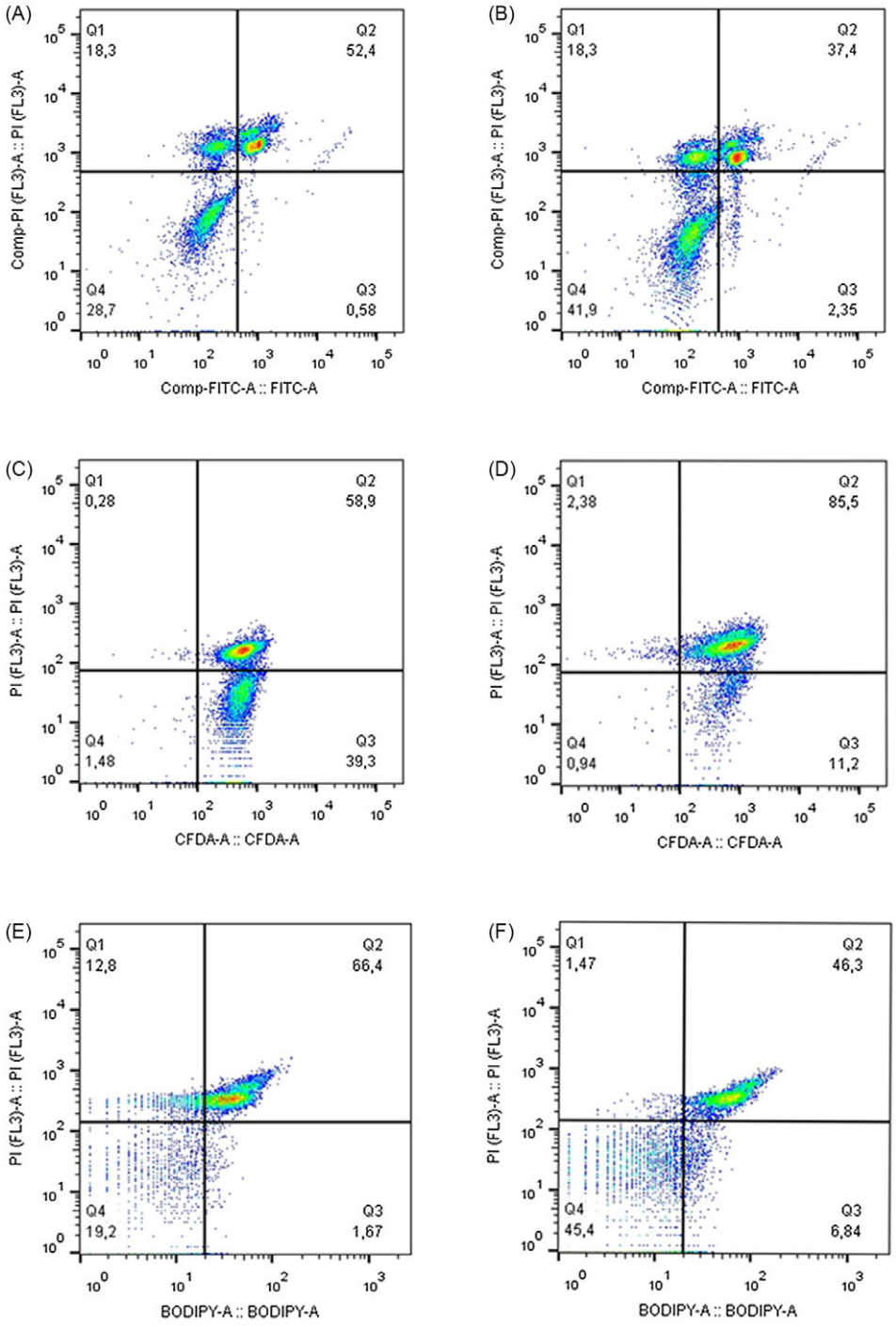

Figure 1. Spermatozoa populations analyzed by flow cytometry for plasma membrane integrity using a propidium iodide (PI) probe together with a fluorescein isothiocyanate (FITC) probe for acrosomal membrane integrity (A, B); with a dichlorofluorescein (DCF) probe for hydrogen peroxide presence (C, D); and with a boron-dipyrromethene (BODIPY) probe for lipid peroxidation (E, F). (A, C, E) first ejaculate. (B, D F) second ejaculate.

Flow cytometry

In addition to semen analysis, sperm quality was performed using a FACSVerse™ cytometer (BD Biosciences, San Diego, CA, USA). The flow cytometer was equipped with 488 and 640 nm excitation lasers, with automatic compensation. Acquisitions were achieved using BD FACSuite™ software (v.1.0, BD Biosciences). The FC calibration was performed using a fresh semen aliquot without any probe. Sperm cells were first identified based on a dot plot of the brightfield area compared with the aspect ratio. A gate was drawn around the population containing putative single cells, the non-sperm events were gated out and, therefore, not analyzed, sorting only the population of interest. For each sample, 10,000 events were acquired, at a rate of approximately 200 eV/s in a medium flow. Data were analyzed using FlowJo™ software (v.10.0.7, BD Biosciences, Ashland, OR, USA). Dot-plot graphs were used to evaluate the percentage of cells double-stained (double positive) and non-labelled (double negative).

Evaluation of plasmatic and acrosomal membrane integrity (propidium iodide (PI)/FITC–PSA)

Integrities of acrosomes and plasma membranes (Figure 1C,D) were evaluated using fluorescein isothiocyanate–*Pisum sativum* (FITC–PSA) and propidium iodide (PI). An aliquot of 150 µl of semen diluted in PBS (5×10^6 cells/ml) was added to a microtube,

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Table 2. Mean ± SEM of the macroscopic and microscopic evaluations of fresh first and second ejaculates of Alpine kid goats

Variable	Ejaculate		*P*-value
	First	Second	
Macroscopic parameters			
Ejaculate volume (ml)	0.77 ± 0.05	0.68 ± 0.04	0.007*
Colour	A (24.0%), B (52.0%), C (24.0%)	A (28.8%), B (57.7%), C (13.5%)	0.0001**
Appearance	A (28.0%), B (66.0%), C (6.00%)	A (30.8%), B (61.5%), C (7.69%)	0.0001**
Microscopic parameters			
Mass activity (1–5)	2.82 ± 0.15	2.67 ± 0.16	NS
Sperm motility (0–100)	78.6 ± 1.37	74.7 ± 2.56	NS
Sperm vigour (1–5)	3.22 ± 0.09	3.11 ± 0.11	NS
Sperm concentration (10^9 sperm/ml)	2.25 ± 0.16	1.83 ± 0.12	0.007*

*Significant by Student's *t*-test ($P < 0.05$).
**Chi-squared test ($P < 0.05$). Colour (A: yellowish; B: yellow-whitish; and C: white). Appearance (A: watery; B: milky; and C: creamy).

heated in a 37°C water bath, and was incubated with 10 µl of FITC–PSA (0.1 mg/ml) for 10 min. There were 5 µl of PI (0.5 mg/ml) added and samples were incubated for an additional 5 min. The FITC–PSA and PI were excited using a 20 mW 488 nm laser and FITC–PSA fluorescence was detected on Channel 1 (Ch01: LP 507 nm/BP 527/32 nm), whereas PI fluorescence was detected on Channel 3 (Ch03: LP 665 nm/BP 700/54 nm).

Evaluation of the production of intracellular hydrogen peroxide (DCFDA/PI)

For the evaluation of intracellular peroxide concentrations (ROS; Figure 1A,B), an aliquot of 500 µl of the sample diluted in PBS (5×10^6 cells/ml) was added to a microtube heated in a 37°C water bath and stained with 0.5 µl of 2′,7′-dichlorofluorescein diacetate (DCFDA; 1 mg/ml) for 30 min. Subsequently, 150 µl of the sample was transferred to another microtube, stained with 5 µl of PI, 0.5 mg/ml) for an additional 5 min. The DCFDA and the PI were excited using a 20 mW 488 nm laser; DCFDA fluorescence was detected on Channel 1 (Ch01: LP 507 nm/BP 527/32 nm), whereas PI fluorescence was detected on Channel 3 (Ch03: LP 665 nm/BP 700/54 nm).

Evaluation of sperm membrane peroxidation (BODIPY)

The C11-BODIPY compound was used to detect lipid peroxidation in sperm plasma membranes (Figure 1E,F). Briefly, an aliquot of 500 µl of the sample diluted in PBS (5×10^6 cells/ml) was added to a microtube heated in a 37°C water bath and stained using 0.5 µl of C11-BODIPY (1 mg/ml) for 30 min. There was subsequently 150 µl of the sample transferred to another microtube, and sperm were stained with 5 µl PI, 0.5 mg/ml for an additional 5 min. The C11-BODIPY and PI compounds were excited using a 20 mW 488 nm laser with the C11-BODIPY fluorescence being detected on Channel 1 (Ch01: LP 507 nm/BP 527/32 nm), whereas PI fluorescence was detected on Channel 3 (Ch03: LP 665 nm/BP 700/54 nm).

Statistical analysis

Statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., San Jose, CA, USA). Normality was assessed using the Shapiro–Wilk test and was normally distributed. Levene's test was used to assess the equality of variances, which was similar between groups. A comparison of means between treatments was analyzed using the independent-sample *t*-test (Student's *t*-test). One-way analysis of variance (ANOVA) was used plus post hoc Tukey's test to evaluate the thermoresistance test data. The semen colour and appearance, and sperm reactivity in the hypo-osmotic medium were analyzed using a chi-squared test. Data are presented as means (±SEM) and percentages, and the statistical significance was set at $P < 0.05$.

Table 3. Mean ± SEM of the thermoresistance test in the frozen–thawed first and second ejaculates of Alpine kid goats

Time	Sperm motility		Sperm vigour	
	First	Second	First	Second
0	38.7 ± 1.78^A	35.8 ± 1.83^A	2.88 ± 0.07^A	2.79 ± 0.08^A
30	33.2 ± 1.81^B	33.3 ± 1.87^B	2.72 ± 0.09^B	2.66 ± 0.10^B
60	28.3 ± 2.08^C	29.4 ± 2.15^C	2.52 ± 0.13^C	2.38 ± 0.13^C
90	24.0 ± 2.18^D	22.3 ± 2.25^D	2.22 ± 0.15^D	1.96 ± 0.15^D
120	21.3 ± 2.18^E	19.9 ± 2.25^E	1.86 ± 0.16^E	1.49 ± 0.16^E
150	17.3 ± 2.00^F	14.90 ± 2.06^F	1.57 ± 0.14^F	1.06 ± 0.15^F
180	13.0 ± 1.73^G	10.3 ± 1.78^G	1.13 ± 0.13^G	0.76 ± 0.13^G

$^{A–G}$ Uppercase letters differ in the same column by Student's *t*-test ($P < 0.05$).

Results

Semen analysis and thermoresistance test

The ejaculate volume and sperm concentration were greater in the first ejaculate than in the second ejaculate. However, the second ejaculate was whiter ($P = 0.0001$) and creamier ($P = 0.0001$) than the first ejaculate (Table 2). There was no difference in mass activity, and sperm motility and vigour.

Motility and vigour were evaluated using a thermoresistance test. Both parameters showed gradual reduction in these parameters with time. All times were different from each other but were equal between ejaculates (Table 3).

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Table 4. Mean of the spermatozoa count in hypo-osmotic medium in the first and second cryopreserved ejaculates of Alpine kid goats

Semen		Ejaculate	
		First (%)	Second (%)
Fresh	Reactive	22[A]	23[A]
Thawed	Reactive	27[A]	26[A]

[A–B]Uppercase letters differ on the same line by *F*-test ($P < 0.05$).

Table 5. Mean ± SEM of the major, minor and total spermatozoa defects in the first and second cryopreserved ejaculates of Alpine kid goats

Semen	Spermatozoa defects		
	Major	Minor	Total
Fresh	4.78 ± 0.34[A]	4.86 ± 0.30[A]	9.65 ± 0.57[A]
First	5.65 ± 0.52[A]	6.45 ± 0.28[B]	12.1 ± 0.64[B]
Second	4.40 ± 0.30[A]	5.55 ± 0.16[A]	9.95 ± 0.44[A]

[A–B]Uppercase letters differ in the same column by Tukey test ($P < 0.05$).

Functional plasmatic membrane integrity and sperm morphology

The evaluation of fresh and frozen semen in hypo-osmotic medium (Table 4) identified differences between the percentage of reactive spermatozoids, in the first and second ejaculates. There was no difference between treatments. When compared by morphology (Table 5), the second ejaculate did not differ from fresh semen in any of the variables evaluated. However, the first ejaculate had a higher percentage of minor and total defects than the second one.

Plasmatic and acrosomal membrane integrity

Propidium iodide (PI) (Mátyus *et al.*, 1984) and fluorescein isothiocyanate-labelled *Pisum sativum* agglutinin (FITC–PSA) (Cross *et al.*, 1986) were used to analyze the integrity of the spermatic plasma and acrosomal membranes (Figure 1A, B).

There was only difference between the ejaculates in the population in which the spermatozoa were alive and with membrane damage, being higher in the second one. When compared with the populations, the spermatozoa in this analysis presented, in the most part, were dead and had damage to the plasma and acrosomal membranes (Table 6).

Intracellular hydrogen peroxide production

The production of intracellular hydrogen peroxide was evaluated using the fluorophore dichlorodihydrofluorescein diacetate (DCFDA), which, upon penetration into the cell, was oxidized by intracellular hydrogen peroxide, emitting green fluorescence (Fig. 1C,D).

There was only one difference in the population of spermatozoa that was dead and without hydrogen peroxide, being higher in the first ejaculate than in the second. In this analysis, most spermatozoa were dead and contained hydrogen peroxide, which did not differ between treatments (Table 6).

Sperm membrane peroxidation

The evaluation of sperm membrane peroxidation was performed using C11-BODIPY$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-*bora*-3*a*,4*a*-diaza-s-indacene-3-undecanoic acid), a probe that is readily incorporated into biological membranes (Neild *et al.*, 2005) and responds to the attack of free radicals with an irreversible reaction that is quantified by FC as green fluorescence (Aitken *et al.*, 2007) (Figure 1E, F).

All populations differed between the first and second ejaculates. The second ejaculate presented a higher number of live spermatozoa, but also higher peroxidation. When the populations of each ejaculate were evaluated within each probe, all of them differed for all probes and ejaculates (Table 6).

Discussion

The animals used in this study were Alpine kids at 6 months of age, considered as pubescent. Puberty is defined as the age at which the male goat exhibits an ejaculate that contains live and viable spermatozoa to impregnate a female goat (Bezerra *et al.*, 2009). The following were observed, for the first and second ejaculates, respectively: sperm volume (0.77 ± 0.05 and 0.68 ± 0.04; $P = 0.007$), sperm concentration (2.25 ± 0.16 and 1.83 ± 0.12; $P = 0.007$), sperm motility (78.6 ± 1.37 and 74.7 ± 2.56; $P > 0.05$), and mass activity (2.82 ± 0.15 and 2.67 ± 0.16; $P > 0.05$). Mojapelo and Lehloenya (2019), in experiments with 40 Saanen kids at 5.5 months of age, reported similar results of sperm concentration, mass activity and sperm motility to our data. It is important to highlight that these related seminal parameters were obtained with a seminal analyses protocol resembling ours. Furthermore, regarding sperm motility, several studies with male adult goats showed comparable results, both obtained with subjective-related seminal analysis protocols (Shamsuddin *et al.*, 2000; Barkawi *et al.*, 2006; Kumbhar *et al.*, 2019; Ebenezer *et al.*, 2021; Gopinathan *et al.*, 2021). Sperm quality can be evaluated as sperm characteristics before and after freezing and thawing. To assess this, sperm motility is the most common microscope evaluation. Nevertheless, using an objective approach can be more accurate, avoiding the high variability among experimental units or evaluators for the seminal parameters (Verstegen *et al.*, 2002). However, the sperm motility verified in our data was similar to that described with an objective protocol for seminal analysis of fresh and frozen–thawed buck semen using a computer-assisted semen analysis (CASA) system (Jiménez-Rabadán *et al.*, 2012; Anand and Yadav, 2016; Falchi *et al.*, 2020). Therefore, these findings indicated that the seminal characteristics observed in our data did not differ to those described. Despite the kids' ages, good seminal characteristics were attested.

Variations in seminal quality were reflected directly in reproductive capability. The assessments of semen characteristics included an evaluation of seminal attributes to determine the fertilizing potentialities of the spermatozoa (Agossou and Koluman, 2018). Parameters such as sperm volume, sperm motility, morphology, and concentration are commonly used to achieve this qualification. The production of ROS by spermatozoa is a normal physiological process necessary for the occurrence of sperm capacitation and the acrosomal reaction (Olivares *et al.*, 2015). However, long periods of storage in the epididymis can generate oxidative stress and the consequent DNA damage, as well as lipid peroxidation of the sperm membrane, leading to a reduction in sperm motility (Silva, 2011). The integrity of the plasma and acrosomal

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Table 6. Mean ± SEM of the fluorescent probes in the first and second cryopreserved ejaculates of Alpine kid goats by flow cytometry

	FITC		DCFDA		BODIPY	
Variable	First	Second	First	Second	First	Second
PI+/probe+	57.1 ± 2.34^{aB}	55.0 ± 2.89^{aB}	73.5 ± 2.18^{aB}	73.0 ± 2.01^{aB}	36.1 ± 3.67^{aB}	24.0 ± 3.92^{bB}
PI+/probe−	18.1 ± 1.22^{aC}	17.2 ± 1.42^{aC}	2.78 ± 0.56^{aC}	1.92 ± 0.42^{bC}	0.91 ± 0.26^{aC}	0.03 ± 0.02^{bC}
PI−/probe+	3.00 ± 1.54^{aA}	6.13 ± 2.73^{bA}	19.4 ± 2.00^{aA}	20.5 ± 1.77^{aA}	37.6 ± 3.77^{aA}	54.7 ± 3.19^{bA}
PI−/probe−	21.9 ± 1.44^{aD}	21.7 ± 1.96^{aD}	4.28 ± 0.79^{aD}	4.58 ± 0.79^{aD}	25.4 ± 2.64^{aD}	21.3 ± 1.75^{bD}

Lowercase letters ($^{a-b}$) differ on the same line, while uppercase ($^{A-D}$) letters differ in the same column by Tukey test ($P < 0.05$).
BODIPY, boron-dipyrromethene (membrane lipid peroxidation); DCFDA, dichlorofluorescein (hydrogen peroxide presence); FITC, fluorescein isothiocyanate (acrosomal membrane integrity).

membranes is also of paramount importance for the evaluation of an animal's reproductive capability (Borg *et al.*, 1997).

The reduction in sperm quality has also been correlated with cryopreservation (Watson, 2000). Freezing of semen could result in the production of ROS and the formation of ice crystals, both factors that damage the cell (Azevedo *et al.*, 2000). Thawing is also stressful for the sperm cell, causing increased oxidative stress and decreased antioxidant enzymes. The reduction in enzyme concentration and elevation of ROS can generate lipid peroxidation, leading to a loss in sperm function (Valença *et al.*, 2007).

To perform freezing, the semen is pre-cooled, reducing the temperature from approximately 37°C to 5°C, which often causes changes in the normal pattern of sperm motility and vigour, as well as damage to plasma and acrosomal membranes (Squires *et al.*, 1999). The evaluation of the longevity of the semen is made through the TRT (Santos *et al.*, 2006), in which the functionality of the plasma membrane is verified. The CBRA recommends that the thawed semen can be used only when it has sperm motility of more than 30% and vigour of more than 2 (CBRA, 2013).

In the present data, recommendations were met only within the first 30 min of TRT (Table 2). The presence of spermatozoa in the semen of kid goats could be very variable (Trejo *et al.*, 1988), as well as their quality. In this case, this was corroborated by the low reactivity of these cells to the hypo-osmotic test, both in fresh and frozen semen (Table 3), therefore evidencing low cell membrane integrity, as defined by Santos *et al.* (2001). The data were lower than the values obtained by Santos *et al.* (2006), however they were similar to those obtained by Oliveira *et al.* (2013), working with adult bucks.

Concerning the ejaculate order, our data demonstrated no difference between first and second ejaculate for mass activity, sperm motility and vigour. Recently, some authors reported similar results using adult bucks for sperm motility and mass activity with no difference in ejaculate order (Gopinathan *et al.*, 2021; Morrel *et al.*, 2022). However, we observed that the ejaculate volume and sperm concentration presented significant differences, being greater for the first ejaculate. Similarly, Mayorga-Torres *et al.* (2016) verified a reduction in sperm volume and concentration from one collection to another, in a short period of time, as in the present study. Even so, these results do not seem consistent with the published literature. Shamsuddin *et al.* (2000) studying male adult goats evaluated ejaculate order from 1 to 4, verifying no difference in sperm collected for mass activity, and sperm motility and concentration until the third ejaculate. Only the fourth ejaculate demonstrated difference in sperm volume. Therefore, the observed differences between ejaculate order in sperm volume and concentration could be due to the age of the kids. Despite that, sperm volume, motility, and concentration in both ejaculates were above the minimum recommended by the CBRA and the last two parameters agreed with the literature describing Alpine kid goats (CBRA, 2013; Turri *et al.*, 2016).

Sperm viability was lower in the first ejaculate, which presented higher sensitivity for membrane integrity. The morphology analysis (Table 4) demonstrated a higher percentage of minor and total spermatozoa defects in the first ejaculate compared with fresh semen and the second ejaculate. This indicates that the development of these spermatozoa may have been compromised in the spermatogenesis (Garcia, 2004) or there was increased susceptibility for these to cryopreservation (Nöthling and Irons, 2008), especially with respect to the integrity of plasma and acrosomal membranes (Ozkavukcu *et al.*, 2008).

Our data indicated decreased cellular or dead cell damage in the second ejaculate compared with the first, however there was increased membrane peroxidation (Table 5). Lipid peroxidation can be used as an indicator of the ROS action (Del Rio *et al.*, 2005), associated with oxidative stress and its pathologies (Bagis *et al.*, 2005). However, in response to lipid peroxidation of the membrane, cells can also promote defence systems of antioxidants that stimulate cellular survival or activation of signalling pathways. An increase in the regulation of antioxidant proteins occurs in response to oxidative stress (Ayala *et al.*, 2014), which can be directly related to the results of the DCFDA analysis, despite the BODYPI analysis.

Therefore, the second ejaculate presented as more viable and had less membrane damage, nonetheless showing higher lipid peroxidation, whereas the first ejaculate seemed to be more sensitive to cryopreservation, presenting more minor and total defects. Thereby, the ejaculates seemed to be affected by different factors of viability, which did not compromise the sperm quality in both cases.

In conclusion, for Alpine kid goats without a sexual resting period, the cryopreserved first and second ejaculates demonstrated similar seminal qualities. So, discarding the first ejaculate is not recommended.

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