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Effect of the donor's age and type of extender (egg yolk versus clarified egg yolk) over the sperm quality of Majorera bucks preserved at 4°C: In vitro results and fertility trials

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Abstract

This study assessed the effect of donor´s age and two different extenders in the sperm quality of chilled semen in Majorera bucks. In experiment 1, semen was individually processed from 5 young (10–12 months old) and 4 mature (3–5 years old) bucks and then was diluted in two different extenders: EY (Tris-glucose, 12% egg yolk) and CEY (Tris-glucose, 12% clarified egg yolk) and cooled at 4°C; semen quality (sperm motility, percentages of alive spermatozoa, acrosome status and abnormal spermatozoa) was evaluated at 24, 48, 72 and 96 hr after cooling. In experiment II, 72 Majorera goats were assigned to four experimental groups: for groups 24-EY ($n = 18$) and 24-CEY ($n = 18$), goats were inseminated with EY and CEY cooled semen for 24 hr, respectively, while for groups 72-EY ($n = 18$) and 72-CEY ($n = 18$), goats were inseminated with EY and CEY cooled semen for 72 hr, respectively. In vitro results confirmed that only ejaculate volume and sperm concentration were significantly different between young and mature bucks. In addition, semen quality was similar between both diluents, presenting values for the first 48 hr similar to that recorded in fresh samples. The fertility rate was around 70% after 24 hr (4°C) in both groups, but the kidding rate was significantly lower (44.4%, $p < .05$) in goats inseminated with EY diluent preserved for 72 hr. Our results showed that the semen samples may be stored at 4°C in media with egg yolk or clarified egg yolk, and, therefore, the use of clarified egg yolk may represent a valid alternative to chill semen samples. Finally, young bucks (older than 10–12 months) of Majorera breed could be successfully used in breeding programmes with similar efficacy to older males.

KEYWORDS
age, buck, cooling, egg yolk diluent, semen

1 | INTRODUCTION

Semen preservation in bucks has become a basic tool for reproduction technology, but its success is directly linked to many quality factors such as semen quality, type of extender quality or sample handling during the semen preservation. It is necessary to decrease the metabolism of spermatozoa, just before the cooling or freezing of semen, and so, adding substances to protect it from cold shock is necessary (Bashawat et al., 2021). The aim of adding extenders to semen is to create a protective atmosphere, where plasma-membrane functionality, spermatozoa motility and acrosome integrity remain unchanged (Allai et al., 2018; Memon et al., 2013; Salvador et al., 2006).

In buck semen, the components of seminal plasma could affect the cooling and cryopreservation of semen samples (Cseh et al., 2012). Fresh egg yolk is a common component in sperm preservation media, basically used to prevent cold shock, but some components of seminal plasma may interact with egg yolk substances (Salamon & Maxwell, 2000; Tabarez et al., 2017). Buck semen present lipases secreted by the bulbourethral glands, and it has been defined a toxic interaction causing coagulation of egg yolk and hydrolyses lecithin to fatty acids and spermicidal lysolecithins that inhibit the motility of spermatozoa, producing toxic substances and reducing its freezing ability (Bashawat et al., 2021; Leboeuf et al., 2000). For this reason, the procedure of semen preservation in bucks is more complex than in other species and usually, and sperm washing by centrifugation is usually done in order to remove the seminal plasma (Paulenz et al., 2005; Salvador et al., 2007).

In addition, it is highly desirable to study the components of egg yolk to determine the less-noxious usable, obtaining a better sperm protection capacity (Tabarez et al., 2017). A recent study (Swelum et al., 2018) assessed the use of egg yolk of different avian species as freezing extenders and concluded that chicken egg yolk extender is recommended for the cryopreservation of buck semen. In addition, to reduce the bio-hazard potential risk associated with the use of fresh egg yolk as an extender media, different studies have proposed the replacement of the fresh egg yolk by similar components with similar sperm protection capacity (García et al., 2016; Tabarez et al., 2017).

Different studies have proposed the use of clarified egg yolk for preservation of semen in different species, such boar (Orrego et al., 2019), camel (Desantis et al., 2021), equine (Ferrante et al., 2018), cattle (El-Sheshtawy et al., 2016) and canine (Lockyear et al., 2009; Sichtar et al., 2016). In small ruminants, few research has assessed the use of clarified egg yolk to cryopreserve semen, showing similar efficacy to egg yolk diluents, regarding to maintain fertilizing capacity of frozen semen (García et al., 2017; Lukusa et al., 2021; Tabarez et al., 2017). However, most of the reported studies have tested the use of clarified egg yolk as a freezing extender, but its efficacy to preserve semen at 4°C has not been deeply assessed and fertility trials have been not carried out in small ruminants.

The age of the males represents a basic factor that influences the semen quality. In this sense, sexual maturity and fertilizing capacity range depending on the goat breeds, with variations between 6 and 12 months (Al-Ghalban et al., 2014; Roca et al., 1991; Tabarez et al., 2017). Different studies have assessed the influence of donor´s age over the subsequent preservation of the semen in small ruminants, whether it is cooled or frozen (Lymberopoulos et al., 2010; Roca et al., 1991; Tabarez et al., 2017). However, most of these studies only assess in vitro fertility, not conducting in vivo fertility trials, which are necessarily more conclusive.

The present study aimed to compare the use of two different cooling extenders (egg yolk versus clarified egg yolk) over the seminal quality of Majorera bucks, by conducting in vitro tests and fertility trials. In addition, the influence of the age of the male on the quality of fresh and chilled semen and fertility was also valued.

2 | MATERIAL AND METHODS

2.1 | Animals

Nine bucks and seventy-two dairy goats of the Majorera breed were involved in the present study. Animals were housed and managed at the experimental farm of Gran Canaria Council. Feeding was a mix of dehydrated lucerne and concentrates (maize, soy and dehydrated beetroot), mineral salts and water were offered ad libitum. A sanitary programme including anti-parasitic treatments and vaccination was periodically carried out. All animals were clinically healthy before and during the experimental period. Before synchronization, an ultrasound scanning confirmed no alterations of the reproductive tract. All experimental work was carried out according to the European laws for animal and was approved by the Welfare Animal Committee of University of Las Palmas de Gran Canaria.

2.2 | Experimental design

In experiment I, semen collection was performed from 9 bucks, classified as young (n = 5; 10–12 months old) and mature (n = 4; 3–5 years old). After collection, semen was individually processed and semen samples were divided in two aliquots. These aliquots were diluted in two extenders: EY (Tris-glucose, 12% egg yolk) and CEY (Tris-glucose, 12% clarified egg yolk) and cooled at 4°C for different times (24, 48, 72 and 96 hr) and were assessed for sperm motility, percentages of alive spermatozoa, acrosome status and abnormal spermatozoa.

In experiment II, semen was collected from 4 bucks (2 youngs and 2 mature) and processed individually in a similar way to that described in experiment I. Once the second washing was completed, semen was pooled and then diluted in two different diluents (EY and CEY) to reach a final concentration of 200×10^6 spermatozoa/Ml and cooled. All samples remained chilled for 24–72 hr, until insemination. Seventy-two goats were assigned to four experimental groups and were inseminated with EY cooled semen (24 and 72 hr, n = 36) and CEY cooled semen (24 and 72 hr, n = 36).

2.3 | Semen collection and semen evaluation

Between May and July 2020, semen was collected from each buck, once a week for 6 consecutive weeks, using an artificial vagina. Immediately after collection, semen samples were assessed for volume, sperm concentration and percentages of motile spermatozoa, alive spermatozoa, sperm cells with intact acrosome membrane integrity and abnormal morphology. The ejaculated volume was defined directly in a calibrated tube. The sperm concentration was assessed using a Spermacue® spectrophotometer (Minutübe GmbH). The percentages of total and progressive fast motile spermatozoa were evaluated with a computer-assisted sperm analysis (CASA) system (Sperm Vision Lite®, Minitüb Ibérica). The percentages of abnormal spermatozoa and acrosome-intact spermatozoa were estimated with the Spermac® staining technique (Minitübe) under a phase-contrast microscope at 1000× magnification, examining a minimum of 200 sperm cells per slide. Sperm viability was defined with supravital staining (eosine-nigrosin) at 1000 magnification, assessing between 100 and 200 sperm cells.

2.4 | Semen processing

Buck semen processing was performed as described Batista et al. (2011). Briefly, the ejaculates were diluted in washing solution (250 mM Tris, 28 mM glucose, 104 mM citric acid) at 37°C and centrifuged twice at 700 *g* for 15 min (room temperature, 20–22°C). At this time, the sperm pellets were diluted in two extenders to obtain a final sperm concentration of 400 × 10^6 spermatozoa/ml; first aliquot was diluted in a Tris-yolk extender (250 mM Tris, 28 mM glucose, 104 mM citric acid, 12% egg yolk, 0.05% streptomycin, 500 UI penicillin/mL and distilled water to 100 ml). The second diluent was diluted in a similar Tris-yolk extender but including clarified egg yolk (250 mM Tris, 28 mM glucose, 104 mM citric acid, 12% clarified egg yolk, 0.05% streptomycin, 500 UI penicillin/mL and distilled water to 100 ml). Clarified egg yolk was obtained by mixing similar volume of egg yolk and saline solution and centrifugated at 10,000 g, for 30 min, at 4°C; then, the supernatant was centrifugated once in a similar way to obtain clarified egg yolk. Finally, semen samples were cooled at 4°C for different amounts of time: 24, 48, 72 and 96 hr.; after each chilling period, aliquots were assessed for sperm motility, alive/dead spermatozoa, abnormal sperm cells and acrosome-intact spermatozoa, using similar techniques to those described above.

2.5 | Oestrus synchronization and heat detection

During June and September 2020 (breeding season), the goats (n = 72, 12–14 months old and weighing 32.3 ± 5.4 kg, mean ± *SD*) were synchronized with intravaginal progestagen-impregnated sponges (45 mg fluorogestone acetate, Chrono-gest®, Intervet) for 11 days. Forty-eight hours before the sponge withdrawal, the goats received an intramuscular injection 7.5 mg of a Prostaglandin-F2α analogue (Luprostiol, Prosolvin®, Intervet) and eCG (200 UI, Foligon®, Intervet).

Oestrus detection was carried out two times daily (08.00 am and 08.00 pm), starting 18 hr after the sponge removal. Goats showing an onset of heat >60 hr after removal of the sponges were excluded from the study.

2.6 | Artificial insemination and pregnancy diagnosis

Semen was collected from 4 bucks (2 mature and 2 youngs), assessed and processed individually, and once the second washing was completed, semen was pooled and then diluted in two different diluents (EY and CEY) to reach a final concentration of 200 × 10^6 spermatozoa/Ml and finally samples remained chilled for 24–72 hr, until insemination.

Goats were assigned to four experimental groups: in group 24-EY (n = 18), goats were inseminated with EY cooled semen (24 hr); in group 24-CEY (n = 18), goats were inseminated with CEY cooled semen (24 hr); in group 72-EY (n = 18), goats were inseminated with EY cooled semen (72 hr), and finally, in group 72-CEY (n = 18), goats were inseminated with CEY cooled semen (72 hr).

Between 18 and 24 hr after the beginning of the oestrous, artificial insemination was performed using an insemination pipette (IMV technologies). Semen samples were deposited as deeply as possible in the genital tract, and inseminations were performed by one experimented technician. A minimum of 200 × 10^6 spermatozoa showing progressive motility was used for each goat.

Pregnancy diagnosis was performed by transabdominal ultrasound scanning on day 30 after insemination. The goats were considered pregnant when an embryonic heart beat was observed. Goats were re-evaluated to check the foetal viability at day 60. Finally, after parturition, kidding rate and prolificacy were recorded.

2.7 | Statistical analysis

Data are presented as means ± standard error of the mean (*SEM*). Data of sperm viability from experiment 1 were analysed using the general linear model procedure (ANOVA) of SPSS 20.0 (IBM SPSS Statistics for Windows, Version 20.0). The lineal model included the effects of age donor´s (young and mature), the cooling extenders (EY and CEY) and the time of preservation (1, 2, 3 and 4 days), as well as the interactions between them. Dependent variables expressed as percentages (sperm motility, sperm viability, acrosome integrity and abnormal sperm cells) were arcsine-transformed before analysis. Differences between means were analysed by the Duncan test. Fertility data were compared by Fisher´s exact test. Values were considered to be statistically significant when $p < .05$.

3 | RESULTS

Fresh semen characteristics are shown in Table 1. The ejaculated volume and the total spermatozoa concentration showed differences between both groups, being higher ($p < .05$) in older males. The other seminal characteristics showed a high uniformity between adults and young males, although within each group there were individual differences, especially in the percentages of progressive motility, alive spermatozoa and acrosome-intact spermatozoa. No differences were found either between groups or within the same group, regarding the masal motility and the percentage of abnormal sperm cells.

The progressive sperm motility throughout the cooling period in adult and young males is expressed in Table 2. No differences were detected between both diluents, although the CEY extender always presented slightly higher values. Within each diluent, the progressive motility values hardly changed during the first 2 days of cooling with respect to the values observed in fresh semen, but after 72 hr, significant differences (72 hr, $p < .05$; 96 hr, $p < .01$) were detected for both diluents. The progressive sperm motility in young males showed a similar behaviour, with higher mean values in the samples preserved in CEY, but never showing significant differences. Likewise, during the first 48 hr of preservation, the mean values of sperm motility remained practically unchanged with respect to the fresh samples, but a reduction in the mean values became evident during day 3 ($p < .05$) and day 4 ($p < .01$) Finally, when adult and young males were compared, no differences were detected, with practically similar values, in the samples preserved in the same diluent.

The percentages of live sperm in adult and young males are expressed in Table 3. In older males, the mean values hardly changed throughout the cooling period, practically similar to those recorded in fresh semen (72.8%) and, consequently, no differences were detected. Similarly, in young animals, the mean values of live sperm remained uniform throughout the experimental period, also without observing the influence of the type of diluent. Finally, when adult and young males were compared, no differences were detected between both groups.

Regarding the percentage of acrosome-intact spermatozoa in adult bucks (Table 4), no differences were detected between both diluents. Within each extender, the mean values remained higher than 72% during the first 2 days of cooling similar to that observed in fresh semen; however, significant differences were detected for both diluents, after 72 ($p < .05$) and 96 ($p < .01$) hours after cooling. Similar to older males, the acrosome-intact spermatozoa percentage in young males did not show differences during the first 48 hr of preservation, but a slight decrease was detected in the samples, after 3 ($p < .05$) and 4 days ($p < .01$) of chilling, with respect to the fresh values. Finally, no differences were detected when the different groups of males and the same diluent were compared.

The last seminal parameter studied was the percentage of sperm with morphoanomalies (Table 5). The mean value remained practically unchanged (between 18% and 19%), both in young males and in adults, after 96 hr of cooling and for both diluents.

Finally, Table 6 shows the fertility rate observed after insemination. No differences were detected between groups when goats were inseminated with semen preserved for 24 hr at 4°C; however, after, 72 hr of cooling, a lower fertility rate ($p < .05$) was recorded in goats inseminated with semen diluted with EY. In addition, kidding rate and prolificacy were slight higher ($p < .05$) in goats inseminated with EY extender chilled for 72 hr.

4 | DISCUSSION

This study has defined the seminal quality in adults and young bucks of Majorera breed, comparing a standard extender (Tris-glucose-egg yolk) and a modified extender (Tris-glucose-clarified

TABLE 1 Semen characteristics in fresh samples in adult and young bucks (mean ± *SEM*)

	Adults bucks	Young bucks
Volume (ml)	1.89 ± 0.08^{a} (1.2–2.1)	1.41 ± 0.10^{b} (1.1–1.7)
Concentration/ml ($\times 10^{9}$ spermatozoa)	1.55 ± 0.20 (1,3–1.8)	1.45 ± 0.14 (1,1–1.6)
Total concentration ($\times 10^{9}$ spermatozoa)	2.89 ± 0.12^{a} (2.7–3–1)	2.03 ± 0.9^{b} (1.6–2.1)
Masal motility (0–5)	4.25 ± 0.17 (3.5–4.5)	4.21 ± 0.12 (3.5–4.5)
Progressive motility (%)	66.67 ± 1.76 (50–75)	64.37 ± 1.03 (55–70)
Alive spermatozoa (%)	72.9 ± 1.29 (64–84)	72.7 ± 1.36 (60–83)
Acrosome-intact spermatozoa (%)	76.50 ± 1.09 (66–88)	75.92 ± 1.30 (66–87)
Abnormal spermatozoa (%)	18.05 ± 0.89 (11–25)	17.83 ± 0.48 (14–22)

[ab]Different superscripts within the same row denote significant differences ($p < .05$).

TABLE 2 Sperm progressive motility (%) in EY and CEY extenders of adult and young bucks throughout the cooling period (mean ± *SEM*)

		Cooling time				
Males	**Extender**	**0 hr**	**24 hr**	**48 hr**	**48 hr**	**96 hr**
Adult	EY	66.67 ± 1.76^{a}	64.21 ± 1.60^{a}	62.8 ± 1.54^{a}	57.05 ± 1.67^{b}	54.72 ± 2.04^{bc}
	CEY		66.12 ± 1.55^{a}	65.2 ± 1.65^{a}	60.52 ± 1.79^{b}	58.24 ± 1.96^{bc}
Young	EY	64.37 ± 1.03^{a}	62.76 ± 1.54^{a}	61.95 ± 1.43^{a}	56.07 ± 1.78^{b}	55.54 ± 1.88^{bc}
	CEY		64.23 ± 1.62^{a}	63.65 ± 1.76^{a}	58.79 ± 1.19^{b}	58.11 ± 1.90^{bc}

Note: Different letter superscripts within the same row denote significant differences ($^{ab}p < .05$; $^{ac}p < .01$).
Abbreviations: CEY, semen samples preserved in clarified egg yolk; EY, semen samples preserved in egg yolk.

TABLE 3 Percentage of sperm viability (alive spermatozoa) in EY and CEY extenders of adult and young bucks throughout the cooling period (mean ± *SEM*)

		Cooling time				
Males	**Extender**	**0 hr**	**24 hr**	**48 hr**	**72 hr**	**96 hr**
Adult	EY	72.9 ± 1.29	71.32 ± 1.45	70.60 ± 1.09	69.90 ± 1.47	68.92 ± 2.24
	CEY		72.8 ± 1.35	71.58 ± 1.15	70.44 ± 1.47	69.54 ± 1.88
Young	EY	72.7 ± 1.36	71.50 ± 1.24	71.68 ± 1.33	69.87 ± 1.46	68.95 ± 1.97
	CEY		71.05 ± 1.22	71.08 ± 1.26	71.01 ± 1.39	69.71 ± 1.85

Abbreviations: CEY, semen samples preserved in clarified egg yolk; EY, semen samples preserved in egg yolk.

TABLE 4 Acrosome-intact spermatozoa (%) in EY and CEY extenders of adult and young bucks throughout the cooling period (mean ± *SEM*)

		Cooling time				
Males	**Extender**	**0 hr**	**24 hr**	**48 hr**	**72 hr**	**96 hr**
Adult	EY	76.50 ± 1.09^{a}	74.32 ± 1.5^{a}	72.67 ± 1.09^{ab}	68.6 ± 1.6^{bc}	65.44 ± 2.01^{c}
	CEY		73.8 ± 1.48^{a}	71.58 ± 1.15^{ab}	69.75 ± 1.55^{bc}	68.24 ± 1.79^{c}
Young	EY	75.82 ± 1.30^{a}	75.71 ± 1.64^{a}	72.69 ± 1.83^{a}	70.05 ± 1.24^{ab}	67.44 ± 1.77^{b}
	CEY		72.75 ± 1.60^{a}	71.18 ± 1.66^{a}	70.25 ± 1.05^{ab}	68.31 ± 1.35^{b}

Note: Different letter superscripts within the same row denote significant differences ($^{ab}p < .05$; $^{ac}p < .01$).
Abbreviations: CEY, semen samples preserved in clarified egg yolk; EY, semen samples preserved in egg yolk.

TABLE 5 Sperm abnormalities (%) in EY and CEY extenders of adult and young bucks throughout the cooling period (mean ± *SEM*)

		Cooling time		
Males	**Extender**	**0 hr**	**48 hr**	**96 hr**
Adult	EY	18.05 ± 0.89	18.77 ± 0.39	18.55 ± 0.74
	CEY		19.02 ± 0.54	18.33 ± 0.82
Young	EY	17.83 ± 0.48	18.76 ± 0.81	19.11 ± 0.55
	CEY		17.98 ± 0.42	18.79 ± 0.38

Abbreviations: CEY, semen samples preserved in clarified egg yolk; EY, semen samples preserved in egg yolk.

egg yolk) not usually described to cooling semen in bucks. Semen quality was similar between both diluents throughout the cooling period (96 hr), presenting values for the first 48 hr similar to that recorded in fresh samples. In addition, this study tried to define if the age of donors affected the quality of cooled semen and the fertility; in vitro and in vivo findings clearly showed not influence of the donor's age over the seminal features or the subsequent fertility.

TABLE 6 Pregnancy rate and kidding rate after artificial insemination with cooled semen stored for 24 and 72 hr in egg yolk (EY) and clarified egg yolk (CEY) extenders

Cooling protocols	Pregnancy rate Day 30 (%)	Pregnancy rate Day 60 (%)	Kidding rate (%)	Prolificacy
24-EY[1]	72.22[a] (13/18)	72.22[a] (13/18)	72.22[a] (13/18)	1.54[a] (20/13)
24-CEY[2]	77.77[a] (14/18)	72.22[a] (13/18)	72.22[a] (13/18)	1.57[a] (22/14)
72-EY[3]	50.00[b] (9/18)	44.44[b] (8/18)	44.44[b] (8/18)	1.33[b] (12/9)
72-CEY[4]	61.11[ab] (11/18)	61.11[a] (11/18)	61.11[a] (11/18)	1.54[a] (17/11)

Note: [1]24-EY: semen diluted in egg yolk for 24 hr; [2]24-CEY: semen diluted in clarified egg yolk for 24 hr. [3]72-EY: semen diluted in egg yolk for 72 hr; [4]72-CEY: semen diluted in clarified egg yolk for 72 hr. [ab]: Different letter superscripts within the same column denote significant differences ($p < .05$).

Fresh seminal characteristics observed in adult males were comparable to that previously described in the same breed (Batista et al., 2002, 2014) and in other caprine breeds (Leboeuf et al., 2000). Different studies have confirmed the influence of the photoperiod over the seminal characteristics especially regarding sperm concentration (Coloma et al., 2010), but, in our study, semen was obtained at the beginning of the breeding season, and this can explain the high quality of fresh semen. Regarding young bucks, the only differences observed in fresh semen quality were related to the ejaculate volume and the total concentration; these findings are completely consistent with testicular size and body weight, since both characteristics are closely related to seminal production (Roca et al., 1991; Tabarez et al., 2017). Different studies (Fuerst-Waltl et al., 2006; Furstoss et al., 2009) have defined that the sperm production is hardly relationship with the amount of testicular tissue (grams) and increases the quantity of sperm production. The other seminal parameters measured did not show significant differences, indicating that Majorera males reach a sufficient maturity around 12 months of age. For untrained young bucks, the semen collection requires training and time and the seminal quality is usually worse than those obtained in adult males and a transitional puberty period has been defined affecting the morphometric parameters and semen quality (Arangasamy et al., 2018). However, in our study, young bucks were previously trained before starting the experimental period and, for this reason, the influence of age over the seminal quality was exclusively limited to the ejaculate volume and sperm concentration. Finally, individual differences between males were observed in both young and adult males, indicating that these individual differences are already defined since the males reach sexual maturity (Morales et al., 2006).

Progressive sperm motility was slightly modified for the first 48 hr of cooling, and no significant differences between young and adult bucks were detected. Previous studies developed in the same breed (Batista et al., 2011, 2014) showed practically similar values in sperm motility of stored semen at 4°C for 24–48 hr and were comparable when the sperm quality was assessed in chilled semen, whether the diluent used was based on egg yolk (Tabarez et al., 2017) or skimmed milk (Mocé et al., 2020; Sadeghi et al., 2020; Salvador et al., 2006) with medium values between 50% and 65% for the first 48 hr of chilling. In our study, sperm motility was reduced after 72 hr of storage, regardless of the diluent used or the age of the males; as a general rule, as the preservation time increases, a decrease in sperm motility is observed, probably as a consequence of metabolic depletion of sperm (Bashawat et al., 2021), being a fact to take into account, in order to reach an adequate fertility rate. Although the kinetic parameters of sperm motility were not assessed in our study, the subjective assessment showed that the spermatozoa moved more easily probably due to the lower number of particles present in the CEY extender, and, consequently, the spermatozoa found fewer obstacles and moved, apparently, quickly.

Sperm viability and the percentages of abnormal spermatozoa remained practically unaltered throughout all the experimental period. In addition, no significant differences were observed between extenders and bucks (young versus mature). Usually, these parameters did not change during the cooling procedure (Bashawat et al., 2021; Batista et al., 2011, 2014), suggesting that the storage at 4 °C did not increase the development of sperm abnormalities and the sperm viability does not change substantially, when the semen is refrigerated for a few days. However, the percentage of spermatozoa with membrane intact acrosomes showed a similar behaviour to the sperm motility, without detecting differences based on the extender used or the donor´s age. In this way, this parameter showed values, during the first 48 hr of storage at 4°C, similar to those observed in fresh semen and then, started a slight decrease, being more evident after 96 hr of chilling. These results were slightly lower to those reported when semen was stored at 4°C (Batista et al., 2011, 2014; Roca et al.; 1997), but notably higher compared when a milk-based extender was used to preserve buck semen (Salvador et al., 2006). Therefore, in vitro results confirmed that all seminal parameters presented similar behaviour, independently of the type of diluents and the donor´s age and only sperm motility and acrosome status were modified when the preservation time increased.

The fertility rate after artificial insemination was around 70% in goats after 24 hr of preservation at 4°C. These values agree with that reported in other studies, where inseminations were performed with chilled semen (Arrébola et al.; 2016; Gore et al.; 2020; Mocé et al., 2020) and were comparable to the fertility rate obtained after artificial insemination with fresh-diluted semen (Leboeuf et al., 2000). After 72 hr, the kidding rate was slightly lower in goats inseminated with CEY diluent but significant differences were observed in EY group; anyway, our results were comparable to different studies after artificial insemination with frozen-thawed ranging between 30% and 65% (Batista et al., 2009; Dorado et al., 2007; Karatzas et al., 1997; Salvador et al., 2006). Usually, artificial

insemination with fresh or cooled semen takes place in the first 24 hr after collection, and the fertility of semen stored (at 4°C) after 72 hr has not been frequently assessed. In some geographical areas, such as the Canary Islands, the transport of semen over long distances is a common procedure and the long-term preservation of cooled semen can be an optimal alternative to the use of frozen semen, reducing the inconveniences (equipment and cost) associated with the use of frozen semen.

In small ruminates, few studies have evaluated the use of clarified egg yolk-based extender for the preservation of semen, and most of these studies have focussed on its usefulness and efficacy to cryopreserve semen (García et al., 2017; Tabarez et al., 2017) and not as a diluent to store semen at 4 °C. In our study, both diluents showed similar efficacy to preserve chilled semen, as shown by in vitro and fertility trails results. Although the kinetic parameters of the semen were not determined, the subjective assessment of spermatozoa movement was apparently higher in CEY samples and this finding may be related to the higher fertility rate observed at 72 hr in the goats inseminated with the CEY extender. Tabarez et al. (2017) concluded that the use of clarified egg yolk did not show any advantage over fresh egg yolk and requires a more complex procedure, but our results suggested its usefulness as a viable alternative for the cooling of semen in the short and medium term.

Based on our results, it may be concluded that the semen samples may be stored at 4°C in media with egg yolk or clarified egg yolk, with no differences in semen quality throughout 96 hr of preservation. In the same way, although fertility studies with a greater number of animals are necessary, the use of clarified egg yolk as a media to preserve buck goat semen is shown as a valid alternative to other diluents (fresh egg yolk, powdered egg yolk skimmed milk) to refrigerate or freeze seminal samples. On the contrary, in vitro results confirmed that the age of the male had an effect on the volume and sperm concentration, but not over other seminal parameters assessed or on the ability to be preserved (at 4°C) by the two diluents used. Therefore, young bucks (older than 10–12 months) of Majorera breed could be successfully used in breeding programmes. Finally, further studies are necessary to assess the efficacy of clarified egg yolk to preserve frozen semen and if the freezability of semen samples of young bucks are comparable to that obtained in fresh and chilled samples.

CONFLICT OF INTEREST

All authors agree to this submission and declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Kseniia Iusupova and Miguel Batista-Arteaga designed the experiment. Kseniia Iusupova and Sergio Martín-Martel collected semen and assessed semen evaluations. Artificial inseminations and pregnancy diagnosis were performed by María Luisa Díaz-Bertrana, Miguel Batista-Arteaga and Oliver Rodríguez-Lozano. Miguel Batista-Arteaga and Kseniia Iusupova analysed data and drafted the paper. Sergio Martín-Martel and Oliver Rodríguez-Lozano revised critically the manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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