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Original Research

Comparison of Whole and Centrifuged Egg Yolk Added to Kenney's and Lactose-EDTA Extenders for Donkey Semen Cryopreservation

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ABSTRACT

Egg yolk (EY) has been the most widely used external cryoprotectant for donkey spermatozoa; however, it interferes with semen evaluation. Using clarified EY in equine freezing extenders has been successful, but it is difficult to apply in mountainous areas where mules are bred. Thus, the objective of this study was to evaluate the use of centrifuged EY for deep-freezing donkey spermatozoa. Twelve ejaculates were collected from four jacks of proven fertility. Seminal plasma was removed and, before freezing, samples were rediluted in four extenders, each with 7% dimethylformamide (DMF): (1) Kenney extender with 20% EY (Kenney-Y); (2) Kenney extender with 20% centrifuged EY (Kenney-CY); (3) EDTA-glucose extender with 20% EY (EDTA-Y); and (4) EDTA-glucose extender with 20% centrifuged EY (EDTA-CY). After thawing, motility, membrane function, and acrosome status were evaluated. Total and progressive motility observed in Kenney-Y and EDTA-Y was higher ($P < .05$) than that in Kenney-CY and EDTA-CY. The percentage of frozen-thawed sperm with functional membranes and intact acrosomes was higher ($P < .05$) in the samples extended with EY when compared with those extended with centrifuged EY. In addition, samples extended with centrifuged EY showed a higher ($P < .05$) proportion of sperm with nonfunctional membranes and percent of detached acrosomes. This study has shown that it is possible to freeze donkey semen with 7% DMF using a rapid curve of temperature decrease and a manual freezing procedure. However, addition of centrifuged EY to semen freezing extenders had an unexpected detrimental effect on sperm motility, membrane function, and acrosome status.

1. Introduction

Current interest in donkey reproduction is centered on the use of breeding males to produce hybrids (*Equus mulus*) with mares (*Equus caballus*). The importance of producing mules in Argentina, Brazil, other South American countries, and the United States resides in the fact that these animals are the most efficient means of transportation for mountainous regions and areas of difficult access, as they combine the best characteristics of both the donkey and the horse and for agricultural activities such as herding cattle, especially in poor rural regions, where mules are also used because of their physical strength [1,2]. Other mentioned uses of donkeys are for entertainment and leisure, for example, in the UK and Northern Europe [3]. Despite the decrease in the donkey population over the years, the recent inclusion of donkeys in animal-assisted therapies for different human pathologies (onotherapy) is an emerging use of this species. Compared with horses, donkeys are smaller and slower; they have long soft hair that enhances tactile sensations, making the simple contact with this animal easy and therapeutically profitable. Hence, onotherapy is now considered a suitable and effective alternative to equine therapy [4]. In addition, there is now an increasing interest in the Republic of China to produce donkeys both for direct consumption of their meat, for their hypoallergenic milk and its by-products, and to obtain a gelatinous substance that is found in their hides, as it is claimed to have important medicinal attributes [5]. Therefore, the preservation of the genetic pool is mandatory [6].

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Currently, there are approximately 185 donkey breeds worldwide [7], and the number of individuals in certain breeds is very low, thus demanding an intervention to guarantee their survival [8,9]. Consequently, in the light of both the current low donkey population and aforementioned increased interest in donkey production, the use of reproductive biotechnologies becomes relevant. Artificial insemination (AI) is a reproductive technique that most influences animal production as it is a biotechnological instrument that directly impacts genetic improvement. This is because breeding males can leave hundreds of descendants over their lifetime and even after deceased if their semen has been frozen [10].

Artificial insemination can be carried out using raw, cooled, or frozen-thawed semen. Frozen semen presents multiple advantages such as permitting conservation and commercialization of genetic material [11], ease of distribution of desirable genes because of the simplicity of transport, and it also allows the control of certain pathogens from being disseminated via semen [12].

However, to date, there is not a standardized procedure for deep-freezing semen from equids, with techniques varying from facility to facility and from country to country, contributing in this way to the high variation observed both in experimental and field results [1,13,14].

The first study on freeze-thawing donkey semen was carried out by Polge and Minotakis [15]. These authors used an egg yolk (EY)-glycerol–based extender and implemented the cryopreservation method used in bulls. Since then, much has been carried out over the years varying the packaging used and the freezing media and protocols. Kreuchauf in 1984 [16] used EDTA-glucose-EY in 5 mL macrotubes and compared it with freezing in pellets using Nagase and Graham's extender for bovines [17] and did not observe any significant differences in post-thaw motility. A report from China, involving a large number of AI both in horses and jennies between 1982 and 1987 (90,000), also used an extender with EY and glycerol [18]. Trimeche et al. [19] compared the use of hen versus quail EY in INRA 82 extender and reported better results with 10% quail EY for freezing donkey semen. In this same study, they also evaluated the addition of L-glutamine to the INRA 82 freezing extender and reported that post-thaw motility improved significantly with the addition of 80 mM L-glutamine. The use of both L-glutamine and 10% quail EY was supported by further studies from these authors who also reported that removal of glycerol from the extender before AI seems to be important for obtaining pregnancies in jennies [6]. Penetrating cryoprotectants are most effective after reaching an intracytoplasmic equilibrium across the sperm membrane and therefore the best cryoprotectant is the one that enters the cells at the fastest rate, independent of the external temperature, and presents the lowest cell toxicity [20]. Glycerol is highly soluble in water and can cross the sperm plasma membrane but at a slow rate [21]. Amides, such as formamide, methyl and dimethylformamide, acetamide, methyl and dimethylacetamide on the other hand, present greater membrane permeability and cause less osmotic stress, mainly due to their lower molecular weight and viscosity as compared with glycerol [22]. In 2004, Álvarez et al. [23] assessed different concentrations of amides as cryoprotectants and compared them with 2.5% glycerol (control). They concluded that the extender with 5% dimethylformamide (DMF) resulted in superior post-thaw membrane integrity. However, Oliveira et al. [24] did not find significant differences either in post-thaw motility or membrane integrity when comparing dimethyl sulfoxide and different amides for freezing donkey semen, and although they obtained good in vitro post-thaw results, they were not able to obtain pregnancies after AI in jennies and obtained only a 40% pregnancy rate (PR) in mares with the same samples. Vidament et al. [25] also froze donkey semen and inseminated both mares and jennies, but with poor fertility results, and they concluded that high percentages of glycerol have deleterious effects on donkey spermatozoa, especially if AI is carried out before ovulation. Canisso et al. [26] added Orvus-ES-paste (sodium lauryl sulfate) to the freezing medium for donkey sperm and obtained a 53% PR after AI in mares. Although whole EY has been the component most widely used as the external cryoprotectant for donkey semen extenders with any degree of success, among its disadvantages is that the large granular material and debris it provides may interfere with sperm metabolism and with semen evaluation as it is of the same size and shape as sperm. Egg yolk also interferes with the biochemical and metabolic evaluation of semen. A reduction of some of these disadvantages may be achieved by removing the larger components of EY using centrifugation [27]. Centrifugation has been used to clarify EY by separating the plasma from the granules. Egg yolk plasma is composed of 85% low-density lipoproteins (LDLs) and 15% livetins [28]. Centrifugation is easy, and the LDLs have been attributed with the cryoprotective properties of the EY in many species [29–31] and are now regarded as the main components contributing to the cryoprotective properties of EY [28]. This centrifugation has the added advantage of producing a clearer extender that facilitates evaluation and appears to be suitable for conducting several sperm metabolic tests [32].

Mule production in Argentina is located in high mountainous areas with difficult access, where the existing laboratories are not fully equipped with centrifuges capable of achieving high forces and speed (10,000 g) as recommended by Pillet et al. [28] to perform proper EY plasma extraction. However, most laboratories freezing semen from equids are equipped with centrifuges capable of achieving 1,000 g; therefore, a simplified method to centrifuge EY using Kenney's skim milk–based extender [33], to separate the largest granular material and debris, has already been successfully used to preserve chilled donkey semen [34]. Thus, we hypothesized that this same method could be applied to successfully freeze donkey semen, as it is simple and more applicable to field conditions.

Therefore, the objective of this study was to compare the use of centrifuged EY versus whole EY added to the lactose-EDTA-glucose [35] and the Kenney [33] extenders for donkey semen cryopreservation using 7% DMF as the penetrating cryoprotectant.

2. Materials and Methods

2.1. Reagents

Unless otherwise stated, all reagents were from Sigma-Aldrich Co (St Louis, MO).

2.2. Animals and Semen Processing

A total of 12 ejaculates were collected from four jacks ($n = 4$; $r = 3$) of proven fertility of the Remonta Argentino breed, using a Missouri model artificial vagina. Once obtained, the ejaculates were filtered through sterile gauze to separate detritus and the gel fraction and then they were routinely evaluated both macroscopically (volume and color) and microscopically (progressive sperm motility and concentration) before being processed. Progressive motility was evaluated on a slide, using a phase contrast microscope and a warm stage (37°C), and concentration was calculated, in all instances, by hemocytometry using a Neubauer chamber. Only, ejaculates with a minimum of 50% progressive motility were processed.

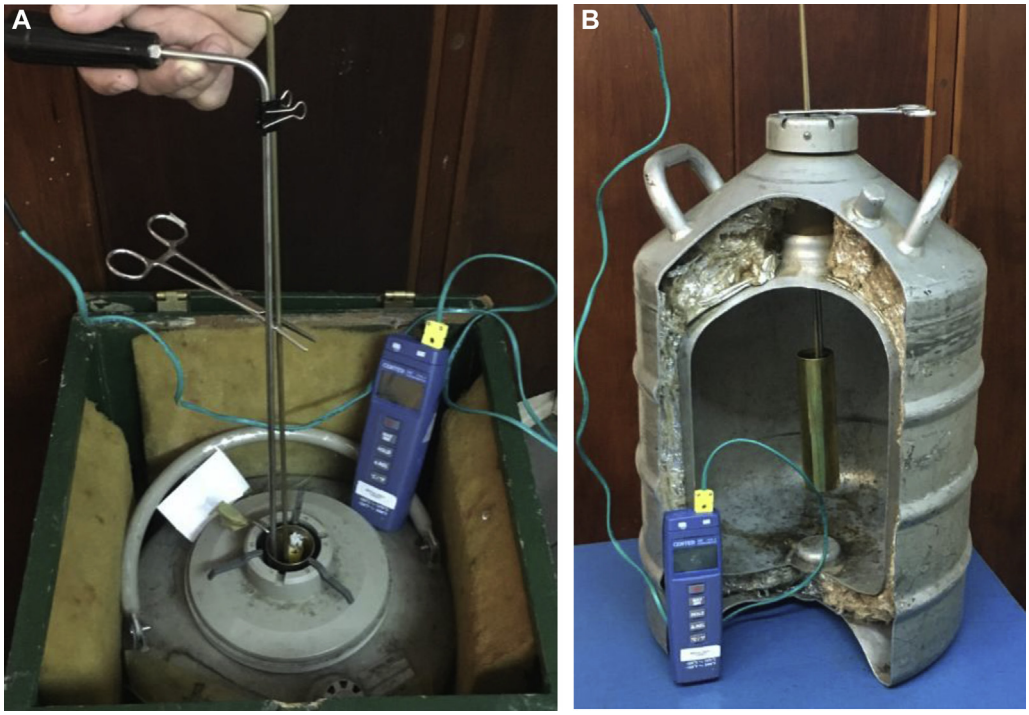

Fig. 1. Freezing semen in a liquid nitrogen tank. (A) Seen from above; (B) open tank to visualize how the bronze canister is positioned inside the tank, above the level of liquid nitrogen.

2.3. Egg Yolk Centrifugation

Egg yolk centrifugation was adapted from P. J. Burns [36]. Briefly, 5 mL of EY were centrifuged (1,000 × g) together with 25 mL of Kenney extender (4.9 g glucose, 2.4 g skim milk, 0.1 g ticarcillin-clavulanic acid, distilled water for 100 mL) [33] for 15 minutes. The supernatant obtained was added to the respective freezing extenders according to the experimental design.

2.4. Freeze-Thawing

Freezing was carried out according to Martin et al. [35] modified by Cristanelli et al. [37] and adapted by Miragaya et al. [38]. Ejaculates were diluted in a milk-based extender and divided into four aliquots, then they were centrifuged at 800 × g for 15 minutes, and the pellets were resuspended to 800 × 10^6 sperm with progressive motility/dose (six straws/dose) with different extenders according to the following treatments: (1) nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex, and 20% EY (Kenney-Y); (2) nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex, and 20% centrifuged EY (Kenney-CY); (3) lactose-EDTA-glucose extender (5.99 g glucose, 0.37 g citrate sodium, 0.37 g EDTA, 0.12 g sodium bicarbonate, 0.1 g ticarcillin-clavulanic acid and distilled water for 100 mL) with 7% dimethylformamide, 11% lactose, 0.5% Equex, and 20% EY (EDTA-Y); and (4) lactose-EDTA-glucose extender with 7% dimethylformamide, 11% lactose, 0.5% Equex, and 20% centrifuged EY (EDTA-CY). Extended semen was equilibrated 30 minutes at room temperature before being manually loaded into 0.5 mL straws and sealed with polyvinyl alcohol. The straws were placed in plastic goblets and submerged in a mixture of ethanol:acetone (1:1) in a bronze canister. Temperature was rapidly decreased inside a 9–10 L liquid nitrogen tank (Fig. 1A), according to Miragaya et al. [38]. Briefly, the bronze canister was held 6 cm over liquid nitrogen vapors, within the liquid nitrogen tank (Fig. 1B), from room temperature until −15°C was reached, with a temperature decrease of −10 to 15°C/min, and then, the canister is lowered to the level of the liquid nitrogen increasing the rate to −25 to 40°C/min until −120°C was reached. Finally, the straws were plunged into the liquid nitrogen (−196°C).

Table 1
Mean total, progressive sperm motility, and rapid motility in post-thaw donkey semen evaluated using a CASA system (AndroVision) together with mean percentages of the different staining patterns observed in frozen-thawed donkey sperm with the HOS/Coomassie blue technique (n = 4; $r = 3$).

Extenders	Total Sperm Motility (%)	Progressive Sperm Motility (%)	Rapid Motility (%)	H+/CB+ (%)	H+/CB− (%)	H−/CB+ (%)	H−/CB− (%)
Kenney-Y	41 ± 6[a]	34 ± 6[a]	37 ± 4[a]	47 ± 6[a]	8 ± 1[a]	22 ± 2[b]	22 ± 7[a]
Kenney-CY	13 ± 3[b]	10 ± 3[b]	20 ± 5[bc]	18 ± 2[b]	8 ± 1[a]	31 ± 4[a]	42 ± 4[b]
EDTA-Y	40 ± 4[a]	35 ± 4[a]	34 ± 4[ab]	47 ± 3[a]	10 ± 2[a]	21 ± 4[a]	22 ± 5[a]
EDTA-CY	0.5 ± 0.3[b]	0.4 ± 0.2[b]	7 ± 1[c]	10 ± 2[b]	9 ± 2[a]	22 ± 6[a]	59 ± 4[b]

Abbreviations: EDTA-CY: EDTA-glucose extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% centrifuged egg yolk; EDTA-Y, EDTA-glucose extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% egg yolk; Kenney-CY, nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% centrifuged egg yolk; Kenney-Y, nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% egg yolk; H+/CB+, hypoosmotic swelling test positive (functional membrane) and Coomassie blue positive (acrosome present); H+/CB−, hypoosmotic swelling test positive (functional membrane) and Coomassie blue negative (acrosome absent); H−/CB+, hypoosmotic swelling test negative (nonfunctional membrane) and Coomassie blue positive (acrosome present); H−/CB−, hypoosmotic swelling test negative (nonfunctional membrane) and Coomassie blue negative (acrosome absent); SD, standard deviation.
[a,b,c]Different letters indicate significant differences between treatments ($P < .05$).
Values are mean ± SD.

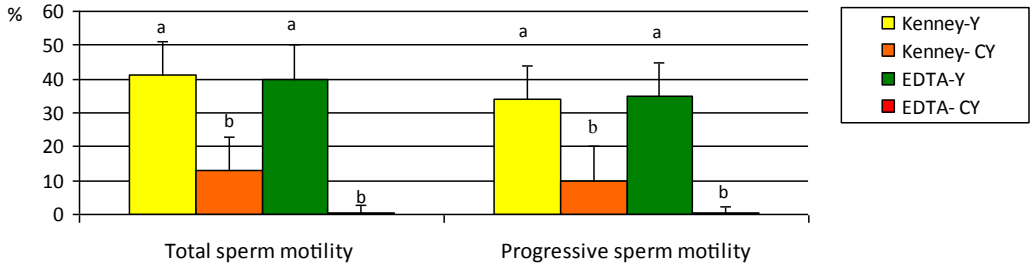

Fig. 2. Percentages of total and progressively motile post-thaw donkey sperm evaluated using a CASA system (AndroVision). [a,b]Different letters indicate significant differences between treatments ($P < .05$).

Samples were thawed in a 37°C water bath for 1 minute before their evaluation.

2.5. Post-Thaw Evaluations

2.5.1. Sperm Motility

Sperm motility was evaluated using a computer analysis system using the stallion semen set-up according to the manufacturer (AndroVision; Minitüb GmbH, Tiefenbach, Germany). The analysis was carried out using a warmed count chamber, in which the system identifies sperm cells using a correction that takes cell length and width into account (minimum and maximum particle size: 5 μm and 80 μm respectively; minimum and maximum shape: 1.1 μm and 5 μm, respectively) and thus avoids other general particles present in the media. In addition, the samples were diluted 1:3 before evaluation. In all cases, total and progressive motility were registered. Motility settings used were as follows: amplified lateral head displacement < 4 μm and beat cross frequency < 4 μm for immotile sperm. For progressively motile sperm: curvilinear velocity (VCL) > 40 μm/sec; straight line velocity (VSL): > 20 μm/sec; linearity > 75%; rapid motility > 120 μm/sec; slow motility < 120 μm/sec.

2.5.2. Membrane Function and Acrosome Status

Simultaneous evaluation of sperm membrane function and acrosome status was carried out using the Coomassie blue (CB) stain on samples that were previously subjected to the hypoosmotic swelling test (HOS) (H/CB) according to Ferrante et al. [39]. Briefly, sperms were subjected to a hypoosmotic lactose solution (50 mOsm) according to Neild et al. [40], centrifuged (800 g/3 min) and fixed with 2% paraformaldehyde. After two more centrifugations with phosphate buffered saline (800 g/3 min each) to wash the sperm, 5 μL of the samples were placed on a slide (contained in a well made with a Dako pen) and stained 5 minutes with CB. After washing excess stain with phosphate buffered saline, samples were air-dried, and they were evaluated using a phase contrast microscope under oil (1,000×), and a minimum of 200 cells were counted.

2.5.3. Statistical Analysis

Shapiro–Wilk test was used to test the normality of the data, and a split plot experimental design was used, blocking the males, and a Kruskal–Wallis analysis of variance followed by Chi square test to identify significant differences ($P < .05$) was applied.

3. Results

3.1. Post-Thaw Motility

Total and progressive motility observed in Kenney-Y and EDTA-Y was significantly higher ($P < .05$) than that of the samples frozen with centrifuged EY (Kenney-CY and EDTA-CY). The results can be seen in Table 1 and Fig. 2.

Regarding sperm velocity, rapid motility in Kenney-Y was not different to that observed in EDTA-Y; however, it was significantly higher ($P < .05$) in Kenney-Y when compared with both extenders with the addition of centrifuged EY (Kenney-CY and EDTA-CY) (see Table 1).

The results from the remaining sperm motion characteristics evaluated with the AndroVision can be seen in Table 2.

3.2. Sperm Membrane Function and Acrosome Status (HOS/CB)

The same staining patterns that were reported for equine spermatozoa were observed in the frozen-thawed jack semen samples. The patterns can be observed in Fig. 3.

The percentage of frozen-thawed sperm with functional membranes and intact acrosomes (H+/CB+) was significantly higher ($P < .05$) in the samples extended with EY (Kenney-Y and EDTA-Y) when compared with the samples extended with centrifuged EY (Kenney-CY and EDTA-CY). In contrast, samples extended with centrifuged EY showed a significantly higher ($P < .05$) proportion of sperm with nonfunctional membranes and

Table 2
Sperm motion characteristics in post-thaw donkey semen evaluated using a CASA system (AndroVision) (n = 4; $r = 3$).

Extender	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	BCF (Hz)	ALH (μm)	STR (VSL/VAP)	LIN (VSL/VCL)	WOB (VAP/VCL)
Kenney-Y	58.11 ± 20.6	24.95 ± 9.1	30.43 ± 11.1	6.99 ± 3.2	0.58 ± 0.2	0.82 ± 0.02	0.43 ± 0.03	0.53 ± 0.04
Kenney-CY	19.36 ± 8.5	8.03 ± 3.1	9.66 ± 3.8	1.52 ± 1.5	0.22 ± 0.1	0.83 ± 0.05	0.44 ± 0.1	0.52 ± 0.1
EDTA-Y	56.65 ± 16.4	26.23 ± 8.3	30.94 ± 9.2	7.19 ± 2.5	0.57 ± 0.1	0.84 ± 0.03	0.46 ± 0.04	0.54 ± 0.03
EDTA-CY	12.09 ± 2.2	5.75 ± 2.1	6.72 ± 2.1	0.33 ± 0.2	0.12 ± 0.02	0.84 ± 0.06	0.47 ± 0.1	0.55 ± 0.09

Abbreviations: ALH, amplified lateral head displacement; BCF, beat cross frequency; EDTA-CY, EDTA-glucose extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% centrifuged egg yolk; EDTA-Y, EDTA-glucose extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% egg yolk; Kenney-CY, nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% centrifuged egg yolk; Kenney-Y, nonfat milk-based extender with 7% dimethylformamide, 11% lactose, 0.5% Equex and 20% egg yolk; LIN, linearity (straight line velocity/curvilinear velocity); SD, standard deviation; STR, straightness (straight line velocity/average path velocity); VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, average path velocity/curvilinear velocity.
Values are mean ± SD.

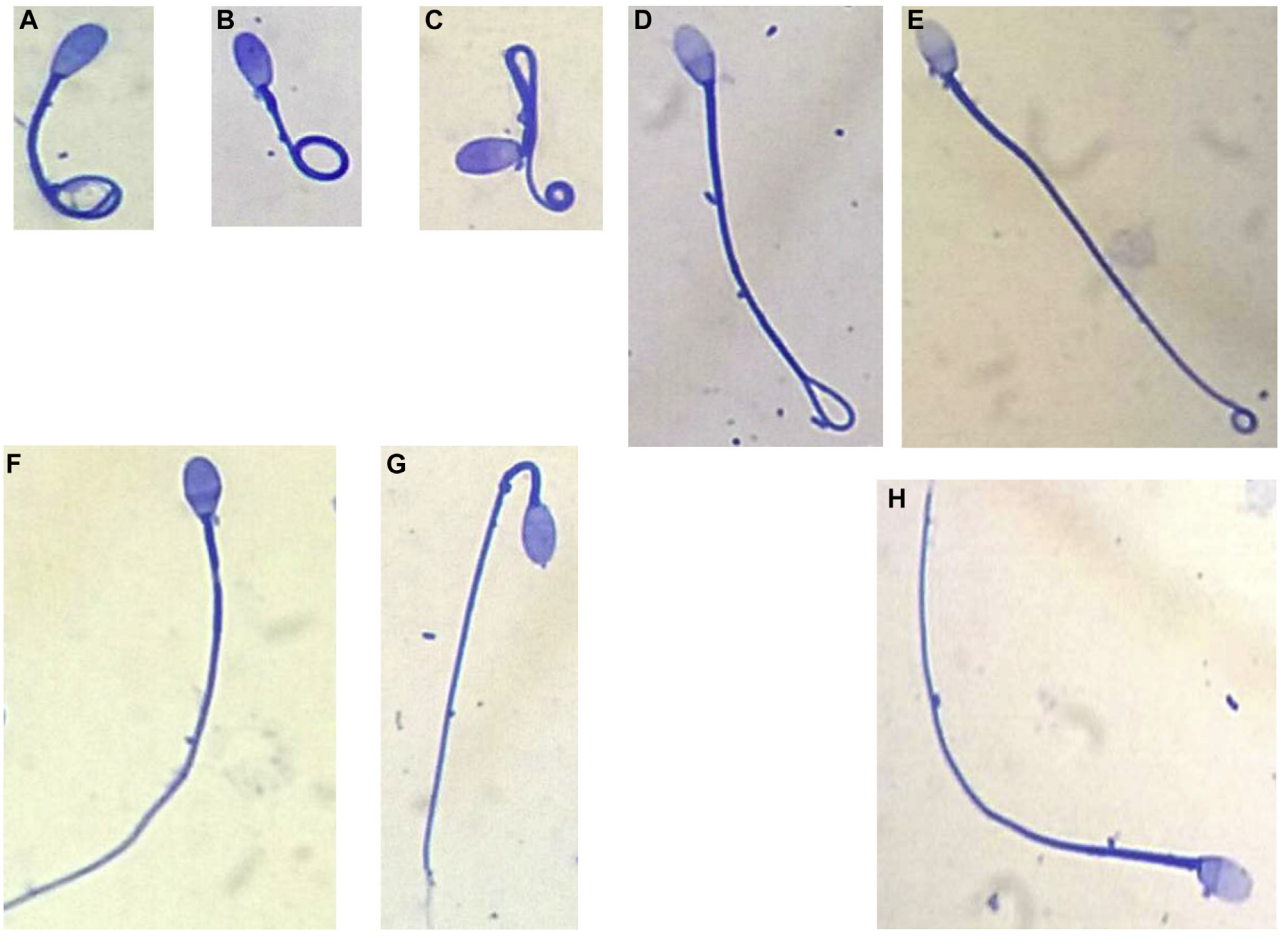

Fig. 3. Staining patterns of the combined HOS-CB technique in donkey sperm. (A, B, and C) sperm with functional membranes and intact acrosomes (H+/CB+); (D and E) sperm with functional membranes and no acrosomes (H+/CB−); (F and G) sperm with nonfunctional membranes and intact acrosomes (H−/CB+); and (H) sperm with nonfunctional membranes and no acrosome (H−/CB−). CB, Coomassie blue; HOS, hypoosmotic swelling test.

no acrosomes (H−/CB−) when compared with those with whole EY. These results can be seen in Table 1 and Fig. 4.

4. Discussion

Centrifuged EY has been used with good in vitro results for preserving donkey semen at 5°C [34], and our objective was to verify if similar results could be obtained with frozen-thawed semen. However, this does not seem to be the case when subjecting the spermatozoa to more rigorous temperatures such as those used in freeze-thawing. Our results with the addition of centrifuged EY to freezing extenders for donkey semen showed that this modification was unable to preserve neither total or progressive motility nor membrane function and acrosome status when compared with the control whole EY. Neither was there any improvement in cryoprotection for any of the motion parameters studied. Perhaps some crucial components, at least for donkey sperm motility, membranes and acrosomes, were lost in the centrifugation processing of the EY. These disappointing findings could be due to insufficient EY as the final concentration of EY after centrifuging and rediluting was approximately 3.2% for Kenney-CY and EDTA-CY. However, this was contrary to what has been reported by Burns in 1992 [36], who used 4% clarified EY and 3.5% glycerol when comparing the addition of different sugars as external cryoprotectants to Kenney's extender [33] for freezing stallion semen and did not observe any differences between the different extenders. Pillet et al. [28], also in stallion frozen-thawed semen, concluded that plasma EY was as efficient as EY for freezing spermatozoa, despite reporting slightly lower, yet significant, membrane integrity for samples frozen in plasma EY. However, their conclusion was supported by their results from fertility trials in the field. Most probably, the different result obtained in our study is due to the different treatment applied to the EY in either study, as in this study only a simple centrifugation was carried out (whereas both Burns and Pillet used ultracentrifugation).

Freeze-thawing protocols have still not established the ideal curve of temperature decrease for spermatozoa, possibly due to the wide variety of extenders and cryoprotectants used [41] and the high variability across species in sperm susceptibility to being exposed to low temperatures. Our study shows that it is possible to freeze donkey semen using a rapid curve of decrease in temperature and obtain post-thaw sperm with satisfactory in vitro quality. De Oliveira et al. [42] reported similar results in donkey frozen semen after comparing a rapid curve (in an automated system) versus a conventional freezing curve (using a manual method in a Styrofoam box). However, the protocols used by these authors are considerably longer than the one used in the present study (approximately 46 minutes for the conventional manual method and 26 minutes for the automated system vs. 6 minutes in our manual method). Our study combined the rapid curve of the automated system with a nonconventional manual method and dispensed with the prior step of cooling to 5°C for 20 minutes with

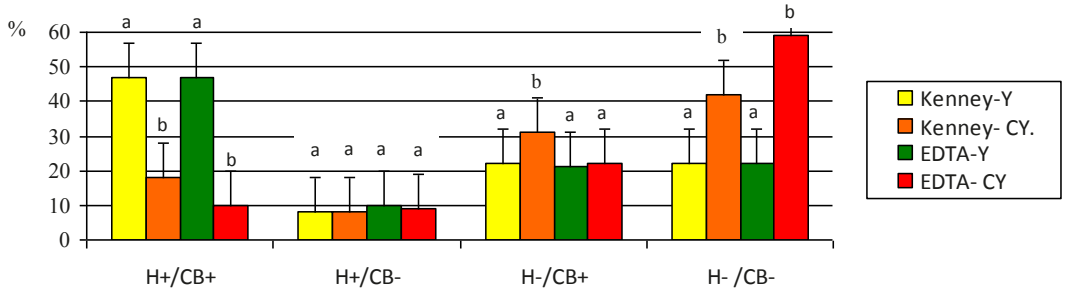

Fig. 4. Percentages of the different staining patterns of the HOS/Coomassie blue technique in frozen-thawed donkey sperm. [a,b]Different letters indicate significant differences between treatments ($P < .05$). HOS, hypoosmotic swelling test.

good repeatable results. Thus, this nonconventional manual method does not need a refrigerator and in addition costs are lower, as less liquid nitrogen is used, both when compared with the automated system (which requires a constant influx of nitrogen) and to the conventional manual method which requires both filling a Styrofoam box and an extra nitrogen tank for storing the frozen samples. Rota et al. [43] also froze donkey semen using an automated system and a rapid decrease in temperature with satisfactory post-thaw results; however, despite the temperature curve being similar to both our and de Oliveira's study, their protocol was even longer as it included equilibration at 4°C for approximately 60 minutes. Consequently, our method of semen cryopreservation is simple, inexpensive, with a repeatable temperature curve, and is easy to transport, making it very appropriate for our mule production conditions.

Trimeche et al. [6] evaluated four different extenders in Poitou donkey semen, with or without the removal of glycerol after thawing and before insemination and reported that no jennie became pregnant when glycerol was present in the extender, but a 38.9% PR was obtained when glycerol was removed before AI. Vidament et al. [25] also evaluated the effect of various concentrations of cryoprotectants on the fertility of frozen-thawed donkey semen and concluded that high concentrations of glycerol are deleterious for donkey spermatozoa, especially when AI is carried out before ovulation. These authors also propose that there could be an interaction between the milk-based extender with glycerol and the jennies' genital tract. In 2009, Vidament et al. [25] proposed that high concentrations of glycerol might affect the motility and fertility of donkey and stallion spermatozoa as early as the prefreezing process. Álvarez et al. [23] assayed different amides (methylformamide, DMF, and dimethylacetamide) in concentrations ranging between 2.5 and 5% and compared them to the use of glycerol in Zamorano-Leonés donkeys and found that 5% DMF gave the best results. As in South American Camelids, higher concentrations of DMF (7%) have been used successfully. Therefore, in this study, we used this same concentration of DMF. Despite this being higher than the concentration used by Álvarez et al. [23], this did not seem to adversely affect the post-thaw progressive motility (34%–35%), VSL: 25–26 μm/sec, VCL: 56–58 μm/sec, average path velocity (VAP: 30–31 μm/sec), viability (HOS: 55%–57%), or acrosome integrity (CB: 68%–69%) of the spermatozoa when combined with whole EY in the extenders. However, these positive results obtained in vitro using 7% DMF need to be corroborated with fertility trials.

Regarding the warming method, it has been reported that if a very rapid curve of temperature decrease is used, the thawing should be equally fast to counterbalance the fast temperature decrease, as an improvement in progressive motility was observed [37,44–46]. In our study, a rapid freezing curve, without equilibration, was performed, and samples were thawed at 37°C for 1 minute. For this reason, it would be interesting to evaluate the ultra-rapid warming method used in equine semen cryopreservation (7 seconds at 75°C followed by 30 seconds at 37°C) [37] and compare the results with the ones obtained in this study.

5. Conclusions

This study has shown that it is possible to freeze donkey semen with 7% DMF as the permeable cryoprotectant using a rapid curve of decrease in temperature and a manual freezing procedure in the nitrogen tank. It would be interesting to assay the ultra-rapid thawing protocol to see if this improves the post-thaw results obtained with this protocol. However, addition of 3.2% centrifuged EY to donkey freezing media showed no benefits, on the contrary, it proved counter-productive as evidenced by the detrimental effect on sperm motility, membrane function, and acrosomes.

We are currently aiming at correlating the in vitro results obtained in this study with an in vivo fertility trial.

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