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Donkey semen cryopreservation: Alternatives with permeable, non-permeable cryoprotectants and seminal plasma

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Abstract

Cryopreservation of semen is an important technique to preserve genetic material. Yet, pregnancy rates in jennies after artificial insemination with frozen–thawed donkey semen are poor. This condition has been attributed to the impact of permeable cryoprotectants, that could cause high post-breeding endometritis. Removal of seminal plasma (SP) prior to semen freezing process is another contributing factor. SP is involved in a multitude of sperm functions and events preceding fertilization and has a mediating effect of sperm capacitation and postcoital uterine inflammatory response. The aim of this study was to evaluate different alternatives in donkey semen cryopreservation with permeable, non-permeable cryoprotectants, BSA and SP. Thirty ejaculates from 10 donkeys were cryopreserved with different combinations of dimethylformamide (DMF, 5%), sucrose (SUC, 200 mM) and homologous SP (10%): DMF (T1), DMF/SP (T2), SUC/BSA (T3), SUC/BSA/SP (T4), DMF/SUC/BSA (T5), DMF/SUC/BSA/SP (T6), DMF/BSA (T7) and DMF/BSA/SP (T8). After thawing, sperm motility and kinetics were assessed by computerized semen analysis. Sperm vitality (SV) was evaluated by fluorescence microscopy, functional membrane integrity (FMI) by the HOST test, abnormal morphology by eosin-nigrosin staining and sperm membrane stability by flow cytometry. For statistical analysis, sperm quality indexes (SQi) were obtained, general linear models were carried out and mean comparisons were made by the Tukey test. T1, T2, T5, T6, and T7 had higher and equivalent results for motility, most kinetic parameters and function membrane integrity. Cryopreservation of donkey semen without permeable cryoprotectant (T3 and T4) showed a reduction in motility, kinetics, SV, FMI and SQi. T5 showed a reduction in progressive motility, sperm velocities, IMF and SQi compared to other DMF treatments. T6 and T8 achieved higher SQi values compared to T1, but they were not different compared to T2 and T7. T1 had a smaller sperm population with low-M540 compared to T3. It is concluded that the use of permeable cryoprotectant is essential to achieve higher post-thaw quality of donkey semen. In addition, the combined use of BSA, SUC and/or PS may provide additional sperm protection compared to the individual use of DMF.

KEYWORDS
bovine serum albumin, dimethylformamide, membrane stability, seminal quality, sucrose

1 | INTRODUCTION

Donkey has been one of the most affected livestock species by industrialization, depopulation of rural districts, and mechanization of agriculture; this has led to a donkey population decline of up to 80% in the 20th century (Camillo et al., 2018). Environment and biodiversity preservation of domestic species resources is crucial and research should be focused on those reproductive procedures aiming to preserve available individuals (Diaz-Jimenez et al., 2019). The improvement of semen cryopreservation protocols for endangered donkey breeds would, therefore, allow the establishment of sperm banks that contributes to preserve valuable genetic material and a more efficient use of donkey semen for insemination (Bottrel et al., 2018; Hidalgo et al., 2020). For this reason, some studies have been carried out that evaluate methods of freezing donkey semen (De Oliveira et al., 2016), the use of non-permeable cryoprotectants such as whole and centrifuged egg yolk (Ferrante et al., 2018; Zhang et al., 2018), sucrose, bovine serum albumin (Diaz-Jimenez et al., 2018, 2019) and some additives as L-Proline (Li et al., 2021) to the freezing extender, yield promising results. But then, the low reproductive efficiency of jennies has become an important challenge to overcome (Li et al., 2021). Unfortunately, pregnancy rates in jennies after artificial insemination with frozen–thawed donkey semen are poor (Diaz-Jimenez et al., 2021). This condition could be attributed to the impact of permeable cryoprotectants (Vidament et al., 2009), which could induce high post-breeding uterus inflammation and consequently, affect fertility (Diaz-Jimenez et al., 2021). The inflammation caused by spermatozoa is characterized by a rapid influx of polymorphonuclear neutrophils (PMNs) into the uterine lumen after artificial insemination (Troedsson et al., 2000). But then, it is known that in the equine species, seminal plasma (SP) significantly reduces the proportion of spermatozoa phagocytosed by PMNs (Alghamdi et al., 2004). SP is a mixture of fluid secreted from the testes, epididymis and accessory sex glands, and it is involved in a multitude of sperm functions and events preceding fertilization (Kareskoski & Katila, 2008), such as activation of motility, antimicrobial action, neutralization of metabolites and a mediating effect of sperm capacitation and postcoital uterine inflammatory response (Rodríguez-Martínez et al., 2011). Nevertheless, SP in *Equidae* must be removed for preservation processes of sperm in artificial conditions such as cryopreservation (Miró et al., 2013; Monteiro et al., 2022; Vilés et al., 2013), since has been associated with a deleterious effect on sperm capacitation and fertilization capacity (Morrell et al., 2014). Yet, some studies have reported promising results regarding the improvement of post-thaw sperm quality when it is supplemented with small amounts of SP, which has been related to the contribution of SP to the antioxidant capacity of the cryopreserved semen (Usuga et al., 2020). Bovine serum albumin (BSA) has been successfully described as a scavenger of free radicals against oxidative stress and protection of the plasma membrane against thermal shocks generated in the freezing and thawing of ram spermatozoa (Uysal & Bucak, 2007) and the addition of sucrose (SUC) to the extender for stallion sperm vitrification, results in a greater viscosity of the solution that suppresses ice crystal formation (Consuegra et al., 2019). Yet, there are no studies with the combination of dimethylformamide (DMF), SP, BSA and SUC in the freezing of donkey semen. Therefore, the aim of this research was to evaluate the use of permeable, non-permeable cryoprotectants, bovine serum albumin and SP, on post-thaw sperm quality of donkeys.

2 | MATERIAL AND METHODS

2.1 | Animals and semen collection

The study included 10 Colombian Creole donkeys (*Equus asinus*) located in Antioquia (Colombia). Animals were active breeding donkeys whose age ranged from 8 to 12 years (10.8 ± 3.67, mean ± SD). Likewise, their fertility had been confirmed by their live offspring, and their handling and stabling conditions were similar. Their body condition scores ranged from 7 to 8 (using a scale from 1 to 9). Samples of semen were collected using a Missouri model artificial vagina (Minitube) previously lubricated with non-spermicidal gel. Three ejaculates were collected from each donkey with a difference of 1 week, for a total of 30 ejaculates. A mare or a jenny was used to increase sexual stimulation. The gel fraction of the ejaculate was removed through filtration. The volume of each ejaculate was evaluated with a graduated cylinder. Spermatozoa concentration was assessed from a drop of 20 µl of fresh semen using a photometer (Spermacue, Minitube) and sperm motility using a phase contrast microscope Eclipse E200 (Nikon Inc.), thus obtaining an average of five observation fields (400×). Semen was separated into two fractions, the first one was extended at a ratio of 2:1 using EquiPlus® (Minitube) for semen maintenance during transport to the laboratory; the extender was preheated for this dilution to the same temperature as semen. The second fraction was centrifuged at 800 ***g*** for 15 min to obtain the SP used in the treatments described later. The sperm and SP were transported for 2 h at 15°C in an Equitainer (Hamilton Biovet). As inclusion criterion, semen samples with total motility equal to or greater than 80% were included for the study. In this sense, 20% of the samples were excluded, since they did not meet this criterion and their collection had to be repeated. This study included only those samples whose total motility was 80% or higher. The development of this research was approved by the Ethical Committee for Animal Research of the Colombian Polytechnic Jaime Isaza Cadavid (Approval number: 20610801).

2.2 | Semen freezing and thawing

For freezing process, each ejaculate was split into eight aliquots which were randomly assigned to one of eight treatments. Each aliquot was first centrifuged at 800 ***g*** for 10 min (Mikro 220R; Hettich); then, the pellet was resuspended for a total sperm concentration of 100×10^6 per ml in EquiPlus® (Minitube), supplemented with 5% of clarified egg yolk (CEY; Nouri et al., 2013) and the additives

TABLE 1 Treatments for cryopreservation of donkey semen

Treatment	Permeable cryoprotectant	Non-permeable cryoprotectants		Seminal plasma
	DMF (%)	SUC (mM)	BSA (%)	SP (%)
T1	5	–	–	–
T2	5	–	–	10
T3	–	200	1	–
T4	–	200	1	10
T5	5	200	1	–
T6	5	200	1	10
T7	5	–	1	–
T8	5	–	1	10

Abbreviations: BSA, bovine serum albumin; DMF, dimethylformamide; SP, seminal plasma; SUC, sucrose; T1, DMF; T2, DMF/SP; T3, SUC/BSA; T4, SUC/BSA/SP; T5, DMF/SUC/BSA; T6, DMF/SUC/BSA/SP; T7, DMF/BSA; T8, DMF/BSA/SP.

corresponding to each treatment (Table 1). Supplemented SP was homologous. Semen was packaged in 0.5 ml straws (MRS1 Dual V2; IMV Technologies) and was cooled at 5°C for 60 min. Then straws were placed at a distance of 4 cm above the level of liquid nitrogen and exposed to liquid nitrogen vapours for 15 min, and finally were stored in liquid nitrogen. After a week of storage, the cryopreserved straws were thawed by plunging into a water bath at 37°C for 1 min. Ten straws per treatment were frozen, of which 4 straws were randomly selected and evaluated per treatment, for a total of 32 straws per ejaculate, 96 straws per animal and 960 straws for the study.

2.3 | Semen quality assessment

Sperm motility and kinetics was assessed by the Sperm Class Analyser computer system (Microptic S.L). An Eclipse E200 phase-contrast microscope (Nikon Inc.) with a digital camera (Scout SCA780 Basler) were used. A specific configuration established for the software was: 20×20 mm glass slide, optics in negative phase, drop of 5 µl, equine species, thermal plate at 37°C and a particle size of 20–72 mm. A minimum of 500 spermatozoa were evaluated in five observation fields. The parameters analysed were total motility (TM), progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), linearity index (LIN), straightness index (STR), average path velocity/curvilinear velocity (WOB), amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF). The sperm vitality (SV) was evaluated using the Live/Dead Sperm Viability kit (Invitrogen; Gamboa et al., 2010). A drop of 50 µl of semen was mixed with 0.3 µl of SYBR-14 at a final concentration of 6 µM and incubated for 8 min at 35°C. Following this, PI (0.48 mM) was added to the mixture and incubated for 8 min at 35°C. Samples of 7 µl of semen were placed on a glass slide and covered with a cover slip. Then, 200 spermatozoa were counted using an Eclipse E200 microscope with HBO fluorescence (Nikon Inc.). Abnormal morphology (AM) was assessed using the modified eosin–nigrosin test (Brito et al., 2011). A droplet of semen and a droplet of eosin–nigrosin (Sigma–Aldrich) were placed on a microscope slide, mixed, smeared and placed on a warming plate at 37°C. Subsequently, 200 spermatozoa were assessed individually in an Eclipse E200 phase-contrast microscope (Nikon Inc.). Functional membrane integrity (FMI) was assessed using the hypo-osmotic swelling test (HOST; Neild et al., 1999). To provide a concentration of 100 mOsmol/L, 20 µl of semen was added to 200 µl of a hypo-osmotic 5.4% sucrose solution. This mixture was incubated at 38.5°C for 30 min. Then, 200 spermatozoa were evaluated using an Eclipse E200 phase-contrast microscope (Nikon Inc.). The above tests were performed on both extended and frozen–thawed semen.

Membrane stability of frozen–thawed semen was determined using the lipid fluorochrome merocyanine-540 (M-540), as reported by Thomas et al. (2006). A semen sample was extended in HANKS' solution at a concentration of 1×10^6/ml. Subsequently, merocyanine-540 was added to a final concentration of 0.68 µM and incubated at 25°C for 10 min. Then, samples were analysed by flow cytometry (LSRFortessaTM, BD Biosciences) as low, medium and high sperm membrane stability. Samples were excited using a 488 nm solid phase laser, and fluorescence was detected at 575/26 nm. Data were analysed using FlowJo software version 7.6.2 (FlowJo, LLC).

2.4 | Statistical analysis

A general linear model was fitted for each dependent variable. In each statistical model, the fixed effect of ejaculate nested within the donkey (Ej[Dn]) and the fixed effect of treatment were included. Normal distribution of variables was validated with the Kolmogorov–Smirnov´s test. In order to express the seminal quality in a consolidated criterion, a seminal quality index (SQi) was calculated, according to the following formula: $SQi = \Sigma Sp/100n$, where Sp is a sperm parameter and n is the number of parameters (Ortiz et al., 2015), being $SQi = \Sigma(TM, PM, SV, AM, FMI)/100\times5$. Values for SQi ranged between 0 and 1, where SQ = 1 represents the maximum level of sperm quality (Ortiz et al., 2015). Means were compared by the Tukey's test. Results were expressed as mean ± standard error of the mean (SEM). The significance level used for all assessments was $p < .05$. All analyses were conducted using SAS Version 9.2. Software (SAS Inst. Inc.).

3 | RESULTS

Volume, sperm concentration and motility of raw donkey semen were 51.20 ± 5.68 ml, 310.70 ± 21.90 million of cells/ml and 85.16 ± 1.40%, respectively. Table 2 shows the quality parameters of extended donkey semen. With respect to frozen–thawed samples, it was found that cryopreservation of donkey semen without permeable cryoprotectants (T3 and T4) caused a decreased in sperm motility compared to treatments with DMF, as permeable cryoprotectant ($p < .05$;

TABLE 2 Semen parameters of extended donkey semen

Variable	Mean	CV	SD	SEM	Min	Max
TM (%)	86.51	11.32	9.79	1.79	66.20	98.27
PM (%)	57.62	28.26	16.28	2.97	33.12	83.64
VCL (μm/s)	97.54	21.15	20.63	3.77	66.46	131.90
VSL (μm/s)	66.58	28.45	18.94	3.46	39.50	106.51
VAP (μm/s)	42.11	25.27	10.64	1.94	27.56	69.78
STR (%)	60.17	7.17	4.31	0.79	52.74	68.46
LIN (%)	40.66	13.91	5.66	1.03	31.07	53.64
WOB (%)	64.77	12.45	8.07	1.47	51.98	78.31
ALH (μm)	3.55	20.16	0.72	0.13	2.56	5.40
BCF (Hz)	8.12	26.42	2.15	0.39	4.35	12.85
SV (%)	81.33	10.30	8.37	1.53	65.00	93.00
AM (%)	25.50	35.92	9.16	1.67	11.00	50.00
FMI (%)	74.50	22.40	13.03	1.67	50.00	89.00

Abbreviations: ALH, mean lateral head displacement; AM, abnormal morphology; BCF, frequency of head displacement; CV, coefficient of variation; FMI, functional membrane integrity; LIN, linearity index; Max, maximum value; Min, minimum value; PM, progressive motility; SD, standard deviation; SEM, standard error of the mean; STR, straightness index; SV, sperm vitality; TM, total motility; VAP, average pathway velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, average path velocity/curvilinear velocity.

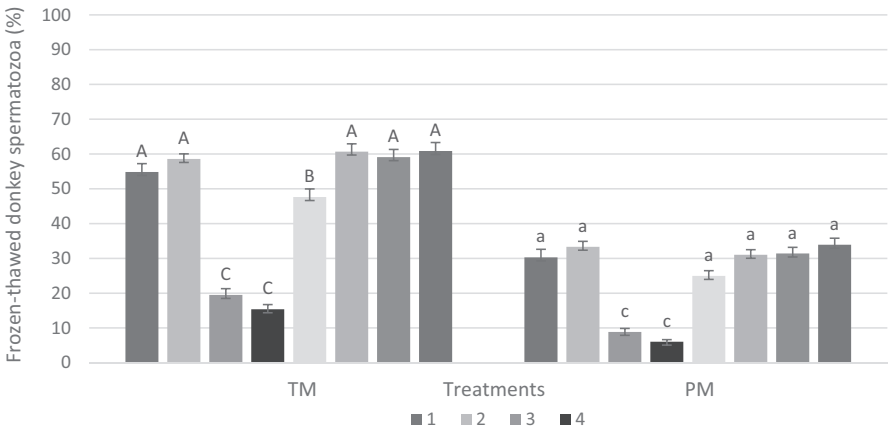

FIGURE 1 Total (TM) and progressive (PM) motility of post-thaw donkey spermatozoa according to treatment (T1: DMF, T2: DMF/SP, T3: SUC/BSA, T4: SUC/BSA/SP, T5: DMF/SUC/BSA, T6: DMF/SUC/BSA/SP, T7: DMF/BSA and T8: DMF/BSA/SP). BSA, bovine serum albumin; DMF, dimethylformamide; SUC, sucrose; SP, seminal plasma. Values are expressed as mean ± standard error of the mean (SEM) ($n = 10$, $r = 3$). ^{A,B,C}Different capital letters indicate significant differences for TM between treatments ($p < .05$). ^{a,b,c}Different lowercase letters indicate significant differences for PM between treatments ($p < .05$).

Figure 1). Additionally, a decrease in TM and PM was observed for T5 (DMF/SUC/BSA) compared to the other treatments that included DMF ($p < .05$). In addition, a significant decreased in sperm viability and FMI was observed in treatments T3 and T4 (without permeable cryoprotectant) compared to other samples ($p < .05$; Figure 2).

In Table 3 some differences between treatments are evident in sperm kinetics; VSL, VCL and VAP showed lower values in treatments without permeable cryoprotectants (T3 and T4) ($p < .05$). No differences for STR and LIN ($p \geq .05$) were observed. Similarly, no differences were found between treatments for the membrane stability population (Figure 3), which corresponds to sperm stained with Merocyanine-540 and bright fluorescence (M540HIGH) ($p \geq .05$). Yet, the percentage of low membrane stability (M540LOW) was lower for T1 compared to T3 ($p < .05$; Figure 3). Finally, for the seminal quality index, T6 and T8 achieved higher SQi values compared to T1 ($p < .05$), but they were not different compared to T2 and T7 ($p > .05$), while treatments without permeable cryoprotectant (T3 and T4) had the lowest results for this parameter that integrates seminal quality ($p < .05$; Figure 4).

4 | DISCUSSION

To date, there is not a standardized procedure for freezing semen from donkeys, contributing in this way to the high variation observed

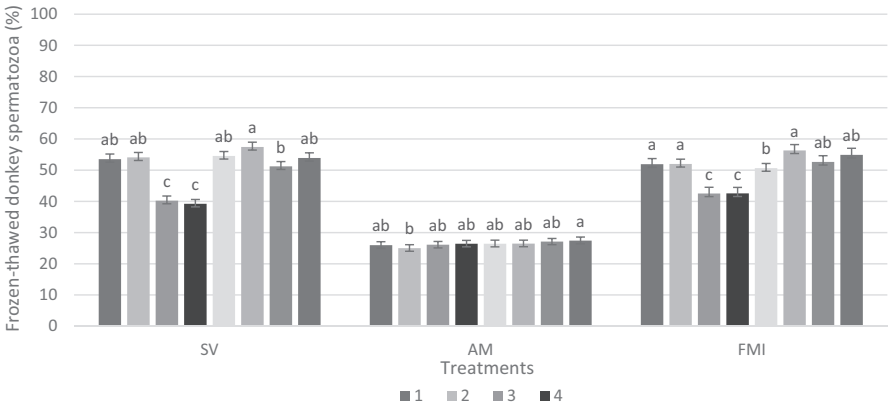

FIGURE 2 Evaluation of sperm viability (SV), functional membrane integrity (FMI) and abnormal morphology (AM) of post-thaw donkeys' semen according to treatment (T1: DMF, T2: DMF/SP, T3: SUC/BSA, T4: SUC/BSA/SP, T5: DMF/SUC/BSA, T6: DMF/SUC/BSA/SP, T7: DMF/BSA and T8: DMF/BSA/SP). BSA, bovine serum albumin; DMF, dimethylformamide; SUC, sucrose; SP, seminal plasma. Values are expressed as mean ± standard error of the mean (SEM) ($n = 10$, $r = 3$). ^{a,b,c}Different letters indicate significant differences between treatment for each sperm parameter ($p < .05$).

both in experimental and field result (Ferrante et al., 2018). The present study compared alternatives for donkey semen freezing with permeable, non-permeable cryoprotectants, a protein source such as bovine serum albumin and homologous SP. We found that post-thaw semen quality when using DMF, regardless of whether or not it was with other components, was superior in semen quality parameters. Similarly, in previous research with the use of DMF and SP for donkey semen freezing, TM greater than 60%, PM near 40% and FMI above 50%, were found (Montoya et al., 2017). Likewise, Acha et al. (2016) reported that using DMF as a cryoprotectant in sperm from Andalusian donkeys had a positive effect on TM, PM, and FMI with values of 66.21 ± 2.60%, 57.09 ± 2.99% and 64.95 ± 3.77%, respectively. For canine semen freezing, it has been shown that 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 (Rota et al., 2006). Dimethylformamide present greater membrane permeability and cause less osmotic stress in equine spermatozoa, mainly due to their lower molecular weight and viscosity, than other permeable cryoprotectants (Ball & Vo, 2001; Ortega-Ferrusola et al., 2009); besides, it is not a toxic substance for the stallion sperm during the freezing process (Alvarenga et al., 2005).

In the present study we found that treatments with DMF, SUC, BSA and/or SP had the highest protective effect for functional and structural integrity of the plasmatic membrane of post-thaw donkey spermatozoa. Previous studies in bulls have reported that BSA could decrease the lipid peroxidation in the plasma membrane caused by oxygen reactive species and protect the plasma membrane efficiently (Fu et al., 2017). Plasma membrane is important to maintain sperm metabolism, it is highly correlated with sperm motility and survival index in swine spermatozoa (Zhang et al., 2015). In buffaloes, BSA has shown a protective effect related to specific interaction with plasma membrane phospholipids, which decrease the degree of sperm injury during semen storage; additionally, this may be attributed to extraction of cholesterol from the sperm membrane which leads to high fluidity of sperm membranes before freezing and enhancing the cryosurvival of spermatozoa (El-Kon, 2011). Furthermore, SUC is not able to diffuse across the plasma membrane, creating an osmotic pressure that induces cell dehydration and a lower incidence of intracellular ice formation of domestic animal sperm cells (Barbas & Mascarenhas, 2008).

During capacitation, various signalling pathways at the sperm membrane and cytoplasm must be induced in the spermatozoa, leading to physiological and biochemical modifications (Maitan et al., 2021). Only capacitated spermatozoa have the ability to fertilize the mature oocyte (Gervasi & Visconti, 2016). Capacitation is induced generally by Ca^{2+} and a cholesterol acceptor such as albumin (Leemans et al., 2019). In the present study, treatments with BSA did not denote differences in the values of sperm capacitation in thawed semen of donkeys. Sperm cryocapacitation represents an altered state of sperm functionality triggered by cooling, freezing and thawing stress, so-called due to similarities to the capacitation process, which happens precociously before the insemination, impeding fertilization (Córdova et al., 2012). Kavak et al. (2003) evaluated the capacitation state of frozen–thawed equine spermatozoa with Merocyanine 540 staining and found a percentage of 36.8 ± 9.1% of the non-capacitated spermatozoa, being comparable to the result found in this research with donkey semen.

Frozen semen leads to greater post-artificial insemination fluid accumulation than insemination with fresh semen or natural service (Vidament et al., 2009). It has been suggested that permeable cryoprotectants agents (CPAs) generate an important inflammatory reaction in the reproductive tract of jennies, causing low fertility rates (Diaz-Jimenez et al., 2018; Serres et al., 2014). Besides donkey sperm seems to be sensitive to glycerol and its toxic effect has been shown to start as early as the pre-freezing process begins (Rota et al., 2012; Serres et al., 2014; Vidament et al., 2009). For this reason, non-permeable agents could be an alternative for donkey

TABLE 3 Evaluation of donkey post-thaw sperm kinetics using different freezing alternatives (T1: DMF, T2: DMF/SP, T3: SUC/BSA, T4: SUC/BSA/SP, T5: DMF/SUC/BSA, T6: DMF/SUC/BSA/SP, T7: DMF/BSA and T8: DMF/BSA/SP)

Variable	Treatments							
	T1	T2	T3	T4	T5	T6	T7	T8
VCL (μm/s)	71.24 ±1.57^{ab}	73.68 ±1.60^{a}	52.51 ±1.55^{e}	50.65 ±1.24^{e}	65.31 ±1.51^{d}	65.86 ±1.46^{cd}	68.29 ±1.82^{c}	70.39 ±1.81^{abc}
VSL (μm/s)	47.12 ±1.38^{abc}	51.13 ±1.38^{a}	35.22 ±1.39^{e}	32.64 ±1.09^{e}	42.44 ±1.20^{d}	44.15 ±1.18^{cd}	46.21 ±1.51^{bcd}	49.33 ±1.51^{ab}
VAP (μm/s)	37.71 ±1.24^{abc}	40.84 ±1.30^{a}	29.06 ±1.40^{d}	26.49 ±1.13^{d}	34.14 ±1.14^{c}	34.66 ±1.12^{c}	36.59 ±1.33^{bc}	38.89 ±1.34^{ab}
STR (%)	69.40 ±0.87^{a}	70.21 ±0.85^{a}	70.85 ±1.24^{a}	70.20 ±1.08^{a}	71.10 ±0.78^{a}	69.27 ±0.84^{a}	68.19 ±0.94^{a}	69.40 ±0.84^{a}
LIN (%)	45.88 ±1.08^{a}	48.80 ±1.06^{a}	48.16 ±1.45^{a}	46.17 ±1.22^{a}	47.60 ±0.92^{a}	47.30 ±0.96^{a}	46.36 ±1.11^{a}	48.73 ±1.05^{a}
WOB (%)	61.67 ±0.22^{c}	65.14 ±0.80^{ab}	63.39 ±1.08^{abc}	61.43 ±0.92^{c}	63.09 ±0.70^{abc}	64.08 ±0.70^{abc}	62.65 ±0.77^{bc}	65.68 ±0.78^{b}
ALH (μm)	2.64 ±0.04^{ab}	2.74 ±0.05^{ab}	2.22 ±0.05^{b}	2.22 ±0.04^{b}	2.62 ±0.05^{ab}	2.63 ±0.05^{ab}	4.42 ±1.28^{a}	2.65 ±0.06^{ab}
BCF (Hz)	8.80 ±0.86^{a}	8.53 ±0.20^{ab}	7.14 ±0.25^{a}	7.14 ±0.25^{a}	7.97 ±0.19^{b}	7.61 ±0.17^{bcd}	9.05 ±0.96^{a}	8.05 ±0.17^{ab}

Abbreviations: ALH, mean lateral head displacement; BCF, frequency of head displacement; BSA, albumin bovine serum; DMF, dimethylformamide; LIN, linearity index; SP, seminal plasma; STR, straightness index; SUC, sucrose; VAP, average pathway velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, average path velocity/curvilinear velocity.

^{a,b,c,d,e}Different letters indicate significant differences between treatments for each sperm parameter ($p < .05$).

sperm cryopreservation (Diaz-Jimenez et al., 2018). Diaz-Jimenez et al. (2018) found that the extender with sucrose 0.25 M combined with BSA (1%) improved the TM of post-thaw donkey semen, with values (mean ± SD) of 31.4 ± 12.9% compared to the control of 22.9 ± 9.8%, being considered as an alternative to conventional extenders with glycerol for donkey sperm cryopreservation. Yet, our results showed that treatments that only had non-permeable cryoprotectants such as SUC and BSA, had a lower sperm quality index and a decrease in most of the parameters evaluated. Similarly, Diaz-Jimenez et al. (2019) found that sperm freezing in absence of permeable cryoprotectants may not be still an option for cryopreservation of subfertile donkey sperm. In the same way, De Oliveira, Budik, et al. (2017) reported that the complete removal of glycerol and replacement by a combination of urea, proline and betaine in freezing extender for stallions, resulted in lower post-thaw semen quality than Gent control, a commercial extender (Minitube, glycerol concentration 5%).Yet, they demonstrate that equine semen can be successfully frozen after reduction of glycerol content to a final concentration of 1.25% when compensated by addition of two other alternative cryoprotectants namely glucose and urea (De Oliveira, Teixeira, et al., 2017).

Seminal plasma removal is another divergent issue about successful sperm cryopreservation. One since of the main negative impacts on SP is the formation of reactive oxygen species (ROS; Monteiro et al., 2022). Quantities bigger than 10% SP in semen samples, in horses, cause negative effects on spermatozoa submitted cryopreservation and ROS participation is attributed in these cases (Len et al., 2020). But then, SP contains a complex mix of ions, enzymes, sugars, lipids, amino acids, proteins, and hormones (Neuhauser et al., 2019; Wood et al., 2016) that have an important role in physiological processes for sperm functions such as protection, nutrition, transport, regulation of the inflammatory response in the uterus and interaction with the oocyte (Portus et al., 2005; Töpfer-Petersen et al., 2005). Rota et al. (2012) found that the re-extension in homologous SP of thawed donkey semen before artificial insemination showed an improvement of fertility, since appears to have a modulation on the endometrial response (Miró et al., 2013). Also, SP has a role in facilitating transport of spermatozoa and in protecting them in the genital tract and thereby extending their survival time (Katila, 2001). Our results showed that supplementation with 10% of SP did not decrease the quality of donkey thawed semen, except for the treatment that did not include CPA. Even two of the treatments that included SP and BSA (T6 and T8) showed the highest seminal quality index. The calculation of seminal indexes allowed integrating the results of quality of the spermatozoa. Previously, the importance of using indexes to express seminal quality and even freezability has been highlighted (Yeste et al., 2015). In this way, since sperm motility is one of the key parameters to evaluate the quality of frozen semen, hypo-osmotic test and supra vital staining can be considered good predictive indicators in raw semen for donkey semen freezability (De Oliveira, Teixeira, et al., 2017). It has even been reported that hypo-osmotic test has the same accuracy as fluorescence probes regarding the

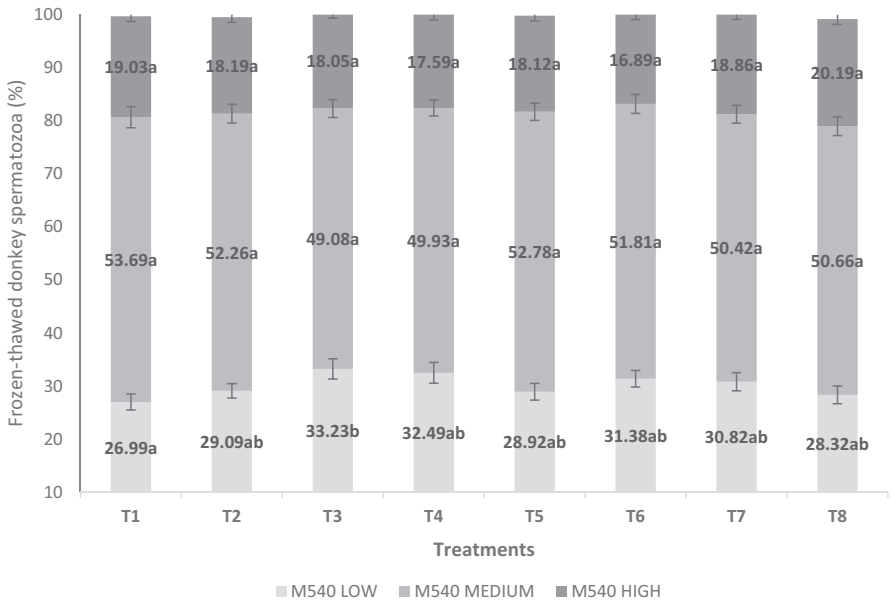

FIGURE 3 Sperm membrane stability analysis evaluated with Merocyanine 540 (M540) in post-thaw donkey semen according to treatment (T1: DMF, T2: DMF/SP, T3: SUC/BSA, T4: SUC/BSA/SP, T5: DMF/SUC/BSA, T6: DMF/SUC/BSA/SP, T7: DMF/BSA and T8: DMF/BSA/SP). BSA, bovine serum albumin; DMF, dimethylformamide; M540HIGH, % HIGH membrane stability; M540LOW, % LOW membrane stability; M540MEDIUM, % MEDIUM membrane stability; SP, seminal plasma; SUC, sucrose. Values are expressed as mean ± standard error of the mean (SEM) ($n = 10$, $r = 3$). [a,b]Different letters indicate significant differences between treatments in each sperm parameter ($p < .05$).

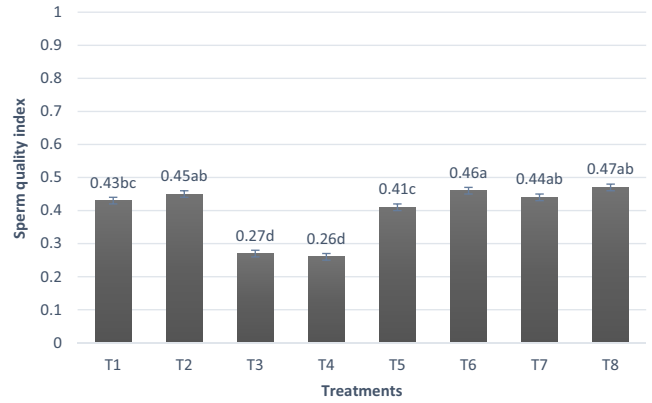

FIGURE 4 Sperm quality index in post-thaw donkey semen according to treatment (T1: DMF, T2: DMF/SP, T3: SUC/BSA, T4: SUC/BSA/SP, T5: DMF/SUC/BSA, T6: DMF/SUC/BSA/SP, T7: DMF/BSA and T8: DMF/BSA/SP). BSA, bovine serum albumin; DMF, dimethylformamide; SUC, sucrose; SP, seminal plasma. Values are expressed as mean ± standard error of the mean (SEM) ($n = 10$, $r = 3$). [a,b,c,d]Different letters indicate significant differences between treatments ($p < .05$).

plasma membrane integrity in donkey sperm (De Oliveira, Teixeira, et al., 2017) and according to Brito et al. (2003) hypo-osmotic test could be considered to be a more accurate plasma membrane test to predict the fertilizing capacity of a semen sample. For these reasons, the simultaneous use of multiple parameters, including other aspects of sperm integrity and functionality, could be considered as a more objective method (Restrepo et al., 2022). Ortiz et al. (2015) reported a seminal quality index of 0.65 ± 0.3 in thawed Andalusian donkey semen, being a higher value than those found in the present research for all treatments. This could be due to the fact that post-thaw semen quality and sperm freezability seem to vary consistently among donkeys (Ortiz et al., 2015) and aspects such as breed, age, seasonality and frequency of collections, affect this variability (Carluccio et al., 2013; Ebel et al., 2021). In addition, the freezing protocol used by Ortiz et al. (2015), as well as the sperm quality parameters used to calculate the seminal quality index, were different from the present study, which could generate different results. T6 and T8 achieved higher SQi values compared to T1, but they were not different compared to T2 and T7. This can be explained by the interaction in extenders that contain a protein source such as BSA and sucrose adequately preserved important sperm physiological attributes, including viability, motility, progressive motility, morphology, acrosome, sperm tail membrane integrity, and DNA integrity (Pipan et al., 2020).

5 | CONCLUSIONS

The use of permeable cryoprotectant is essential to achieve higher post-thaw quality of donkey semen, non-permeable cryoprotectants for freezing donkey semen, results in a lower post-thaw semen quality. In addition, the combined use of bovine serum albumin, sucrose and homologous SP may provide additional sperm protection compared to the individual use of DMF.

It is proposed to carry out studies where the inflammatory reaction of the reproductive tract of jennies can be evaluated, as well as studies that allow to confirm the fertilizing capacity of donkey sperm under different freezing alternatives. Additionally, to study the relationship of the components of SP with the fertilizing capacity of sperm in donkeys, will be promising to create germplasm banks and increase pregnancy rates in jennies.

AUTHOR CONTRIBUTIONS

JD Montoya contributed to all sections. G Restrepo and A Usuga contributed to the study design, preparation and final approval. All the authors were involved in revision and approval of the final version of the manuscript.

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CONFLICT OF INTEREST

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

DATA AVAILABILITY STATEMENT

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

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