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Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses

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The aim of this study was to determine the effect of single layer centrifugation (SLC) using Androcoll-E-Large on donkey sperm quality parameters after 24 h of cool-storage. Ejaculates were collected from Andalusian donkeys and then cooled at 5°C. SLC was carried out after 24 h of cool-storage using Androcoll-E-Large. In the first experiment, all sperm parameters assessed (total and progressive sperm motility, viability, sperm morphology and sperm kinematics VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) were statistically compared between semen samples processed or not with Androcoll-E-Large. Significant differences ($P < 0.05$*) were found between SLC-selected and unselected semen samples for all parameters assessed, obtaining better results after SLC. In the second experiment, semen samples were classified in two groups according to their sperm progressive motility (PM) before SLC. Then, the increments obtained in semen quality parameters after SLC were compared between groups. No significant differences were found between groups, indicating that SLC improved the sperm quality parameters of entire set of semen samples processed with independence to their original PM. In conclusion, SLC with Androcoll-E-Large can be used in donkeys, increasing the sperm quality of cooled-stored donkey semen doses after 24 h of cool storage.*

Keywords: single layer centrifugation, donkey semen, sperm cooling, Androcoll-E-Large

Implications

Andalusian donkey has lost its traditional role, which has resulted in the inclusion of this breed in the UN Food and Agricultural Organization endangered species list. Nowadays, the importance of donkey is increasing again and it is essential that the jackasses with desirable genetic features have as good sperm quality as possible. Sperm selection by single layer centrifugation (SLC) through silica colloids has shown to improve the sperm quality in other animal species. Recently, Androcoll-E-Large has been developed as a colloid suitable for processing large volumes of semen; however, there are no studies that substantiate the potentially beneficial effects of SLC on donkey semen doses.

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Introduction

The donkey's relationship with human populations is well-documented (Rossel *et al*., 2008). The earliest domesticated donkey bones identified archeologically date to 4600 to 4000 BC. Thenceforth, donkey has been used as a valuable pack animal. Nonetheless, in the last century, in industrialized countries donkeys are losing their traditional role and the number of animals is decreasing rapidly. For example, the number of animals from the Spanish donkey breeds (Andalusian, Balear, Catalonian, Encartaciones, Majorera and Zamorano-Leonés) has decreased dramatically during recent years. As a result, all of them have been included in the UN Food and Agricultural Organization (FAO) list of domestic animals to be conserved (FAO, DAD-IS http://dad.fao.org/). In 2011, the Andalusian donkey population was 760 individuals with only 101 breeding males. Nowadays, the role of these preserved animals is changing. In this sense, donkeys are used in the production of hypoallergenic milk, as

pet therapy for human beings to treat several diseases such Alzheimer and as draught animals in those areas in which the use of machines is banned. All this has increased the interest in donkey reproduction.

Artificial insemination (AI) with cooled-stored semen doses is considered to be one of the most important assisted reproductive technique to increase the number of individuals of many species, in order to improve gene distribution and reduce inbreeding. Suboptimal pregnancy rates (45%) after AI using cooled donkey sperm was obtained in a previous study (Vidament *et al.*, 2009). It may result from breeding with low quality or not adequately processed for shipment semen. The quality of sperm samples is crucial when cooled-stored semen is used for AI. It is documented that the semen cooling process causes several changes in mammal spermatozoa known as a whole as 'cold shock' (Watson, 2000). It is particularly focused on changes in sperm membranes (Peña *et al.*, 2011); however, this negative effect alters cellular metabolism and organelles, decreases motility and induces irregularities in the sperm motility pattern (Sieme *et al.*, 2008). As a consequence, sperm quality decreases during the cooling process and so do pregnancy rates (Varner *et al.*, 1989).

In order to increase the sperm quality of semen samples, a number of sperm selection techniques have been developed (Morrell, 2012). Recently, single layer centrifugation (SLC) using Androcoll^{TM} (SLU, Uppsala, Sweden) has been successfully used for sperm selection in different animal species (Morrell *et al.*, 2009e; Thys *et al.*, 2009; Chatdarong *et al.*, 2010). In this SLC technique, spermatozoa are centrifuged through a column (single layer) of silane-coated silica colloid in a species-specific formulation. The formulation for stallions is Androcoll-E (Johannisson *et al.*, 2009) and has been recently commercialized by Minitüb GmbH (Tiefenbach, Germany). Androcoll-E^{TM} has been used to select robust spermatozoa in terms of motile and morphologically normal sperm, with intact membranes and good chromatin integrity (Morrell *et al.*, 2009c). On the basis of all this sperm parameters to perform the sperm quality analysis, Androcoll-E has improved the quality of fresh (Morrell *et al.*, 2009a), frozen (Macias Garcia *et al.*, 2009a; Macias Garcia *et al.*, 2009b; Hoogewijs *et al.*, 2011) and cooled-stored (Morrell *et al.*, 2009d; Bergqvist *et al.*, 2011) stallion sperm samples with a shorter preparation time and less complicated process than the conventional density gradient centrifugation (Morrell *et al.*, 2009b). However, a specific formulation for donkey semen has not been developed yet. Moreover, this procedure was developed to process small volumes of semen, which could be useful for example, to increase the sperm quality of frozen-thawed stallion semen samples. However, this technique is unsuitable to prepare cool semen doses for equine AI, where large volumes of semen are required. In order to solve this problem, recently a new presentation of Androcoll-E has been developed for large volumes of stallion semen: Androcoll-E-Large. Using this new formulation, up to 15 to 18 ml of semen can be processed easily and quickly. Sperm quality parameters, such as sperm motility, morphology and chromatin integrity are also improved in SLC-selected stallion semen samples using Androcoll-E-Large (Morrell *et al.*, 2011a). However, to our knowledge, only preliminary results of the use of SLC to improve sperm motility in cooled donkey semen samples have been published by our research group (Ortiz *et al.*, 2012). Despite common belief, the transfer of knowledge and procedures from horses to donkeys often achieves poor results (Contri *et al.*, 2010b); so additional studies should be performed to evaluate if SLC previously used with great success in stallion semen (Morrell *et al.*, 2011b) is suitable for donkey semen samples as well.

Thus, the aim of this study was to determine if the sperm quality parameters of cooled-stored donkey semen samples can be improved after SLC using Androcoll-E-Large.

Material and methods

Animals

Four healthy, mature, Andalusian donkeys, aged from 6 to 15, were used as semen donors. One of the jackasses was owned by 'Donkey's House Foundation' (Rute, Córdoba, Spain) and was housed in individual paddocks placed at the Veterinary Teaching Hospital (VTH) of the University of Córdoba (Spain). The feeding consisted of alfalfa hay and water *ad libitum*. The other three donkeys were housed at the Equine Center for Assisted Reproduction Services (CENSYRA) in Badajoz (Spain), where they usually live, and they were fed with a mixture of grasses hay.

Semen collection

Semen was collected using a Missouri artificial vagina with an in-line gel filter (Minitüb) in the presence of a jenny in natural or induced estrus to stimulate copulatory activity. Three to four ejaculates per animal were collected twice a week obtaining a total number of 13 ejaculates. Total and progressive sperm motility was evaluated from fresh semen by CASA. Gel free volume (ml) was measured in a collector. Sperm concentration ($\times 10^6$ spermatozoa/ml) was assessed with a sperm photometer (Spermacue®; Minitüb).

Semen processing

Immediately after collection, an aliquot of raw semen was extended with INRA96 (IMV, l'Aigle, France) at 37°C until a final concentration of 100×10^6 sperm/ml . Extended semen was maintained at room temperature (~22°C) for 15 min in a 50 ml corning tube. Semen samples were slowly cooled (0.3°C/min) for 2 h in an equitainer at 5°C. After that, 20 ml of each semen sample were loaded in syringes previously cooled at 5°C in a fridge. Syringes were then placed in a Styrofoam box at 5°C (Minitüb) previously loaded with two cold packs. Cooled-stored semen doses were shipped to the Animal Reproduction Laboratory if the ejaculates were collected at CENSYRA or cooled and stored following the same methodology if semen was collected at the VTH. All semen samples were evaluated after 24 h of cool storage at 5°C in a shipping box for the following sperm quality parameters.

Computer-assisted sperm motility analysis

Sperm motility was objectively evaluated using the Sperm Class Analyzer (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain). This system consists of an optical phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan), a warm plate at 37°C (OK 51-512, Osaka, Digifred SL, Barcelona, Spain) and a high-speed digital camera (A312fc, Basler^{TM} AG, Ahrensburg, Germany), which captures a total number of 25 consecutive digitalized frames in 1 s per captured field (image-capture rate, one photograph every 40 ms and a PC (Intel Inside®, Pentium 4®, Intel Labs, Barcelona, Spain) to analyze and save data. CASA settings were as follows: cell size from 15 to 75 μm^2; connectivity 12; progressive spermatozoa >75% of the straightness coefficient (STR). An aliquot of each semen sample was extended with INRA96 (IMV Technologies, L'Aigle, France) to a final concentration of 25×10^6 sperm/ml and then incubated at 38°C for 10 min. After that, 5 μl of each diluted semen sample were placed in a Mackler counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel). Three drops, with two randomly microscopic fields per drop, were analyzed in each semen sample. The trajectory of each individual sperm was determined by the SCA software obtaining CASA sperm kinematic parameters: total motility (TM), progressive motility (PM), sperm curvilinear velocity (VCL, μm/s), sperm linear velocity (VSL, μm/s), average path velocity (VAP, μm/s), linear coefficient (LIN, VSL/VCL × 100), straightness coefficient (STR, VSL/VAP × 100), wobble coefficient (WOB, VAP/VCL × 100), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) were assessed.

Sperm morphology

Sperm morphology was performed by visual examination on slides stained with Diff-Quick® (Baxter DADE AG 3186, Düdingen, Switzerland) as described previously (Hidalgo *et al*., 2006). At least 200 sperm were evaluated from each semen sample and the percentage of normal and abnormal forms was recorded.

Sperm viability

Sperm viability was assessed using a supravital stain (Cortes-Gutierrez *et al*., 2008) based on the red/green emission of two fluorescent dyes: acridine orange and propidium iodide, respectively (Duo-Vital Kit; Halotech DNA SL, Madrid, Spain). At least 200 sperm were counted, considering green spermatozoa as 'live sperm', and red or red-green as 'dead sperm'.

SLC with Androcoll-E-Large

Before SLC, both cooled-stored semen samples and colloid were allowed to equilibrate at room temperature (about 22°C) for 30 min to avoid temperature fluctuations. Fifteen milliliters of cooled semen were carefully layered on top of 15 ml Androcoll-E-Large located in 50 ml corning tubes, taking care not to disrupt the interface. The suspension was centrifuged at 300 × **g** for 20 min without brake (Eppendorf, 5702 RH; Eppendorf AG, Hamburg, Germany). The supernatant (semen extender, seminal plasma and colloid) was removed and the sperm pellet recovered and transferred to a clean tube containing INRA96. According to the protocol described by Morrell *et al.* (2011a). Concentration of the sperm pellets was measured using the SCA system. After that, semen samples were adjusted to a final concentration of 25 million sperm/ml and then sperm motility, morphology and viability were analyzed as described above. The yield of selected spermatozoa was calculated according to the following formula:

$$\text{Yield} = (\text{number of spermatozoa in sperm pellet} / \text{number of spermatozoa in initial load}) \times 100$$

Experimental design

Experiment 1. Effect of sperm selection using Androcoll-E-Large in cooled-stored donkey semen doses for 24 h at 5°C. Two aliquots of each semen sample were taken. One of them was immediately evaluated for sperm quality parameters following the methodology described above. The other one was subjected to SLC with Androcoll-E-Large as described previously and then evaluated. The results of the sperm quality parameters assessed in uncentrifuged semen samples (unselected) were compared with those obtained after SLC centrifugation with Androcoll-E-Large (SLC-selected).

Experiment 2. Relationship between PM of uncentrifuged samples and improvement of sperm parameters in SLC-selected samples. Semen samples were divided into two groups according to the original sperm PM of unselected samples (Group 1: PM ⩽ 43.7%; Group 2: PM ⩾ 43.8%). Increment obtained in each semen quality parameter after SLC-selection was compared between groups.

Statistical analysis

Data analyses were performed using SPSS v20.0 for Mac OS X (IBM, SPSS Statistics, Armonk, NY, USA) and SAS v9.0 for Windows (SAS Institute Inc, Cary, NC, USA). Analysis of the data was carried out using a general linear model (GLM), with animals, treatments and ejaculates as fixed effects. Differences between treatments in each animal were also assessed using GLM, with the fixed effects being ejaculates and treatment. A two-step cluster procedure was performed to classify the cooled semen samples according to their initial progressive sperm motility (before SLC-treatment). Comparisons between groups were performed using a one-way ANOVA. Normality of the data distributions and variance homogeneity were checked by the Kolmogorov–Smirnov and Cochran tests, respectively. Values were expressed as mean and root mean square error (RMSE). Statistical significance was set at $P < 0.05$.

Results

Sperm parameters from all the ejaculates assessed in fresh semen aliquots were between the ranges considered as physiologic when evaluating donkey sperm (Table 1). The sperm yield (%) of total sperm obtained after SLC with Androcoll-E-Large was 25.4.

Table 1 *Sperm parameters immediately after collection and yield when SLC was carried out after storage of extended semen at 5°C for 24 h from all animals*

	Donkey 1 ($n=3$)		Donkey 2 ($n=3$)		Donkey 3 ($n=3$)		Donkey 4 ($n=4$)		Mean values ($n=13$)	
Fresh semen parameters	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Gel-free volume (ml)	55.9	4.9	85.0	41.4	44.0	2.1	44.0	2.1	58.7	24.0
Sperm concentration ($\times 10^6$ per ml)	288.7	43.3	409.7	203.2	405.3	22.9	405.3	22.9	353.2	101.9
Total sperm count ($\times 10^9$)	15.9	1.3	29.9	6.9	17.8	0.9	17.8	0.9	19.8	6.7
TM (%)	95.3	1.5	90.3	6.1	94.3	3.1	94.3	3.1	94.6	4.0
PM (%)	73.0	3.0	70.7	4.7	67.3	4.9	67.3	4.9	75.9	9.6
Yield after SLC (%)	26.5	2.7	30.7	6.1	24.4	3.5	24.4	3.5	25.4	5.0

n = number of ejaculates; TM = total motility; PM = progressive motility; SLC = single layer centrifugation.
Values are expressed as mean and s.d. (standard deviation).

Table 2 *Parameters of semen quality for uncentrifuged (n = 13) and SLC-selected (n = 13) donkey sperm samples stored at 5°C for 24 h*

	Treatments			
Parameters	Uncentrifuged	SLC-selected	RMSE	Statistics (*P*-value)
TM (%)	69.0	79.9	9.0	<0.001
PM (%)	46.6	63.1	13.4	<0.01
Live (%)	66.7	71.1	5.8	<0.01
Normal (%)	79.6	87.0	6.9	<0.05

SLC = single layer centrifugation; RMSE = root mean square error; TM = total motility; PM = progressive motility; Live = live sperm; Normal = normal forms.
Values are expressed as mean and RMSE.

Comparison of sperm quality parameters between uncentrifuged and SLC-selected samples after 24 h of cool storage

All sperm parameters assessed (motility, viability and morphology) were higher in the SLC-selected samples compared with uncentrifuged controls (Table 2).

Mean total sperm motility was significantly higher ($P < 0.001$) in SLC-selected samples in comparison to unselected samples (79.9% *v.* 69.0%), which means an increment of 10.9%. Moreover, sperm PM was also significantly ($P < 0.01$) higher in SLC-selected samples (63.1% *v.* 46.6%). This parameter increased 16.5% when compared with uncentrifuged samples (Table 2).

The statistical analysis performed to assess viability showed significantly increased values ($P < 0.01$) in the live sperm percentage from SLC-selected aliquots (71.1% *v.* 66.7%). In this case SLC-selected samples had an increment of 4.4% in viability values (Table 2).

Normal sperm morphology in SLC-selected samples was also significantly higher ($P < 0.05$) than uncentrifuged semen (87.0% *v.* 79.6%), increasing by 7.4 the percentage of normal forms (Table 2).

Most of the sperm kinematics parameters assessed (VCL, VSL, VAP, LIN, STR, WOB and BCF) was significantly improved ($P < 0.001$) in the SLC samples compared with the uncentrifuged controls (Table 3).

Relationship between PM of uncentrifuged samples and improvement of sperm parameters in SLC-selected samples

Significant differences ($P < 0.001$) were found between the progressive sperm motility of the two groups obtained (Table 4); however, no significant differences in the improvement of sperm quality parameters after SLC were seen between groups (Table 5).

Discussion

The objective of the present study was to determine if sperm quality parameters from cooled donkey semen doses stored for up to 24 h could be improved after SLC using Androcoll-E-Large, which has been used successfully in previous studies to process large volumes of stallion semen (Morrell *et al.*, 2011a).

According to the results obtained in this study, SLC using Androcoll-E-Large significantly improved total and PM, vitality and normal sperm morphology in donkey sperm doses after 24 h of cool-storage. Kinematic sperm parameters were also improved.

These results are in agreement with previous studies which supported SLC using Androcoll-E-Large is an effective method to select motile sperm in stallions (Morrell *et al.*, 2008, 2009d; Johannisson *et al.*, 2009). Preliminary results of the use of SLC in donkey semen samples have been published by our research group (Ortiz *et al.*, 2012); however, to our knowledge, this is the first full research article in which the effect of colloid centrifugation is tested in cooled-stored donkey sperm doses.

Total and PM are traditionally considered as essential indicators to evaluate the quality of a sperm semen sample (Love, 2011). In this study, both parameters were lower in the unselected samples than those obtained in previous

Table 3 *Kinematic parameters for uncentrifuged (n = 13) and SLC-selected (n = 13) donkey sperm samples when SLC was carried out after storage of extended semen at 5°C for 24 h*

	Treatments			
Parameters	Uncentrifuged	SLC-selected	RMSE	Statistics (*P*-value)
VCL (μm/s)	108.1	113.4	58.3	<0.001
VSL (μm/s)	73.7	86.1	55.1	<0.001
VAP (μm/s)	92.7	99.5	56.1	<0.001
LIN (%)	58.5	65.7	28.7	<0.001
STR (%)	72.4	77.5	26.3	<0.001
WOB (%)	76.5	80.3	19.0	<0.001
ALH (μm)	2.7	2.6	1.2	<0.001
BCF (Hz)	7.7	8.6	4.4	<0.001

SLC = single layer centrifugation; RMSE = root mean square error; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = mean lateral head displacement; BCF = frequency of head displacement.
Values are expressed as mean and RMSE.

Table 4 *Groups obtained according to the original sperm progressive motility of cooled-stored semen samples before SLC (uncentrifuged samples)*

		Progressive sperm motility before SLC (%)	
Group	*n*	Mean	Range
G1	7	27.7[A]	0–43.7
G2	6	68.5[B]	43.8–100

n = number of semen samples; SLC = single layer centrifugation.
[A-B]Indicate significant differences ($P < 0.001$).

Table 5 *Comparison of the increment of sperm parameters of cooled-stored semen after SLC between semen samples grouped on the basis of their original progressive motility*

	Semen sample group according to original PM		
Increment of sperm parameters after SLC	Group 1 (PM = 27.7)	Group 2 (PM = 68.5)	RMSE
TM-I (%)	13.5	8.3	6.4
PM-I (%)	20.1	12.3	9.1
L-I (%)	4.2	4.6	7.6
NF-I (%)	9.1	5.2	6.7
Yield (%)	24.5	26.5	5.1

SLC = single layer centrifugation; RMSE = root mean square error; TM = total motility; PM = progressive motility; L = live sperm; NF = normal forms
No significant differences were found between groups ($P > 0.05$).
Values are expressed as mean and RMSE.
TM-I (TM increment) = (TM% SLC-selected)–(TM% uncentrifuged).
PM-I (PM increment) = (PM% SLC-selected)–(PM% uncentrifuged).
L-I (L increment) = (L% SLC-selected)–(L% uncentrifuged).
NF-I (NF increment) = (NF% SLC-selected)–(NF% uncentrifuged).

studies (Mello *et al*., 2000; Rota *et al*., 2008; Contri *et al*., 2010a), however, total and PM were significantly enhanced when comparing unselected with SLC-selected samples. These results agree with studies performed with cooled stallion semen doses (Morrell *et al*., 2011a). In this way, the capacity of SLC with Androcoll-E-Large to increase total and progressive stallion sperm motility after 24 h of cool storage compared to uncentrifuged semen samples has also been shown.

Sperm viability represents the integrity of sperm plasma membrane. It is supposed that all motile sperm should be alive; however, these parameters are not always related (Love *et al*., 2003). Our results showed a lower percentage

of live sperm than motile sperm. This fact is explained in a previous study by Cortes-Gutierrez *et al.* (2008) in which they discovered that some live sperm remained unstained with Duo-Vital® staining, becoming 'invisible' to the evaluator and the final percentage of live sperm is lower than the true value. Nevertheless, sperm viability percentage was significantly higher in the samples centrifuged with Androcoll-E-Large. Another study has also reported that SLC using this colloid improves cooled stallion semen doses (Morrell *et al.*, 2009b). This is quite an interesting finding bearing in mind that the temperature drop triggers several changes in the spermatozoa known as cold shock (Watson, 2000), mainly in the acrosomal and plasma membrane decreasing sperm quality because of death or shortening of sperm life (Petrunkina *et al.*, 2005; Peña *et al.*, 2012). Consequently, pregnancy rates after insemination of cool semen decrease (Heckenbichler *et al.*, 2011) implying this last motive a good reason to consider colloid centrifugation as an option in order to improve sperm viability of cooled semen samples.

Sperm morphology was also improved after SLC, being the proportion of normal forms higher in SLC-selected samples in comparison to uncentrifuged cooled-stored semen doses. These results correspond to those from other studies in stallions (Morrell *et al.*, 2011a). Percentage of normal forms has been related to pregnancy rates (Morrell *et al.*, 2008). This could suggest that samples processed with Androcoll-E-Large which presented better morphology would be more fertile than unprocessed ones.

Mean sperm yield obtained was 25.4% being the range in stallions from 20% to 69%. Although the recovery rate is small compared with those obtained in stallions (Morrell *et al.*, 2009c, 2011a), these differences can be explained by this colloid formulation, which has been previously used with stallions and not with donkeys. This fact can be explained attending to previous studies performed with horses (Morrell *et al.*, 2009b), where it is described that volume and concentration must be adjusted to a specific colloid. If this requirement is not fulfilled significant differences in the yield are obtained. Further studies are needed in order to develop more accurate protocols to use in donkeys (testing different sperm concentrations, volumes of sperm and colloid, centrifugation times and densities) or a specific colloid for donkey sperm to increase the yield obtained.

On the other hand, previous studies have calculated AI donkey semen doses based on sperm concentration excluding other parameters. These authors concluded that a sperm dose of 400 millions of total sperm in 10 ml was followed with acceptable pregnancy rates (Vidament *et al.*, 2009). This number of sperm per dose could be decreased if sperm parameters like motility, viability or morphology are taken into consideration. In our study, the mean yield obtained was ~381 million of sperm in each semen sample processed (15 ml sperm $\times$ 100 million sperm per ml $\times$ recovery rate/100). However, donkey sperm concentration of raw semen is much larger than horse sperm concentration (Miró *et al.*, 2009). If we scaled-up the yield to the number of sperm in the whole ejaculate, we could obtain around 5000 million of SLC-selected sperm. It makes possible to prepare more than 12 cooled doses for AI with 400 million of sperm per dose.

Sperm velocities, mainly VCL and VSL are the most important kinematic parameters related to potential fertility (Olds-Clarke, 1989). Results obtained in this study revealed a highly significant ($P < 0.001$) enhancement of both parameters after SLC. In the same way, the remaining kinematic parameters assessed were also significantly higher ($P < 0.001$) after SLC except for ALH, which was significantly smaller. Irregular trajectories are mainly induced by two causes: (1) low linearity (LIN = VSL/VCL $\times$ 100) or (2) a high degree of lateral deviation of the head (ALH), both related to very low VSL and very high values of VAP (Mortimer, 2000). In our study, VSL and VAP increases in SLC-selected samples accompanied with lower mean values of ALH. This implies that SLC excludes sperm with an irregular trajectory. Since differences between treatments in each animal have shown different results, individual factors should be considered when processing samples by SLC. Previous studies performed in stallions reported an improvement of all sperm kinematics parameters in frozen-thawed semen, obtaining similar results to fresh semen evaluation prior freezing (Macias Garcia *et al.*, 2009a). To conclude, SLC with Androcoll-E-Large improves both general and kinematic motility parameters in donkey sperm samples, indicating better sperm motility.

In experiment 2, Androcoll-E-Large improved every sperm quality parameter regardless of the PM of the original sample, and there were no differences in the yield between groups. This result suggests that cooled semen samples are suitable for processing with Androcoll-E-Large. Furthermore, even semen samples with higher progressive sperm motility after cool storage are capable of being improved using Androcoll-E-Large. However, as mean values for the increment of different sperm quality parameters and yield were different, maybe a larger number of ejaculates and animals could show significant differences between groups.

In short, this general improvement of sperm quality in donkey cooled semen doses based on the enhancement of sperm parameters could be related to selection of the most robust 'fertile spermatozoa' from the entire sperm population. Nevertheless, we could think that a number of some good spermatozoa that still remained some potential fertility ability may be lost during the SLC process. Consequently, it could also affect fertility after SLC. However, it is important to highlight the fact that SLC not only selected the robust 'fertile' sperm but also removed dead and immotile sperm. Removing dead or immotile sperm from a semen sample means that sources of reactive oxygen species are also removed and that should improve fertility of AI doses (Morrell *et al.*, 2013). Since previous studies have obtained moderate pregnancy rates (45%) in jennies inseminated with unselected cooled semen (Vidament *et al.*, 2009), SLC could be used to try to increase this percentage. The relationship between SLC-selected semen and fertility has been reported before in stallions (Morrell *et al.*, 2011b). However,

no experiences have been performed in donkeys. Once SLC has been shown to be an effective method to improve sperm quality parameters of cooled-stored donkey semen doses *in vitro*, further studies are needed to relate sperm quality enhancement of donkey semen doses to pregnancy rates after AI.

In conclusion, SLC with Androcoll-E-Large improved total and PM, viability, morphology as well as most of sperm kinematics parameters assessed over the entire set of donkey semen doses processed after 24 h of cool storage.

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