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ScienceDirect
Theriogenology 72 (2009) 1017–1022
Theriogenology
www.theriojournal.com

Effects of dilution and centrifugation on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey semen

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Received 23 September 2008; received in revised form 17 June 2009; accepted 27 June 2009

Abstract

The aim of this work was to study the effects of dilution and centrifugation (i.e., two methods of reducing the influence of the seminal plasma) on the survival of spermatozoa and the structure of motile sperm cell subpopulations in refrigerated Catalonian donkey (*Equus asinus*) semen. Fifty ejaculates from nine Catalonian jackasses were collected. Gel-free semen was diluted 1:1, 1:5 or 1:10 with Kenney extender. Another sample of semen was diluted 1:5, centrifuged, and then resuspended with Kenney extender until a final dilution of 25×10^6 sperm/ml was achieved (C). After 24 h, 48 h or 72 h of refrigerated storage at 5 °C, aliquots of these semen samples were incubated at 37 °C for 5 min. The percentage of viable sperm was determined by staining with eosin-nigrosin. The motility characteristics of the spermatozoa were examined using the CASA system (Microptic, Barcelona, Spain). At 24 h, more surviving spermatozoa were seen in the more diluted and in the centrifuged semen samples (1:1 48.71%; 1:5 56.58%, 1:10 62.65%; C 72.40%). These differences were maintained at 48 h (1:1 34.31%, 1:5 40.56%, 1:10 48.52%, C 66.30%). After 72 h, only the C samples showed a survival rate of above 25%. The four known donkey motile sperm subpopulations were maintained by refrigeration. However, the percentage of motile sperms in each subpopulation changed with dilution. Only the centrifuged samples, and only at 24 h, showed exactly the same motile sperm subpopulation proportions as recorded for fresh sperm. However, the 1:10 dilutions at 24 and 48 h, and the centrifuged semen at 48 h, showed few variations compared to fresh sperm. These results show that the elimination of seminal plasma increases the survival of spermatozoa and the maintenance of motility patterns.

The initial sperm concentration had a significant ($P < 0.05$) influence on centrifugation efficacy, but did not influence the number of spermatozoa damaged by centrifugation. In contrast, the percentage of live spermatozoa in the fresh semen significantly influenced the number of spermatozoa damaged by centrifugation, but not centrifugation efficacy.

Keywords: Seminal plasma; Sperm subpopulations; Cooled semen; Donkey

1. Introduction

The Catalonian donkey (*Equus asinus*) is a large donkey found in a number of Pyrenean and pre-Pyrenean areas of Catalonia (northeastern Spain). It is the forerunner of several large donkey breeds around the world, including the Martina Franca, Pantelleria, or Ragusana breeds in Italy, the Pyrenean donkey of France, the Mammoth Jackass of the United States, the Mallorquin donkey of Spain, the Cyprus donkey, and others. The value of the Catalonian donkey in mule breeding has been known for centuries. Nonetheless, this breed is now in a danger of extinction [1]. Knowledge regarding the conservation of its semen is therefore not only important with respect to breeding

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0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.06.012

programs but also to the maintenance of its numbers and the prevention of its disappearance.

Making use of computer-assisted motility analysis (CASA), other studies have examined the sperm motility patterns of Catalonian donkey semen and report four specific motile sperm subpopulations [2]. Subpopulations of motile spermatozoa with specific motility characteristics have been reported in several species such as the common marmoset, gazelle, pig, dog, horse, and red deer [3–12]. The existence of such a structure in mammals of very different phylogenetic origin suggests the existence of a relationship between changes in the subpopulation structure of an ejaculate and its fertilizing ability [9,10].

The cooling, storing, and transport of semen for use in subsequent insemination is very important in the reproductive management of a number of species. Several authors report that sperm survival and the motility of refrigerated semen increases with the removal of the seminal plasma, and a number of seminal plasma proteins have been identified as a cause of reduced survival and motility loss in pig [13], cattle [14], goat [15], and horse [16] semen. These proteins make contact with the spermatozoa during ejaculation, interact with the spermatozoid membrane, and influence different characteristics, including motility [17]. The detrimental effects of seminal plasma on spermatozoa during cold storage may also be related to the action of several enzymes [16]. In horses, these undesirable effects can be reduced by semen dilution or the total or partial removal of the seminal plasma [18]. In the Zamorano-Leonés donkey, Serres et al. [19] observed that total motility, progressive motility, and HOST (Hipoosmotic Swelling Test) results were higher for centrifuged semen than for noncentrifuged semen.

The aims of the current study were to determine the effect of the seminal plasma on refrigerated Catalonian donkey semen, to investigate the maintenance of motile sperm subpopulation structure over time in different dilutions of semen and in centrifuged sperm (i.e., with the seminal plasma removed), and thus to determine the best protocol for refrigerating Catalonian donkey semen. The information obtained might help to increase more easily the population of Catalonian donkeys and avoid the extinction of this breed.

2. Materials and methods

2.1. Experimental animals

The study was performed at the experimental farm of the Veterinary Faculty, Autonomous University of Barcelona, between April 2006 and June 2007. Fifty ejaculates were collected from nine healthy, mature Catalonian jackasses of proven fertility aged 6 to 10 yr. Collections were made using an artificial vagina with an in-line gel filter. A jenny in natural or induced estrus was used to induce copulatory activity.

2.2. Sperm evaluation: Computer-assisted motility analysis

The sperm concentrations in aliquots of ejaculate were determined using a hemocytometer [20]; the pH was determined using a pH meter (micropH2000; Crison, Strumenti S.p.A., 41012 CARPI (MO), Italy). Gel-free semen was immediately diluted 1:1, 1:5, and 1:10 with dry skimmed milk extender [21] previously kept in a 37 °C water bath and stored in air-free 50-mL Corning tubes (Corning Incorporated, Corning, NY, 14831 USA). Another sample of gel-free semen was diluted 1:5 and centrifuged at $660 \times g$ for 15 min at 20 °C. The pellet, along with 5% to 20% of the supernatant, was resuspended with Kenney extender in air-free Corning tubes at a final dilution of 25×10^6 sperm/mL. All samples were then maintained at 5 °C for either 24, 48, or 72 h before analysis.

After storage, percentage viability was determined by eosin-nigrosin staining, examining 200 spermatozoa at ×1000 magnification, as described by Bamba [22]. Five-milliliter aliquots of semen samples were incubated for 5 min in a water bath at 37 °C. The pH was determined using a pH meter (micropH2000; Crison). Prior to sperm motility analysis, the above 1:1 and 1:5 samples were further diluted with Kenney extender until a 1:10 ratio was reached with respect to fresh semen. Sperm motility characteristics were determined using the Sperm Class Analyzer (Microptic; Barcelona, Spain) as described Miró et al. [2] for donkey semen. Three consecutive 5-μL samples of all of the studied ejaculates was observed using an optical phase-contrast microscope with a heatable plate (37 °C). Two fields per drop were examined; the number of spermatozoa examined in each field (including those not motile) was 50 to 100. The CASA system used is based on the analysis of 24 consecutive, digitalized photographic images obtained from a single field at ×200 magnification with dark-field illumination. These photographs were taken in a total time of 0.64 sec (image-capture rate, 1 photograph every 40 msec).

The CASA settings were as follows: cell size, 4 to 75 μm^2; connectivity, 12; progressive spermatozoa, >75% of the straightness coefficient (STR); and minimum number of images required to assess the

mean lateral head displacement (ALH), 10. The CASA system used takes into account 21 sperm motility descriptors. However, as described by other authors [2], only six of these variables are required to explain overall donkey sperm movement: mean velocity (VAP), mean lateral head displacement (ALH), the linear coefficient (LIN), the frequency of head displacement (BCF), the minor harmonic oscillation of the head (HLO), and the algebraic angular mean displacement (MAD). With these six variables, the FASTCLUS clustering procedure was used to separate the spermatozoa into their different motility subpopulations [9,10].

2.3. Statistical analysis

Data were processed using the SAS statistical package (version 8.2; Statistical Analysis System, SAS Institute Inc., Cary, NC, USA). Normality was assessed by the Shapiro-Wilks test (W) included in the UNIVARIATE procedure.

A general linear model (the PROC GLM routine) was used to test for significant differences in motility ($P < 0.05$) among the samples subjected to different treatments and refrigerated storage times. The LSMEANS procedure was used to identify significant differences. The chi-squared test was used to detect differences in viability and pH.

Finally, the centrifugation efficacy ([centrifuged spermatozoa mL^{-1}/fresh semen spermatozoa mL^{-1}] $\times$ 100) and the index of spermatozoa damaged by centrifugation ([fresh live spermatozoa/centrifuged live spermatozoa] $\times$ 100) were calculated. Correlations between the initial concentration and number of live spermatozoa mL^{-1} and the concentration and the number of live spermatozoa after centrifugation were determined by linear regression.

3. Results

Table 1 shows the sperm characteristics for the collected ejaculates. The composition of the semen and the values of the sperm motility variables varied between donkeys and even between ejaculates of the same animal ($P < 0.001$; data not shown).

The FASTCLUS procedure detected four subpopulations of motile sperm from the data for fresh semen. Table 2 shows the mean values for each motility variable in each subpopulation.

The spermatozoa of Subpopulation 1 showed the greatest progressiveness and were highly active (as inferred from the very high LIN and VAP values). The spermatozoa of Subpopulation 2 showed nonlinear trajectories, low progressiveness, and a very low VAP value. Subpopulation 3 contained spermatozoa with high VAP values, although their trajectories showed intermediate linear coefficients. Subpopulation 4 included spermatozoa with the least progressiveness and linearity.

Table 1
Mean values of the semen quality analysis.

Variable	Mean ± SEM
Filtered volume (mL)	64.1 ± 2.6
Sperm count ($\times 10^6$/mL)	338.2 ± 1.7
Total motility	81.3 ± 0.5
Progressive motility	68.4 ± 1.3
pH	7.6 ± 0.4
Sperm viability (%)	84.1 ± 1.5
Sperm immature tail (%)	10.3 ± 0.3
Sperm coiled-tail (%)	1.1 ± 0.2
Sperm head abnormality (%)	3.2 ± 0.4
Tailless spermatozoa (%)	2.5 ± 0.1
Immature sperm with proximal cytoplasmic droplets (%)	1.6 ± 0.0
Immature sperm with distal cytoplasmic droplets (%)	0.2 ± 0.1
Total abnormalities (%)	20.1 ± 0.2
Sperm curvilinear velocity (VCL; μm/sec)	82.7 ± 0.4
Sperm linear velocity (VSL; μm/sec)	90.9 ± 0.4
Mean velocity (VAP; μm/sec)	57.9 ± 0.1
Linear coefficient (LIN; %)	52.3 ± 0.5
Straightness coefficient (STR; %)	73.8 ± 0.6
Wobble coefficient (WOB; %)	79.2 ± 0.6
Mean lateral head displacement (ALHmed; μm)	2.5 ± 0.06
Frequency of head displacement (BCF; Hz)	12.3 ± 0.2
Minor harmonic oscillation of the head (HLO; μm)	0.4 ± 0.05

Table 3 shows the viability of the sperm after each treatment (different dilutions and centrifugation) and refrigerated storage time. Significant differences were seen between treatments for the same refrigeration time. Sperm cell survival increased with dilution, but the best results were recorded for the centrifuged semen. After 72 h of refrigerated storage, only the centrifuged samples showed greater than 25% survival.

The pH of the semen fell significantly between at 24 and 48 h when diluted by more than 1:1, with the lowest values seen for centrifuged semen. However, no significant differences were observed between the 1:5 and 1:10 dilutions at 24 and 48 h (Table 4).

The sperm subpopulation structure was perfectly maintained at 24 h only in the centrifuged semen (Table 5), although the 1:10 dilutions at 24 and 48 h and the centrifuged semen at 48 h showed few variations

Table 2
Fresh semen sperm subpopulations and motility descriptors.

Sperm subpopulation	Sperm motility descriptors					
	VAP (μm/sec)	LIN (%)	ALHmed (μm)	MADalg (°)	BCF (Hz)	HLO (μm)
1	154.4 ± 1.7	75.2 ± 2.2	4.89 ± 0.24	–1.72 ± 1.85	12.9 ± 0.6	1.11 ± 0.10
2	28.5 ± 0.9	44.3 ± 1.5	2.64 ± 0.12	4.85 ± 0.98	12.1 ± 0.3	0.32 ± 0.05
3	81.9 ± 0.8	59.8 ± 1.1	4.00 ± 0.12	0.57 ± 0.90	13.1 ± 0.3	0.67 ± 0.05
4	27.8 ± 1.2	26.4 ± 1.6	3.19 ± 0.17	–33.26 ± 1.30	15.0 ± 0.4	0.25 ± 0.07

Note: The motility descriptors are described in Section 2. Results are expressed as mean ± SEM for 50 semen samples from 9 Catalonian donkeys. The total number of spermatozoa analyzed was 5828.

Table 3
Sperm viability percentages at 24, 48, and 72 h for each treatment (dilutions 1:1, 1:5, 1:10, and centrifuged semen [C]).

Hours	Treatment			
	1:1	1:5	1:10	C
24	48.71 ± 23.42[a]	56.58 ± 21.42[b]	62.65 ± 19.87[c]	72.40 ± 14.50[d]
48	34.31 ± 22.46[e]	40.56 ± 19.96[e]	48.52 ± 23.28[f]	66.30 ± 17.97[d]
72	<25	<25	<25	42.10 ± 20.17[e]

[a–f]Different superscripts in the same row indicate significant differences. Results are expressed as mean ± SEM.

compared with that of fresh sperm. At 72 h, only the centrifuged semen maintained a population structure similar to that of fresh sperm, although with a predominance of Subpopulations 2 and 3.

Finally, the initial sperm concentration ($338.2 \times 10^6 \pm 1.7 \times 10^6$ spermatozoa/mL) had a significant influence on centrifugation efficacy (86.98 ± 1.93%) but no influence on the number of spermatozoa damaged by centrifugation (1.33 ± 1.06%), whereas the percentage of live spermatozoa in the fresh semen (84.1 ± 1.5%) significantly influenced the number of spermatozoa damaged by centrifugation but not the centrifugation efficacy (Table 6).

4. Discussion

In horses, the negative effects of refrigerating sperm can be diminished when a suitable extender is added to the semen; it can then be stored at 4 to 5 °C for 1 to 2 d [23–25]. In addition, the harmful effects of seminal plasma can be reduced by using high ratios (≥3:1) of extender to whole ejaculate semen [26,27] or totally or partially removing the seminal plasma [17]. Brinsko et al. [28] reported centrifugation and the partial removal of seminal plasma to be beneficial in the refrigerated storage of horse semen, especially for ejaculates with poor cooling and storage tolerance. In agreement, the results of the current study show that donkey sperm viability after storage is greater when more diluted or when the seminal plasma partially has been removed by centrifugation.

Serres et al. [19] reported that centrifugation and the removal of seminal plasma have a significant, positive effect on total motility, progressive motility, and HOST test results in Zamorano-Leonés donkey spermatozoa cooled in INRA (Institute National Recherche Agricole, France) 82. However, Rota et al. [29] indicated that the total removal of seminal plasma during in vitro preservation did not seem to offer any advantage over using semen diluted in INRA 82 + 2% egg yolk. Compared with the study by Rota et al. [29], the current conditions were different. The latter authors used only 12 ejaculates from four Amiata donkeys compared with the 50 ejaculates from nine Catalonian donkeys used here. A different extender was also used in the current work, and the different centrifugation conditions, initial dilution, temperature, and the almost complete removal of seminal plasma mark further variations in conditions.

Table 4
Semen sample pH at 24, 48, and 72 h in each treatment (dilutions 1:1, 1:5, 1:10, and centrifuged semen [C]).

Hours	Treatment			
	1:1	1:5	1:10	C
24	7.14 ± 0.15[a]	7.05 ± 0.12[b]	7.02 ± 0.14[b]	6.92 ± 0.10[c]
48	7.15 ± 0.17[a]	7.06 ± 0.11[b]	7.04 ± 0.08[b]	6.91 ± 1.15[c]
72	—	—	—	7.05 ± 0.10[a]

[a–c]Different superscripts indicate significant differences. Results are expressed as mean ± SEM.

Table 5
Sperm subpopulation proportions (SP) in donkey semen and their change with dilution or centrifugation and time (0, 24, 48, and 72 h).

SP	Fresh semen	24 h				48 h				72 h
		1:1	1:5	1:10	C	1:1	1:5	1:10	C	C
1	9.24[a]	3.37 [a]	1.66 [b]	1.31 [b]	3.71 [a]	5.26 [a]	2.56 [b]	2.11 [b]	1.60 [b]	0.70 [b]
2	33.01[a]	56.13[b]	40.85 [a]	39.02 [a]	35.43 [a]	45.61 [b]	48.08 [b]	42.11 [a]	40.52 [a]	51.75 [b]
3	39.07 [a]	7.36 [b]	24.77 [b]	33.77 [a]	39.57 [a]	21.05 [b]	14.10 [b]	34.42 [a]	30.34 [a]	27.97 [a]
4	18.68 [a]	33.13 [b]	32.72 [b]	25.89 [a]	21.29 [a]	28.07 [b]	35.26 [b]	17.37 [a]	27.54 [b]	19.58 [a]

C, centrifuged semen.
[a,b]Different superscripts indicate significant differences.

Table 6
Significance of correlations between sperm concentration or viability and centrifugation efficacy (initial concentration/postcentrifugation concentration × 100) and spermatozoa damaged by centrifugation (initial live spermatozoa/postcentrifugation live spermatozoa × 100).

	Centrifugation efficacy	Damaged spermatozoa
Fresh-semen spermatozoa/mL	0.023*	0.996
Percentage (%) fresh-semen live spermatozoa	0.910	0.000*

* $P < 0.05$.

Finally, Rota et al. analyzed their CASA results globally, which is nowadays thought to be inadequate.

Very different mammals have been found to have similar motile sperm subpopulation structures [3–12], the maintenance of which could be important in the general functioning of ejaculates. The four known donkey motile sperm subpopulations are maintained with freezing [30] and, according to the current results, with refrigerated storage. Nevertheless, the distribution of these subpopulations showed variations. Only the centrifuged samples, and only at 24 h, showed the same distribution of subpopulations as in fresh semen. However, the 1:10 diluted semen at 24 and 48 h, as well as the centrifuged semen at 48 h, showed relatively few variations compared with the fresh sperm. The centrifuged semen at 72 h showed a significant reduction in Subpopulation 1 (SP1) and an increase in Subpopulation 2 (SP2); overall sperm survival was good. In fresh semen, SP2 and Subpopulation 3 (SP3) showed the highest numbers (33.01% and 39.07% of the total number of sperm, respectively). The percentage of these subpopulations did not differ ($P < 0.05$) in the 1:10 diluted semen or the centrifuged semen at 24 or 48 h. At 72 h, however, whereas SP2 increased significantly, SP3 did not differ; nonetheless, both subpopulations remained the largest.

Brinsko et al. [28] observed a trend toward a reduction in the sperm curvilinear velocity (VCL) in diluted (1:3) and centrifuged horse semen after 24 or 48 h of refrigerated storage. The present diluted and centrifuged donkey semen showed an increase in the proportion of SP2 and Subpopulation 4 (SP4), but both showed low VAP values; the proportion of SP1 and SP3 were reduced, but their members showed higher VAPs.

Sperm survival and motile subpopulation structure in Catalonian donkey semen are therefore influenced by the presence of seminal plasma. High dilution ratios, but with an adequate number of normal motile spermatozoa ($>25 \times 10^6$ spermatozoa/mL), allow for optimum sperm cell survival and the maintenance of motile subpopulation structure in semen refrigerated for 24 or 48 h. If storage needs to be longer than 48 h, centrifugation should be performed to remove the seminal plasma. However, semen centrifugation can damage spermatozoa; the presence of semen extender and the concentration of spermatozoa are very important in reducing this damage [18,31]. The dilution rate normally used for horse semen centrifugation is 1:1 or 1:2, and centrifugation results in a recovery rate of about 75% of the spermatozoa in the pellet [25]. Matás et al. [32] (for pig semen) and Ecot et al. [33] (for horse semen) used a cushioned centrifugation method to minimize sperm loss. However, this is not easy to perform, and the cushion solution must be removed separately after centrifugation [25]. In the current study, the 1:5 dilution for centrifugation provided good sperm recovery results. However, the number of sperm damaged in centrifugation was not related to the precentrifugation dilution ratio. Rather, initial sperm viability correlated inversely with the numbers damaged; good initial semen results in good centrifuged semen.

Acknowledgments

The authors thank Mr. José Manuel Fernández, the owner of the jackasses used in our study, for his kind

collaboration. This work was funded by Grant RZ-03-021 from Instituto Nacional de Investigaciones Agrarias, Spain.

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