



Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro*

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ABSTRACT

An experiment was designed to study the interaction between fresh/frozen-thawed donkey spermatozoa and zona pellucida (ZP)-free bovine oocytes in an attempt to develop a model for assessing cryopreserved Catalonian donkey sperm function. Semen from five donkeys was collected using an artificial vagina. Sperm motility and viability were immediately assessed and the semen sample cryopreserved. Sperm viability and motility were then reassessed immediately after thawing. The motion characteristics of the fresh and frozen-thawed spermatozoa were determined using a computer-assisted sperm analysis system. *In vitro*-matured cow oocytes were inseminated with different percent live donkey sperm (high (>60%) or low (<40%) viability donkey sperm). After 18 h of co-incubation, the oocytes were fixed, stained with 4',6-diamidino-2-phenylindole (DAPI) and examined for sperm penetration, the number of penetrated spermatozoa per oocyte, and male pronucleus formation. Frozen-thawed spermatozoa from high viability semen showed significantly lower VCL, VAP and mean ALH values than did high viability fresh spermatozoa. In contrast, frozen-thawed spermatozoa of low viability had significantly higher velocity values than fresh spermatozoa of low viability. A significant positive correlation ($P < 0.01$) was detected between percentage fertilization and viability ($r = 0.84$), and between percentage fertilization and certain CASA parameters (VAP, $r = 0.56$; VCL, $r = 0.61$ and mean ALH, $r = 0.68$). Fresh or frozen-thawed high viability spermatozoa penetrated 90.1% and 85.4% of bovine oocytes respectively. Lower rates of penetration were observed for fresh and frozen-thawed low viability spermatozoa (34% and 22.5% respectively). The donkey spermatozoa were able to fuse with the oolema and even to decondense and form the male pronucleus (85–94%). Larger numbers of penetrated spermatozoa per oocyte were recorded when high viability sperm samples were used, whether fresh (3.02 vs. 1.12 for low viability sperm) or frozen-thawed (3.41 vs. 1.47). Consequently, low viability sperm samples showed higher percentages of monospermic penetration (91.17% and 61.97% for fresh and frozen-thawed sperm samples respectively). These findings suggest that bovine oocytes provide a useful model for assessing the penetration potential of frozen-thawed donkey sperm.

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1. Introduction

The laboratory assessment of sperm quality is essential if assisted reproduction in domestic species is to be efficient. However, it is becoming ever more apparent that the

standard variables of sperm motility, morphology and concentration are insufficient for predicting fertility or even for identifying subfertile individuals (Gadea et al., 2004). The search for more appropriate variables that can be measured quickly by sensitive and repeatable methods is therefore the focus of increasing interest.

In vitro fertilization (IVF) tests might afford an adequate means of assessing fertility. This type of procedure allows the evaluation of sperm–oocyte interactions that

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occur during *in vivo* fertilization, and permits different endpoints in the early stages of embryo development to be monitored. Some authors have used homologous IVF assays to predict male fertility using zona pellucida (ZP)-intact oocytes (Papadopoulos et al., 2005; Schneider et al., 1999; Zhang et al., 1997). However, it is often difficult to obtain oocytes of the same species as the sperm donor, especially if this species is wild or endangered. In such animals, heterologous IVF would appear to be an attractive method for evaluating the fertilizing capacity of fresh or frozen-thawed sperm. Further, compared to artificial insemination, heterologous IVF requires fewer sperm cells, allowing sperm function to be thoroughly investigated while sparing valuable male gametes. Nonetheless, the potential of heterologous IVF remains largely unexplored.

Mammalian oocytes have been used as a model for assessing human sperm functionality (Canovas et al., 2007; Liu and Baker, 1992; Terada et al., 2004; Yanagimachi et al., 1976), and cross-species fertilization of the oocytes of domestic farm and laboratory species by the cryopreserved sperm of endangered felids (Baudi et al., 2008; Swanson and Wildt, 1997), non-domestic bovines (McHugh and Rutledge, 1998), oryx (Kouba et al., 2001) and equines (Brackett et al., 1982; Campos-Chillón et al., 2007; Choi et al., 2003) has been reported. However, the literature contains no references on the interaction between donkey spermatozoa and bovine oocytes.

The aim of the present work was to determine whether the functionality of Catalanian donkey sperm (fresh and frozen-thawed) could be assessed by studying its interaction with bovine oocytes at the oolema and ooplasm levels. The objectives were: (i) to evaluate the ability of donkey spermatozoa to fuse with *in vitro*-matured bovine ZP-free oocytes (sperm–oolema interaction), (ii) to assess the ability of donkey spermatozoa to decondense and transform into a male pronucleus following the *in vitro* fertilization of bovine oocytes (sperm–ooplasm interaction), and (iii) to study the correlation between donkey spermatozoa motility and viability and its ability to penetrate bovine oocytes.

2. Materials and methods

2.1. Reagents and laboratory supplies

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark).

2.2. Bovine oocyte collection and *in vitro* maturation

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in phosphate buffered saline (PBS) at 36–38 °C. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and showing homogeneous cytoplasm were selected for maturing *in vitro*. Groups of up to 50 COCs were placed in 500 µL of maturation medium in four-well dishes and cultured for 24 h at 38.5 °C in a 5% CO₂ humidified air

atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v/v) foetal calf serum (FCS), 10 ng/mL epidermal growth factor and 50 µg/mL gentamicin.

2.3. Animals, semen collection and cryopreservation

The donkeys used in this study were five Catalanian jackasses between four and eight years of age. All were in good condition and were known to be fertile. Semen was collected using a Hanover model artificial vagina with an in-line gel filter. Collections were performed using an ovariectomized female donkey brought into oestrus with estrogens. After collection, gel-free semen from each donkey was immediately diluted 1:5 (v/v) with prewarmed (37 °C) dry skimmed milk extender. Semen samples were evaluated upon collection for volume, viability, morphology and motility. Aliquots were then taken as needed for analysis. The sperm concentration of the ejaculate was determined using a Neubauer haemocytometer. Sperm viability was classified and determined using a standard percent live spermatozoon assay. Samples were stained with eosin–nigrosin as described by Bamba (1988). Following this stain, viable spermatozoa were defined as those that showed an uniform, white colour under observation, whereas non-viable sperm were defined as those which showed any sign of both partial and total pinkish-purple staining.

Sperm processing and cryopreservation were conducted as previously described (Flores et al., 2008). Briefly, the diluted semen samples were centrifuged at 660 × g for 15 min at 20 °C in a programmable refrigerated centrifuge to remove the seminal plasma. The supernatant was eliminated and the spermatozoa re-suspended in Gent A diluent[®] (Minitüb, Tiefenbach, Germany). The sperm cell concentration was then re-determined and Gent B diluent[®] (Minitüb, Tiefenbach, Germany) added to obtain a final concentration of 200 × 10⁶ viable sperms/mL (50% final volume). Diluted semen was packaged into 0.5 mL polyvinyl chloride plastic straws (Minitüb, Tiefenbach, Germany). These were positioned horizontally on a metal rack and cooled in a programmable liquid nitrogen freezer (Ice-Cube 14S; Minitüb, Tiefenbach, Germany) at a rate of 0.25 °C/min from 20 °C to 5 °C, and 5.50 °C/min from 5 °C to –120 °C. The straws were then plunged into the liquid nitrogen (–196 °C) for storage. Frozen semen was thawed by immersing the straws in a water bath at 37 °C for 30 s. Sperm motility and viability was then determined.

2.4. Sperm motility

The motion characteristics of fresh and frozen-thawed sperm samples were determined using a computer-assisted sperm analysis system (CASA system) (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain). Samples were incubated for 5 min in a water bath at 37 °C. Five microlitres aliquots of these samples were then observed using a phase contrast microscope equipped with a heat stage (37 °C). Three fields per drop were analysed. The CASA system is based on the analysis of 50 consecutive, digital images of a single field at a magnification of 200 ×

(dark ground). These 50 consecutive images were obtained over 1 s – an image capture rate of one photograph every 20 ms. The sperm motility descriptors obtained by CASA are described below:

Curvilinear velocity (VCL)	$\mu\text{m/s}$	Measures the sequential progression along the true trajectory
Linear velocity (VSL)	$\mu\text{m/s}$	Measures the straight trajectory of the spermatozoa per unit time
Mean velocity (VAP)	$\mu\text{m/s}$	Measures the mean trajectory of the spermatozoa per unit time
Linearity coefficient (LIN)	%	$\text{VSL/VCL} \times 100$
Straightness coefficient (STR)	%	$\text{VSL/VAP} \times 100$
Wobble coefficient (WOB)	%	$\text{VAP/VCL} \times 100$
Mean lateral head displacement (mean ALH)	μm	Measures the mean head displacement along the curvilinear trajectory
Frequency of head displacement (BCF)	Hz	The frequency with which the sperm trajectory crosses the average path trajectory

Total motility was defined as the percentage of spermatozoa with a VAP of $>10 \mu\text{m/s}$, and progressive motility as the percentage of spermatozoa with a VAP of $>90 \mu\text{m/s}$ plus an STR of $>75\%$.

2.5. Sperm preparation and ZP-free fertilization

For *in vitro* fertilization, cow oocytes were collected and matured as described above. After maturation, they were washed twice in PBS and then denuded (removal of the cumulus cells) by gentle pipetting. Oocytes with polar bodies were then selected and incubated in $50 \mu\text{L}$ droplets of prewarmed 0.3% pronase (w/v) in fertilization medium (Tyrode's medium supplemented with 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin sodium salt [Calbiochem, Darmstadt, Germany]) for 2–3 min to remove the ZP. Its digestion was observed continuously using a stereomicroscope. When the ZP was no longer visible, the oocytes were immediately removed from the pronase solution, washed, and transferred in groups of up to 20–25 to four-well plates containing $250 \mu\text{L}$ of fertilization medium per well. The oocytes were then allowed to recover for 30 min prior to insemination. ZP-free oocytes were randomly assigned to two groups; those of one were inseminated with fresh spermatozoa, while those of the second were inseminated with frozen-thawed spermatozoa. Presumptive zygotes were fixed 18–20 h after insemination and examined for sperm cell penetration. In order to exclude parthenogenetic activation of bovine oocytes, a sample of oocytes was cultured in presence of ionomycin without sperm cells (data not shown).

Motile spermatozoa were obtained by centrifuging fresh or frozen-thawed sperm in HEPES-buffered Tyrode's medium for 5 min at $700 \times g$ at room temperature. The supernatant was removed and the pellet resuspended in 8 mL of HEPES-buffered Tyrode's and centrifuged again at $700 \times g$ for 5 min. Spermatozoa were counted in a haemocytometer and diluted in an appropriate volume of fertilization medium to give a final concentration

of 2×10^6 spermatozoa/mL. In order to induce *in vitro* capacitation, the spermatozoa were treated with $0.1 \mu\text{M}$ ionomycin for 15 min (adding $1 \mu\text{L}$ of 0.1 mM ionomycin to 1 mL of the final diluted sperm). A $250 \mu\text{L}$ aliquot of this suspension was then added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/mL. Plates were incubated for 18–20 h at 38.5°C in a 5% CO_2 humidified air atmosphere. In order to exclude parthenogenetic activation of bovine oocytes, a sample of oocytes ($n=51$) was cultured with a $250 \mu\text{L}$ aliquot of $0.1 \mu\text{M}$ ionomycin without sperm cells.

2.6. Evaluation of penetration

At 18–20 h post-insemination, the oocytes were washed three times, fixed for 30 min in 2% (v/v) paraformaldehyde in PBS, stained with DAPI (4',6-diamidino-2-phenylindole; Vysis Inc., Downer's Grove, IL, USA) (125 ng/mL) and mounted on glass slides. Nuclear stage, sperm penetration and the formation of the male pronucleus were assessed at $400\times$ under an epifluorescence microscope (see Fig. 1).

2.7. Experimental design

Fifteen ejaculates – three from each of the five donkeys – were collected and sperm viability and motility immediately assessed. All collected semen was cryopreserved as described above. After thawing, viability and motility were checked again. The motion characteristics of the fresh and thawed spermatozoa were determined using a CASA system. The ZP of *in vitro*-matured cow oocytes was removed and the oocytes fertilized with fresh or frozen-thawed spermatozoa from the five donkeys. Four experimental groups were established: (1) fertilization attempts involving fresh, high viability (HV; $>60\%$) semen, (2) fertilization attempts involving low viability (LV; $<40\%$) semen, (3) fertilization attempts involving frozen-thawed HV ($>60\%$) semen and (4) and fertilization attempts involving frozen-thawed LV ($<40\%$) semen. Criteria for HV ($>60\%$) and LV ($<40\%$) were established according to Flores et al. (2008). After an 18 h co-incubation period, the oocytes were fixed and examined for sperm penetration, the number of penetrated spermatozoa per oocyte, and male pronucleus formation.

2.8. Statistical analysis

All calculations were performed using the Statistical Analysis Systems Package (SAS, 2000). The normality of the distribution of the sperm motility results was assessed using the Shapiro–Wilks test (W) included in the UNIVARIATE procedure. The PROC GLM procedure was used to determine the mean values of the motility variables and to analyse differences between the results for the sperm motility descriptors. The total number of spermatozoa analysed following this protocol was 1322. Total *in vitro* penetration and the mean number of penetrated sperm per oocyte were analysed by ANOVA. All experiments were replicated three times. Percentage data were subjected to arcsine transformation before analysis. When

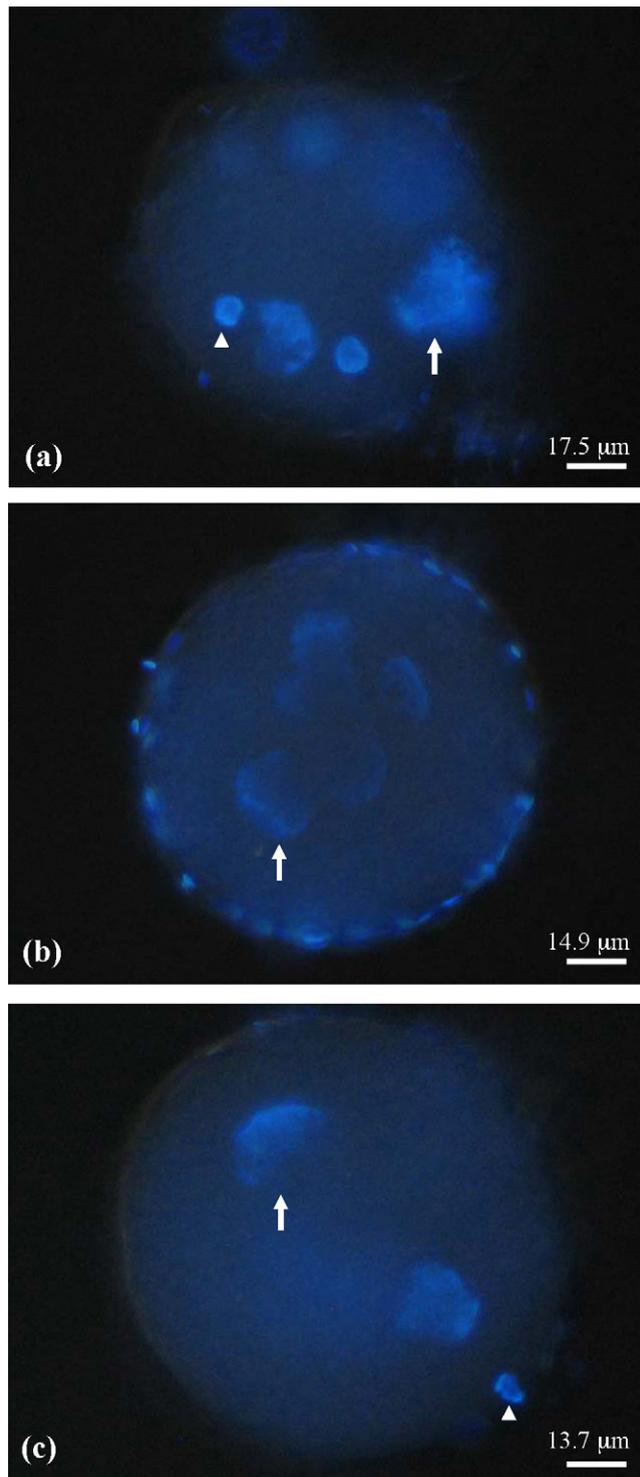


Fig. 1. Epifluorescence microscope images of *in vitro*-matured bovine oocytes after 18 h of co-incubation with donkey spermatozoa. Oocytes were immunocytochemically stained using DAPI to visualize the nuclear stage, sperm heads and the male pronucleus (blue). (a) Oocyte with five pronuclei (PN) (arrow) and one enlarged sperm head (arrowhead); (b) Oocyte with 5 PN; (c) Oocyte showing 2 PN and 1 enlarged sperm head.

Table 1
Quality analysis of high and low viability fresh and frozen-thawed donkey sperm.

	Fresh HV	Fresh LV	F-T HV	F-T LV
Viability (%)	69.30 ± 3.81 ^a	22.00 ± 4.66 ^b	71.26 ± 2.95 ^a	33.87 ± 3.30 ^b
Total motility (%)	71.66 ± 15.81	62.00 ± 19.36	82.40 ± 12.24	43.75 ± 13.69
Progressive motility (%)	31.33 ± 10.59	37.5 ± 12.97	34.20 ± 8.20	23.25 ± 9.17
VCL (μm/s)	121.02 ± 0.84 ^a	73.89 ± 1.15 ^b	93.15 ± 0.83 ^c	78.79 ± 0.93 ^d
VSL (μm/s)	64.30 ± 0.82 ^a	52.36 ± 1.12 ^b	62.21 ± 0.82 ^{ac}	59.11 ± 0.91 ^c
VAP (μm/s)	88.76 ± 0.68 ^a	58.99 ± 0.93 ^b	76.41 ± 0.68 ^c	66.85 ± 0.76 ^d
LIN (%)	48.30 ± 0.99 ^a	62.48 ± 1.35 ^{bc}	60.39 ± 0.98 ^b	66.73 ± 1.10 ^c
STR (%)	67.82 ± 1.01 ^a	80.31 ± 1.38 ^{bc}	76.54 ± 1.00 ^b	82.45 ± 1.12 ^c
WOB (%)	68.41 ± 0.69 ^a	75.42 ± 0.94 ^b	76.41 ± 0.68 ^{bc}	78.63 ± 0.76 ^c
Mean ALH (μm)	3.90 ± 0.05 ^a	2.44 ± 0.06 ^b	2.75 ± 0.05 ^c	2.19 ± 0.05 ^d
BCF (Hz)	8.95 ± 0.16 ^a	8.13 ± 0.22 ^b	8.49 ± 0.16 ^{ab}	8.85 ± 0.18 ^{ab}

Results are expressed as means ± SEM of four different semen preparations involving a total of 1322 spermatozoa. Fresh HV and fresh LV: fresh semen of high and low viability respectively; F-T HV and F-T LV: frozen-thawed semen of high and low viability respectively; VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement; BCF: frequency of head displacement. Different superscripts between rows indicate significant differences ($P < 0.05$).

ANOVA revealed a significant effect, the results for the different treatments were compared using Fisher's protected LSD test. Significance was set at $P < 0.05$.

3. Results

3.1. Quality of fresh and frozen-thawed donkey sperm

Table 1 shows the characteristics of the fresh and frozen-thawed sperm from the five Catalanian donkeys used in this study. Significant differences were seen in the viability of HV and LV sperm, although their total and progressive motility values were not significantly different. The overall motility of the frozen-thawed HV samples was lower but more linear than that of the fresh HV samples ($P < 0.05$). Further, the frozen-thawed HV samples presented lower mean ALH and VCL values than the fresh HV samples ($P < 0.05$). Moreover, the STR and WOB values were significantly higher in the frozen-thawed HV samples. In contrast, frozen-thawed LV samples showed significant higher velocity variables (VAP, VCL and VSL) and linearity coefficients than the fresh LV samples. The WOB was significantly higher in the LV samples, while the mean ALH was significantly lower in the LV samples. The BCF values of the different types of sample were not significantly different.

3.2. Penetration of ZP-free bovine oocytes by donkey sperm

Results are presented in Table 2. When ZP-free bovine oocytes were inseminated with HV sperm, significantly higher penetration rates were obtained – for both fresh (90.12%) and frozen-thawed (85.40%) samples – than with

LV sperm (34% and 22.47% for fresh and frozen-thawed samples respectively). High viability sperm were associated with a larger number of penetrated spermatozoa per oocyte ($P > 0.05$) than LV sperm – both for fresh and frozen-thawed samples. Consequently, LV sperm samples showed higher percentages of monospermic. The donkey spermatozoa were seen to be able to fuse with the oolema, decondense and form male pronuclei (85–94%). Parthenogenetic activation of bovine oocytes was 7% (4/51) and only 1 PN was observed on activated oocytes.

3.3. Correlation between sperm characteristics and penetration rate

Table 3 shows the correlations detected between sperm motion characteristics, viability and the penetration rate. Progressive motility was strongly correlated with the CASA variables (VCL, VSL, VAP, WOB, mean ALH and BCF). Penetration frequency was found to be strongly and positively correlated ($P < 0.01$) with percentage viability ($r = 0.84$). Only the VCL and mean ALH values correlated strongly with percentage fertilization ($r = 0.61$ and 0.68 , respectively; $P < 0.01$). The VAP and total motility correlated with the penetration rate ($P < 0.05$). The remaining CASA variables were not correlated with penetration percentage. Finally, percentage viability correlated only with total motility and mean ALH ($P < 0.05$).

4. Discussion

The Catalanian donkey is an endangered local donkey breed that can be found in a number of Pyrenean and pre-Pyrenean areas in Catalonia (northeastern Spain).

Table 2
Results of *in vitro* heterologous oocyte penetration testing involving fresh and frozen-thawed donkey sperm of different viability.

Sperm type	Examined, <i>n</i>	Penetration, <i>n</i> (%)	Monospermic, <i>n</i> (%)	N sperm/oocyte
Fresh HV	81	73 (90.12) ^a	21 (28.77) ^a	3.02 ± 0.52 ^{ab}
Fresh LV	100	34 (34) ^b	31 (91.17) ^b	1.12 ± 0.06 ^{ac}
F-T HV	226	193 (85.40) ^a	61 (31.61) ^a	3.41 ± 0.97 ^b
F-T LV	316	71 (22.47) ^b	44 (61.97) ^b	1.47 ± 0.09 ^c

Data are means (SEM of three replicates). Letters a–c denote significant differences within columns ($P < 0.05$). Fresh HV and fresh LV: fresh semen of high and low viability respectively; F-T HV and F-T LV: frozen-thawed semen of high and low viability respectively.

Table 3

Pearson's correlation coefficient between motion characteristics, viability and penetration rate.

	%Penetration	%Viability	PM	TM	VCL	VSL	VAP	LIN	STR	WOB	ALH
%Viability	0.84**										
PM	0.41	0.32									
TM	0.57*	0.60*	0.91**								
VCL	0.61**	0.48	0.73**	0.66**							
VSL	0.42	0.30	0.71**	0.52*	0.91**						
VAP	0.56*	0.43	0.75**	0.63**	0.98**	0.97**					
LIN	-0.07	-0.18	0.43	0.14	0.40	0.73**	0.55*				
STR	-0.23	-0.39	0.23	-0.07	0.22	0.59*	0.38	0.97**			
WOB	0.13	0.07	0.61**	0.36	0.57*	0.84**	0.70**	0.96**	0.88**		
ALH	0.68**	0.56*	0.65**	0.66**	0.94**	0.74**	0.87**	0.12	-0.05	0.30	
BCF	0.38	0.34	0.81**	0.69**	0.88**	0.89**	0.90**	0.60*	0.46	0.71**	0.78**

PM: progressive motility; TM: total motility; VCL: curvilinear velocity; VSL: linear velocity; VAP: mean velocity; LIN: linearity coefficient; STR: straightness coefficient; WOB: wobble coefficient; mean ALH: mean lateral head displacement, BCF: frequency of head displacement.

* $P < 0.05$.** $P < 0.01$.

Knowledge regarding the cryopreservation of its semen is important for breeding programs, the maintenance of the breed's numbers, and the prevention of its disappearance. Sperm cryopreservation can, however, damage sperm cells, reducing their fertilization capacity (Bailey et al., 2000). The damage caused to membranes during cooling to 5 °C or freezing is often subtle and difficult to detect in laboratory assays.

Sperm penetration assays can assess a number of sperm functions simultaneously (e.g., motility, ability to undergo an acrosome reaction, oocyte penetration and DNA decondensation) and may better assess sperm quality than assays that evaluate a single sperm characteristic (Bousquet and Brackett, 1981, 1982; Bousquet et al., 1983; Brahmkshtri et al., 1999). The ZP-free oocyte penetration assay has been used to assess the spermatozoa of many species, including horses (Blue et al., 1989; Graham et al., 1987; Okolski et al., 1987; Samper et al., 1989; Zhang et al., 1990) and cattle (Bousquet and Brackett, 1981, 1982; Bousquet et al., 1983; Eaglesome and Miller, 1989; Graham and Foote, 1987a,b). Wilhelm et al. (1996) reported that frozen-thawed horse spermatozoa could penetrate ZP-free hamster oocytes after treatment with dilauroylphosphatidylcholine (PC12) to induce the acrosome reaction, and indicated the percentage of penetrated hamster oocytes to be highly correlated to stallion fertility. When bovine ZP-free oocytes were used, the penetration rates for fresh HV stallion sperm (capacitated with 8-bromoadenosine cyclic monophosphate (8BrcAMP) + 0.1 μM of ionomycin in PVA-containing media) varied from 18% to 47% depending on the concentration of equine preovulatory follicular fluid added to the bovine oocyte maturation medium (Choi et al., 2003). These authors concluded that ZP-free bovine oocytes might be useful for assaying the *in vitro* capacitation and fertilization potential of stallion sperm. However, as far as we know, the literature contains no studies on heterologous bovine fertilization involving donkey sperm. The present work, shows that donkey sperm can penetrate bovine oocytes, fuse with the oolema, decondense, and form male pronuclei.

The penetration rates of fresh (90.12%) and frozen-thawed (85.40%) HV donkey sperm were higher than those obtained by Choi et al. (2003) and Landim-Alvarenga et al. (2001) for equine spermatozoa. It is known that spermato-

zoa from different species can have different penetration rates depending on the ZP-free oocyte used. For example, Graham et al. (1986) and Landim-Alvarenga et al. (2001) observed that bull spermatozoa penetrated a higher percentage of ZP-free hamster oocytes than did stallion spermatozoa. One reason for the higher penetration capacity of the donkey spermatozoa observed in the present work compared to that of stallion spermatozoa may be the differences in the lipid composition of the sperm cell membranes. Differences in the membrane lipid composition of mammalian spermatozoa have been studied in an attempt to understand the susceptibility of spermatozoa to cold shock. Parks and Lynch (1992) observed that cholesterol was the major sterol present in spermatozoa membranes of boar, bull, stallion and rooster spermatozoa, and that the molar ratio of cholesterol to phospholipid was higher in bull spermatozoa than in stallion spermatozoa. Cholesterol may help regulate the fluidity and permeability of the lipid bilayers of the membrane. During capacitation, cholesterol efflux from the spermatozoa increases the fluidity and permeability of their membranes, eventually leading to membrane fusion and the acrosome reaction. Davis (1981) first suggested that differences in the capacitation rates of human spermatozoa from different individuals might be related to differences in the cholesterol:phospholipid ratio of the membranes.

Stallion spermatozoa can be capacitated and the acrosome reaction induced by treatment with 8BrcAMP, heparin, PC-12, lysophosphatidyl-serine, ionophore calcium and ionomycin (Choi et al., 2003; Landim-Alvarenga et al., 2001; Wilhelm et al., 1996). In the present work, spermatozoa were incubated with ionomycin (0.1 μM) for a short time (15 min) to induce capacitation and thus allow the oocyte penetration capacity to be assessed (Choi et al., 2003). Spermatozoa that did not undergo ionomycin treatment were incapable of penetrating the present ZP-free oocytes (data not shown), while those that were thus treated penetrated them at rates of 22.5–90.1%. This study was not, however, designed to test which compound is most effective at inducing the acrosome reaction. In the present work, use was made of a concentration of ionomycin previously shown to induce the acrosome reaction in stallion and bull spermatozoa.

Motility is essential for the transport of sperm cells through the female reproductive tract and for oocyte penetration. The mean sperm motility values obtained in this study are comparable to those indicated in previous reports (Flores et al., 2008; Miro et al., 2005). However, the BCF recorded in the present work was higher in all experimental groups. The values of the velocity variables for the fresh HV sperm were higher than those recorded in other works, perhaps due to variability between the animals used. The frozen-thawed sperm motion characteristics recorded in the present study are dissimilar to those reported in previous reports (Flores et al., 2008). The velocity and linearity variables had higher values in the present work, whereas the mean ALH and BCF values were lower. This discrepancy might also be due to variability between the animals used. However, the changes in VCL and VAP before and after freezing were similar. Whereas freezing/thawing is reported to cause a significant increase in the values of boar sperm motility characteristics, the same was not seen for the present donkey cells. This discrepancy might be related to the notable differences observed in the physiological and metabolic characteristics of boar and donkey spermatozoa.

Relationships between motility characteristics in human and bovine spermatozoa, measured by CASA in both *in vitro* and *in vivo* fertility studies, have been reported by different authors. Fetterolf and Rogers (1990), working with human spermatozoa, showed that the total motility and VCL were highly correlated with the homologous IVF rate. Similarly, in bovines (*in vivo*), Farrell et al. (1998) reported a strong correlation between several motility characteristics (BCF, LIN, VAP, VSL and VCL) and fertility ($r^2 = 0.97$), where fertility was defined as 59 days of non-return service in cows inseminated with frozen-thawed semen. More recently, Kathiravan et al. (2008) reported that the progressive motility, VAP and VSL of bull spermatozoa were highly correlated with penetration in ZP-free hamster oocytes. Wilhelm et al. (1996) also reported that the percentage viability was correlated with stallion fertility ($r = 0.68$). In agreement with the above reports, the present penetration rates were correlated with viability, total motility, VAP and VCL. In contrast, no correlation was reported between penetration rate and sperm motility in the pig (Martinez et al., 1993; Suzuki et al., 1996). It has been suggested that the lack of correlation between conventional semen quality tests and sperm penetration assays is due to the fact that they measure different aspects of sperm fertilizing capacity (Gadea, 2005). Certainly, the influence of sperm characteristics on the success of *in vitro* fertilization is incompletely understood (Ivanova and Mollova, 1993; Martinez et al., 1993; Vazquez et al., 1993) and this lack of correlation may be due to differences in protocol, the number of penetrated sperm per oocyte, and differences among ejaculates selected.

In conclusion, this work shows that fresh and frozen-thawed donkey spermatozoa are able to fuse with *in vitro*-matured bovine ZP-free oocytes, and to decondense and form male pronuclei. Some of the CASA motility characteristics (VAP, VCL, mean ALH and total motility) and sperm viability are highly correlated with the *in vitro* fertilization rate. Heterologous IVF would seem to be a good way to evaluate the quality of frozen-thawed donkey semen and

to verify the storage quality of banked sperm samples. Further studies are needed to correlate sperm variables and heterologous penetration with *in vivo* fertility.

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