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Supplementary Material

Head morphology of ram spermatozoa is associated with their ability to migrate in-vitro and correlates with fertility.

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Materials and methods

1. Sperm motility evaluation

The sperm were diluted in INRA 96 solution to 10 to 20 x 10^6 spermatozoa/ml and loaded into a Makler counting chamber (10-µm depth; Sefi Medical Instruments, Haifa, Israel) at 39 °C. The CASA system consisted of a trinocular optical phase-contrast microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) with a 10 X negative phase-contrast objective, equipped with a warming stage set to 39 °C and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured at 50 frames/sec and were analyzed using a Computer-Assisted Semen Analysis (CASA: ISAS; Proiser, Valencia, Spain) with specific settings for ram spermatozoa. The software rendered the following parameters: (1) percentage of motile spermatozoa (PM), and (2) for each spermatozoon, three velocity parameters (VCL, velocity according to the actual path; VSL, velocity according to the straight path; and VAP, velocity according to the smoothed path), three track-linearity parameters (LIN, linearity; STR, straightness; and WOB, wobble), the amplitude of the lateral displacement of the sperm head (ALH), and the head beat-cross frequency (BCF).

2. Assessment of sperm quality using flow cytometry

We used flow cytometry to assess the quality of fresh sperm and of the spermatozoa that traversed the cervical mucus barrier. The non-sperm particles and debris were excluded using viability staining (Petrunkina & Harrison, 2011).

2.1. Acrosomal status and viability

Triple staining with PNA-FITC (Sigma-Aldrich, Madrid, Spain), propidium iodide (PI; Sigma-Aldrich, Madrid, Spain), Hoechst 33342 (Sigma Aldrich, Madrid, Spain) was conducted. The staining was performed by diluting the sperm sample (1-2 x 10^6 spermatozoa/ml) with 300 µL of PBS containing 1 µg/ml of PNA-FITC, 1.5 µM PI and 5 µM Hoechst 33342. After 10 min at

room temperature in the dark, the samples were analyzed using flow cytometry. The spermatozoa (Hoechst+) were classified into three sperm subpopulations, as follows: PI+ (not viable), PI-/PNA+ (viable with a damaged acrosome, dACRO), and PI-/PNA- (viable with an intact acrosome, VIAB).

2.2. Mitochondrial status

Triple staining with YO-PRO-1, Mitotracker Deep Red (Invitrogen) and Hoechst 33342 was conducted. The sperm samples were diluted (1-2 x 10^6 spermatozoa/ml) in 300 µL of PBS and 100 nM YO-PRO-1, 100 nM Mitotracker Deep Red and 5 µM Hoechst 33342 were added. After 10 min at room temperature in the dark, the samples were analyzed using flow cytometry. One population of spermatozoa (Hoechst+) was considered, as follows: Mitotracker+/YO-PRO-1-, which were classified as viable spermatozoa with active mitochondria.

2.3. Flow cytometric analysis

Flow cytometric analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, Miami, FL, USA) equipped with 405 nm, 488 nm and 635 nm lasers. YO-PRO-1, PNA-FITC and PI were excited at 488 nm, Mitotracker Deep Red was excited at 635 nm and Hoechst 33342 was excited at 405 nm. The fluorescence emitted by YO-PRO-1 and PNA-FITC was analyzed using the FL1 photodetector (530/40-nm band pass filter), the PI and Mitotracker Deep Red fluorescence was analyzed using the FL3 photodetector (670-nm long-wavelength pass filter) and Hoechst 33342 fluorescence was analyzed using FL6 photodetector (450/50-nm band pass filter). The signals of the forward scatter/side scatter (FSC/SSC) and the Hoechst 33342 fluorescence were used to discriminate the sperm population from other events and debris. For each sample, we acquired 5000 spermatozoa using Summit v 4.3 software (Beckman Coulter). The analysis of the flow cytometric data were performed using the Weasel v.2.6 program (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

Results

Experiment 1: Qualitative analysis of the spermatozoa that migrated through the artificial mucus penetration test, evaluated using pooled semen.

The motility, viability (VIAB), acrosomal damage (dACRO) and mitochondrial activity (hMITO) of the fresh sperm sample and the spermatozoa that migrated (except motility) were evaluated (**Table S1**). The spermatozoa that migrated presented a higher level of acrosomal damage and lower mitochondrial activity and viability than did the control sample ($P<0.05$). The main components obtained after PCA of the four primary morphometric parameters obtained using all the spermatozoa (pre-and post-migrating samples) in the same analysis (3 Pools, 3 replicates) are shown in **Table S2**.

Experiment 3: Evaluation of the sperm subpopulations defined by head morphology after performing the penetration test using semen samples damaged by oxidation

The quality of the sperm of the fresh samples, the samples after 2-h incubation with the oxidant substance and the spermatozoa from the oxidized sample that migrated through the mucus surrogate in the penetration test are shown in **Table S3**. The motility, viability, acrosomal status and mitochondrial-membrane potential were negatively affected by the oxidant treatment. The spermatozoa in the oxidized sample that migrated showed a lower level of viability and a higher mitochondrial membrane potential than those of the control and oxidized samples ($P<0.05$), but the percentage of spermatozoa with damaged acrosomes differed only from that of the control sample.

Experiment 4: Relationship between performance in the artificial mucus-penetration test, the head morphometry values and fertility

The motility, viability, acrosomal damage and mitochondrial activity of spermatozoa that migrated in the fresh sperm of 7 males were evaluated (**Table S4**) and significant differences were observed among the males.

Discussion

In experiment 1, we observed that the spermatozoa that migrated through the synthetic mucus showed a modest reduction in viability (10%) and a significant reduction in the percentage of spermatozoa with a high mitochondrial membrane potential (33%), as in a previous study (Martínez-Rodríguez e*t al.* 2014). Windsor & White (1995) observed that the mitochondrial activity in ram spermatozoa decreased during procedures associated with frozen storage due to the effects of various stresses and suggested that this could be a factor in the reduced fertility of frozen-thawed ram semen. This decrease in the mitochondrial membrane potential was also observed by Martin e*t al.* (2007) during the cryopreservation of bovine spermatozoa, although they found it to be a reversible process because a significant proportion of the spermatozoa showed a normal mitochondrial membrane potential when returned to physiological conditions.

The energy required for mammalian sperm motility is generated from intracellular ATP. This energy can be provided either through mitochondrial oxidative phosphorylation or glycolysis, independent of one another (Storey 2008). Most of the energy needed for mouse sperm motility is generated by glycolysis (Miki e*t al.* 2004), although in bull sperm, oxidative phosphorylation is the predominant pathway for energy generation; therefore, the performance of similar studies using sperm from different mammals might reveal species-specific differences (Storey 2008).

Bull spermatozoa are dependent upon respiratory metabolism during passage through the cervix (Storey 2008) and consequently, if ram sperm have the same behaviour, that fact would explain the reduction in mitochondrial activity observed in the present study. The synthetic mucus barrier present in the *in-vitro* test incurred physiological stress on the spermatozoa, which may explain the decrease in the mitochondrial activity in the spermatozoa that migrated. Therefore, as described for bull semen (Martin e*t al.* 2007), it can be hypothesized that a proportion of the viable spermatozoa with low mitochondrial membrane potential that migrated in the present

study might recover a normal mitochondrial membrane potential when returned to the resting conditions.

2. Oxidative stress and sperm head morphometry

Oxidative stress negatively affected the total motility, viability, and the acrosomal and mitochondrial membrane status of the spermatozoa. Despite this, the number of spermatozoa that migrated was similar in the oxidized semen and the control sample, which is consistent with the similarities in the progressive motility and kinetic parameters observed in both samples.

REFERENCES

Martin G, Cagnon N, Sabido O, Sion B, Grizard G, Durand P, Levy R 2007 Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum Reprod* 22 380-388.

Martínez-Rodríguez C, Alvarez M, López-Urueña E, Gomes-Alves S, Anel-López L, A Chamorro C, Anel L, de Paz P 2014 Ram spermatozoa migrating through artificial mucus in vitro have reduced mitochondrial membrane potential but retain their viability. *Reprod Fertil Dev* 2014; http://dx.doi.org/10.1071/RD13377

Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA 2004 Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc Natl Acad Sci USA* 101 16501-16506.

Petrunkina AM, Harrison RAP 2011 Mathematical analysis of mis-estimation of cell subsets in flow cytometry: Viability staining revisited. *Journal of Immunological Methods* 368 71–79.

Storey BT 2008 Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* 52 427-437.

Windsor D, White I 1995 Mitochondrial injury to ram sperm during procedures associated with artificial insemination or frozen storage. *Animal Reproduction Science* 40 43-58.

Table S1. Analysis of pooled semen (mean values ± SEM). Motility parameters of the viable spermatozoa (VIAB), spermatozoa with acrosomal damage (dACRO) and spermatozoa with a high mitochondrial-membrane potential (hMITO) in the fresh sample (Control) and of the spermatozoa that migrated through the mucus surrogate (Migrating) during a 30-min incubation period at 39°C (3 pools, 3 replicates).

	Control		**Migrating**	
TM	87.6 ± 2.2			
PM	66.5 ± 2.1			
VAP	140.9 ± 4.7			
VCL	215.4 ± 6.7			
VSL	111.1 ± 4.3			
LIN	51.9 ± 1.4			
STR	76.1 ± 0.8			
WOB	66.4 ± 1.2			
ALH	3.8 ± 0.1			
BCF	26.3 ± 0.6			
VIAB	76.4 ± 1.14	A	68.9 ± 0.8	B
dACRO	8.4 ± 0.6	A	13.6 ± 1.1	B
hMITO	70.8 ± 2.9	A	47.5 ± 1.2	B

TM, total motility (%); PM, progressive motility (%); VAP, average path velocity (μm s^{-1}); VCL, curvilinear velocity (μm s^{-1}); VSL, straight-line velocity (μm s^{-1}); LIN, linearity index (%); STR, straightness (%); ALH, amplitude of lateral head displacement (μm); BCF, head beat-cross frequency (%); VIAB, viable spermatozoa (IP-/PNA-); dACRO, acrosomal damage (IP-/PNA+); hMITO, spermatozoa with a high mitochondrial-membrane potential (Mitotracker+/YO-PRO-1-).

The letters indicate significant differences between the samples (Control and Migrating).

Table S2. Analysis of pooled semen. A) Variances (eigenvalues) and eigenvectors for the three main components obtained after PCA of the four primary morphometric parameters obtained for the fresh and migrating samples (3 Pools, 3 replicates for each). The values for the morphometric parameters (mean values ± SEM) that defined each of the three clusters obtained using PCA and data clustering of the values for the fresh samples (the relative size of each cluster is noted as the proportion) are shown. B) Variances of the three main components obtained using PCA of the values for six morphometric parameters of the fresh and migrating samples (3 Pools, 3 replicates for each).

A) 4-PARAMETER					
VARIANCES	**EXPLAINED**		**CUMULATIVE**		
Prin1	0.78		0.78		
Prin2	0.17		0.96		
Prin3	0.03		0.99		
EIGENVECTORS	**AREA**	**PERIMETER**	**LENGTH**	**WIDTH**	
Prin1	0.552	0.540	0.460	0.437	
Prin2	0.070	-0.107	-0.661	0.739	
Prin3	0.155	-0.809	0.481	0.297	
PARAMETERS	**AREA**	**PERIMETER**	**LENGTH**	**WIDTH**	**L/W**
CLUSTER 1 (40.8%)	33.6 ± 1.7	22.1 ± 0.6	8.6 ± 0.3	3.7 ± 0.1	2.4
CLUSTER 2 (17.1%)	36.8 ± 1.8	23.3 ± 0.6	8.9 ± 0.3	4.1 ± 0.1	2.2
CLUSTER 3 (42.1%)	36.5 ± 2.1	22.8 ± 0.6	8.9 ± 0.3	3.9 ± 0.1	2.3
B) 6-PARAMETER					
VARIANCES	**EXPLAINED**		**CUMULATIVE**		
Prin1	0.53		0.53		
Prin2	0.28		0.81		
Prin3	0.17		0.98		

The variances, which were derived from the eigenvalues, indicate the proportion of the total variance explained by each principal component (Prin). The eigenvectors are a measure of the association of the original parameters with the resulting principal components.

The parameters (mean values and SEM) are the morphometric descriptors of the sperm head for each cluster (subpopulations) found using PCA-cluster analysis (L/W is the Length/Width ratio). The relative number of spermatozoa in the seminal sample belonging to each cluster is indicated in parentheses as a percentage.

Table S3. Analysis of oxidized semen samples (3 Pools, 3 replicates). The values for the motility parameters, VIAB, dACRO and hMITO for the three following types of samples (mean value ± SEM): 1) fresh semen (Control); 2) semen sample after a 2-h incubation period with an oxidizing substance (OXI_Damage), 3) spermatozoa from the oxidized sample that migrated through the mucus surrogate during a 30-min incubation period at 39 °C (OXI_Migrating) are shown.

	Control		**OXI_Damage**		**OXI_Migrating**	
TM	90.9 ± 0.6	a	82.6 ± 7.2	b		
PM	71.1 ± 1.2		71.1 ± 3.1			
VAP	140.5 ± 3.8		136.5 ± 10.1			
VCL	219.3 ± 6.7		216.2 ± 8.9			
VSL	107.1 ± 3.3		113.6 ± 10.7			
LIN	49.6 ± 1.2		52.6 ± 3.9			
STR	74.7 ± 0.9	a	81.8 ± 2.2	b		
WOB	65.1 ± 1.2		63.6 ± 3.5			
ALH	3.8 ± 0.1		3.5 ± 0.2			
BCF	27.5 ± 0.4		32.1 ± 1.9			
VIAB	81.2 ± 2.5	a	70.5 ± 1.4	b	62.8 ± 1.6	c
dACRO	6.3 ± 0.4	a	18.7 ± 1.7	b	17.7 ± 1.3	b
hMITO	66.2 ± 5.1	a	51.8 ± 5.2	b	43.6 ± 2.6	c

See description for Table S1.

The letters indicate significant differences between the samples (Control, OXI_Damage and OXI_Migrating).

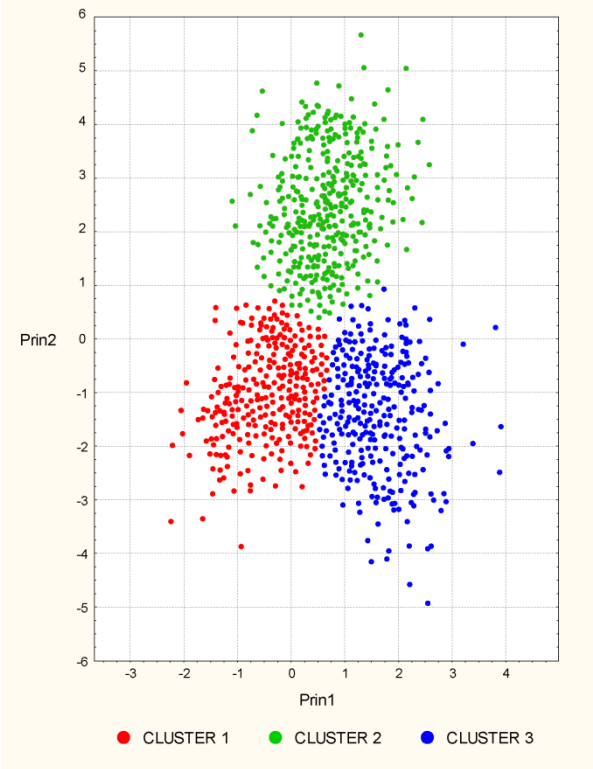
Table S4. Analysis of the semen samples used for insemination (7 males, 3 replicates) for motility, viability, damaged acrosomes and a high mitochondrial-membrane potential of the spermatozoa (mean values ± SEM).

MALE	TM		PM		VIAB		dACRO		hMITO	
M131	93.9 ± 1.7	a	74.5 ± 2.6	a	90.7 ± 1.1	A	7.8 ± 0.8	a	73.9 ± 9.1	ab
M190	80.9 ± 7.3	b	60.9 ± 6.7	a	87.8 ± 1.8	ab	8.4 ± 1.5	a	79.9 ± 4.3	ab
M239	94.1 ± 2.2	a	73.8 ± 1.1	a	90.6 ± 2.1	ab	7.5 ± 1.6	a	70.6 ± 9.3	a
M507	85.3 ± 1.5	ab	63.6 ± 5.7	a	84.5 ± 1.9	ab	13.3 ± 1.6	b	75.2 ± 8.6	ab
M785	76.2 ± 16.2	b	60.8 ± 12.1	a	81.5 ± 5.2	B	14.6 ± 3.1	ab	70.1 ± 8.3	b
M919	85.5 ± 9.6	ab	58.2 ± 6.5	a	89.9 ± 1.1	ab	7.2 ± 0.8	a	75.4 ± 9.9	ab
M920	74.1 ± 11.1	b	58.8 ± 10.1	a	84.7 ± 4.1	B	10.8 ± 2.1	ab	80.1 ± 5.9	b

Different letters indicate significant differences among the males' specimens.

Figure S1. Plots of PC1 and PC2 (3 Pools, 3 replicates) showing the three clusters defined in the analysis for the fresh (a) and migrating (b) samples. The plots were obtained with STATISTICA 9 (StatSoft Inc. Tulsa, OK, USA) and merged using Illustrator CS6 (Adobe, San José, CA, USA)

a)



b)