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Relationship Between the Characteristics of Epididymal Red Deer Spermatozoa and Penetrability Into Zona-Free Hamster Ova

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ABSTRACT: A heterologous (zona-free hamster oocytes) in vitro fertilization (IVF) system was used to evaluate the relationship between sperm factors and penetration capacity of epididymal red deer spermatozoa. The sperm parameters evaluated in 36 sperm samples obtained postmortem from stags selectively shot during the rutting season were sperm motility, functional integrity of plasma membrane by means of the hypo-osmotic swelling test (HOST), and, simultaneously, viability and acrosomal status via a triple-stain technique. Zona-free hamster oocytes were used to evaluate the capacity of the different sperm assays to predict in vitro penetration. In order to increase the variability in sperm quality, we recovered samples from stags at different intervals between the death of the male and the collection of the genitalia. All measures of sperm quality declined progressively ($P < .001$) with increasing intervals between death and sample collection. In addition, many sperm parameters were related to penetration ability in vitro. Subsequently, sperm samples were rearranged in 2 categories according to the interval that had elapsed between death and the collection of the genitalia (group 1, short interval = 0–12 h; group 2, large interval = 18–40 hours). When samples were grouped, less correlation achieved significance, especially for group 1, than when samples were not divided. Also, correlation between the number of sperm per oocyte and sperm parameters for group 1, which had the highest values of sperm quality, failed to reach significance. It is concluded that the classical parameters accepted in assessing the viability of deer spermatozoa can be good predictors of the penetrating ability of the spermatozoa when satisfactory in vitro conditions are used for the development of the IVF system. Also, this study demonstrates that compatible heterologous gamete interaction allows thorough assessment of sperm function in a wild deer.

Key words: *Cervus elaphus hispanicus,* heterologous IVF, postmortem, sperm function.

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Assisted reproductive techniques have demonstrated to be successful tools for use in propagating wild and endangered species. In addition, these techniques allow the production of embryos from gametes obtained from dead, injured, or old males (Wildt et al, 1986; Comizzoli et al, 2001a). This interest in preserving germ plasm of wild species has resulted in greater attention to the possible recovery of sperm from the epididymides of dead animals. Most of the studies have used the mouse as a model (Songsasen et al, 1998; An et al, 1999). Furthermore, in many cervid species, hunter-killed males may represent a readily available source of sperm for captive breeding programs (Hishinuma et al, 2003). Such is the situation of the Iberian red deer (*Cervus elaphus hispanicus*), which is one of the most appreciated wild ungulate species in Spain. Germ plasm conservation of Iberian red deer offers the possibility of genetic variability preservation via biotechnological reproduction programs.

We have demonstrated that viable epididymal spermatozoa can be retrieved from dead stags maintained at room temperature (approximately 20°C) as long as 24 hours after death (Garde et al, 1998). In addition, we have recently reported that viable spermatozoa can be recovered from deer epididymides stored at 5°C as long as 4 days after death (Soler et al, 2003). However, before we can use them to obtain offspring it is necessary to understand the characteristics and viability of sperm cells obtained from dead animals and to examine the most important factors that affect their fertilizing ability. The fertilizing ability of spermatozoa is governed by various factors. Spermatozoa that lack at least one of those factors are not capable of fertilizing an oocyte in vivo. In this sense, assessment of sperm quality by evaluation of standard semen parameters is a routine procedure in the prediction of male fertility. However, the best assessment of in vitro function involves sperm-oocyte interaction (Bavister, 1990).

Heterologous in vitro fertilization (IVF) is an attractive method for evaluating the fertilizing capacity of sperm

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samples in rare or wild species because it does not require the use of valuable homologous gametes. Cross-species fertilization of oocytes from domestic farm species has proven successful using cryopreserved sperm from deer (Comizzoli et al, 2001b), and nondomestic Bovidae (McHugh and Rutledge, 1998; Roth et al, 1999). Recently, sperm function was evaluated in an endangered African antelope, the scimitar-horned oryx (*Oryx dammath*), by means of a heterologous IVF system with zona-intact domestic cow oocytes (Roth et al, 1998; 1999). Human sperm function is assessed by the sperm penetration assay (SPA) with zona-free hamster oocytes (Yanagimachi, 1984), and also has been used in several domestic species (Davis et al, 1987; Berger and Parker, 1989; Hammitt et al, 1989; Choudhry et al, 1995; Berger et al, 1996) and wild species (Lambert et al, 1991). Standardized SPA protocols are highly successful (Berger and Parker, 1989), and, if deer sperm are capable of interacting with hamster oocytes, this heterologous IVF could offer a valuable method of studying sperm functionality in this species.

As a consequence of the references cited above, the interest in the conditions of wild ungulates using IVF has led to studies designed to evaluate the factors that support successful IVF of oocytes, such as maturation and fertilization media, culture conditions, the male effect, and semen dilution. Nevertheless, knowledge of sperm factors related to penetration capacity is limited, especially in wild ungulate species. Thus, the main purpose of the present study was to determine the relationship between sperm characteristics in a semen deer specimen and the potential ability of spermatozoa from that sample to fertilize an oocyte as measured by the sperm's ability to penetrate a zona-free hamster ovum in vitro. In addition, we analyzed the influence on sperm parameters of the time lapse between the male death and the collection of the genitalia.

Materials and Methods

Animals and Preparation of Testes

Sperm samples were collected from stags that were selectively shot during the rutting season (September–October). A total of 36 mature males (age >3.5 years, weight >135 kg) were used. Game keepers collected the complete male genitalia and provided the hour of the death and a tooth to assess the age of the individuals. Stags were legally culled and hunted in their natural habitat in accordance with the harvest plan of the game reserve. The harvest plans followed the Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union regulations. These plans aim to keep the ecological equilibrium of the game populations. Male genitalia were removed at different intervals after the death of the individuals, but in all cases genitalia were transported to the laboratory in portable box at room temperature immediately after removal. The gonads arrived at the laboratory within 2 hours after being removed. For collection of epididymal spermatozoa, testes and epididymides were removed from the scrotal sac. Cauda epididymides, including about 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes.

Collection of Epididymal Spermatozoa

Sperm samples were recovered by injecting 2 mL of sodium citrate-egg yolk medium (Krzywinski and Jaczewski, 1978) retrogradely through the ductus deferens and collecting the epididymal spermatozoa from the proximal end of the severed ductus epididymidis (Amann et al, 1982; Garde et al, 1998). Most of the sperm present in the cauda epididymis presumably were recovered using the described procedure (Amann et al, 1982). The samples were collected directly into this medium, and thus the color and the consistency of the sperm samples could not be estimated. This procedure was necessary because sperm samples at the height of the rutting period have a consistency similar to that of thick honey and stick to the plastic dish walls, thus making subsequent dilution very difficult. Samples obtained from different epididymides from the same individual were mixed and analyzed jointly, because our previous observations showed that no differences appeared to exist in cell quality between testes belonging to the same individual (Garde et al, 1998).

To develop this work, the sperm samples were recovered from the cauda epididymidis because previous studies in several species have demonstrated that these cells are functionally mature and have a fertilizing potential equivalent to that of ejaculated sperm (reviewed in Robaire and Hermo, 1988; Cooper, 1998).

Determination of Traditional Semen Characteristics

After spermatozoa incubation at 37°C for 30 minutes, a routine semen evaluation was performed. Sperm concentrations of the original suspensions were determined using a hematocytometer. Sperm motility; functional integrity of plasma membrane through the hypo-osmotic swelling test (HOST); and, simultaneously, viability and acrosomal status, by a triple-stain technique (TST), were assessed for each sample to determine standard sperm characteristics in vitro. Percentage of individual motile sperm (motility) was noted and quality of motility was assessed using a scale of 0 (lowest), to 5 (highest). A sperm motility index [SMI; = % individual motility + (quality of motility × 20) × 0.5] was calculated following the method used by Comizzoli et al (2001b) for deer semen.

Plasma membrane integrity was assessed using a HOST as described by Garde et al (1998). The osmotic swelling technique consisted of mixing 0.01 mL of diluted sperm sample with 0.1 mL of hypo-osmotic solution (100 mOsmol/kg) and incubating the mixture at room temperature for 30 minutes. The samples were then fixed in 3% glutaraldehyde-buffered solution and evaluated by phase-contrast microscopy at ×400. The sperm membrane was considered intact in cases in which the sperm tail was coiled, according to the report by Jeyendran et al (1984).

Sperm viability and acrosomal status were simultaneously determined by TST. The details of the TST have been described previously (Garde et al, 1997). Briefly, 100 μL of the sperm suspension were incubated for 10 minutes at 37°C with an equal volume of TCM-199 containing 1% Trypan blue, a vital dye that

selectively stains dead sperm. Afterward, the samples were smeared on prewarmed glass slides and air-dried. The slides were rinsed in water and blotted. The smears were then fixed in 3% glutaraldehyde solution, in 0.1 M cacodylate buffer pH 7.4, at room temperature for 30 minutes, and then were rinsed with water and air-dried. The smears were stained in 0.8% Bismark brown at 40°C for 10 minutes, rinsed in water, and air-dried. Finally, smears were stained with 0.8% rose Bengal at 24°C for 20 minutes, thoroughly rinsed in water, and air-dried. After mounting, the slides were examined at 1000× with a light microscope. Four classes of deer spermatozoa can be distinguished with TST: 1) live spermatozoa with normal acrosomes (light brown post-acrosomal regions with red acrosomes), 2) live spermatozoa without normal acrosomes (true acrosome reaction, light brown postacrosomal regions with unstained acrosomal regions), 3) dead spermatozoa with normal acrosomes (dark brown postacrosomal regions with dark red acrosomes), and 4) dead spermatozoa without normal acrosomes, false acrosome reaction, dark brown postacrosomal regions with unstained or dark blue acrosomal regions).

Sperm Penetration Assay

The sperm samples were preincubated in Biggers, Whitten, and Whittingham (BWW) medium to induce the acrosome reaction, as previously described for ram spermatozoa (Garde et al, unpublished data). Briefly, the sperm samples were resuspended in 5 mL of BWW + 0.3% bovine serum albumin (BSA; Fraction V, Sigma, Madrid, Spain) at a concentration of 5×10^6 cells/mL and preincubated at 37°C for 6 hours in an atmosphere of 5% CO_2 in air. Following preincubation, the spermatozoa were used for insemination of zona-free hamster oocytes.

The sperm penetration assay was developed following the procedure described previously for human spermatozoa (Yanagimachi et al, 1976) and modified by our own group for ram semen (Garde et al, unpublished data). Zona-free hamster oocytes were prepared as described below. Prepubertal female golden hamsters were induced to superovulate by i.p. injection of 30 IU equine chorionic gonadotropin (Folligon, Intervet, Madrid, Spain), followed 51–54 hours later by an i.p. injection of 30 IU human chorionic gonadotropin (hCG; Chorulon, Intervet). Oocytes were recovered 15–17 hours after hCG injection from the ampullary portion of the oviducts and were treated with 0.1% (w/v) hyaluronidase (Type I, Sigma) to remove the cumulus oophorus. The oocytes were then washed 3 times with fresh BWW medium, transferred to a droplet containing 0.1% (w/v) trypsin (Type III, Sigma) to dissolve the zona pellucida, and then washed 3 times with BWW medium. Groups of about 15 oocytes each were transferred into 0.35 mL of BWW + 0.3% BSA under paraffin oil in a plastic culture dish. The preincubated sperm (0.05 mL) were added to the fertilization medium to make a final concentration of 6.2×10^5 spermatozoa/mL. After culture for 5 hours (5% CO_2 in air at 37°C), the oocytes were washed with BWW medium by gentle and repeated aspiration with a Pasteur pipette, mounted on a glass slide, and examined microscopically for sperm penetration. The occurrence of penetration was determined according to the criteria described by Yanagimachi et al (1976). The presence of an enlarged sperm head, male pronucleus (or both) with a tail in the vitellus was taken as evidence of sperm penetration. The percentage of penetrated eggs (PP, number of eggs penetrated/number of eggs examined) and the penetration index (PI, number of decondensed sperm heads/number of eggs penetrated)—to reflect polyspermy—were recorded. Two independent replicates of 15 eggs each were developed per sample.

Experimental Design

In order to have a great variability in sperm quality, we recovered samples from stags at different intervals between the death of the individuals and the collection of the male genitalia. These time lapses were <3, 3–6, 6–12, 12–18, 18–24, and 24–40 hours. Subsequently, sperm samples were rearranged in 2 categories according to the interval elapsed between death and the collection of the genitalia (group 1, short interval = 0–12 hours; group 2, large interval = 18–40 hours).

The entire experiment was repeated 5 times; thus, there were 6 replicates (ie, 6 males/postmortem interval) in this study. Two hundred sperm cells were assessed in each sample and for each sperm evaluation technique.

Statistical Analysis

All statistical analyses were performed with the use of the general linear models procedures of the Statistical Analysis Systems Institute (1989). Data were expressed as least squares means (LSM) ± SEM. Analysis of variance (ANOVA) was used to compare the effects on sperm parameters of the interval lapse between the death of the individual and the collection of the sample. When ANOVA revealed a significant effect, means were compared with the Fisher least significant difference procedure.

Linear regression was used to further investigate relationships between the sperm characteristics and the results of the SPA. Pearson coefficients of correlation were calculated to determine the relationship among variables.

Results

We obtained spermatozoa from epididymides of all stags. Thus, a total of 36 epididymal sperm samples were collected from 36 hunter-killed mature males. The LSM ± SEM for the sperm parameters assessed in the 36 samples are outlined in Table 1. Overall, the values for the semen parameters evaluated showed a large variability among samples. In this sense, the results of the ANOVA procedure revealed that the quality of samples was significantly ($P < .001$) affected by the time elapsed between the death of the individuals and the collection of the male genitalia.

Characteristics of sperm samples collected at various postmortem times are shown in Table 2. Overall, all measures of sperm quality declined progressively with increasing elapsed time between death and sample collection. In this sense, the percentage of live spermatozoa decreased from ~83% at hour 0 to ~40% at 40 hours. Similar results were obtained for the percentage of swollen sperm by HOST (range = ~75% at hour 0 to ~23%

Table 1. *Overall assessment results of sperm characteristics in epididymal samples collected from red deer cadavers at different intervals**

Sperm characteristics	Overall LSM ± SEM	Range
Total number of sperm ($\times 10^9$)	1.6 ± 0.3	0.8–4.5
Motility (%)	50.0 ± 0.7	18.0–72.8
SMI (%)	44.7 ± 0.8	15.2–70.2
Viability (%)	68.7 ± 0.6	37.2–86.2
Normal acrosomes (%)	74.7 ± 0.4	55.0–90.5
HOST, swollen sperm (%)	56.3 ± 0.5	21.3–77.5
True acrosome reaction (%)	3.4 ± 0.2	0.6–6.9
False acrosome reaction (%)	21.5 ± 0.5	5.1–39.7
Percentage of penetration	55.7 ± 0.6	10.3–75.7
Penetration index	2.8 ± 0.1	0.7–4.2

* Data were obtained from epididymal sperm samples collected from 36 stags.

Table 3. *Correlations between results of sperm penetration assay and standard sperm characteristics in epididymal red deer sperm samples**

	Correlation with PP		Correlation with PI	
Sperm characteristics	*r*	*P* value	*r*	*P* value
SMI (%)	.84	<.001	.63	<.001
Viability (%)	.91	<.001	.67	<.001
Normal acrosomes (%)	.83	<.001	.63	<.001
HOST, swollen sperm (%)	.91	<.001	.68	<.001
True acrosome reaction (%)	−.61	<.001	−.38	<.021
False acrosome reaction (%)	−.80	<.001	−.61	<.001

* Data were obtained from epididymal sperm samples collected from 36 stags.

at 40 hours). Moreover, the percentage of normal acrosomes deteriorated from ~88% at hour 0 to ~57% at 40 hours. However, plasma membrane (evaluated through HOST or TST) and acrosome integrities of spermatozoa retrieved within the first 12 hours after death were similar to those collected immediately after death. In contrast, the SMI appeared to be much more sensitive to the interval elapsed between the death and sample collection. Thus, the SMI decreased significantly from the initial average value of ~66% to ~59% (despite only 6 hours since postmortem; $P < .05$), up to less than 20% 24 to 40 hours postmortem.

Results of the SPA developed with spermatozoa collected at various times after the stags had been culled are shown in Table 2. There were no significant differences in percentage of penetrated oocytes when the spermatozoa used for SPA had been collected from 0 to 12 hours postmortem. However, the PP was significantly lower when oocytes were inseminated with spermatozoa retrieved from 18 to 40 hours after the death of the stag ($P < .05$). Similar results were obtained for the PI, as this parameter remained unaffected within the first 18 hours after the death of the stag. This parameter, however, decreased significantly thereafter ($P < .05$).

The correlation coefficients between the standard semen parameters and the results of SPA are listed in Table 3, and all were statistically significant. For the percentage of penetrated oocytes, the highest correlation coefficients ($r = .91$, $P < .001$) were obtained with plasma membrane integrity evaluated through HOST or TST. Similarly, good correlations were found between PP and the percentages of spermatozoa with normal acrosomes ($r = .83$, $P < .001$) and motile cells ($r = .84$, $P < .001$). However, our results showed that there were good, but inverse correlations between PP and true acrosome reaction ($r = -.61$, $P = .001$), and between PP and false acrosome reaction ($r = -.80$, $P < .001$). By contrast, the relationship between the standard sperm parameters and the PI was lower than between these and the PP (Table 3). Finally, the relationship between PP and PI is shown in Figure 1 ($r = .66$, $P < .001$).

Sperm samples collected 18–40 hours (group 2) postmortem showed that values of PP and PI were significantly lower ($P < .001$) than those recovered 0–12 hours

Table 2. *Sperm parameters from epididymal sperm samples collected at different intervals after death of stag**

	Postmortem interval (h)†					
Sperm characteristics	0–3	3–6	6–12	12–18	18–24	24–40
Motility (%)	69.2 ± 1.7^{a}‡	69.2 ± 1.7^{a}	66.7 ± 1.7^{a}	37.5 ± 1.7^{b}	35.8 ± 1.7^{b}	21.6 ± 1.7^{c}
SMI (%)	66.2 ± 1.9^{a}	59.6 ± 1.9^{b}	55.0 ± 1.9^{b}	37.1 ± 1.9^{c}	31.2 ± 1.9^{d}	19.2 ± 1.9^{e}
Viability (%)	82.8 ± 1.4^{a}	83.1 ± 1.4^{a}	80.2 ± 1.4^{a}	63.2 ± 1.4^{b}	63.0 ± 1.4^{b}	40.2 ± 1.4^{c}
Normal acrosomes (%)	88.5 ± 1.0^{a}	86.8 ± 1.0^{a}	84.8 ± 1.0^{a}	67.3 ± 1.0^{b}	65.7 ± 1.0^{b}	57.0 ± 1.0^{c}
HOST, swollen sperm (%)	75.0 ± 1.2^{a}	75.5 ± 1.2^{a}	74.5 ± 1.2^{a}	52.6 ± 1.2^{b}	39.3 ± 1.2^{c}	23.8 ± 1.2^{d}
True acrosome reaction (%)	1.8 ± 0.6^{a}	2.3 ± 0.6^{ab}	3.5 ± 0.6^{ab}	3.3 ± 0.6^{ab}	3.8 ± 0.6^{b}	5.6 ± 0.6^{c}
False acrosome reaction (%)	7.5 ± 1.1^{a}	10.8 ± 1.1^{ab}	13.5 ± 1.1^{b}	29.3 ± 1.1^{c}	30.5 ± 1.1^{c}	37.3 ± 1.1^{d}
Percentage of penetration	72.5 ± 1.6^{a}	70.2 ± 1.6^{a}	71.0 ± 1.6^{a}	65.3 ± 1.6^{b}	45.7 ± 1.6^{c}	13.3 ± 1.6^{d}
Penetration index	3.4 ± 0.3^{a}	3.5 ± 0.3^{a}	3.2 ± 0.3^{ab}	2.9 ± 0.3^{ab}	2.4 ± 0.3^{b}	1.4 ± 0.3^{c}

* Data are expressed as least squares means ± SEM.
† Six samples belonging to 6 different stags were used for each interval analyzed.
‡ Values with different superscripts within each row are significantly different ($P < .05$).

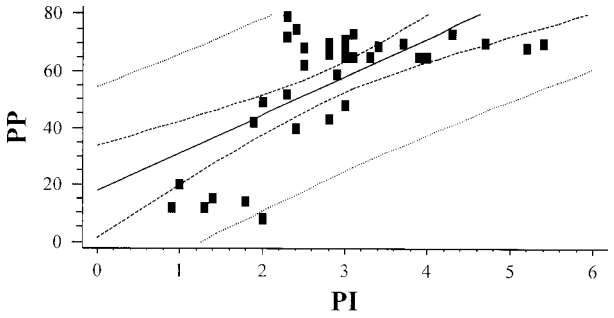

Figure 1. The penetration index (solid points) of epididymal sperm samples plotted as a function of the percentage of penetration into hamster oocytes of the same epididymal samples. The correlation coefficient is $r = .66$ (n = 36; $P < .0001$).

(group 1) after death (41.5 ± 5.3 vs 69.0 ± 0.9 and 2.2 ± 0.1 vs 3.4 ± 0.2, respectively). Nevertheless, when the relationships between sperm characteristics and SPA were studied in the 2 groups, relationships were lower for group 1 than for group 2 (Tables 4 and 5). For PP, correlation coefficients were statistically significant in both groups, but the relationships for group 2 were stronger than those for group 1. However, standard sperm parameters were related to PI only for group 2. The relationships between PP and PI for both groups of samples are shown in Figure 2. For group 2 (Fig. 2b), there was a good correlation between the number of sperm per oocyte and the penetration percentage ($r = .83$, $P < .001$). However, the relationship was not significant for group 1 (Fig. 2a; $r = -.24$, $P = .32$).

Discussion

The interest in preserving germ plasm of wild species has resulted in an greater attention to the possible recovery of sperm from the epididymides of dead animals. Such is the situation of many cervid species, in which killed-hunter males may represent a readily available source of sperm for captive breeding programs. However, up to now, almost no information was available on the relationship between the characteristics of deer spermatozoa obtained from epididymides and the potential ability of spermatozoa from that sample to fertilize oocytes in vitro.

Table 4. *Linear regressions of semen measurements with average percentage of oocyte penetration**

Sperm characteristics	Group 1† Short interval		Group 2 Large interval	
	r	*P* value	*r*	*P* value
SMI (%)	.56	.01	.89	<.001
Viability (%)	.58	.01	.88	<.001
Normal acrosomes (%)	.72	.0006	.88	<.001
HOST, swollen sperm (%)	.66	.002	.95	<.001
True acrosome reaction (%)	−.32	.19	−.54	.02
False acrosome reaction (%)	−.66	.002	−.78	<.001

* In each group, data were obtained from sperm samples collected from 18 stags.

† Short interval, sperm samples obtained from male genitalia collected 0–12 hours after death; large interval, sperm samples obtained from male genitalia collected 18–40 h after death.

Table 5. *Linear regressions of semen measurements with average number of sperm per penetrated oocytes**

Sperm characteristics	Group 1† Short interval		Group 2 Large interval	
	r	*P* value	*r*	*P* value
SMI (%)	−.03	.87	.85	<.001
Viability (%)	−.04	.85	.79	.001
Normal acrosomes (%)	−.02	.93	.77	.0002
HOST, swollen sperm (%)	.03	.90	.82	<.001
True acrosome reaction (%)	.10	.68	−.45	.05
False acrosome reaction (%)	.01	.94	−.68	.001

* In each group, data were obtained from sperm samples collected from 18 stags.

† See Table 4 for explanation of experimental groups.

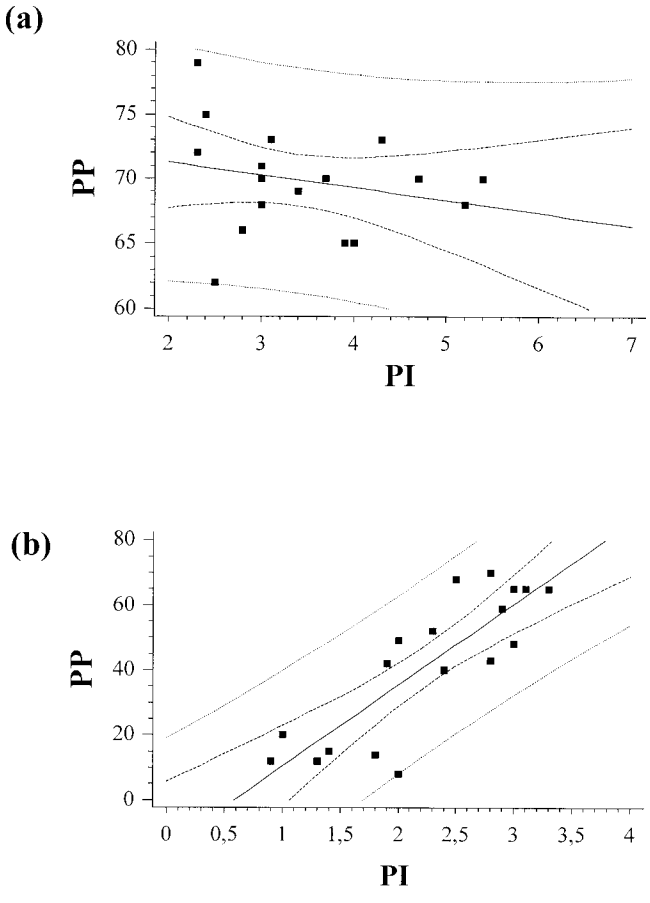

Figure 2. The penetration index (solid points) of group 1 **(a)** and group 2 **(b)** are plotted as a function of the percentage of penetration into hamster oocytes. The correlation coefficient for groups 1 and 2 are, respectively, $r = -.24$ (n = 18; $P = .32$), and $r = .83$ (n = 18; $P < .0001$).

Therefore, in the present study we have determined the relationship between sperm factors and the penetration capacity of spermatozoa to fertilize zona-free hamster oocytes, as an estimation of the sperm in vitro function.

To evaluate the influence of the different sperm factors on in vitro fertilizing capacity, samples were collected from stags at different intervals between the death of the individuals up to the collection of the male genitalia. Our results show that the quality of sperm samples is significantly influenced by the time elapsed between death and collection of these (Table 2; $P < .05$). Such a decrease in sperm quality with increasing interval between death of the individual and collection of the gametes might be due to a process of degeneration in spermatozoa stored in the male genitalia after death. These results agree with those reported by An et al (1999) and Songsasen et al (1998) for mice spermatozoa.

Generally few relationships were found between standard sperm characteristics and in vitro penetration ability (Martinez et al, 1996), and when they were significant, the regression coefficient was low (Hammitt et al, 1989). Few single sperm parameters appear to correlate significantly with in vitro penetration, especially when the quality of the samples is within acceptable ranges of normality. In addition, it has been proposed that the lack of correlation between conventional sperm parameters and the SPA suggest that these assays measure different aspects of the viability and fertilizing capacity of spermatozoa (Jeyendran et al, 1984). However, in this study we used sperm samples with very different initial quality and the results showed highly significant correlation coefficients between the results of the SPA and the standard semen characteristics, with higher coefficients for PP than for PI (Table 3).

For PP, the highest correlation coefficients ($r = .91$, $P < .001$) were obtained with plasma membrane integrity evaluated through HOST or TST (viability). Similar results have been reported when homologous (Van der Ven et al, 1986) and heterologous (Jeyendran et al, 1984) IFV systems were used to evaluate human spermatozoa. Suitable sperm metabolism and function requires an intact plasma membrane. The good correlations obtained in our study provide evidence that plasma membrane integrity is important in the fertilization process. The membrane swelling was particularly noticeable at the sperm tail. In this sense, membranes of sperm tail and sperm head may function independently from each other (ie, measuring sperm tail swelling may not be indicative of normal functional activity of the head membranes). This is apparently not the case in our study. Capacitation and fusion with the oocyte involves many changes in the sperm head without which these processes cannot occur. Because a good correlation was present between these events and the ability of sperm tails to swell in a hypo-osmotic solution, it is clear that sperm swelling generally indicates normal head membrane function as well.

With regard to sperm motility, SMI seems to be a good indicator of sperm penetration ability and was highly correlated with PP ($r = .84$). Similar results have been reported previously in several species under specific IVF conditions and systems (Hall, 1981; Xu et al, 1996; Songsasen et al, 1998; Gadea and Matas, 2000; Morris et al, 2001). In this sense, Clarke and Johnson (1987) reported that the ability of boar spermatozoa to penetrate zona-free hamster oocytes decreased when their motility was lower. However, in other studies, no correlation was found between the penetration rate and sperm motility (Martinez et al, 1993).

The acrosome reaction is required for sperm penetration through the zona pellucida of the oocytes and subsequent fusion with the plasma membrane. Our results show a negative relation between the initial percentages of sperm with damaged acrosomes (false AR or true AR) and the penetration rates of zona-free hamster oocytes. However, a strong and positive correlation was found between PP and the initial percentage of normal apical ridge in sperm samples. Generally, no correlations were found between the acrosome status in sperm samples during IVF and the ability of those spermatozoa to fertilize homologous (Vazquez et al, 1993) or heterologous (Berger et al, 1992) oocytes. However, we can see from the results in this study and from other studies developed with boar semen and homologous IVF (Gadea and Matas, 2000) that the acrosome status of fresh spermatozoa affected the results of the in vitro penetration test. Thus, the greater the rate of NAR in the initial sample, the greater the percentage of oocyte penetration. In contrast, the greater the percentage of spermatozoa either that were damaged or acrosome-reacted in fresh semen samples, the worse the results of the sperm penetration test. This happens because sperm death occurs soon after the acrosome reaction has occurred (Kusunoki et al, 1990).

As has been previously described for PP, highly significant correlation coefficients were found between standard sperm factors and PI (Table 3). Likewise, a good correlation was also found between PI and PP (Fig. 1). Similar results were obtained by Hammitt et al (1989), when the SPA was developed with boar spermatozoa ($r = .72$). The number of sperm cells per penetrated oocyte mainly depends on sperm concentration during coculture (Martin and Taylor, 1983; Martínez et al, 1993; Comizzoli et al, 2001a; Berg et al, 2002). In our experimental conditions, the sperm concentration used (6.2×10^5 spermatozoa/mL) provided good information about sperm penetration ability determined by the two parameters (PP and PI).

Although a great number of studies have been carried out in homologous and heterologous IVF, only a few of

them are related with the sperm factors implied in the penetration ability. The literature regarding the influence of sperm factors on in vitro penetration success is confusing. In this sense, in the only study conducted so far regarding heterologous IVF of zona-free bovine oocytes with deer spermatozoa, no relationship was found between standard semen characteristics and in vitro penetration (Comizzoli et al, 2001b). However, in our study, we used a high number of samples and we did not preselect them, so a great number of seminal parameters were found to be related to in vitro penetration ability.

Our results are contrary to those reported in previous studies regarding the influence of sperm factors on in vitro penetration. The contradictory results would be caused by great experimental differences, a few number of ejaculates or IVF trial analyzed, a high number of sperm concentration in the IVF system that could mask the relations or by a preselection of the sperm samples. In our experimental conditions, the sperm concentration used (6.2×10^5 spermatozoa/mL) provided strong relationships between results of SPA and standard sperm parameters. These relationships were not found when authors used higher sperm concentration ($2–10 \times 10^6$ spermatozoa/mL) for the in vitro penetration test (Chan et al, 1985; Comizolli et al, 2001b). The high correlations found in our work between SPA values and standard semen parameters might occur because when sperm concentration in the coculture of the IFV is low, as occurred here, the quality of the semen samples, as determined by standard sperm characteristics, will be shown in the percentage and penetration index of hamster oocytes. In contrast, at high sperm concentrations, the sperm quality of the samples will not result in different sperm penetration rates, because there will always be enough spermatozoa to fertilize oocytes in any time lapse.

Finally, our results also showed that when samples were allocated to 2 groups according to the interval elapsed between male death and the collection of the genitalia, the relationships between standard sperm parameters and PI, and between PP and PI, were significant only for group 2, which was the group that had the lowest sperm quality. These findings emphasize our previous results related to the effect of sperm concentration on in vitro penetration rates. Thus, the group with the worse semen quality, in turn, was the one with the lowest concentration of viable/motile spermatozoa during IFV, and is also the group in which the semen standard factors better predict the fertilizing ability of its samples in vitro. This is also the group that achieved the higher correlations between PI and PP. This may happen because when the concentration of spermatozoa that are able to fertilize decreases in the sample, the relationship between oocyte and viable spermatozoa is remarkably lower than that of group 1, and this indicates that the quality of the sperm sample is clearly shown in the results of the SPA. In contrast, such a close relationship for group 1 either does not appear or it is not very high, because there will always be large number of spermatozoa available for fertilizing oocytes.

In conclusion, the results of the present study showed that when low sperm concentrations were used for the coculture, the classical sperm parameters accepted in assessing the viability of deer spermatozoa can be good predictors of the penetrating ability of the spermatozoa in vitro. In these conditions, this study also demonstrates that compatible heterologous gamete interaction allows thorough assessment of sperm function in a wild deer.

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