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Short communication. Post-thawing evaluation of Spanish ibex *(Capra pyrenaica hispanica)* epididymal spermatozoa recovered *postmortem*

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Abstract

The current study was conducted to describe the sperm parameters of post thawed epididymal spermatozoa collected *postmortem* from 14 Spanish ibex males. All samples were recovered in the rutting season established for this species (December-January). The spermatozoa from 11 Spanish ibex were diluted at environmental temperature (13-14ºC) in a commercial medium (Triladyl®-20% egg yolk). In three males, the sperm mass of each epididymis was diluted in a Triladyl®-20% egg yolk and a Tris-6% egg yolk medium, respectively. A strong individual variation was found for whole sperm parameters: motility (mean ± SD, 48 ± 21.5%), normal morphology (69.4 ± 9.9%), normal acrosomes (75.7 ± 10%), viability (69.4 ± 18.6%). The sperm parameters were significantly ($p < 0.05$) correlated between them. No significant effect of diluents was observed.

Key words: cryopreservation, diluents, epididymis, freezing, spermatozoa.

Resumen

Nota corta. Valoración post-descongelación de células espermáticas epididimarias de macho montés *(Capra pyrenaica hispanica)* recogidas *postmortem*

En el presente estudio se describen los parámetros espermáticos post-descongelación de espermatozoides epididimarios obtenidos *postmortem* de 14 machos monteses. Todas las muestras se obtuvieron dentro de la estación reproductiva definida para esta especie (enero-diciembre). En 11 machos, los espermatozoides se diluyeron, a temperatura ambiente (13-14ºC), en un diluyente comercial (Triladyl®-20% yema de huevo). En otros 3 se utilizó un diluyente diferente para el material espermático procedente de cada testículo (Triladyl®-20% yema de huevo, y un diluyente a base de Tris-6% yema de huevo). Se apreció una importante variabilidad individual en todos los parámetros analizados: motilidad (media ± DE, 48 ± 21,5%), formas normales (69,4 ± 9,9%), acrosomas intactos (75,7 ± 10%), viabilidad (69,4 ± 8,6%). Los parámetros espermáticos estudiados presentaban correlaciones significativas entre ellos ($p < 0,05$). No se apreció un efecto significativo del diluyente.

Palabras clave: criopreservación, diluyentes, epidídimo, congelación, espermatozoides.

The Spanish Ibex (*Capra pyrenaica,* Schinz 1838) is one of the major big game species in our country. This is because it is endemic to Spain and is only found in this country in mountainous regions characteristic of Spanish geography. As well as its attraction as a game species, where it corresponds to the species with the highest economic value, it is also of great ecological value reflecting the special zoological biodiversity characteristic of Spain. Of the four original subspecies defined by Cabrera in 1911 *(Capra pyrenaica hispanica, C. p. victoriae, C. p. lusitanica, C. p. pyrenaica*), only two currently exist. *C. p. lusitanica* became extinct more than one century ago and *C. p. pyrenaica* («bucardo») finally disappeared a few years ago. Owing to outbreaks of mange over the last few decades, combined with the risk of homozygosis because of fragmentation of the habitat, the International Union for the Conservation of Nature and Natural Resources (IUCN) have classified this species

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as «vulnerable». Because of this situation, the development of reproductive technologies aimed at creating banks of genetic resources of Spanish Ibex, constitutes a guarantee to preserve its different populations and ecotypes when faced with situations of population instability that can affect its viability and can also lead to its extinction. The freezing of epididymal spermatozoa obtained after an animal's death is a technique that has been established in other game ungulates such as the mouflon (*Ovis gmelini musimon*) and the red deer (*Cervus elaphus*) (Garde *et al.,* 1995; Soler *et al.,* 2003a), and is very useful to create germplasm banks in non domesticated species.

The aim of the present work was to assess the technique used to collect and freeze epididymal spermatozoa of the Spanish Ibex, collected *postmortem*, using *in vitro* analysis of the sperm parameters after thawing.

Sperm samples were obtained in the National Game Reserve of Almijara and Tejeda (Málaga), during the months of December and January, coinciding with the rutting season (Santiago-Moreno *et al*., 2003), and using 17 animals hunted by stalking. Sperm samples were obtained from a total of 14 male Spanish Ibex aged between 8 and 13 years. Another three males hunted during this period were excluded for orchitis or severe testicular traumatism. The animal's age was determined by the marks of growth on the horns (Losa-Huecas, 1993). The testicles extracted were kept, during transport and during the laboratory processing, at room temperature (11-14ºC, over the study period). The working temperature in the laboratory was similar to that used for collection and transport of the gonads (13-14ºC). After dissection of the testicular envelopes, the diameters of both testicles were measured with a measuring tape, and the sperm material was collected by making small cuts with a scalpel at the tail of the epididymus (Soler *et al*., 2003b). The samples obtained were diluted in Petri dishes in which a small amount (1 ml) of diluent had been previously introduced (Ortiz, 1999). Spermatozoa were obtained in a variable time after the animal's death, ranking from 2 to 32 h. For freezing, the sperm mass was diluted in a medium made up of Tris (3.8% w v^{-1}), glucose (0.6% w v^{-1}), citric acid (2.2% w v^{-1}), egg yolk (6% v v^{-1}) and glycerol (5% v v^{-1}) and in another commercial diluent (Triladyl®-20% egg yolk). In 11 males, only Triladyl® was used while in 3 animals, the sperm from each testicle was diluted, respectively, in each of the previously mentioned diluents. The sperm concentration was determined by a Neubauer hematocytometric plate (Evans and Maxwell, 1990). Spermatozoa were diluted in a single step, with the corresponding volume of medium to achieve a sperm concentration of 800×10^6 spz ml^{-1}. After, the samples were cooled for 60 min to a temperature of +5ºC (cooling rate = –0.17ºC min^{-1}), and were kept, for equilibration, for 150 min at this temperature. Samples were packed in 0.25 ml French straws (IMV®), and were frozen in liquid nitrogen vapors, placed horizontally on a grid kept 5 cm above the level of liquid nitrogen for 10 min (freezing rate = –12ºC min^{-1}) and stored in cryogenic tanks. The sperm samples, containing 200×10^6 spermatozoa, were thawed after 3 months (60 s in a water bath at 37ºC), followed by analysis of the different sperm parameters. The percentage of sperm with progressive mobility was analysed according to the technique described by Evans and Maxwell (1990). The spermatozoa morphology was evaluated using a phase contrast microscope (×400) by counting 200 spermatozoa per sample, fixed in a glutaraldehyde solution at 2% in BL-1 (Ortiz, 1999). The presence of cytoplasmatic droplets was not considered as a morphoanomaly, given the epididymal origin of the spermatozoa, and was evaluated independently. The integrity of the acrosome was determined by phase contrast microscope (×1,000), after fixation of the samples in a glutaraldehyde solution at 2% in BL-1 (Pursel and Johnson, 1974). Spermatic viability was evaluated by staining the samples with eosin-nigrosin (Soler *et al*., 2003a). Sperm parameters after thawing were studied by linear correlation (Pearson). The influence of the time of collection (hours *postmortem*) on the sperm parameters studied was analysed by ANOVA. Differences in the sperm values on thawing between the two diluents were compared by a paired *t*-test, calculating the coefficient of variation (CV) for each parameter studied.

In the males studied, the diameter of the testicular parenchyma (free from scrotal sac) was 4.9 ± 0.4 cm (range: 4.2-5.7 cm). Analysis of the sperm samples after thawing revealed a wide individual variability in the different parameters studied. In the 13 animals in which the sperm was collected within the first 9 h *postmortem* (range: 2-9 h), sperm parameters after thawing, in samples diluted with Triladyl® are recorded in Table 1. The motility was correlated with the percentage of spermatic viability ($R = 0.7$; $p < 0.01$), with the percentage of normal forms ($R = 0.7$; $p < 0.01$) and of intact acrosomes ($R = 0.5$; $p < 0.05$). The percentage of viable spermatozoa was correlated with that of

Table 1. Sperm parameters in thawed sperm collected *postmortem* (2-9 h) (means ± SD and ranges in parentheses) in samples diluted in Triladyl® taken from 13 adult male Ibex (8-13 years)

%MT	%NS	%NAR	%VI	%CD
48 ± 21.5 (4.6-80.2)	69.4 ± 9.9 (47.5-81.8)	75.7 ± 10 (48.1-91.4)	69.4 ± 18.6 (30.4-92.3)	75.4 ± 23 (10-94.6)

MT: motility. NS: normal spermatozoa. NAR: intact acrosomes. VI: viability. CD: cytoplasmatic droplets.

spermatozoa of normal morphology ($R = 0.7$; $p < 0.01$) and with intact acrosomes ($R = 0.5$; $p < 0.05$).

Although the results could suggest a better response to thawing with the diluent Tris-6% egg yolk compared to Triladyl-20% egg yolk (Table 2), these differences were not significant ($p > 0.05$). The low number of observations (3 males) and the high variability obtained, as reflected by the coefficients of variation (Table 2), make these results inconclusive. Nonetheless, coefficients of variation were lower for the percentage of intact acrosomes and viable sperm cells with the diluent Tris-6% egg yolk, reflecting greater homogeneity of the results with this medium.

Within the first 9 h *postmortem*, no effect was found of the time of collection on the parameters studied. In the male Ibex, for which sperm was collected 32 h *postmortem*, post-thawing analysis of the epididymal sperm cells revealed a motility of 40%, 83.1% of normal forms, 70.7% of intact acrosomes and a viability of 83.9%. The incidence of cytoplasmatic droplets in this animal was 13.9%, i.e., it was lower than the mean value observed in samples obtained within the first 9 h *postmortem*. The number of samples collected over a wide interval of collection times after death (≥ 32 h) was insufficient to make an accurate statistical evaluation of this factor.

Table 2. Comparative analysis of the sperm parameters of thawed sperm collected *postmortem* (2-9 h) (means ± SD) between samples diluted in commercial diluent Triladyl®-20% egg yolk and in a diluent Tris-6% egg yolk (samples obtained from 3 adult male Ibex: 9-10 years). The coefficients of variation for each parameter are given in parentheses

	%MT	%NS	%NAR	%VI
Triladyl®-20% ey	30.6 ± 14.9 (CV = 48.7)	62.9 ± 13.9 (CV = 22.1)	67.1 ± 16.5 (CV = 24.6)	51.9 ± 32.1 (CV = 61.8)
Tris-6% ey	52.6 ± 32 (CV = 60.8)	70.3 ± 14.2 (CV = 20.2)	84.6 ± 7 (CV = 8.3)	81.0 ± 11.5 (CV = 14.2)

ey: egg yolk. MT: motility. NS: normal spermatozoa. NAR: intact acrosomes. VI: viability.

The results show the possibility of obtaining, in the male Ibex, *postmortem* sperm samples which are viable after thawing. Similarly, previous works on other wild species, such as mouflon (*Ovis gmelini musimon*), have reported the possibility of obtaining viable sperm material up to 40 h after the animal's death (Garde *et al*., 1995). Also, in red deer, viable sperm can be obtained up to 24 h after the animal's death (Garde *et al*., 1998). As well as the period between death and sperm collection, the storage temperature of the testicles has also been shown to affect sperm quality (Soler *et al*., 2003b). In the ram (*Ovis aries*), a significant reduction of the percentage of abnormal forms has been recorded in refrigerated samples (5ºC) compared to those kept at room temperature (Kaabi *et al*., 1999). In this experiment, sperm was kept during collection, transport and processing in the laboratory at an ambient temperature that oscillated around 13ºC (lower than isothermal conditions). Although this storage temperature seemed to be suitable, the possible increase in sperm quality by storing the testicles in refrigerated conditions (5ºC) should be studied.

In conclusion, the results show the viability of the technique used to collect and store sperm cells *postmortem*, obtaining sperm doses available for use with some guarantee of success in artificial insemination. *In vitro* studies of sperm viability on thawing constitute an important step forward in the development of germplasm banks for the male Ibex in the National Game Reserve of Almijara and Tejeda.

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