Fertility of undiluted ram epididymal spermatozoa stored for several days at 4°C

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In vitro preservation of the male gamete is a challenge in the development of artificial insemination techniques for domestic animals. Specific strategies and diluents have been developed for the preservation of the fertilizing ability of the semen for each species. However, the epididymal medium has been demonstrated to be the best sperm environment to maintain sperm viability over several days and weeks for mammals. The aims of this study were to evaluate the motility and in vivo fertility of ram epididymal spermatozoa when the semen was stored for up to 4 days at 4°C undiluted in epididymal plasma. The study was undertaken with two ovine breeds (Ile de France and Corriedale). The motility of epididymal spermatozoa was better preserved in the undiluted epididymal fluid than when epididymal spermatozoa were diluted in classic ovine extender such as skim milk. During storage, the decrease in the percentage of motile sperm was lower if the epididymal spermatozoa were collected immediately after epididymal sampling than 24 h after castration or animal death. The fertility obtained after cryopreservation of the stored sperm and subsequent intrauterine insemination ranged from 55% to 24% following 24 to 96-h sperm storage. There was a linear regression relationship between fertility and the number of motile sperm inseminated for both breeds. These results show that it is possible to keep epididymal sperm motile and fertile for several days without dilution. Such a method of sperm preservation could be a final possibility for animals of high genetic value or for endangered species when the collection of semen before death of the animal is not possible.

Keywords: ram, epididymal semen, cryopreservation, fertility

Implications
This study shows that it is possible to keep sperm motile and fertile in the epididymis for several days when semen is maintained at 4°C without dilution. The direct application of such findings may be postmortem collection of epididymal semen from animals of high genetic value or endangered species when collection of semen before death is not possible.

Introduction
The conservation of fertile spermatozoa was a major contribution to the development of artificial insemination (AI) for many species. Traditionally, semen collected in an artificial vagina from trained domestic species and by electroejaculation from several wild species is diluted in appropriate medium stored at either 5°C to 15°C or frozen and kept in nitrogen. Improving sperm storage for AI is a significant challenge for ovine species. Indeed, in contrast to most other species, very low fertility is achieved after cervical insemination with frozen–thawed ejaculated ram spermatozoa, although deposition of thawed semen in the uterus or oviducts provides high fertilization rates. As intrauterine insemination is time consuming, large flocks of ewes are inseminated cervically with fresh semen. However, as the fertility of liquid stored sperm declines at a rate of 20% to 40%/day (Maxwell and Salamon, 1993; Salamon and Maxwell, 2000), most cervical insemination is performed on the day of sperm collection. Such a short storage period imposes significant constraints on the transport of ovine semen between collection centers and farms.

The fertilizing ability of spermatozoa for all mammals is achieved during transit through the epididymis, a long tubule that can reach up to 70 m in some species such as the bull, boar and horse. The spermatozoa become fertile and motile for all species in the last part of the organ (for the ram, see (Fournier-Delpech et al., 1979), and review by (Dacheux and Dacheux, 2014)). Although mature spermatozoa are known
to have a short life span, because they have no capacity for synthesis, they can remain viable and fertile in the epididymis for several weeks in vivo (e.g. 20 to 30 days in the guinea pig (Young, 1929), 21 days in the rat (White, 1933), 28 to 35 days in the rabbit (Tesh and Glover, 1969), and 20 to 25 days in the hamster (Lubicz-Nawrocki et al., 1973)). The epididymal environment is thus an efficient storage medium, and understanding of its preservation conditions might be a way to improve in vitro sperm preservation.

Furthermore, as the number of fertile sperm present in the epididymal cauda can reach 5 to 15 times the number of spermatozoa contained in an ejaculate (Jones, 1998), the epididymal spermatozoa might also be an ultimate source of spermatozoa for genetically valuable animals untrained for collection and wild animals that die unexpectedly.

Sperm collected from caudae epididymides has been used in reproductive techniques developed for several species, including the mouse (An et al., 1999), dog (Yu and Leibo, 2002; Garcia-Macias et al., 2006a), deer (Hishinuma et al., 2003; Martínez-Pastor et al., 2006), pig (Kikuchi et al., 1998), ram (Kaabi et al., 2003), bull (Foote, 2000), horse (Vieira et al., 2012), bison (Garcia-Macias et al., 2006b) and even humans (McLachlan, 1998). The sampling of epididymal spermatozoa has been reported after mincing or cutting the epididymal tubule and collecting the exudate directly (Martins et al., 2009; Tamayo-Canuel et al., 2011b) or diluting in a medium solution (Hori et al., 2005b; Ehling et al., 2006). To avoid any blood contamination and to obtain a maximum number of spermatozoa, epididymal sampling has also been performed by retroflushing the epididymal lumen content from the deferent duct with air (Kikuchi et al., 1998; Vieira et al., 2012), with physiological solution (Dacheux, 1980; Martínez-Pastor et al., 2006), sodium citrate-egg yolk (Soler and Garde, 2003) or paraffin oil (Dacheux et al., 2006). Although some differences have been reported between caudal and ejaculated sperm (Garcia-Macias et al., 2006a), the fertility of epididymal sperm used either fresh or frozen–thawed for insemination has been found to be similar to ejaculated samples (Ehling et al., 2006; Varisli et al., 2009; Alvarez et al., 2012). However, the preservation of fertility of epididymal sperm may be influenced by many factors such as the conditions under which the epididymis is manipulated, the environment of the sperm during and after sampling or the postmortem period of storage of the epididymis before epididymal semen is collected. Different strategies have been developed for epididymal sperm preservation such as refrigerating the whole epididymis or storage of undiluted or extended epididymal sperm. Although storage of the spermatozoa inside the epididymis seems to be the easiest strategy (Tamayo-Canuel et al., 2011b), a progressive decline in sperm quality, including motility and viability, is observed as early as at the end of 24 h of storage whatever the methods of preservation. However, few studies have been performed to evaluate the preservation of fertility of such stored epididymal sperm. Epididymal spermatozoa collected from the mouse cauda epididymis stored for 3 days were able to fertilize 16% to 25% of oocytes in vitro (Jishage et al., 1997), and spermatozoa retrieved from cadavers stored for more than 10 days at 4°C were still able to fertilize 3% of oocytes in vitro (Kishikawa et al., 1999). The in vitro fertility of ram epididymal sperm declined significantly after 24-h storage (Kaabi et al., 2003). For red deer, 24 to 48-h stored epididymal sperm were able to penetrate 13% of zona-free hamster oocytes in vitro (Soler and Garde, 2003). Cleavage and blastocyst formation using epididymal sperm stored for 24 to 72 h did not differ significantly in bovines (Martins et al., 2009) and blastocysts were also obtained with epididymal sperm stored for 7 days (Martins et al., 2007). Pronuclear formation obtained by ICSI in equines and penetration of bovine zona-free oocytes was obtained in vitro with epididymal stallion sperm stored for up to 72 h in the epididymis at 4°C. However, few studies have been performed to estimate in vivo the fertility of stored epididymal spermatozoa, except for canines for which a high conception rate was obtained by intrauterine insemination with epididymal spermatozoa stored for 48 h (Hori et al., 2005a).

The aim of the study presented here was to compare the motility of ram epididymal sperm stored either diluted in the most commonly used extenders or undiluted in their epididymal fluid for long periods (up to 96 h) at 4°C. As in most previous studies involving in vitro characterization of such stored epididymal sperm, we evaluated the effects of storage of undiluted epididymal sperm on in vivo fertility by intrauterine AI of stored epididymal frozen–thawed spermatozoa.

Material and methods

Animals, sperm sampling, storage and cryopreservation

The collection, storage and insemination of sperm were undertaken in France with Ile de France rams and in Uruguay with Corrienedale rams and ewes. The experiment was performed with the agreement of the respective Ethics committees. All the protocols were performed during the ovine reproductive season: during July for Ile de France animals in France and during December for Corrienedale animals in Uruguay. Epididymides were collected either after castration in the INRA laboratory (21 Ile de France rams) or immediately after slaughter at the local abattoir (7 Corrienedale rams). Epididymal semen was sampled at 4°C to 5°C by retroperfusion from the deferent duct of the caudal epididymal tubule with 1.5 to 2.0 ml of fluid and non-toxic mineral oil (M 3516; Sigma, Saint Quentin Fallavier, France), (Dacheux et al., 2006).

Three sperm storage protocols at 4°C in a cool room were used.

Experiment 1 involved the storage of epididymal sperm from six Ile de France rams diluted in a concentration of 800 × 10⁶/ml either in skim milk or in ‘Bel24’ medium extender (170 mM glucose, 50 mM potassium bicarbonate, 30 mM glutamic acid, 50 mM glycine, 10 mM proline, 10 mM myo-inositol, 20 mM α-mannitol, 0.4% BSA and 20% egg yolk) developed in the laboratory for liquid sperm preservation of ram semen.
In Experiment 2, undiluted epididymal sperm from seven Ile de France rams was collected from one epididymis immediately after castration and 1 ml of each undiluted epididymal ram sample was stored in a closed 1.5 ml Eppendorf tube at 4°C. An aliquot was then taken from each tube at 0, 24, 48 and 72 h of storage and sperm motility was analyzed. Undiluted epididymal spermatozoa were also collected from the second epididymis after 24-h storage at 4°C, and an aliquot was sampled from each tube after 24 and 48-h storage, corresponding to 48 and 72 h after castration.

In Experiment 3, mixed undiluted epididymal sperm from both epididymides of eight Ile de France rams was obtained and stored as described above in closed 1.5 ml tubes at 4°C for up to 96 h. An aliquot of 120 µl was taken from each of the eight tubes at 0, 24, 48 and 72 h of storage, motility was then analyzed and 100 µl of the sperm suspension was deep frozen according to the methods described below and stored in nitrogen until being used for intrauterine insemination.

In Experiment 4, a similar protocol to Experiment 3 was applied for seven Corriedale rams, except that ejaculated sperm was collected with an artificial vagina the day before collecting sperm from the epididymis and immediately deep frozen. An aliquot of 120 µl of undiluted epididymal sperm were taken at 0, 24, 48, 72 and 96 h of storage from tubes collected from each animal, and treated as above.

For all experiments, the percentages of total and progressive motile spermatozoa were estimated after dilution (1 : 20) in 1 M sodium citrate dehydrate at pH 8.5 and 10 min incubation at 38°C either by microscope observation (Corriedale) or by a computer-assisted sperm analysis system (IVOS Hamilton-Thorn, Lisieux, France) (Ile de France). For frozen–thawed spermatozoa, motility was assessed just before AI after 10-min incubation.

Ejaculated and epididymal sperm samples stored at 4°C were cryopreserved in 0.25 ml straws according to method of Colas (Colas, 1975). For both species, sperm was cryopreserved with skim milk diluent at a concentration of 100 × 10^6 spz straw for Ile de France rams and 60 × 10^6 spz for Corriedale rams.

**Fertility of stored semen**

The fertility of cryopreserved ejaculated and stored epididymal sperm was estimated by intrauterine insemination. AI was performed after estrus synchronization of 757 ewes. Ovulation was induced with 40 mg fluorogestone acetate for 300 ‘INRA 401’ ewes and 60 mg medroxyprogesterone acetate for 427 Corriedale ewes using impregnated sponges for 12 to 14 days, and an injection of 200 to 500 IU of PMSG according to the breed. Before insemination, straws were plunged into a water bath at 38°C for 30 s. All the ewes were then inseminated with one straw of thawed semen 60 h after withdrawal of the vaginal sponges. The dose inseminated was 100 × 10^9 total spermatozoa/ewe (50 × 10^9 gametes in each uterine horn) for ‘INRA 401’ ewes (raised in the INRA laboratory of Physiology of Reproduction and the INRA Experimental Unit in Bourges, France) and 60 × 10^9 total spermatozoa (30 × 10^9 gametes in each uterine horn) for the Corriedale ewes (raised in the ‘Dr A. Gallinal’ Experimental and Research Centre (CIEDAG)).

In all, 3 Ile de France and 7 Corriedale rams were used to evaluate fertility in 3 to 10 ewes inseminated per ram for each storage time. The conception rate (referred to as ‘% pregnant’) was assessed either by the blood level of pregnancy associated glycoproteins 30 days post-insemination according to Beckers (El Amiriet al., 2007) for ‘INRA 401’ ewes, or by ultrasonography 45 days post-insemination for the Corriedales. Fertility was also estimated by the lambing rate (% lambing), and by the ‘fecundity index’ as the average of the number of lambs per 100 ewes.

**Statistical analysis**

Data are presented as mean ± s.e.m. and with significance at P < 0.05. For each experiment, the sperm status data were evaluated using GLM procedures of the SAS™ program. Conditions and/or storage times were the factors of variation in one- or two-way ANOVA for motility. Mean comparisons were obtained at 95% confidence level using Tukey HSD test and also by paired Student’s t test. Linear regression curves were calculated and drawn with SigmaPlot 10 (Systat Software, Inc.) for all the graphs. The lambing rates were compared using the χ^2 test.

**Results**

Retroflushing the posterior part of the cauda epididymis with paraffin oil provided total epididymal spermatozoa numbers per epididymis of 8.7 ± 1.8 × 10^9 spermatozoa for Corriedale rams to 13.2 ± 2.1 × 10^9 spermatozoa for Ile de France rams, corresponding to 0.8 ± 0.2 and 1.6 ± 0.3 ml of epididymal fluid collected from Corriedale and Ile de France rams, respectively. Most of the spermatozoa present in the posterior epididymal cauda were collected by this method (Figure 1).

**Characteristics of epididymal sperm collected and stored at 4°C under different conditions**

In Experiment 1, epididymal sperm diluted in two different extenders (skim milk and Bel24), (Figure 2a) showed that the
decrease in the percentage of motile sperm when the epididymal sperm diluted and stored in skim milk was statistically different and more rapid than when the gametes were incubated in ‘Bel24’ extender (milk × Bel24 interaction, \( P = 0.006 \)). After 72 h of storage, 18% of spermatozoa were still motile in ‘Bel24’ but no motile gametes were found in milk extender (Figure 2a).

When the epididymal spermatozoa were collected and stored undiluted in their own epididymal fluid from the castration (Figure 2b), the percentage of motile spermatozoa decreased about 10% to 12% during the 3 days of storage. After 72 h, the percentage of motile spermatozoa varied between 40% to 45%.

For the semen collected from epididymis stored intact at 4°C for 24 h after castration, the decrease in the percentage of motile sperm stored undiluted 48 h at 4°C was around 18% to 20%/day. Only 25% of motile sperm were observed at 72 h after castration. This value was significantly lower than those observed for sperm collected immediately from the epididymis and stored for 72 h (45% ± 6.0 v. 25.7 ± 5.1, \( P < 0.05 \)) (Figure 2b).

The mean motility observed for the undiluted sperm collected from the epididymis immediately and stored for 72 h was significantly greater than when the epididymal sperm was diluted in Bel24 extender (45% ± 1.9 v. 18% ± 0.8 \( P < 0.08 \)).

**Survival and fertility of undiluted epididymal sperm after 4 days of storage at 4°C**

In Experiments 3 and 4 (Figures 3 and 4), the percentage of motile epididymal sperm stored undiluted decreased linearly over the 4 days of storage at 4°C, with a mean decrease of 15%/day for Ile de France and 10% for Corriedale rams. Thus, after 96 h, 22% and 45% of the sperm were still motile for the Ile de France and Corriedale samples, respectively (Figures 3a and 4a). No difference was observed between ejaculated and fresh epididymal sperm (Figure 4a).

After the cryopreservation process, the mean loss of motile sperm between pre- and post-freezing was about 50% for Ile de France rams during the storage period. For the Corriedale
ram sperm, the loss of motile sperm increased significantly during the 4 days storage (from 30% to 45%) (Figure 4a). The decrease in motile sperm after thawing was about 7%/day for the Corriedale and 12% for Ile de France rams. After 96-h storage following by freezing–thawing, 13% and 24% of spermatozoa were still motile for Ile de France and Corriedale sperm, respectively.

The fertility of the spermatozoa, estimated in vivo after intrauterine insemination by the percentage of pregnant and lambing ewes, decreased regularly during storage of epididymal sperm, corresponding to 7% loss/day of lambing for the Corriedale (Figure 4b) and 12% for the Ile de France rams (Figure 3b). With the 96-h-stored spermatozoa, 10% of lambing ewes were obtained with Ile de France and 25% for Corriedale rams. The fecundity index did not vary significantly with the sperm storage for the Ile de France rams (211.2 ± 3.6) but slightly decreased for Corriedale rams after 24 h of storage (112 ± 0.4 v. 102 ± 0.48, P < 0.5).

Discussion

The results of this study demonstrate that the preservation effect of epididymal fluid within the epididymis is partly retained when epididymal semen is incubated undiluted in vitro. When the epididymal semen was stored undiluted in vitro the percentage of motile sperm was greater than in samples diluted in skim milk, the extender routinely used for AI, or in a synthetic diluent, Bel24, developed to increase ejaculated ovine sperm survival at 4°C. Thus, after 72 h of storage, the survival of undiluted sperm was twice that obtained in diluted samples. Such a preservation effect of the epididymal fluid on sperm motility has been already described for ram sperm (Tamayo-Canulet et al., 2011b) and other species (Garde et al., 1998; Songsasen et al., 1998; Hishinuma et al., 2003; Kaabi et al., 2003; Soler et al., 2003; Hori et al., 2005b; Fernandez-Santos et al., 2009b; Martins et al., 2009; Vieira et al., 2013), including humans for whom we obtained progressively motile spermatozoa from an epididymis stored at 4°C for 3 days postmortem (Dacheux, unpublished data). Such a protective effect of the epididymal fluid on spermatozoa has also been demonstrated to maintain membrane integrity and DNA integrity for several days, although motility decreases (Fernandez-Santos et al., 2009a).
Tamayo-Canul et al. (2011a) found that sperm quality was better if sperm were stored at 5°C within the epididymis after collection rather than stored undiluted in a glass tube. However, we found that the motility of the sperm collected immediately after castration and stored undiluted in vitro for 72 h was better preserved than that of sperm collected from the epididymis previously stored for 24 h at 4°C. Such differences may be related to the differences in the method of collecting epididymal spermatozoa (samples obtained by cutting v. epididymal tubule flushing, which avoided any epididymal tissue and blood contamination) or to the in vitro storage conditions such as the characteristics of the storage tube, which should avoid desiccation or too much oxygen exchange.

After freezing–thawing, the reduction in percentage of motile spermatozoa was of the same order (about 35% to 50%) for epididymal spermatozoa as for ejaculated sperm, although other studies have shown that epididymal spermatozoa are more resilient than ejaculated spermatozoa (Tamayo-Canul et al., 2011a; Alvarez et al., 2012).

The fertility of the epididymal spermatozoa estimated after intrauterine insemination with the same number of stored sperm decreased regularly over the storage period for the two breeds studied, as was also observed for the motility percentage. The decrease in fertility began to be significant from 48 h of storage, as observed by Kaabi et al. (2003) for ram epididymal sperm with in vitro fertilization. Furthermore, when the total number of motile sperm inseminated was considered, a significant linear relationship was established between the fertility rate and the number of motile spermatozoa inseminated. This suggests that most of the spermatozoa, which remain motile during storage, also retain their fertilizing ability. This was also corroborated by the finding that early embryo loss did not increase with sperm storage, and little difference was observed between % pregnant and % lambing. Furthermore, the number of lambs born per ewe (fecundity index) was unchanged by the sperm storage period even when the number of motile sperm inseminated was very low. For ram epididymal spermatozoa, embryo loss has previously been observed only for motile spermatozoa for which the fertilizing was not optimal (Fournier-Delpech et al., 1979). Such resistance of sperm during the storage period could be linked to the presence of different sperm populations in the cauda epididymis, as described by García-Macias et al. (2006a).

The epididymal sperm environment was surprisingly highly protective if we consider that the sperm concentration in caudal epididymal semen reaches nearly $10^{10}$ cells/ml and that such a high sperm concentration should be highly detrimental to sperm in common semen diluents. It is suggested that the protective effect of epididymal plasma is linked to the presence of several antioxidant systems (e.g. GPX5, thioredoxin peroxidase, glutathione S-transferase P, thioredoxin peroxidase and superoxide dismutase), to the presence of powerful protease inhibitors (e.g. macroglobulin, α-1-antitrypsin, epin, cystatin; review in Dacheux et al., 2009) and also to the presence of several antimicrobially active compounds, such as lactoferrin (Jin et al., 1997) and several β-defensins (Hall et al., 2007). The presence of in vitro antioxidants in refrigerated epididymal sperm has been also found to maintain quality similar to sperm kept within the epididymis (Fernandez-Santos et al., 2009a).

In conclusion, our findings showed that fertile spermatozoa can be preserved for several days when the epididymal spermatozoa are stored at 4°C without any dilution. Furthermore, these spermatozoa can be cryopreserved after such storage. The exact epididymal conditions that contribute to preservation of such a mass of spermatozoa remain to be clarified. However, our study demonstrated that successful reproduction with offspring could be obtained if enough motile sperm could be obtained from undiluted epididymal spermatozoa after several days of storage at 4°C. The direct application of such results could be the postmortem collection of epididymal semen from animals of high genetic value and from endangered species when the collection of semen before death is not possible.

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