In vitro oocyte maturation, fertilization and culture after ovum pick-up in an endangered gazelle (Gazella dama mhorr)


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Abstract

The recovery of immature oocytes followed by in vitro maturation, fertilization and culture (IVMFC) allows the rescue of biological material of great genetic value for the establishment of genetic resource banks of endangered species. Studies exist on sperm cryopreservation of endangered Mohor gazelle (Gazella dama mhorr), but no work has been carried out yet on oocyte collection, fertilization and culture in this or related species. The purpose of this study was to develop a protocol for ovarian stimulation for the recovery of oocytes and subsequent IVMFC in the Mohor gazelle using frozen-thawed spermatozoa. Ovum pick-up was performed after ovarian stimulation with a total dose of 5.28 mg of ovine FSH. A total of 35 oocytes were recovered from 56 punctured follicles (62%) (N = 6 females). Out of 29 cumulus-oocyte complexes matured in vitro, 3% were found at germinal vesicle stage, 7% at metaphase I, 21% were degenerated, and 69% advanced to metaphase II. Fertilization and cleavage rates of matured oocytes were 40 and 30%, respectively. Embryos cleaved in vitro up to the 6–8 cell stage but none progressed to the blastocyst stage, suggesting the existence of a developmental block and the need to improve culture conditions. Although more studies are needed to improve hormonal stimulation and oocyte harvesting, as well as IVMFC conditions, this study demonstrates for the first time the feasibility of in vitro fertilization with frozen-thawed semen of in vitro matured oocytes collected by ovum pick-up from FSH-stimulated endangered gazelles.

Keywords: Oocyte; Sperm; Fertilization; Embryo; Gazelle

1. Introduction

Habitat preservation is a priority in biodiversity conservation, but it is often difficult to avoid habitat destruction and, in some endangered species, other causes such as over-hunting are responsible for the declines in population size. Therefore, complementary strategies for biodiversity conservation, such as captive breeding, the establishment of genetic resource banks (GRBs) and the development of assisted reproductive techniques (ARTs) are needed. Genetic resource banks preserve current levels of genetic variability, facilitate genetic flow between natural and captive populations...
[1–4], and could be regarded as a form of “life insurance” in case wild populations continue their decline.

The World Conservation Union has recognized the potential role that captive breeding may play in conservation [5] and has recommended that taxa with low numbers of individuals in the wild should be considered for captive breeding. However, reproductive processes may be impaired in captivity as a consequence of behavioral or genetic incompatibilities, inadequate diet, disease, or due to the risks inherent to small population size such as inbreeding depression [6]. Reproductive biotechnologies can improve the reproduction of wild animals in captivity, allowing for the recovery and future use of reproductive biomaterials, and facilitating the exchange of gametes both within and between populations thus avoiding the negative effects of inbreeding and overcoming behavioral incompatibilities between individuals. Various ARTs have been applied successfully in several species of wild mammals [7–12].

The Parque de Rescate de la Fauna Sahariana of the Estación Experimental de Zonas Áridas (EEZA), in Almería, Spain, was created in 1971 with the objective of avoiding the extinction of the large ungulates of the Western Sahara. Mohor gazelle (Gazella dama mhorr Bennet 1833) is a subspecies of dama gazelle for which a breeding program has been established. The mhorr subspecies occupied the western strip of the Sahara desert and became extinct in the wild in 1968 due to excessive hunting. The Red List of Threatened Species [13] categorizes G. dama as “critically endangered” and the species is included in Appendix I of CITES. The captive population of Mohor gazelles was founded with two males and nine females [14], and this conservation program has been managed carefully to minimize inbreeding effects. At present the captive population is distributed among several institutions in Europe and North America, all derived form the original population bred in Almería [14]. At the end of 2003, the total captive population of Mohor gazelle consisted of 277 individuals, with 183 individuals (66.5%) within the European Breeding Program (EEP), and 92 individuals (33.5%) in the North American Breeding Program (SSP) [14].

The success of captive breeding programs initiated with a small founding population relies to a great extent on avoiding the deleterious effects of inbreeding on reproduction [15]. There is evidence showing the deleterious effect of inbreeding on reproductive success both in male and female Mohor gazelles and in other related endangered North African gazelles [16]. Inbreeding adversely affects ejaculate quality in gazelles with relatively high coefficients of inbreeding [17,18]. Reproductive performance is negatively affected as the coefficient of inbreeding increases in Cuvier’s (G. cuvieri), Mohor and dorcas (G. dorcas neglecta) gazelles [19]. At the EEZA, although efforts have been aimed at minimizing the levels of inbreeding in the captive populations, there are several limiting factors: the population is small in size, effective population size is even smaller because space restrictions do not allow all animals the opportunity to breed, and some individuals die before they can reproduce. In addition, moving animals between different populations is risky and costly. These problems can be overcome by cryopreserving gametes from most of the individuals so that they can be used to increase their reproductive opportunities even beyond death, and to facilitate gene exchange between populations. Previous work has focused on the management of natural breeding and on the development of semen freezing protocols for the three species to initiate a GRB [16,20,21]. No studies have been carried out on the collection, maturation and fertilization of oocytes in endangered gazelle.

The development of procedures in endangered gazelles for in vitro maturation, fertilization and culture (IVMFC) would allow the rescue of biomaterials from females with genetic value for establishing GRBs. Oocyte collection by laparotomy or laparoscopic pick-up, followed by IVMFC, has been extensively studied in domestic ruminants [22,23] and several studies have been performed in non-domestic ruminant species including red or sika deer [24,25], and mouflons [26,27]. Several factors affect the success of follicle growth stimulation [28,29], and ovum pick-up (OPU) [30], including the possibility of repeated collections [31]. In addition, conditions for in vitro maturation and fertilization have been described [22,23].

The aims of the present study on G. dama mhorr were to develop a protocol for (1) ovarian stimulation for the recovery of immature oocytes, (2) in vitro oocyte maturation, fertilization and embryo culture and (3) to evaluate the ability of cryopreserved spermatozoa to fertilize in vitro matured oocytes. We regard the development of these techniques to be a first step towards the rescue of female reproductive biomaterials for the preservation of genetic diversity.

2. Materials and methods

2.1. Reagents

All chemicals used in the present study were purchased from Sigma Chemical Co. (Madrid, Spain), unless otherwise indicated.
2.2. Animals

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609. Procedures also adhered to the Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences, and recommended by the journal.

The study was carried out in December 2004, using seven healthy mature (aged >2 years) female Mohor gazelles from the breeding herd maintained and managed by the Estación de Zonas Áridas (EEZA) at the Parque de Rescate de la Fauna Sahariana (PRFS) in Almería, south-eastern Spain. The PRFS is located at 36°50'10”N and 2°27'48”W and is 100 m above sea level. The animals were kept in a group in the same enclosure without the presence of males or young. The enclosure was visually opened to the exterior and was provided with shadow areas. Their diet consisted of commercial pellets, fresh alfalfa hay and barley grain. Water and mineral salts were available ad libitum.

2.3. Estrous synchronization and stimulation of follicular growth

Estrous cycles in the gazelle were synchronized by insertion of controlled progesterone internal drug release devices (CIDR, type G, 330 mg progesterone; InterAg Hamilton, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days. CIDRs were replaced on day 10, and removed during ovum pick-up (OPU) on day 15. Follicular growth was stimulated by the administration of a total of 5.28 mg of ovine FSH (Ovagen, ICPbio, Auckland, New Zealand) for 15 days.

2.4. Oocyte recovery

Cumulus-oocyte complexes (COCs) were recovered using a semi-laparotomy technique. Female donors were anesthetized with a combination of an intramuscular administration of xylazine (0.2 mg/kg body weight; Rompun, Bayer, Barcelona, Spain) and intravenous injection of ketamine chlorohydrate (15 mg/kg body weight; Imalgene 1000, Merial, Lyon, France) and then secured in a cradle in dorsal recumbency. When necessary, surgical anesthesia was maintained using halothane inhalation. The effects of xylazine were reversed by intravenous administration of yohimbine chlorohydrate (0.03 mg/kg body weight; Sigma, Madrid, Spain).

Ovum pick-up was carried out following procedures described previously [28]. Briefly, an endoscope was inserted into the abdominal cavity about 10–15 cm cranial to the udder, and 4–5 cm to one side of the midline, whileatraumatic grasping forceps were inserted in a contralateral position through a 3–4 cm long laparotomy incision. After endoscopic visualization, the ovaries were fixed at their ligament origin and exposed through the laparotomy incision. Follicles present on the ovarian surface were counted and aspirated with a 2-ml syringe fitted with a 25-gauge needle carrying 0.5 ml of warm medium, placed in a 15 ml plastic tube (Falcon, Becton Dickinson, Lepont De Claix, France) and kept in a water bath at 38 °C until processed. The collection medium was Hapes-buffered TCM-199 supplemented with antibiotics (50 µg/ml streptomycin plus 50 IU/ml penicillin), polyvinyl-alcohol (PVA, 0.1%, w/v) and heparin (15 IU/ml). After each OPU session, the ovaries were washed with warm physiologic solution.

2.5. In vitro maturation

The COCs derived from each female donor were kept separate throughout the in vitro procedures. Following oocyte collection, the content of each tube was poured into a 60 mm Petri dish (Falcon #35-1007, Becton Dickinson) and observed under a stereomicroscope (Nikon SMZ-1500). Oocytes that were degenerated and/or with expanded cumulus cells were removed. After rinsing three to four times in Hapes-buffered TCM-199 with PVA, heparin and antibiotics, the COCs were placed in four-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) containing the in vitro maturation medium (TCM-199 plus 10% v/v heat-treated estrus sheep serum, 10 µg ovine FSH/LH/ml, 1 µg estradiol/ml and 0.1 µg yohimbine chlorohydrate (0.03 mg/kg body weight; Sigma, Madrid, Spain).
glutamine/ml), covered with 250–300 μl of mineral oil and cultured at 38.5 °C under 5% CO₂ in air and maximum humidity.

2.6. In vitro fertilization

After 24 h, oocytes were washed in Hepes-buffered TCM-199 followed by gentle pipetting with the aid of an automatic micropipette to dissociate surrounding cumulus cells. Fertilization medium consisted of synthetic oviduct fluid (SOF) [32] supplemented with heat-inactivated 2% (v/v) estrus sheep serum, 1 μg hypotaurine/ml and 10 μg heparin/ml. Oocytes were inseminated with cryopreserved spermatozoa. To avoid confounding effects due to male-to-male variations, the same male was used to fertilize oocytes from different females. In preliminary trials, semen samples from various males were tested for cryosurvival. semen was collected by electroejaculation using a sine-wave stimulator (PT. Electronics, Boring, OR, USA) and evaluated for volume, concentration, motility, viability, membrane integrity, morphology, and acrosome integrity, as described previously [21]. A sperm motility index (SMI) was calculated as follows: [sperm % motility + (quality of motility × 20) × 1/2]. semen samples were centrifuged at 700 × g for 5 min and resuspended in a Tes-Tris–glucose diluent with 5% egg yolk and 6% glycerol [21]. Sperm suspensions (adjusted to 400 × 10⁶ spermatozoa/ml) were loaded into 0.25 ml plastic straws, cooled to 5 °C for 1.5 h and allowed to equilibrate for an additional 2 h. The straws were frozen in nitrogen vapors for 10 min and then plunged into liquid nitrogen. Straws were thawed for 30 s at 37 °C in a water bath and the content of each straw was poured into a conical glass tube and assessed for sperm motility and acrosome integrity. Spermatozoa were selected by using a swim-up procedure. Briefly, spermatozoa in the cryodiluent (0.25 ml) were placed gently in the bottom of a round-bottom glass tube (15 mm × 75 mm) which contained 1 ml SOF medium (as used for IVF) and incubated at 38.5 °C under 5% CO₂ in air. After 30 min the upper layer of 0.3 ml was removed (aspirating the sperm suspension at a point located 3 mm below the surface), and sub-samples were taken for assessment of motility and acrosome integrity. After 24 h of gamete co-culture, presumptive zygotes were washed in culture medium to remove spermatozoa and cell debris. They were transferred to 500 μl of SOF medium supplemented with 0.4% BSA (w/v) and nonessential and essential amino acids at oviductal concentrations [33] under mineral oil and cultured in a humidified atmosphere at 38.5 °C under 5% O₂, 5% CO₂ and 90% N₂. Cleaving embryos were separated from 1-cell presumptive zygotes after 36 h of culture. embryo development was monitored for several days, every 24 h, until it was arrested.

2.7. In vitro culture

After 24 h of gamete co-culture, presumptive zygotes were incubated in four-well culture dishes under mineral oil for 24 h at 38.5 °C under 5% O₂, 5% CO₂ and 90% N₂. sperm samples obtained at the end of gamete co-culture were examined for motility and acrosome integrity.

2.8. Evaluation of meiosis progression

The resumption and progression through meiosis was evaluated retrospectively after in vitro fertilization. Non cleaved oocytes and embryos were stained with Hoechst 33342 and propidium iodide (10 μg/ml each) and visualized in a microscope (Nikon E-400) equipped with epifluorescence to determine maturation status and/or fertilization by the presence of ≥2 pronuclei or decondensed sperm heads. Fluorescence of Hoechst and propidium iodide was observed simultaneously using an Hg excitation beam and a filter set (UV-2A Nikon) consisting of a UV330-380 excitation filter, a DM400 chromatic beam splitter and a 420 barrier filter.

3. Results

3.1. Oocyte harvesting, culture and fertilization

Of 56 follicles aspirated from six females (9.3 ± 1.7 follicles per female), 35 COCs were recovered (5.8 ± 1.5 oocytes per female), resulting in a overall recovery rate of 62% (mean ± S.E.M., 58.6 ± 9.2% recovery per female) (Table 1). A total of 29 cumulus-surrounded oocytes (Fig. 1) were selected for in vitro maturation (83% of recovered oocytes). When examined after culture, 3% (1/29) were found at the germinal vesicle (GV) stage, 7% (2/29) at metaphase I (MI), 21% (6/29) were degenerated or not classified, and 69% (20/29) advanced to metaphase II (MII) (Table 1, Figs. 1 and 2).

Semen collected from four males was evaluated pre- and post-cryopreservation and one was selected for IVF.
based on superior sperm survival during cryopreservation and subsequent post-thaw incubation for 2 h at 37 °C. Fresh semen characteristics for the male (ND 932) designated to use for IVF were: volume, 4.95 ml; sperm concentration, 600 × 10^6 spermatozoa/ml; motility, 90%; morphologically normal spermatozoa, 86% and intact acrosomes, 95%. Sperm characteristics immediately after thawing and after 2 h incubation were: motility, 65% vs. 50% and intact acrosomes, 62% vs. 62%, respectively. Sperm characteristics after swim-up

### Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Recovery rate a (%)</th>
<th>Maturation rate b (%)</th>
<th>Fertilization rate c (%)</th>
<th>Cleavage rate d (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND 778</td>
<td>3/5 (60)</td>
<td>2/3 (66.7)</td>
<td>2/2 (100)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>ND 917</td>
<td>1/5 (20)</td>
<td>1/1 (100)</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>ND 1158</td>
<td>11/16 (68.8)</td>
<td>8/9 (88.9)</td>
<td>2/8 (25)</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>ND 1160</td>
<td>5/9 (55.6)</td>
<td>3/4 (75)</td>
<td>2/3 (66.7)</td>
<td>1/3 (33.3)</td>
</tr>
<tr>
<td>ND 1191</td>
<td>7/12 (58.3)</td>
<td>3/7 (42.9)</td>
<td>1/3 (33.3)</td>
<td>1/3 (33.3)</td>
</tr>
<tr>
<td>ND 1219</td>
<td>8/9 (88.9)</td>
<td>3/5 (60)</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>35/56 (62.5)</td>
<td>20/29 (68.9)</td>
<td>8/20 (40)</td>
<td>6/20 (30)</td>
</tr>
<tr>
<td>Mean ± S.E.M. (range)</td>
<td>58.6 ± 9.2 (20–88.9)</td>
<td>72.2 ± 8.4 (42.9–100)</td>
<td>54.2 ± 16.9 (0–100)</td>
<td>31.9 ± 14.9 (0–100)</td>
</tr>
</tbody>
</table>

Data are shown for each individual animal and as total and mean ± S.E.M. (range) results of the experiment. Recovery rate is expressed as recovered oocytes over the total of punctured follicles, maturation rate as matured oocytes over the number of COCs included in culture, and fertilization and cleavage rates over the number of matured oocytes.

- a Recovery rate: number of COCs recovered/number of punctured follicles.
- b Maturation rate: number of matured oocytes/number of COCs included in culture.
- c Fertilization rate: number of oocytes fertilized/number of matured oocytes.
- d Cleavage rate: number of cleaving embryos/number of matured oocytes.

Fig. 1. Oocytes recovered by ovum pick-up (OPU) and embryos obtained after in vitro maturation, fertilization and culture from Mohor gazelle. (A) Cumulus-oocyte complex (COC) recovered by semi-laparoscopic OPU with expanded cumulus cells and degenerated oocyte, not selected for in vitro maturation; (B) COC included in maturation medium; (C and D) 4-cell and 8-cell embryos after in vitro culture in SOF supplemented with 0.4% BSA and amino acids under 5% CO₂, 5% O₂, 90% N₂. Bar = 20 µm.
and after co-incubation with COCs are shown in Table 2. The proportion of motile spermatozoa was improved after swim-up, although sperm concentration was reduced from \(400 \times 10^6\) to an average of \(5.6 \pm 1.5 \times 10^6\) (range \(1.6-8.9 \times 10^6\)) spermatozoa/ml before and after swim-up, respectively. The percentages of motile and acrosome-intact spermatozoa were reduced during co-incubation with COCs (Table 2).

Fertilization and cleavage rates of matured oocytes were 40 and 30%, respectively (Table 1, Figs. 1 and 2). Six oocytes cleaved after IVF, yielding two 2-cell embryos, two 4-cell embryos and two 6- to 8-cell embryos (Fig. 1C and D). None of the embryos developed to the morula or blastocyst stage.

### 3.2. Progesterone and estradiol levels during synchronization and FSH stimulation

Plasma levels of progesterone and 17\(\beta\)-estradiol during CIDR and FSH treatment are shown in Fig. 3. Progesterone concentrations increased from the day of CIDR insertion (0.72 ± 0.17 ng/ml) until the day of replacement of the vaginal device (5.3 ± 1.42 ng/ml). Then, plasma concentrations of progesterone decreased from the day of CIDR replacement until the beginning of FSH treatment (0.88 ± 0.17 ng/ml). Henceforth, progesterone levels remained low, despite the presence of CIDRs. Plasma concentrations of 17\(\beta\)-estradiol showed a decreasing pattern, reaching its lowest level...
on the morning of day 14, with a slight increase occurring afterward.

4. Discussion

This study represents, to the best of our knowledge, the first attempt to generate gazelle embryos by in vitro maturation of oocytes recovered by using ovum pick-up, with subsequent in vitro fertilization and culture. Our results indicate that gazelle embryos can be generated following this approach and, furthermore, that cryopreserved gazelle spermatozoa can be successfully used for in vitro fertilization. Although the results reported here are based on a limited number of individuals, a common problem when dealing with endangered species, these results are important for the future development of genetic resource banks for this and related species.

In earlier studies, work on the preservation of gazelle gametes has concentrated on the characterization of semen traits [34,35] and in the development of semen cryopreservation protocols in three endangered gazelle species, including Mohor gazelle [16,20,21,36]. However, none of these studies have explored whether cryopreserved gazelle spermatozoa are able to fertilize in vitro. In the present study we have used a semen preparation method (swim-up) for in vitro fertilization and tested the fertilizing ability of frozen-thawed spermatozoa. We found that after swim-up there was an improvement in the proportion of motile spermatozoa, the quality of motility and, hence, the sperm motility index. On the other hand, after swim-up, sperm concentration was low, indicating poor recovery of spermatozoa during this procedure, and the percentage of cells having an intact acrosome decreased. Incubation of spermatozoa for up to 24 h led to a reduction of motility and acrosome integrity over time. The percentage of spermatozoa with intact acrosomes was about 57% at the start of incubation, 43% after 1 h of incubation, and about 17% at the end of incubation. The value of spermatozoa with intact acrosomes found at the end of sperm incubation and at the end of the in vitro fertilization period were very similar (16–17%).

The percentage of fertilization in vitro was rather low (40%) and it may be due in part to a rapid decrease in spermatozoa with intact acrosomes during incubation. Nonetheless, the present results demonstrate that frozen-thawed Mohor gazelle spermatozoa can function to fertilize oocytes in vitro.

Several staining methods and sperm incubation assays have been used for estimating sperm fertilizing ability. However, in vitro fertilization assays may offer a more accurate assessment of post-thaw sperm function in vitro. A positive relation between the sperm motility index observed after incubating spermatozoa for 3 or

<table>
<thead>
<tr>
<th>Sperm parameters</th>
<th>Upon thawing</th>
<th>After swim-up</th>
<th>After 1 h incubation</th>
<th>After 24 h incubation</th>
<th>At end of IVF (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>53.1 ± 1.9</td>
<td>77.5 ± 3.4</td>
<td>64.2 ± 5.7</td>
<td>5.1 ± 3.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Quality of motility (0–5)</td>
<td>1.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>SMI</td>
<td>43.4 ± 1.6</td>
<td>66.3 ± 2.6</td>
<td>56.5 ± 3.4</td>
<td>3.9 ± 2.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>NAR (%)</td>
<td>66.5 ± 2.7</td>
<td>57.0 ± 2.5</td>
<td>43.0 ± 2.7</td>
<td>16.8 ± 2.9</td>
<td>16.0 ± 2.3</td>
</tr>
<tr>
<td>DAR (%)</td>
<td>30.0 ± 2.6</td>
<td>30.3 ± 2.6</td>
<td>35.5 ± 2.3</td>
<td>38.8 ± 4.4</td>
<td>40.2 ± 5.2</td>
</tr>
<tr>
<td>MAR (%)</td>
<td>3.5 ± 0.3</td>
<td>12.8 ± 0.5</td>
<td>21.5 ± 3.3</td>
<td>44.5 ± 3.9</td>
<td>43.8 ± 7.5</td>
</tr>
</tbody>
</table>

Spermatozoa (400 × 10⁶ cells/ml) in 0.25 ml straws were thawed at 37 °C for 30 s, transferred to a 12 mm × 75 mm glass tube with 1 ml SOF medium, and incubated for 30 min at 38.5 °C under 5% CO₂ in air. The upper 0.3 ml was recovered and evaluated. Aliquots were incubated for 1 or 24 h under similar conditions or were used for co-culture with oocytes during 24 h. See Section 2 for details. Results are means ± S.E.M. (N = 4). SMI, sperm motility index; NAR, normal apical ridge (i.e., spermatozoa with intact acrosomes); DAR, damaged apical ridge; MAR, missing apical ridge.

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**Table 2**

Sperm motility and acrosome integrity after thawing, swim-up and incubation of Mohor gazelle spermatozoa

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**Fig. 3.** Plasma progesterone and 17β-estradiol levels during treatment with CIDR and ovine FSH in Mohor gazelles. Day 0, insertion of CIDR; day 10, replacement of CIDR; day 13 a.m., FSH injection (8:00 h); day 13 p.m., FSH injection (18:00 h); day 14 a.m., FSH injection (8:00 h); day 14 p.m., FSH injection (18:00 h); day 15, ovum pick-up. Results are means ± S.E.M.; N = 6 females.
6 h and fertilization has been found in the scimitar-horned oryx [37]. A similar relationship has been described between sperm longevity and in vitro fertilization success in the cheetah [38] suggesting that sperm longevity could be a better predictor of potential fertility than a single assessment of motility after thawing. These results should be taken in account for future studies of semen evaluation in gazelles.

Administration of FSH enhances the number of oocytes obtained by increasing the number of follicles that, based on their size and morphological characteristics, are adequate for aspiration [39]. The response of female gazelles to treatment with ovine FSH revealed a rather poor rate of follicular growth. In domestic ruminants, variation in ovarian response to superovulatory treatment relates to many factors, such as ovarian status at the beginning of treatment, [40,41], hormonal profile [42], interval between the last exogenous gonadotropin stimulation and follicle aspiration [43,44] and donor treatment [45,46]. Among gazelles, failure to respond could be due either to stress during animal handling or to insufficient FSH doses. With regards to the former, improvements could perhaps be obtained in the future by animal training, with repeated captures and handling, that may eventually lead to reduced stress. Another option to minimize stress could be the use of long-acting neuroleptics. Use of tranquilizers such as perphenazine enanthate or haloperidol chlorohydrate in Spanish ibex (Capra pyrenaica) females has allowed the collection of transferable embryos after a superovulatory treatment with ovine FSH [47]. Moreover, administration of the long-acting neuroleptic flufenazine decanoate prior to collection of oocytes from European mouflon (Ovis orientalis musimon) resulted in a better response to the hormonal treatment, increasing the number of punctured follicles per donor [26]. The only animal that did not receive neuroleptic treatment had been previously submitted to regular handling and showed a good response to superovulatory stimulation [26].

Poor follicular growth could also be due to insufficient FSH doses. In sheep, successful stimulation of follicular growth occurs with four doses of FSH (as used in our study), allowing for a good collection of healthy oocytes with subsequent fertilization and development in culture. Constant doses of ovine FSH seem to produce better results than decreasing ones [28], although other studies have found no differences between FSH administration regimes [48,49]. We found that plasma concentrations of estradiol decreased during exogenous FSH administration, supporting the hypothesis that the amount of FSH was insufficient to adequately stimulate follicular growth. In sheep, FSH treatment leads to an increase in the number and size of gonadotropin-responsive follicles and in a subsequent increase in circulating levels of estradiol [50]. Thus, estradiol is considered to be a reliable marker of follicular function [51]. Decreases in follicular secretion of estradiol are related to deficiencies in follicular health and, moreover, a decrease in the developmental competence of the oocyte [52]. Therefore, a possible explanation for the limited oocyte response seen in our study is that follicles were not responding adequately to FSH, with stress being an additional factor leading to a reduced response.

The proportion of oocytes recovered from punctured follicles (62%) was similar to that obtained using endoscopic recovery in sheep (55%) [53]. However, it was lower than that obtained in sheep using laparotomy (80%) [54] or in goats employing laparoscopy (90%) [55]. In non-domestic ungulates, oocyte recovery rates ranged from 36 to 46% in red deer (Cervus elaphus) [24] and were 57% in sika deer (Cervus nippon) [25], which are closer to the values obtained in our study. More studies are needed to improve results of oocyte harvesting and to explore the possibility of repeated oocyte collection from the same females.

The size of the follicles found on the ovarian surface of gazelles was small (2–3 mm). It is known that an oocyte is unable to resume and progress through meiosis until it reaches a given size. In sheep, a direct relationship exists between follicular size and the ability of oocytes to progress through meiosis [56]. Thus, the capacity to advance to metaphase II increases with follicle and oocyte sizes in lambs and adult sheep [56]. It is not clear whether this relationship exists in gazelles, but it is possible that oocytes contained in small follicles have a reduced maturation capability.

The overall maturation rate obtained at the end of the in vitro incubation period was about 70%, with considerable variation between females, and this result is similar to data reported in other wild ruminants. In red deer, a maturation rate of about 55% was found for oocytes harvested by laparoscopy [24] and a range of 14–85% for oocytes recovered from slaughterhouse ovaries [25]. In sika deer, 14–76% of oocytes aspirated by laparoscopy matured in vitro [25]. In domestic sheep, oocytes recovered by OPU showed a 80–90% maturation rate when conditions similar to those used for gazelles were employed [28]. Clearly, the fertilization rate we obtained with gazelle oocytes in the present study was rather low (40%) as compared to the ~80% fertilization rate achieved in domestic sheep using similar methodology [28].
When gazelle presumptive zygotes were cultured in vitro, we found that although there was cleavage up to the 6- to 8-cell stage, none of the embryos progressed to the morula or blastocyst stage, suggesting the existence of a developmental block. In sheep, about 15–20% of embryos develop to the blastocyst stage when a similar methodology is used [28]. One possible explanation for our results is that embryo culture conditions were not adequate for gazelle embryos. In vitro embryo culture procedures for wild ungulates are currently limited to protocols developed for domestic animals. Using such protocols, blastocysts have been obtained after oocyte in vitro maturation, fertilization and culture in the axis deer, (Axis axis) [57], red deer [25,58], sika deer [25], and European mouflon [26]. Adjustments are therefore needed for improvements in gazelle in vitro embryo culture, but progress in this area will be slow due to the scarcity of gametes and embryos from this endangered species. Additional factors to consider are that differences exist, even between closely related species, in the requirements of embryos for in vitro development [8]. An alternative to overcome the difficulties of in vitro embryo culture could be an early transfer to recipient females. In deer, in vitro matured and fertilized oocytes were capable of establishing pregnancies when embryos were transferred to recipient hinds at the 2- to 4-cell stage, with this transfer being more efficient than that of 8-cell embryos [59]. In future gazelle work we will explore the feasibility of early embryo transfer, i.e., oviductal transfer of 2- to 4-cell embryos.

Another likely explanation for poor embryo development in culture relates to inadequate oocyte quality and limited developmental competence. Studies on ewes treated with progestagen/gonadotropin for in vitro embryo production have indicated that oocyte quality (i.e., the ability to develop into a viable embryo after in vitro maturation, fertilization, and culture) seems to depend on both the reproductive status of the female and the hormonal treatment employed [28,29]. Since, gazelles showed a limited response to FSH treatment, as revealed by poor follicular growth, it is possible that oocytes had a poor degree of cytoplasmic maturation and this had an important bearing on post-fertilization development. The fundamental processes involved in the acquisition of oocyte developmental competence remain unknown, but there is increasing evidence that such competence is acquired while still in the follicle [43]. Oocyte pre-maturation and final maturation occurring during follicular growth are considered crucial steps in determining the outcome of embryo production [60].

Taken together, our results of in vitro maturation, fertilization and culture in the Mohor gazelle, achieved using protocols developed originally for sheep, demonstrate, for the first time, the production of embryos in vitro from this endangered species. Although more studies are needed to improve in vitro conditions, these results reveal the feasibility of achieving in vitro fertilization, with frozen-thawed semen, of in vitro matured oocytes collected from FSH-stimulated endangered gazelles. Aspects that need improvement include oocyte harvesting conditions, stimulation for the development of healthier oocytes, and in vitro culture of embryos to overcome a developmental block that prevents progression to the blastocyst stage. These improvements would allow the generation of a sufficient number of oocytes and embryos for cryobanking or transfer.

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