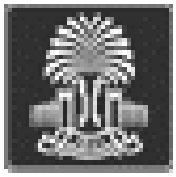

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THE APPLICATION OF ARTIFICIAL REPRODUCTION TECHNIQUES TO THE PROPAGATION OF SELECTED ENDANGERED SPECIES

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ABSTRACT

Artificial reproduction techniques were applied to individual animals not capable of natural reproduction. All individuals represented endangered species. An aged and debilitated Arabian oryx was treated with prostaglandin F2 α (PG) and pregnant mare serum gonadotropin (PMSG) prior to euthanasia. She responded to the treatment with mild superovulation. Four follicular oocytes were recovered, all of which subsequently matured in vitro and were frozen. A group of three Soemmering's gazelles, including a crippled male and a three-legged female, was evaluated for reproductive potential. The male was electro-ejaculated and determined to be producing normal sperm; semen was frozen. Estrus was induced and synchronized in the two females with PMSG and PG prior to the introduction of the male. Copulation attempts were unsuccessful. A second modified estrus induction attempt did not result in behavioral estrus in either female. The handicapped female was subsequently superovulated with PMSG before euthanasia. Four oocytes were recovered but failed to mature in vitro. Two Przewalski's horse mares are not able to mate naturally due to chronic laminitis. Preliminary studies have been undertaken to determine, through ultrasound examination, the estrous cycles and ovarian dynamics of the two horses, in preparation for artificial insemination and embryo transfer. Cycle length and variability, ovarian response to PG and rate of follicle growth have been determined to be similar to the domestic horse.

INTRODUCTION

The Arabian oryx (Oryx leucoryx) and the Przewalski's horse (Equus przewalskii) became extinct in their native habitats around 1967. Both species reproduce well in captivity and neither is in danger of extinction at this time. However, careful genetic management must be applied to these captive populations to maintain their genetic health

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and vigor. As part of this management effort, the Reproductive Physiology program of the Zoological Society of San Diego has instituted a program of germplasm preservation for these and other endangered species.

Another species of interest in this program is the Soemmering's gazelle (Gazella soemmeringi). This rare and beautiful animal is not well described physiologically and there is considerable confusion about its taxonomic place among related species.

Utilizing techniques described for these species or their relatives, specific projects have been undertaken to enhance the reproduction of individuals representing each of the species named above.

MATERIALS AND METHODS

Study I: Arabian Oryx

An 11-year-old, multiparous oryx was separated from the herd for treatment of chronic lameness and anorexia. The deterioration of her general health and failure to respond to treatment resulted in the decision to euthanize her.

The oryx's year-long absence from the male ensured that she was not pregnant, but it was not known if she was experiencing a normal estrous cycle. Earlier work with this and other oryx species (1) has shown the efficacy of an ovulation induction regimen comprising two injections of prostaglandin F2α (PG) ten days apart with pregnant mare serum gonadotropin (PMSG) given two days before the last PG. In this study, 125 µg PG (Estrumate, cloprostenol, Haver-Lockhart, Shawnee, KS, unless otherwise stated) and 3,000 IU PMSG (Gestyl, Diosynth, Chicago, IL) was administered intramuscularly (IM) with a dart gun in an attempt to superovulate the animal prior to follicular oocyte recovery.

Forty-eight hr after the last PG injection, the oryx was euthanized. The ovaries were immediately removed and placed in warm saline for transport to the laboratory. Oocytes were recovered by aspirating all follicles with a 23-ga needle on a 1-cc syringe. The oocytes were incubated in Brinster's Media for Ova Culture ($BMOC_2$) (2) at 37°C and 5% CO_2 in air with cumulus cells intact. One-half ml of fluid with granulosa cells (unquantified) from the aspirated follicles were added to the culture medium.

At the end of a 28-hr incubation the oocytes were frozen slowly (1.3°C/M to seeding, 0.4°C/M to -40°C) in 1.5 M DMSO in $BMOC_2$. At -40°C the oocytes were plunged into liquid nitrogen for storage.

Study II: Soemmering's Gazelle

The Soemmering's gazelle group at the San Diego Wild Animal Park comprised a three-legged animal of each sex and a normal female. The possibility of reproduction within this group was explored.

The right hind leg of an adult male gazelle was plated following fracture of the femur. Refracture with separation of the bone from the plate resulted in ankylosis of the hock. It was important to determine if the handicap would prevent the animal from breeding normally. Semen was collected from this unproven male by electro-ejaculation to evaluate the animal's potential fertility prior to the introduction of the two females. Semen parameters evaluated include volume of ejaculate, concentration per ml, percent motile sperm, speed of progression (SOP; 0-5, 5 being highest) and percent morphologically abnormal forms. Semen collected was frozen at 2°C/min after dilution with Test yolk buffer (A.R. Johnson, personal communication).

The objective of Experiment A was to collect and freeze embryos following natural breeding. One female was a healthy, nulliparous 8-year-old. The left front leg of the other female had been amputated following repeated fractures; she was nulliparous. Prior to their first exposure to the male, each female was given two doses of 125 μg PG ten days apart. Two days before the last PG they received 600 IU PMSG in an attempt to superovulate and synchronize the females. All hormones were administered i.m. using a dart gun. Behavioral observations commenced 24 hr after the last PG injection, at the time of the introduction of the male.

A second ovulation induction regimen (Experiment B) began 30 days after the beginning of the first experiment and was designed to stimulate estrus and breeding followed by natural gestation. PG used in this experiment was Alfavet (125 μg, Alfaprostenol, Roche Chemical Division, Nutley, NJ) and the dose of PMSG was lowered to 200 IU to reduce the possibility of a superovulatory response. As before, behavioral observations were conducted during the females' exposure to the male.

Fifty-two days after the beginning of Experiment B, a third ovulation induction was attempted with the three-legged female (Experiment C). PG was given 11 days apart with 1,200 IU PMSG for superovulation administered two days before the last PG. Twenty-four hr after the second PG the animal was euthanized due to the continued deterioration of her remaining front leg. Ovaries were removed and placed in warm saline for transport to the laboratory. Follicles were classifed as preovulatory, maturing, or developing based on their descending size and prominence on the surface of the ovary. Follicles classified as preovulatory were large (4 - 5 mm in diameter) and protruded from the surface of the ovary by 4 - 5 mm. Maturing follicles measured 4 - 5 mm in diameter but were raised only 2 - 3 mm from the surface. Follicles were classified as developing if they measured more than 3 mm in diameter but did not extend above the surface of the ovary. Follicular fluid and oocytes were aspirated with a 27-ga needle. Recovered oocytes were classified and cultured, cumulus intact, in a 1:1 mixture of $BMOC_2$ and follicular fluid.

Study III: Przewalski's Horse

Two mature, maiden mares are currently being treated for chronic laminitis. They have been separated from the herd and housed

individually since 1983 to facilitate frequent corrective shoeing procedures.

Reproductive tract examinations of the two mares were carried out with sequential linear-array ultrasound scanners producing real-time B-mode images (Pie Data models 400 and 560, Pie Data Medical, Singer Island, FL). Both scanners utilized 5 MHz transducers designed for rectal insertion. Speculum inspection of the cervix and rectal palpation of the reproductive tract accompanied ultrasound evaluation.

All procedures were carried out with the animals in right lateral recumbency following chemical immobilization. Endometrial biopsies were taken for examination for evidence of fibrosis or inflammation. Each animal was evaluated seven times during the three-month preliminary phase of the study.

Normal follicular growth and dynamics, as well as ovarian responses to Pg (3 mg Alfavet) and hCG (3,500 IU, chorionic gonadotropin, Carter-Glogan Labs, Glendale, AZ), was observed in an attempt to define the estrous cycles of the two mares.

RESULTS

Study I: Arabian Oryx

The hormone treatment utilizing one large dose of gonadotropin resulted in one ovulation (as evidenced by a corpus hemorrhagicum) and the development of four maturing follicles on the left ovary. The right ovary contained a corpus albicans and no follicular development.

Each of the four oocytes recovered was classified as immature due to the absence of a polar body in its periviteline space. Following 24-hr incubation, two oocytes matured as evidenced by extrusion of the first polar body. The remaining two oocytes matured by this criterion after 28 hr in culture.

Study II: Soemmering's Gazelle

Experiment A. The results of three electro-ejaculation procedures on the male gazelle are depicted in Table 1. The first attempted semen collection yielded seminal plasma devoid of sperm. At the time, it was thought that the collection may have occurred during a season of reduced sexual function; however, a subsequent collection 25 days later revealed that the animal was indeed producing sperm. A third collection four months after the second attempt also yielded sperm. All evaluated semen parameters were acceptable as indicators of potential fertility, with the possible exception of an elevated percentage of abnormal forms compared to most other mammals (3). However, it would be premature to judge the samples infertile on the basis of morphological abnormalities as no other Soemmering's males have been evaluated.

Table 1. Semen values of a Soemmering's gazelle

Date	Volume (ml)	Conc. x 10^6	Motility, SOP	Abnormal Forms
Mar 14, '85	3.1	0	0	-
Apr 8, '85	1.5	500	95%, 5	39%
Aug 8, '85	6.0	70	95%, 3	56%

The normal female exhibited standing estrus 24 hr following the last PG injection, and although he tried repeatedly, the male was not able to copulate successfully. The three-legged female exhibited signs of estrus (i.e., frequent urination in the presence of the male, tail extension, and allowing the male to sniff her genital region) 72 hr after the last PG. Mating did not occur between the two handicapped gazelles, due to the female's characteristic fearful behavior combined with the male's inability to accomplish copulation quickly.

No behavioral estrus or attempted copulations were observed in Experiment B.

A large dose of PMSG in Experiment C resulted in the development of a total of 39 ovarian structures. Table 2 depicts the distribution of structures on the right and left ovaries.

Table 2. Ovarian response to PMSG in a Soemmering's gazelle

Structure	Right ovary	Left ovary
Preovulatory	2	8*
Maturing	6	9
Developing	9	4
Corpus hemorrhagicum	0	1

*One follicle ruptured during ovariectomy.

In addition to the follicles induced by gonadotropin treatment, two corpora albican were evident on the right ovary.

Four oocytes were recovered from the aspiration of 37 follicles. Dense, sticky cumulus masses were adhered to the oocytes and complicated their recovery and identification. All recovered oocytes were classified as immature and 96-hr incubation did not result in the maturation of any of the four.

Study III: Przewalski's Horse

Initial ultrasound examination revealed active ovaries in both mares. Endometrial biopsies performed at that time indicated mild focal endometritis in both animals. During each mare's two subsequent estruses, she was treated by uterine lavage with antibiotic solution. Uterine flushing resulted in return of 90 - 95% of introduced medium.

Follicular growth proceeded at 3 - 5 mm per day in the two mares with natural ovulation occurring at a follicle size of 48 - 50 mm. Preovulatory irregularity in follicle shape was observed as reported in the domestic horse (A.R. Johnson, personal communication).

Table 3 illustrates preliminary results of the timing of ovulation following PG administration. When PG was given to Mare 1 with a five-day corpus luteum (CL), ovulation occurred six days later. The same mare ovulated ten days after PG was given to lyse a 14-day-old CL. Mare 2 ovulated 7 - 8 days after PG at the time of an 11-day-old CL and in nine days when a 14-day-old CL was lysed.

Table 3. Ovarian response to PG in the Przewalski's horse

Mare No.	Age of CL (days)	Days from PG to Ovul.
1	5	6
	14	10
2	11	7 - 8
	14	9*

*HCG was administered seven days after PG.

HCG administration seven days after PG did not appear to hasten ovulation in Mare 2. The mare had a 33-mm, irregular follicle at the time of hCG. The following day the same follicle had become round and had grown to 40 mm.

DISCUSSION

Study I: Arabian Oryx

A large dose of PMSG resulted in the growth of five follicles--a mild superovulatory response. Greater superovulation has been achieved in the scimitar-horned oryx with twice daily injections of follicle stimulating hormone for several days (D.E. Wildt, personal communication). However, it is not always possible to inject an animal that frequently. In difficult or stressful field conditions, perhaps fewer potential ova or embryos is a reasonable compromise for ease in hormone administration.

The addition of follicular fluid and granulosa cells to culture medium may have enhanced in vitro maturation of aspirated follicular oocytes; identical incubation conditions in our laboratory have not resulted in oocyte maturation in this species when follicular fluid was not added. Follicular fluid collected from animals at necropsy will be frozen for future studies involving in vitro maturation and fertilization. The four frozen ova from this female will be thawed and placed in culture for attempted in vitro fertilization followed by embryo transfer. Although this female was not able to mate naturally, her genetic material has been preserved for addition to the gene pool when it is determined to be most beneficial to the population.

Study II: Soemmering's Gazelle

Experiment A demonstrated that the estrous cycle of the Soemmering's gazelle can be manipulated in a manner similar to the domestic cow (D.E. Wildt, personal communication) and the oryx (1). Estrus of the two females was 48 hr apart, indicating acceptable synchronization.

Experiment B differed from Experiment A in both the form of PG and the dose of PMSG. However, PMSG is not necessary for estrus (and ovulation) induction in cattle (4) and is unlikely to be responsible for the failure of the ovary to respond to PG. It may be that this species is not responsive to all forms of PG or that the females experienced a refractory period during which PG was ineffective. These theories will be tested with this and other gazelle species in future studies.

Experiment C demonstrates the efficacy of PMSG in stimulating follicular growth in this species. Satisfactory superovulation can probably be achieved with a smaller dose of PMSG. The size of the preovulatory follicles, the ease with which they ruptured when touched, and the one recent ovulation indicate that the animal was probably in estrus at the time of ovariectomy. She therefore responded to hormone treatment within 24 hr of the last PG, 48 hr earlier than she responded in Experiment A. The large dose of PMSG and subsequent growth of multiple follicles may explain the animal's hastened response.

Failure of follicle aspiration to recover more than four oocytes may have been due to the abundant, stick cumulus covering of each oocyte and the use of a 27-ga needle. A larger gauge needle would perhaps have allowed the passage of large masses of cumulus cells with their hidden oocytes.

As the world's endangered animals retreat from their threatened habitats into captivity, we, their guardians, must begin to manage them more efficiently. Cryopreservation of germplasm will allow us to limit the size of our living collections without jeopardizing their genetic future. In this study, two non-reproductive animals, unfit for exhibition, were removed from the living collection to make room for individuals capable of reproducing naturally. Germplasm has been preserved from these two valuable gazelles for potential use in future generations.

Study III: Przewalski's Horse

Preliminary evaluation of the estrous cycles of the two mares in this study suggests that this species is reproductively similar to domestic horses. The timing of ovulation has been determined and artificial insemination is being attempted.

The excellent recovery of fluids infused into the uterus during antibiotic treatment verifies that non-surgical embryo recovery from recumbent animals is possible.

Utilizing artificial insemination, non-surgical embryo recovery and domestic recipients for embryo transfer, each of these Przewalski's horse mares could contribute several embryos per year to our captive breeding program. In the absence of appropriate recipients, embryos will be frozen.

Conclusion

The time has come to put the techniques of artificial reproduction to use for the benefit of each species on which it is practiced. Before considering the utilization of high technology propagation, let us ask ourselves two important questions:

1. Will the proposed study benefit the species as well as advance our scientific knowledge toward the goal of conservation?

2. Will offspring produced by the proposed study make valuable genetic contributions to future generations?

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