Successful intrauterine insemination of Eld’s deer (Cervus eldi thamin) with frozen-thawed spermatozoa

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This study tested the efficacy of assisted reproduction (synchronization of oestrus and intrauterine artificial insemination (AI)) in contributing to the captive propagation of an endangered species, the Eld’s deer (Cervus eldi thamin). Semen was collected from males preselected on the basis of under-represented genotype. Motility of spermatozoa after thawing from ejaculates diluted with BF5F extender (8% glycerol), frozen on dry ice in 0.5 ml straws and stored in liquid nitrogen was 60–70%. Intravaginal progesterone-releasing devices (controlled internal drug release, CIDR-type G) were inserted into 20 adult Eld’s deer hinds for 14 days. In all hinds, semen (7.5–10 × 10^6 motile spermatozoa per uterine horn) was deposited by laparoscopy performed 70 h after removal of the CIDR device. Ovarian activity, before and after AI, was monitored by analysing pregnanediol-3-glucuronide (PdG) concentrations in voided urine collected three to seven times per week. During the period of CIDR device insertion, urinary PdG profiles were equal to, or above, normal luteal phase concentrations in all hinds. Within 48 h of device withdrawal, PdG concentrations returned to baseline values in 17 of the 20 females, and the onset of behavioural oestrus occurred at this time in 12 hinds. On the basis of sustained increases in urinary PdG, 9 of the 20 hinds were diagnosed as pregnant by 90 days after AI, all of which delivered offspring after a mean gestation of 241.1 days (range, 235–245). Seven singletons (two females, five males) were born alive and survived, and one singleton and one set of twins were stillborn (three females). This is the greatest number of pregnancies and offspring produced in a single AI trial for any endangered mammal. These results demonstrate that genotype preselection can be combined with assisted reproductive technologies, including use of frozen semen, to produce genetically valuable offspring useful for conserving a rare species.

Introduction

Captive propagation of endangered species is important for preserving rare taxa when in situ conservation efforts are incomplete, fragmented or likely to fail (Soule, 1991). When ex situ tactics are used to manage small populations, a primary goal is to maintain adequate genetic diversity to avoid inbreeding depression. Artificial insemination (AI), in vitro fertilization (IVF) and embryo transfer can be used to enhance captive breeding of rare species; however, these ‘assisted techniques’ have not yet proved to be consistently useful for producing offspring from any endangered mammalian species (Wildt, 1992; Wildt et al., 1993). For wild taxa, AI could be particularly valuable for (i) ensuring reproduction between valuable but behaviourally incompatible pairs; (ii) eliminating the risks of animal transport and (iii) providing an avenue for infusing genes between wild stocks and captive populations, many of which are genetically stagnant (Wildt, 1989).

Of the 40 species in the family Cervidae, 32 contain subspecies that are in danger of extinction (C. M. Wemmer, North American Regional Studbook Keeper, personal communication). The Eld’s deer (Cervus eldi thamin) is a subtropical, endangered species that is primarily distributed in central Myanmar (formerly Burma), and fewer than 4000 individuals are believed to remain in the wild (C. M. Wemmer, personal communication). The North American captive population consists of 140 individuals distributed among three herds (National Zoological Park, New York Zoological Society, San Diego Zoo) separated by as many as 4500 km. This situation typifies the challenges associated with genetically managing small isolated populations. Although Eld’s deer breed well in captivity, their excitable temperament increases the risk of stress and injury during long-distance transport. For this reason, Eld’s deer have become inbred within each individual breeding facility. Germ plasma cryopreservation, combined with AI, has potential for
overcoming these captive management problems while providing insurance against further losses in genetic diversity as a result of disease or other unforeseen catastrophes.

For AI to be successful in any species, detailed prerequisite information must include (i) understanding the reproductive cycle of the female to allow the identification or manipulation of oestrus and ovulation, (ii) safe and reliable methods for collecting and storing viable spermatozoa, and (iii) methods for proper deposition of spermatozoa at the optimal time and site within the female reproductive tract. For a variety of cervid species, the first objective of synchronizing oestrus has been achieved using intravaginal progesterogen-releasing devices, such as medroxyprogesterone acetate sponges or controlled internal drug releasing (CIDR) devices in combination with exogenous gonadotrophins (fallow deer: Asher and Macmillan, 1986; Asher and Smith, 1987; red deer: Haigh et al., 1988; Fennessy et al., 1989; wapiti: Haigh and Bowen, 1991). An alternative approach includes using CIDR devices without gonadotrophin supplements, and this strategy is proving to be the simplest and most promising method for synchronizing oestrus in cervids (Mulley et al., 1988; Asher and Thompson, 1989; Asher et al., 1990a, b).

Pregnancies have been reported in reindeer (Rangifer tarandus; Dott and Uttsi, 1973), white-tailed deer (Odocoileus virginianus; Haigh, 1984) and fallow deer (Dama dama; Asher et al., 1988, 1992; Mulley et al., 1988) following AI with fresh semen. The first offspring produced using AI and frozen–thawed spermatozoa occurred in 1977 (three red deer, Krzywinsky and Jazewski, 1978). Since then, only four other common cervid species (fallow deer: Mulley et al., 1988; Asher et al., 1988, 1990a, 1992; Mylrea et al., 1991; red deer: Fennessy et al., 1989; Haigh and Bowen, 1991; white-tailed deer: Jacobsen et al., 1989; Magyar et al., 1989; axis deer, Axis axis: Mylrea et al., 1992) have been propagated using frozen–thawed spermatozoa. AI using frozen–thawed semen after oestrous synchronization with CIDR devices was shown to be highly effective (60–70% conception rates) in fallow deer (Asher et al., 1990a, 1992).

Our objective was to evaluate the efficacy of oestrous synchronization and laparoscopic intrauterine AI using frozen–thawed semen to produce offspring in Eld’s deer. In addition, we were interested in determining whether assisted reproductive techniques developed in more common cervid species (fallow deer) could be applied directly to an endangered species.

Materials and Methods

Animals

Twenty Eld’s deer hinds (2–9 years old; 50–90 kg body weight, mean 72.2 ± 1.7 kg) and three stags (2–8 years old; 70–120 kg body weight, mean 96.7 ± 14.5 kg) were housed in indoor stalls (3.4 m × 4.6 m with skylights) connected to outdoor enclosures (3.6 m × 36.6 m) that provided exposure to natural fluctuations in photoperiod. Hinds were maintained in pairs within auditory and olfactory proximity to conspecific males that were housed singly. Females were checked twice daily for signs of behavioural oestrus; these included marking of animal handlers with pre-orbital scent glands and assuming a receptive lordotic stance (Monfort et al., 1990).

Sire selection

Semen donors were selected prospectively by the American Association of Zoological Parks and Aquariums’ (AAZPA) Small Population Management Analysis Group (Melissa Rodden, National Zoological Park) to minimize inbreeding coefficients and maximize the genetic contribution of under-represented founders. This was accomplished by calculating mean kinship values (Ballou and Lacy, in press) among all males in the North American population and the 20 recipient females. The genetic value of specific individuals was increased as the corresponding kinship value decreased. A rank order was established, and the three most under-represented males were selected as sperm donors for specific females so that hypothetical matings yielded offspring inbreeding coefficients of <0.25.

Urine collection and assay for pregnanediol-3α-glucuronide

Urinary pregnanediol-3α-glucuronide (PdG) immunoreactivity was assessed to monitor longitudinal ovarian activity and as an index of pregnancy (Monfort et al., 1990). Urine samples (1–5 ml) were collected 3–7 days per week (07:00–11:00 h) by midstream catch or by aspirating samples from the enclosure floor. Samples were centrifuged (1500 g, 10 min) to remove particulate matter, stored frozen (−20°C), and later analysed by radioimmunoassay procedures as described and validated by Monfort et al. (1990). All samples were indexed by creatinine to account for changes in water intake (Monfort et al., 1990). Briefly, 100 µl aliquots (diluted 1:50) of urine from all females were assayed in duplicate. Interassay coefficients of variation for three separate internal controls were 10% (n = 9, 43–59% binding), 10% (n = 9, 62–77% binding) and 13% (n = 9, 78–86% binding). Intra-assay coefficients of variation were less than 10%, and assay sensitivity was 19.5 pg per tube.

Electroejaculation and semen evaluation

A standardized electroejaculation protocol (Howard et al., 1986) was used to collect semen from the three stags after a surgical plane of anaesthesia was induced using combined ketamine HCl (Ketaset: Aveco Co., Fort Dodge, IA, USA, about 2 mg kg⁻¹ body weight) and xylazine HCl (Rompun; Mobay Corp., Shawnee, KA, USA, about 0.25 mg kg⁻¹) administered i.m. Briefly, a sine-wave electrostimulator (AC, 60 Hz) and Teflon rectal probe (PT Electronics, Boring OR) were used to administer 90 incremental stimuli in a 3 s on–off pattern in three series consisting of 30 (10 stimulations at 4, 5 and 6 V, respectively), 30 (10 stimulations at 5, 6 and 7 V), and 30 stimuli (10 stimulations at 6, 7 and 8 V). Each series was separated by a 5 min rest interval at which time aliquots of semen were assessed for pH, volume and sperm concentration. For each series, at least four separate fields (× 250) of undiluted spermatozoa were examined, and the average percentage of motile spermatozoa and status ratings for all three series were calculated. Sperm status was a subjective assessment of forward progressive motility on a graded scale: 0 (no movement) to 5 (rapid, steady forward progression) (Howard et al., 1986). A sperm motility index incorporating motility and status elements was calculated as the product of the progressive status multiplied by 20, plus the percentage sperm motility value, all of
which was divided by two (Howard and Wildt, 1990). After each series, ejaculates were diluted with extender, and combined into a single vial containing semen from all three series. After final mixing, a 20 μl aliquot of semen was fixed in 1% glutaraldehyde, and the structural integrity of 200 sperm cells per ejaculate was assessed by phase contrast (×1000) microscopy (Howard et al., 1986). Spermatozoa were classified as normal or having structural abnormalities of the head, midpiece or flagellar region (Howard et al., 1986).

**Semen freezing and thawing**

Raw semen was transferred into a 10 ml vial and diluted to a concentration of 220–520 × 10⁶ ml⁻¹ with BF5F extender. The latter was a modified BF5 diluent (Pursel and Johnson, 1972) that consisted of 20% egg yolk, 1.6% glucose, 1.6% fructose, 1.2% Tes (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), 0.2% Tris (Tris(hydroxymethyl)aminomethane), 1000 i.u. penicillin G ml⁻¹, 1000 μg streptomycin sulfate ml⁻¹ and 0.5% surfactant mixture of sodium and triethanolamine lauryl sulfate (Orvas ES Paste: Equex, Nova Chemical Sales Inc., Scituate, MA). Diluted ejaculate fractions from each stag were combined in a glass tube and held in a waterbath at 37°C until semen collections were complete. The tube was then placed into a water-insulated container (25°C) and cooled slowly for 1 h to 8°C before being further diluted (1:1) with precooled (4°C) BF5F containing 16% (w:v) glycerol in three steps (final glycerol concentration: 8% w:v). Semen samples were then aspirated into labelled 0.5 ml French straws (IMV International Corp., Minneapolis, MN), heat-sealed and held at 4°C for 1 h. Straws were placed on a dry ice block (−78°C) for 10 min before being transferred to liquid nitrogen (−196°C) and stored for 1–6 months.

**Oestrous synchronization treatment**

A progestosterone-releasing device (CIDR-type G, 9% progestosterone; Agricultural Division, CHH Plastic Products Group Ltd, Hamilton) was inserted intravaginally into each physically restrained, unanaesthetized hind. The device was inserted between 08:00 and 09:00 h or 12:00 and 13:00 h (n = 5 for each group) on either 19 April (n = 10) or 20 April (n = 10). After 14 days (3 and 4 May), CIDR devices were removed from five hinds in each group between 10:00 and 11:00 h and 14:00 and 15:00 h. Females were checked twice a day for overt signs of oestrus, and laparoscopic inseminations were performed 70 h after CIDR device withdrawal (6 and 7 May).

**Sperm thawing and insemination technique**

Approximately 10 min before inducing anaesthesia in each recipient hind (see below), a semen straw was thawed rapidly in a 37°C water bath for 20 s and a 10 μl aliquot examined for sperm motility and progressive status. In addition, post-thaw longevity was assessed by evaluating sperm motility and progressive status in a single replicate straw from each stag immediately after thawing (time 0) and 3 h later. Thawed samples contained at least 60% motile spermatozoa and a status rating of 2.5 or greater. A volume of semen containing 15–20 × 10⁶ progressively motile spermatozoa was aspirated into a 0.25 ml insemination straw and placed in an insemination pipette (UA 111; IMV International Corp.) for AI. Semen from sires 401, 162 and 269 was used to inseminate seven, six and seven hinds, respectively.

For laparoscopic insemination, hinds were induced into a surgical plane of anaesthesia using a combination of ketamine HCl (2.5 mg kg⁻¹) and xylazine HCl (0.25 mg kg⁻¹) administered i.m. After reaching a surgical plane of anaesthesia (usually within 5–10 min of injection), hinds were placed in dorsal recumbency on a laparoscopic trolley, intubated, surgically scrubbed and tilted head-down at 30°. A Verres needle and hand pump was used to inflate the abdominal cavity with air. Two independent trocar–cannula units were inserted through the ventral abdominal wall and peritoneum approximately 10 cm cranial to the mammary glands on either side of the midline. The trocars were removed, and a 5 mm diameter rigid laparoscope was inserted through one cannula and the loaded insemination pipette through the other cannula. Half the volume of each straw was injected into one uterine horn lumen approximately 2–4 cm cranial to the bifurcation; the remaining volume was deposited at the same site in the opposite horn. The presence or absence of pre-ovulatory follicles or corpora lutea was noted when ovaries could be readily observed; manipulation of the reproductive tract to view the ovaries was avoided. After removing the cannulae, the abdominal puncture sites were sutured, and xylazine anaesthesia was reversed with i.v. yohimbine HCl (0.3 mg kg⁻¹, Sigma Chemical Co., St Louis, MO). The average time from induction of anaesthesia to reversal was 28.3 ± 0.9 min (range 21–37 min).

**Statistical analyses**

The effect of stag and hind age on pregnancy outcome (pregnant versus nonpregnant) was determined using a three-way Gtest for independence (Sokal and Rohlf, 1973). An among-group repeated measures analysis of variance, and a posteriori pairwise comparisons (using Fisher’s protected least significant different test) were performed to determine when PdG increased during pregnancy compared with concentrations before conception. Differences in the numbers of hinds experiencing oestrus in each insemination group were tested using a χ² test.

**Results**

Semen quality from the three sperm donors was high. The ranges in sperm motility index values were 60–75 for fresh ejaculates. Post-thaw values for males 401, 162 and 269 were also high (65, 65 and 70, respectively) and had only decreased marginally 3 h later (55, 50 and 55, respectively). The proportion of morphologically normal sperm for males 401, 162 and 269 was similar in fresh (88, 92 and 95%, respectively) and frozen–thawed ejaculates (86, 85 and 90%, respectively). Nine of the 20 hinds (45%) became pregnant following AI with frozen–thawed spermatozoa. Pregnancy outcome was unrelated to sire (P = 0.06), dam age (P > 0.20) or interactions between sire and dam age (P > 0.50).

Urinary PdG excretion profiles from a pregnancy resulting from natural mating (Fig. 1a; adapted from Monfort et al., 1990)
and from a pregnancy resulting from AI are shown (Fig. 1b; this study). There were no differences in PdG profiles during the time of CIDR device insertion in pregnant versus nonpregnant hinds; the devices appeared to stimulate a similar 'false' luteal phase in both groups (Fig. 2). In 17 of the 20 (85%) hinds, urinary PdG concentrations decreased to nadir (<10 ng mg \(^{-1}\) creatinine) within 72 h of CIDR device withdrawal. In the remaining three hinds that failed to conceive, PdG concentrations did not decline to basal concentrations for 4, 11 and 9 days, respectively (Fig. 3a–c). Two preovulatory follicles were observed in one of these hinds (Fig. 3a) at laparoscopy; ovaries were not observed in the other two females. Two hinds (Fig. 3a, c) resumed normal cyclic patterns of PdG excretion (i.e. 15–20 day cycles). One female (Fig. 3b) had a history of prolonged luteal phases from an earlier seasonality study (Monfort et al., 1990), and this individual exhibited persistently increased PdG excretion for more than 30 days. Another nonpregnant hind (Fig. 3d) exhibited a prolonged PdG excretion pattern after oestrous synchronization and AI, but the profile abruptly declined to baseline values approximately 80 days after insemination.

Behavioural oestrus was detected in 12 of the 20 (60%) hinds; the onset occurred in all instances on the second day after CIDR device withdrawal. Of the nine hinds producing offspring at term, eight demonstrated overt oestrus near the time of AI, whereas, seven of eight individuals that failed to exhibit behavioural oestrus failed to conceive. The number of hinds exhibiting oestrus was unevenly distributed (\(P > 0.05\)) among sire groups (401, 6 of 7; 162, 4 of 7; and 269, 2 of 6) resulting in five, three and one pregnancy, respectively.

On the basis of urinary PdG concentrations, nonpregnant hinds (Fig. 2b) resumed normal, spontaneous oestrous cycles of average duration (approximately 18 day) (Monfort et al., 1990). On the basis of sustained increased urinary PdG concentrations (\(P < 0.05\)), pregnancy could be diagnosed by week 12 of gestation (range 400–600 ng mg \(^{-1}\) creatinine) (Fig. 4). However, by tracking PdG patterns in individual females, 9 of 11 hinds were correctly diagnosed as not pregnant by 40 days after AI. Mean (±SEM) gestation time was 241.1 ± 1.1 days (range 235–245). Ten fawns were produced; seven singletons (two females, five males) were born alive and survived, and one singleton and one set of twins were stillborn (three females).
The overall sex ratio was 1:1. Inbreeding coefficients for the ten offspring (including stillbirths) were 0.000 (n = 6), 0.009, 0.031, 0.062 and 0.250.

Discussion

The present study demonstrated that pregnancies can be achieved in Eld’s deer after intrauterine insemination with frozen–thawed semen at a fixed interval from oestrus induced by progesterone-impregnated CIDR devices. Mean PdG profiles in pregnant and nonpregnant hinds revealed that CIDR device insertion always resulted in PdG concentrations that matched, or exceeded, normal luteal phase concentrations (50–150 ng mg⁻¹ creatinine; Monfort et al., 1990). This suggested that CIDR devices resulted in increased circulating progesterone that was metabolized and excreted as urinary PdG. Our results also indicated that spermatozoa from Eld’s deer could be obtained routinely by electroejaculation, and that excellent sperm motility and structural integrity was maintained after one to six months of low temperature storage.

The general AI protocol used here for Eld’s deer is commonly used in farmed fallow deer (Asher et al., 1990b, 1992). This provides an excellent example of how an assisted reproduction technique can be applied to an endangered species if the procedure is already working well in a taxonomically-related ‘model’. Although some refinements may be necessary because of unique species specificities, we anticipate that this approach may have broad applications to other rare cervid species. However, it is worth emphasizing that this study was preceded by a strong pre-emptive effort to establish an integrative database for female and male Eld’s deer before AI was attempted. The detailing of basic life history strategies, seasonality, oestrous cyclicity, behaviour, gametogenesis and semen freezing (Wemmer and Grodinsky, 1988; Monfort et al., 1990, 1993) helped to ensure a higher potential rate of success. For example, detailed information on seasonality ensured that hinds were not inseminated too early or too late in the breeding season, a factor that severely decreases AI success in fallow deer (Asher, 1986). Similarly, seasonal evaluations of males indicated the time of year when optimal semen quality could be obtained (Monfort et al., 1993).

Our results clearly established the usefulness of monitoring of urinary hormones as an important adjunct to this assisted reproduction strategy. Repeated anaesthesia or restraint for collection of blood samples and ultrasonography is usually impractical in deer living under zoo conditions. Because of their timorous nature, deer in general are susceptible to stress, and acute secretions of adrenal progesterone can confound attempts to monitor the secretory dynamics of blood progesterone profiles (white-tailed deer, Plotka et al., 1983; fallow deer, Asher et al., 1989; red deer, Jopson et al., 1990). For many species, urinary and faecal steroid monitoring are the only alternatives for assessing longitudinal endocrine profiles. As such, these approaches provide considerable promise for improving success rates of artificial breeding attempts in other species that are susceptible to stress.

The inability to track longitudinal endocrine rhythms has often prevented researchers from retrospectively determining the reasons for poor synchronization of oestrus or conception.
failures after AI. In the present study, urinary PdG profiles showed that conception failure in three hinds was related to enduring, high progesterone concentrations evident after CIDR device removal. Two of these hinds had histories of persistent corpora lutea (Monfort et al., 1990) that may explain our failure to induce oestrus in these two individuals. In retrospect, given the relatively high incidence of persistent corpora lutea in this species, the routine administration of prostaglandin analogues during CIDR device treatment may be warranted.

Intermittent monitoring of PdG (weekly or biweekly) permitted accurate, routine pregnancy detection by week 12 of gestation in Eld’s deer. By this time, urinary PdG concentrations in pregnant hinds were higher than peak luteal phase concentrations of PdG. However, more frequent sampling (at least three times per week) could be used to detect conception failure 20–40 days after AI. One hind exhibited a prolonged (approximately 80 days) increase in PdG concentrations after AI, suggesting that either corpora lutea function was abnormal or pregnancy was established but terminated with an undetected abortion or fetal resorption.

As is common with many ungulates, detection of overt signs of behavioural oestrus in Eld’s deer can be complicated by missed or misinterpreted observations by animal handlers. However, it was interesting that seven of the eight hinds that failed to exhibit behavioural oestrus in the present study also failed to conceive to AI. Abnormal endocrine profiles explained the absence of oestrus at the expected time in three of these eight hinds. Because daily oestrous detection was temporarily suspended for several days after AI, it is not known whether these three hinds eventually exhibited oestrus when PdG finally decreased to basal concentrations. Furthermore, the pregnancy rate was highest in the sire group in which the greatest number of hinds exhibited oestrus. Overall, these data suggested that expression of behavioural oestrus 48 h after withdrawal of the CIDR device in Eld’s deer was a potentially useful predictor of reproductive–endocrine status, and hence the likelihood of AI success.

Offspring have now been produced from 30 mammalian species using AI with frozen–thawed spermatozoa (see review, Wildt, 1992). Seven of 30 of these species have been cervids (white-tailed deer, fallow deer, red deer, wapiti, reindeer, axis deer and Eld’s deer). Thus, the technology is now available to implement genetic management plans for maximizing the genetic diversity of smallcaptive populations of rare cervid species. Although performed on a limited scale, the present study represents one of the first examples in which prospective sire and dam selection, germ plasm banking, AI and urinary hormone monitoring were used for a specific conservation goal in an endangered species.

The authors thank C. Wemmer for logistical support, M. Rodden and J. Ballou for assistance with sire selection, M. E. Smak, I. Teittinen, M. McClure, A. Brickey, E. Laurent, J. Brown, S. Symnestvedt, D. Roy, V. Armstrong and D. Pavon for assistance with sample collections and behavioural observations, and M. Sorenson for assistance with statistical analyses. This research was funded by grants from the Scholarly Studies Program of the Smithsonian Institution, Friends of the National Zoo, NOAHs Center, the Women’s Committee of the Smithsonian Associates and by a National Institutes of Health Clinical Investigator Award to S.L. Monfort (HD 00903).

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