SUCCESSFUL INDUCTION OF OVARIAN ACTIVITY AND LAPAROSCOPIC INTRAUTERINE ARTIFICIAL INSEMINATION IN THE CHEETAH (*ACINONYX JUBATUS*)

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Abstract: An exogenous gonadotropin regimen and a laparoscopic intrauterine artificial insemination (AI) technique, previously developed in the domestic cat, were adapted and assessed for effectiveness in the cheetah (*Acinonyx jubatus*). Seven female cheetahs were given an injection of either 200 or 400 IU pregnant mares' serum gonadotropin (PMSG) and either 125 or 250 IU human chorionic gonadotropin (hCG) 80 hr later. At 42.5–47.0 hr after hCG, all females were evaluated laparoscopically for fresh ovarian corpora lutea (CL). Ovulation was induced successfully in all cheetahs (range: 3–13 CL among females). However, two morphologically distinct CL types were observed: 1) large-sized CL that appeared more related to the low gonadotropin dose; and 2) small-sized CL that were detected more often in the high gonadotropin dose group. Six of the females were laparoscopically inseminated by depositing electroejaculated/processed sperm transabdominally into the proximal aspect of each uterine horn. The AI procedure was simple and rapid, generally requiring only 30 min after laparoscope insertion. One female, induced to ovulate with 200 IU PMSG and 125 IU hCG and inseminated in utero with 10×10^6 motile sperm at 42.5 hr post-hCG, produced a pregnancy and a single live cub after a 95-day gestation. Laparoscopic AI appears to have considerable potential as a tool for assisting captive propagation of the cheetah.

Key words: Cheetah, *Acinonyx jubatus*, artificial insemination, intrauterine, laparoscopy.

INTRODUCTION

The cheetah (*Acinonyx jubatus*) is well-known for its unique anatomical characteristics (enlarged respiratory and cardiovascular capacities, elongated legs, semiretractile claws) that allow high-speed pursuit of prey. The cheetah is the only surviving species of the genus *Acinonyx* and is threatened by extinction due to the rapid spread of agriculture and subsequent reduction in habitat and ungulate biomass.[28] Legal and illegal killing of cheetahs by farmers attempting to protect livestock also has reduced the population markedly in Namibia and Zimbabwe.[22,27] The species appears to have experienced a severe population bottleneck that was followed by inbreeding in its recent natural history as evidenced by limited genetic variation in modern populations.[29,30] Coincidentally, our laboratory has demonstrated that the cheetah exhibits unusual reproductive traits including a high proportion (~70%) of pleiomorphic sperm in the ejaculate.[18,41,45] Cheetah sperm also may lack some of the robust motility characteristics of other species, like the tiger (*Panthera tigris*),[9] thereby partially explaining why in vitro fertilization (IVF) rates in the cheetah are comparatively low.[7]

Historically, the cheetah has experienced poor reproductive performance in captivity.[3,25] Although the species has been displayed in North American zoos since 1871, the first documented birth of cheetah cubs did not occur until 1956 at the Philadelphia Zoo.[25] Currently, the captive population on this continent is not self-sustaining.[25] Fewer than 20% of all adult cheetahs in North America have ever reproduced, and the

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number of deaths exceeds captive births, primarily because few individuals in the population are reproductively active. Other problems affecting the population dynamics of this species include a high rate of neonatal/juvenile mortality and disease susceptibility, perhaps related to the lack of genetic diversity.[29]

Given the small number of successfully breeding cheetahs, the development of assisted reproductive techniques for this species as well as other felid taxa that exhibit suboptimal fecundity is warranted.[40,44,48,49] One advantage to formulating artificial insemination (AI) strategies for the cheetah is that the species is sensitive to exogenous gonadotropic hormones. Follicular activity and ovulation have been induced routinely in the cheetah using a follicle stimulating hormone preparation (FSH-P, Sigma Chemical Co., St. Louis, Missouri 63178, USA)[48] followed by the administration of human chorionic gonadotropin (hCG). However, our previous attempts to produce pregnancies by depositing a raw ejaculate into the vaginal vault before anticipated ovulation have been unsuccessful.[49] In retrospect, these findings are not surprising because sperm transport[47] and ovulation[16] (see below) appear compromised in anesthetized felids, and others have reported difficulties in producing live felid offspring by AI.[10,26]

Recently, we have made two findings that have altered our AI strategy for nondomestic felid species. The first was associated with the formulation of artificial breeding techniques for the National Black-footed Ferret Recovery Plan.[4,19,42,44] In these studies, an AI technique that relied upon vaginal sperm deposition failed to produce pregnancies. Therefore, we were compelled to develop a laparoscopic intrauterine insemination technique for depositing sperm directly into the uterine horn of ferrets via a catheter inserted through the abdominal wall. This approach proved to be highly successful; pregnancy rates in the domestic ferret (*Mustela putorius furo*), Siberian polecat (*Mustela eversmanni*), and black-footed ferret

(*Mustela nigripes*) were >50% using either fresh or frozen-thawed sperm.[19,42,44] The second major finding was related to adapting the ferret AI technique to the domestic cat.[16] When laparoscopic AI was performed under ketamine hydrochloride, acepromazine, and gaseous halothane anesthesia before ovulation, the pregnancy rate was low (~14%). However, approximately 50% of all cats became pregnant and delivered live offspring when insemination was performed 6–14 hr after ovulation had begun.

The present study assessed the efficacy of laparoscopic intrauterine AI in the postovulatory cheetah while simultaneously evaluating the influence of a "high" versus "low" dosage gonadotropin regimen on inducing follicular development and ovulation.

MATERIALS AND METHODS

Animals

This study was conducted in May 1991 using seven adult (3–8 yr of age) nulliparous female cheetahs at the Caldwell Zoo in Tyler, Texas and the Fossil Rim Wildlife Center in Glen Rose, Texas. Five adult (2.5–5 yr of age) male cheetahs served as sperm donors. Immediately before study onset, females and males were housed in multiple animal exhibits (one male:one to two females/exhibit or two to three females/exhibit), all in outdoor enclosures. Beginning 1 wk before scheduled AI and continuing at least 2 wk after AI, females were housed either singly or only with other females. Before, during, and after ovulation induction and AI, each cheetah was fed a carnivore diet (1–2 kg/day; Nebraska Brand Feline Diet, North Platte, Nebraska 69101, USA; 4 days/wk) supplemented with a whole chicken (1–2 days/wk) and fetal calf meat (1–2 days/wk). Water was available ad libitum.

Induction of ovarian activity and laparoscopic assessment

All gonadotropin hormone injections were delivered by a blow pipe and a projectile

dart. Female cheetahs were given a single i.m. injection of either 200 (n = 4 females) or 400 (n = 3 females) IU pregnant mares' serum gonadotropin (PMSG; Equitech International Ltd., Kerrville, Texas 78029, USA) to induce ovarian follicular development. In previous studies, daily FSH-P injections were used to stimulate ovarian activity.[31,48,49] Based upon domestic cat studies,[11,16] a single PMSG injection was chosen for the present study in anticipation of inducing a follicular response without exposing cheetahs to a daily stress of repeated FSH-P administration. Eighty hr after PMSG, a single injection of either 125 (n = 4 females) or 250 (n = 3 females) IU hCG (Sigma Chemical Co.) was given to induce ovulation. The four females given the low PMSG dose (200 IU) subsequently were injected with the low hCG dose (125 IU), whereas the remaining three females received the high dose of both PMSG (400 IU) and hCG (250 IU).

At 42.5–47.0 hr post-hCG (see details below), females were anesthetized for laparoscopic assessment of ovarian activity and AI. Based upon previous data indicating that anesthesia could inhibit ovulation and reduce pregnancy rates in PMSG/hCG-treated domestic cats,[16] we attempted to coordinate the laparoscopy and AI to the early postovulatory phase. In a preliminary study, each of three female cheetahs at the Caldwell or Metro Toronto Zoo was injected i.m. with 200 or 500 IU PMSG and 125 or 250 IU hCG. At 37–39 hr after hCG, none of these cheetahs had ovulated on the basis of direct ovarian observations, although the ovaries of each contained distinct (≥ 2 mm in diameter) preovulatory follicles (13, 16, and 5 follicles/female, respectively). Therefore, in the present study, laparoscopy was postponed to >42 hr post-hCG in an attempt to coincide with early postovulation.

Laparoscopy

Food was withheld from cheetahs for 24 hr before scheduled laparoscopy. A surgical plane of anesthesia was induced with an i.m. injection of either tiletamine and zolazepam (5.8–6.8 mg/kg body weight; Telazol®, A.H. Robins Co., Richmond, Virginia 23220, USA) or a combination of ketamine hydrochloride (1.1 mg/kg; Vetalar®, Parke-Davis, Morris Plains, New Jersey 07950, USA) and xylazine (1.1 mg/kg; Rompun®, Haver-Lockhart, Shawnee, Kansas 66201, USA) delivered via a projectile dart. Surgical anesthesia was maintained with either isoflurane or halothane gas/oxygen administered via intubation. Each cheetah was placed in a supine head-down position and subjected to laparoscopy as described previously for cheetahs.[31,48] In brief, an intraabdominal pneumoperitoneum was produced using a Verres needle (inserted lateral to midline) attached to a manual insufflator bulb (Richard Wolf Medical Instruments Corp., Rosemont, Illinois 60018, USA). A 12-mm-diameter trocar-cannula was inserted through a 2-cm skin incision made 4 cm cranial to the umbilicus. The trocar was removed and replaced with a rigid, 10-mm-diameter laparoscope (Richard Wolf Medical Instruments Corp.) attached to a light source. Ovaries were examined for preovulatory vesicular follicles (flattened, clear areas with a well-defined border measuring 1.5 mm or more in diameter) and/or postovulatory corpora lutea (CL; opaque structures raised above the ovarian surface).[48] Direct manipulation of the ovary or oviduct was avoided, but an examination of all aspects of each gonad was possible with modest manipulation with the Verres needle on adjacent ligaments or the uterine horn itself. Females exhibiting at least one fresh CL were classified as postovulatory regardless of the number of follicles present.

Semen collection and processing

Anesthesia for semen collection was induced with an i.m. injection of tiletamine and zolazepam (3.2–4.4 mg/kg body weight; Telazol®, A.H. Robins Co.). A standardized electroejaculation procedure, which included the use of a rectal probe (1.6 cm diameter) containing three longitudinal

electrodes and an AC 60-Hz sine-wave ejaculator, has been described previously.[20,41,45,46] Immediately after collection, a 5-μl aliquot of the ejaculate was examined with a phase-contrast microscope ($\times$250) for subjective estimates of sperm percent motility (0–100%) and progressive motility on a 0–5 scale (0 = no movement; 5 = rapid forward progression).[41,46] Sperm concentration/ml of ejaculate was determined using a standard hemacytometer counting procedure.[20,41] Detailed sperm morphology evaluations were performed after fixing ejaculate aliquots (10 μl) in 0.3% glutaraldehyde, followed by phase-contrast microscopic examination of 200 sperm/aliquot at $\times$1,000.[17,41,46]

Remaining ejaculate was transferred rapidly into a sterile 1.5-ml conical tube (Sarstedt Inc., Princeton, New Jersey 08543, USA) and slowly diluted (1:1) with Ham's F10 medium (Irvine Scientific, Santa Ana, California 92705, USA) containing 5% fetal calf serum (Irvine Scientific). Diluted semen was centrifuged (300 g, 10 min), the supernatant discarded, and the sperm pellet resuspended gently into 200 μl of fresh Ham's F10 medium. Sperm concentration, motility, and progressive motility were evaluated, and the sample was maintained at room temperature (23°C) and shielded from light until the time of AI. A single sperm donor was used to provide one inseminate for each female recipient. A total of 250–300 μl of the sperm suspension was used per female (125–150 μl/uterine horn). The total number of motile sperm/AI was calculated by multiplying the sperm concentration by the inseminate volume by the sperm motility rating.

Laparoscopic AI

Following laparoscopic assessment of ovarian activity, the intrauterine AI technique was performed in six of the seven cheetahs as described previously for the domestic cat.[16] An accessory Palmer grasping forceps (Richard Wolf Medical Instruments Corp.) was inserted 3 cm lateral to the um-

bilicus to stabilize each uterine horn. The uterus was elevated toward the ventral abdominal wall and then, under direct laparoscopic viewing, cannulated using a sterile, 18-ga, 5-cm-long canine indwelling catheter (Sovereign®, Sherwood Medical, St. Louis, Missouri 63103, USA). Each catheter was inserted percutaneously into the proximal third of the uterine lumen, the catheter stylette removed, and replaced with sterile polyethylene tubing (PE-50, Intramedic®, Clay Adams, Parsippany, New Jersey 07054, USA) containing the sperm suspension. The PE tubing was inserted beyond the tip of the catheter and into the uterine lumen, and the diluted sperm within the PE tubing was expelled into the lumen using ~0.4 ml of air delivered from a 1-ml plastic syringe. After repeating the entire procedure on the contralateral horn, the catheter, tubing, and laparoscopic instruments were removed and the incision sites were sutured. The entire anesthesia/laparoscopy interval (from anesthesia induction through suturing) was 60–120 min, whereas the AI procedure generally required only 30 min after laparoscope insertion. Semen was deposited into the uterus of each female within 60–100 min of initial ejaculate dilution with Ham's F10 medium.

Estradiol-17β and progesterone analysis

To develop a data base for hormonal values following this exogenous gonadotropin therapy, a blood sample was collected from each female following induction of anesthesia but before the onset of laparoscopy. Serum estradiol-17β was quantified using a double antibody ^{125}I radioimmunoassay kit (ICN Biomedicals, Inc., Costa Mesa, California 32625, USA). In validation tests, serial dilutions of cheetah serum pools were parallel to the standard curve. Addition of 15.0, 50.0, 150.0, and 500.0 pg estradiol resulted in a net recovery of 14.5, 45.6, 157.9, and 513.2 pg, respectively ($y = 1.03x - 1.60$; $r = 0.999$). Assay sensitivity was 5.0 pg/ml, and the intra- and interassay coefficients of variation were <10%.

Table 1. Ovarian activity and serum concentrations of estradiol-17β and progesterone at the time of laparoscopic intrauterine AI in cheetahs following PMSG and hCG stimulation.

Female no.[a]	PMSG/hCG dosage (IU)	Time of AI (hr post-hCG)	No. follicles	Estradiol-17β (pg/ml)	No. and type of CL[b] Total	No. and type of CL[b] Large	No. and type of CL[b] Small	Progesterone (ng/ml)
464	200/125	43.0	6	13.6	5	5	0	68.0
429	200/125	42.5	2	8.4	6	6	0	0.6
539	200/125	43.0	4	11.3	8	4	4	0.9
504	200/125	45.5	6	14.5	3	3	0	0.2
572	400/250	45.0	4	9.0	6	3	3	4.4
503	400/250	43.5	2	11.5	12	0	12	0.3
537	400/250	47.0	3	12.7	13	0	13	0.4

[a] Represents the identification number assigned to each female cheetah in the International Cheetah Studbook.

[b] Two types of CL were observed: 1) large-sized CL that were 5–8 mm in diameter, bright red, and prominently raised above the ovarian surface; and 2) small-sized CL that were 2–4 mm in diameter, pale pink, and flattened or only slightly raised above the ovarian surface.

Serum progesterone was measured using a double antibody ^{125}I radioimmunoassay kit (ICN Biomedicals, Inc.). Serial dilutions of cheetah serum pools were parallel to the standard curve. Upon addition of 0.25, 1.0, 2.5, 5.0, 10.0, and 20.0 ng progesterone, 0.35, 1.03, 2.41, 4.14, 9.97, and 19.71 ng, respectively, were recovered after subtraction of endogenous hormone (y = 0.99x − 1.12; r = 0.999). Assay sensitivity was 0.2 ng/ml, and the intra- and interassay coefficients of variation were <10%.

Data analysis

Values are reported as means ± standard error of the mean (SEM). Differences in the mean number of follicles, total CL, types of CL observed, and serum concentrations of estradiol-17β and progesterone between the low and high dose gonadotropin groups were analyzed using a Student's t-test.[37] Correlation coefficients between specific ovarian activity characteristics and serum hormone data were calculated on a portable calculator.

RESULTS

All seven females demonstrated follicular activity and ovulation following PMSG/hCG treatment (Table 1). Although each cheetah had initiated ovulation at the time of laparoscopy (42.5–47.0 hr post-hCG), the ovaries of each female still contained an average of 3.9 ± 0.6 distinct follicles ranging from 2 to 5 mm in diameter. There was no difference (P > 0.05) in the mean number of follicles between the low (200 IU PMSG/125 IU hCG; 4.5 ± 0.9 follicles/female) and high (400 IU PMSG/250 IU hCG; 3.0 ± 0.6 follicles/female) dose gonadotropin treatment groups. Overall, females produced an average of 7.6 ± 1.4 CL; however, CL number was influenced (P < 0.05) by gonadotropin dosage. Females receiving the higher PMSG/hCG dose produced approximately 2× more ovulations (10.3 ± 1.4 CL/female) compared to females injected with the lower PMSG/hCG dose (5.5 ± 1.0 CL/female).

Gonadotropin treatment also influenced (P < 0.05) the morphologic characteristics of the CL. Two distinct CL types were detected: 1) large-sized CL that were 5–8 mm in diameter, bright red, and prominently raised (>3 mm) above the ovarian surface (Fig. 1a); and 2) small-sized CL that were 2–4 mm in diameter, pale pink, and flattened or only slightly raised (<3 mm) (Fig. 1b). At the time of AI, cheetahs receiving the low dose of PMSG/hCG exhibited more (P < 0.05) large type CL (4.5 ± 0.6 CL/female) than females given the high PMSG/
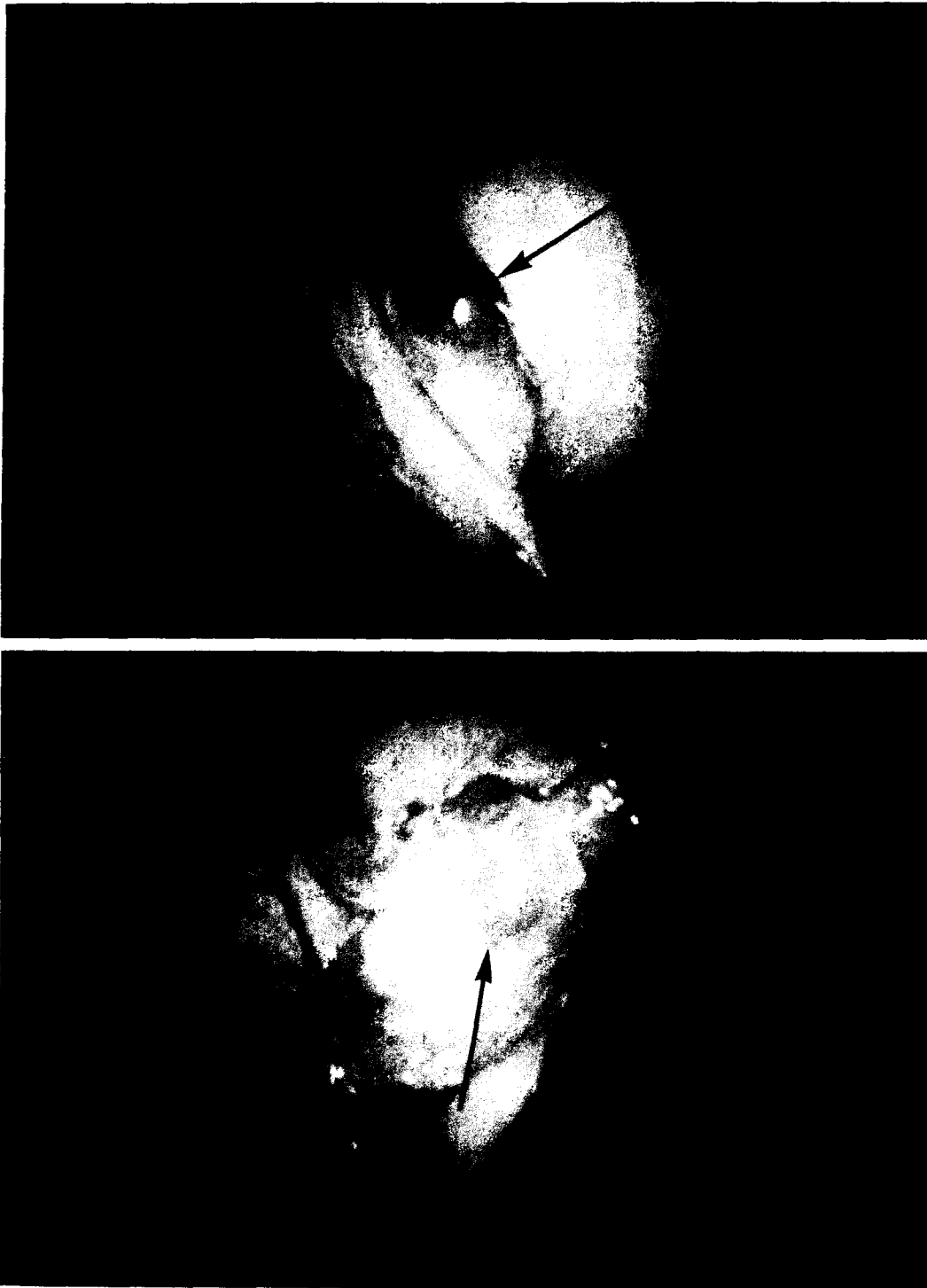
Figure 1. Cheetah ovaries containing two types of corpora lutea (CL) following gonadotropin stimulation. **a.** The large type of CL (arrow) were 5–8 mm in diameter, bright red, and prominently raised above the ovarian surface. **b.** The small type of CL (arrow) were 2–4 mm in diameter, pale pink, and only slightly raised above the ovarian surface.

Table 2. Ejaculate and inseminate traits for individual male cheetahs used as sperm donors for laparoscopic intrauterine AI.

Measure	Male no.[a] 501	536	1774	505	506	1774
Ejaculate traits:						
Ejaculate volume (ml)	2.7	2.5	0.6	1.2	1.8	1.0
Sperm concentration/ml ($\times 10^6$)	16	6	16	22	14	7
Sperm motility (%)	75	65	80	70	80	80
Sperm progressive motility[b]	4.5	4.0	4.0	3.5	4.0	4.0
Structurally normal sperm (%)	41	20	20	18	33	19
Structurally abnormal sperm (%)						
Macrocephalic	0	3	2	0	1	3
Microcephalic	0	4	1	0	3	2
Abnormal acrosome	3	13	5	8	5	5
Midpiece aplasia	0	1	1	0	1	0
Coiled flagellum	18	33	8	33	13	17
Bent midpiece	20	19	45	26	14	37
Bent flagellum	3	3	1	8	1	0
Proximal cytoplasmic droplet	11	1	11	6	26	12
Distal cytoplasmic droplet	4	3	6	1	3	5
Inseminate traits:						
Female no.[a]	464	429	539	504	572	503
Sperm motility (%)	40	65	75	40	10	65
Sperm progressive motility[b]	4.0	4.0	4.5	3.0	1.5	4.0
No. of motile sperm ($\times 10^6$)	9.1	10.0	6.4	13.6	1.5	5.4
Pregnant	−	+	−	−	−	−

[a] Represents the identification number assigned to each cheetah in the International Cheetah Studbook.

[b] Sperm progressive motility was based on a scale of 0–5, where 5 = most rapid forward progression.

hCG dose (1.0 ± 0.9 CL/female). Conversely, the low hormone dose group produced fewer ($P < 0.01$) small type CL (1.0 ± 0.9 CL/female) than females administered the high gonadotropin dose (9.3 ± 3.2 CL/female).

Mean serum estradiol-17β concentrations at the time of AI were similar ($P > 0.05$) among females (range: 8.4–14.5 pg/ml; Table 1). There was no difference ($P > 0.05$) in circulating estradiol-17β concentrations between the two gonadotropin treatment groups. Postovulatory serum progesterone concentrations varied widely among females (mean range: 0.2–68.0 ng/ml; Table 1) and were not influenced ($P > 0.05$) by gonadotropin treatment or CL number or type ($r = -0.3$ for progesterone and total CL number).

Overall, mean ejaculate characteristics for male cheetahs were consistent with published values for the species:[41,45] ejaculate volume, 1.6 ± 0.3 ml; sperm concentration, $13.5 \pm 2.4 \times 10^6$/ml; sperm percent motility, $75.0 \pm 2.6\%$; sperm forward progressive motility, 4.0 ± 0.1; and percent morphologically normal sperm/ejaculate, $25.2 \pm 3.9\%$. Seminal characteristics for individual sperm donors varied among cheetahs (Table 2). However, all males produced relatively high sperm motility ratings (range: 65–80%) and high proportions of structurally abnormal spermatozoa (range: 59–82%) (Table 2). The most prevalent sperm cell abnormalities were a bent midpiece (overall mean = 26.8%), coiled flagellum (20.3%), proximal cytoplasmic droplet (11.2%), and abnormal acrosome (6.5%) (Table 2).

All of the cheetahs were subjected to laparoscopic AI, except female #537 because no male was available to serve as a sperm donor. Overall, the mean percent sperm mo-

tility, progressive motility, and total number of motile spermatozoa inseminated/female were 49.2 ± 9.8%, 3.5 ± 0.4, and 6.7 ± 2.1 × 10⁶, respectively. The mean interval between hCG administration and AI was 43.8 ± 0.5 hr (range: 42.5–45.5 hr) (Table 1). Because cheetah sperm rapidly lose motility after dilution and centrifugation, it was not possible to standardize inseminate characteristics for each female. Nevertheless, each female received from 1.5 × 10⁶ to 13.6 × 10⁶ motile spermatozoa in utero (Table 2).

A single pregnancy was produced and a single live cub was born following a 95-day gestation in cheetah #429, the female producing the greatest number of large-sized CL ($n = 6$) and the fewest unovulated follicles ($n = 2$) (Tables 1, 2). This female was inseminated at 42.5 hr after hCG injection with 10 × 10⁶ motile sperm (overall sperm percent motility, progressive motility, and proportion of structurally normal sperm, 65%, 4.0, and 20%, respectively) from donor male #536. The cub was of normal size and healthy at birth, and nursing was observed. Approximately 18 hr later, the female became agitated during a brief, severe thunderstorm and killed the cub. Necropsy revealed the neonate to be a normal male. Cause of death was attributed to thoracic cavity trauma induced by bite wounds.

DISCUSSION

This represents the first documented birth of a cheetah cub as the result of assisted reproductive technology. Although only one of six inseminated females delivered a live offspring, we are encouraged by the potential of this approach for two reasons. First, emphasis on basic reproductive mechanisms is the key to understanding the physiological processes that eventually allow artificial breeding techniques to become routine,[49] and the domestic cat serves as a model for various wild felid species. During the past decade, laboratory studies of the domestic cat have permitted establishment of the: 1) time course and conditions for sperm capacitation; 2) conditions necessary for high AI, IVF, and embryo culture success; 3) factors regulating oocyte maturation; and 4) impact of teratospermia upon fertilization in vitro.[44] This information not only is invaluable for the domestic cat but allows the more rapid adaptation of knowledge and technology to related, nondomesticated felid species. Secondly, laparoscopic AI is finding utility in other endangered taxa. Leopard cats (Felis bengalensis), black-footed ferrets, a puma (Felis concolor), and a tiger cub have been produced using this approach.[15,36,44] Recently, a leopard cat female inseminated laparoscopically with frozen-thawed sperm became pregnant and delivered two live kittens.[21] Also, nine of 20 Elds deer (Cervus eldi) females produced offspring following laparoscopic insemination with frozen-thawed sperm.[44]

The female cheetah, in which the AI was successful, was a wild-caught individual that had been in captivity since 1985 and exposed to at least three different males including one proven breeder with no observed breeding. In general, a male cheetah exposed to an estrous female will not demonstrate the aggressive behavioral incompatibility observed in species like the clouded leopard (Neofelis nebulosa).[40] On the contrary, most zoo-maintained male cheetahs tolerate exposure and even chronic cohabitation with one or more female conspecifics, but a primary problem appears to be lack of sexual interest by both partners. Recent unpublished data from a nationwide survey of cheetahs in the North American Species Survival Plan reveal that most males are producing spermic ejaculates, but overall semen quality (relative to other felid species) is comparatively low (Wildt et. al., unpubl. data). Likewise, laparoscopic examinations indicate that females appear anatomically sound, but few demonstrate evidence of ovarian follicular activity. Whether the etiology of these observations and the overall poor reproductive performance of the species in captivity are genetic, organic, or simply management related remains to

be determined. Nevertheless, this birth demonstrates the biological competence of sperm from an unproven, unrepresented male in the captive population. More importantly, these results indicate that there may soon be reliable artificial breeding techniques available for assisting in the management of a species that has eluded consistent captive breeding success for centuries. Although our objective is not to manage the entire species by assisted reproductive technology, AI will allow more rapid production of offspring from valuable founders that have failed to reproduce naturally in captivity. Combined with cryopreservation technology, AI also allows for long-term preservation of genetic diversity while offering a feasible approach for transferring genetic material among zoos without the health risks associated with transporting living animals.

There are several possible reasons why laparoscopic AI of felids can generate term offspring in contrast to vaginal sperm deposition that almost inevitably fails to result in pregnancy. Pregnancies have resulted in the domestic cat after vaginal deposition of fresh or thawed sperm into anesthetized females, but the incidence of pregnancy is <11%.[32] This low conception rate may have been related to improper site of sperm deposition and/or timing of insemination with respect to ovulation. Moore et al.[26] reported the first successful AI in any wild felid species (puma), but the procedure required laparotomy to deposit the sperm into the uterine horn. Dresser et al.[10] reported the birth of a stillborn leopard cub after ovulation induction and the nonsurgical deposition of sperm into the uterine body. Previous attempts in our laboratory to produce pregnancies from AI with fresh or thawed sperm and vaginal deposition have been unsuccessful, despite the availability of relatively large numbers of sperm recipients (30 cheetahs[49] and 11 tigers[47]). A preliminary study in female tigers ovariohysterectomized 1 hr after AI revealed no sperm in the oviducts, suggesting that perhaps the anesthesia or some related factor compro-

mised the ability of sperm to reach the site of fertilization.[47] Additionally, in more recent AI studies of ferrets, no pregnancies resulted when sperm were deposited into the vagina of anesthetized females, but at least 70% of anesthetized ferrets delivered live young when fresh or thawed sperm were placed directly in utero using the laparoscopic approach.[19,42,44] In sheep, significant improvements in fertilization rates in superovulated ewes have been reported after intrauterine insemination with fresh or frozen-thawed semen,[1,34,39] and the laparoscopic approach has been used for many years in this species to by-pass the cervical barrier.[24] Similarly, laparoscopic intrauterine AI has been applied and is used routinely in the deer farming industry for the production of fallow deer (Dama dama).[2] An extensive AI study in cattle including >4,000 inseminations revealed that site of insemination within the uterus also appears to influence fertilization. Sperm deposition into the uterine horns of cows resulted in a higher conception rate (65%) than when sperm were deposited transcervically into the uterine body (45%).[35] Recently, we have determined that timing of insemination affects conception rates in anesthetized, gonadotropin-stimulated domestic cats.[16] In that study, ovulation was compromised by the preovulatory administration of ketamine hydrochloride, acepromazine, and gaseous halothane required for laparoscopy and sperm deposition. If anesthesia/laparoscopy was performed after ovulation onset, AI efficiency was enhanced more than 3×, and 50% of the domestic cats became pregnant. Based upon these findings and the present results, it is apparent that the appropriate AI strategy for felids is to deposit sperm as near as possible to the site of fertilization (oviduct), but only after ovulation has commenced. This recommendation probably is essential for species like the cheetah that consistently produce ejaculates containing few normally structured sperm with relatively poor motility characteristics.

For the cheetah, enhancing the AI preg-

nancy rate likely can be achieved by focusing on 1) the ovulation induction regimen and 2) further improvements in inseminate quality. A single injection of PMSG followed by hCG stimulated folliculogenesis and ovulation in each female. However, ovarian activity and the morphological features of the postovulatory CL were influenced by gonadotropin dosage. The higher PMSG/hCG dose produced more ovulations per female, but the resulting CL were small and generally flattened or only slightly raised above the ovarian surface. In contrast, the CL observed following the lower gonadotropin dosage usually were large-sized and prominently raised above the ovarian surface, similar to those observed in an earlier cheetah study[48] when FSH-P was used to stimulate ovarian activity. Although we have no empirical evidence to indicate that the larger-sized CL are more "normal" in structure and function than their smaller-sized counterparts, on the few occasions that naturally mated cheetahs have been subjected to laparoscopy, the resulting CL have been morphologically similar to the large-type luteal structures described here. This CL type apparently exhibited adequate luteal function to sustain embryo development and implantation because the pregnancy occurred in the female producing the greatest number of large-sized CL. Major differences in CL size also are observed in gonadotropin-treated sheep where the induction of estrus is facilitated by the simultaneous use of prostaglandins.[33,34] Small-sized CL in these ewes are associated with premature luteal regression and an inability to sustain pregnancy. It is not known if these smaller-sized CL in cheetahs immediately regress or are functionally normal. Interestingly, in the present study, the difference in CL size had no relationship to circulating progesterone concentrations at the time of AI, and there was nothing obviously unique about the pregnant cheetah that would predict why AI was successful. With the exception of cheetah #464, which produced a rather extraordinary

amount of circulating progesterone on the day of laparoscopy, the remaining females produced progesterone concentrations that were fairly typical of early postovulatory gonadotropin-treated domestic cats.[8,11]

Although both PMSG/hCG regimens were effective in stimulating the production of luteal tissue in all treated cheetahs, not all follicles had ovulated by the day of laparoscopy. Our unpublished observations suggest that these post-hCG "residual" follicles never ovulate and, when aspirated, either contain a degenerate oocyte or no oocyte at all. The origin of residual follicles may be attributed to the long-acting effects of the large molecular weight PMSG or even to hCG, which is known to be partially folliculogenic in the domestic cat.[12,43] The potential adverse impact of residual follicles cannot be ignored. It is possible that gonadotropin stimulation for subsequent AI may perturb temporal endocrine events, perhaps only subtly but sufficiently to compromise conception and implantation. For example, an abnormal estradiol-17β-to-progesterone ratio resulting in inadequate priming of the endometrium appears to be one reason for the high implantation failure rate in women after gonadotropin therapy, IVF, and embryo transfer.[5] Because these mechanisms remain so poorly understood, some human IVF programs provide supplemental progesterone during the early luteal phase of the IVF–embryo transfer cycle.[5] From the limited data generated in the present study, it is impossible to predict accurately whether the residual follicles in cheetahs were producing "excessive" estradiol-17β or somehow were disrupting the estrogen-to-progesterone ratio. Unfortunately, there are no normal baseline values for circulating estrogen concentrations near the time of ovulation in cheetahs. Even so, at least one previous study has demonstrated a weak association between circulating estradiol-17β concentration and quantitative follicular activity in the domestic cat.[8]

As assisted reproductive techniques continue to evolve, it is prudent to develop go-

nadotropin regimens that result in ovarian responses simulating activity observed after a natural estrus and mating. Hyperstimulative responses, whereby the ovaries contain a greater than normal number of CL and multiple unovulated follicles, should be avoided. This goal presents some interesting challenges because reliance upon animal models like the domestic cat does not always ensure rapid deployment of effective technology, primarily because of individual variation among females and unique species specificities. For example, the present data suggest that the timing of ovulation following hCG administration may be different between the cheetah and the domestic cat. Mature ovarian follicles in the domestic cat rupture approximately 25–27 hr after hCG,[13,38] whereas, in our preliminary cheetah study, no evidence of ovulation was detected by 37–39 hr after hCG. However, when ovarian activity was evaluated 42.5–47 hr after hCG, all cheetahs had fresh CL. There also appeared to be species differences with respect to gonadotropin sensitivity. We routinely induce follicular activity and an average of 7.5 CL in the domestic cat using 100 IU PMSG and 75 IU hCG.[16] Given that the cheetah weighs 7–8× the domestic cat mass, then it is extraordinary that multiple ovulations in the former species can be produced after giving as little as 200 IU PMSG (only 2× the domestic cat dose). Therefore, it is mandatory that these species sensitivity differences be examined as gonadotropin regimens are developed for assisted reproduction in wild felids.

CONCLUSION

Intrauterine insemination has been used for treating human infertility for more than 40 yr, primarily because the technique increases the number of sperm reaching the oviduct and the opportunity for fertilization.[6,14,23] The present data demonstrate that it is time to consider seriously the use of laparoscopic AI in certain wildlife species, especially those demonstrating suboptimal reproductive performance in captivity. The cheetah is a prime candidate because of its traditionally poor reproductive history in zoos and its tendency to produce sperm characterized by many abnormal forms and relatively poor viability. All (7 of 7) females responded to a simple, two-injection gonadotropin regimen. The cheetah appeared highly sensitive to the PMSG and hCG preparations, more so than the domestic cat. A lower gonadotropin dose was more effective in mimicking a "normal" ovulatory response, which also included producing CL of a size observed in naturally ovulating cheetahs. Finally, a laparoscopic technique was determined to be simple and effective for cannulating the uterine lumen transabdominally to allow direct deposition of semen. One of six females inseminated using this approach became pregnant and delivered a live cub after a normal length gestation. Laparoscopic AI may be useful for overcoming barriers preventing sperm cells from reaching the site of fertilization, thereby enhancing our ability to manage the cheetah, and other species, in captivity.

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