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In Vitro Fertilization and Embryo Development In Vitro and In Vivo in the Tiger (*Panthera tigris*)[1]

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ABSTRACT

A study was conducted to evaluate the adaptability to the tiger of an in vitro fertilization/embryo culture system previously developed in the domestic cat. In Trial I (July 1989), 10 female tigers were treated with either 2 500 (n = 5) or 5 000 (n = 5) IU eCG i.m. and with 2 000 IU hCG i.m. 84 h later. In Trial II (January 1990), 6 females (5 of which were treated in Trial I) were given 2 500 IU eCG i.m. and 2 000 IU hCG i.m. 84 h later. Twenty-four to twenty-six hours after hCG treatment, all tigers were subjected to laparoscopy, and oocytes were aspirated transabdominally. On the basis of follicular development (follicles ≥ 2 mm in diameter), all females responded to exogenous gonadotropins (range, 6–52 follicles/female). Follicle number and oocyte recovery rate were unaffected ($p > 0.05$) by eCG dose or time of year. A total of 456 oocytes were collected from 468 follicles (97.4% recovery; mean, 28.5 ± 3.4 oocytes/female). Of these, 378 (82.9%) qualified as mature, 48 (10.5%) as immature, and 30 (6.6%) as degenerate. During Trial I, 8 electroejaculates were collected from 7 male tigers, and in Trial II, 3 semen samples were collected from 3 males. Motile sperm were recovered on each occasion; the overall mean (± SEM) ejaculate volume was 7.5 ± 0.7 ml, the number of motile sperm/ejaculate was 105.9 ± 20.6 × 10^6, and the percentage of structurally normal sperm/ejaculate was 81.4 ± 2.0%. After swim-up processing, 0.05 × 10^6 motile sperm were co-cultured with 10 or fewer tiger oocytes in a humidified atmosphere (38°C) of 5% CO_2 in air. Of the 358 mature oocytes inseminated, 227 (63.4%) were fertilized. Oocytes from 2 females became contaminated in culture and, therefore, were excluded from embryo cleavage calculations. Of the remaining 195 fertilized oocytes, 187 (95.9%) cleaved to the two-cell stage. No parthenogenetic cleavage was observed in noninseminated control oocytes (n = 20). Eighty-six good-to-excellent-quality two- to four-cell embryos were transferred surgically into the oviducts of 4 of the original oocyte donors in Trial I and 2 females in Trial II. A pregnancy occurred in 1 female in Trial II, and 3 live-born cubs were delivered by Caesarian section 107 days after embryo transfer. Of the 56 cleaved embryos cultured in vitro in Ham's F10 for 72 h, 14 (25.0%) were at the sixteen-cell stage, and 15 (26.8%) were morulae. Of the 46 embryos cultured for 96 h, 20 (43.5%) advanced to morulae, and 14 (30.4%) to early blastocysts. The data demonstrate the ability of tiger sperm to fertilize follicular oocytes in vitro. The resulting embryos are capable of advancing to morulae and blastocysts in culture and to live-born offspring after embryo transfer.

INTRODUCTION

The tiger is one of 36 threatened or endangered felid species [1]. Since 8 subspecies of tigers were recognized formally in 1969 by the International Union for Conservation of Nature and Natural Resources, 3 (*Panthera tigris vigata, P.t. sondaica, P.t. balica*) have become extinct, and 1 (*P.t. amoyensis*) is critically endangered (only about 50 animals remain) [2]. The primary causes for loss of free-living tigers are extensive habitat fragmentation and a declining prey base [3]. About 250 individuals are required to sustain adequate genetic diversity within a tiger subspecies [3]. Because a natural, free-ranging population of this size would require extensive habitat (~25 000–50 000 km^2) [3], it is necessary to propagate tigers in individual captive breeding programs [4]. In this context, attempts are being made to manage most of the world's captive tigers under guidelines that provide breeding recommendations for sustaining maximum genetic diversity within a given subspecies [3, 4]. Reproductive biotechniques including in vitro fertilization (IVF) and embryo transfer could play an important role in managing tigers, especially when desirable animal pairs are separated geographically by long distances or in selected cases of sexual incompatibility or infertility [5].

There is a growing database on the reproductive physiology of the tiger. In North America, the Siberian tiger (*P.t. altaica*) is seasonally polyestrous; females demonstrate a 5-day estrus every 25.0 ± 1.3 days between January and early June [6]. In contrast, electroejaculation data suggest that Siberian and Bengal (*P.t. tigris*) tigers produce sperm throughout the year [7–9]. Unlike the majority of non-domestic felid species, which produce more than 60% pleiomorphic sperm/ejaculate [8, 10–12], tiger ejaculates

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contain low proportions of morphologically abnormal spermatozoa (range, 21.8–37.5%) [7–9, 13]. There have been a few attempts to stimulate ovarian activity in the tiger with exogenous gonadotropins. Serial injections of commercially available FSH-P have been only partially effective [14, 15], and the need to give repeated injections of FSH-P is a disadvantage when studying stress-susceptible wildlife species. Vaginal artificial insemination (AI) has not resulted in pregnancies [15], perhaps because exogenous gonadotropins alter the endogenous endocrine milieu, thereby compromising cervical sperm transport [7].

One attractive alternative to AI is IVF. The resulting embryo can be seen and characterized and, if pregnancy does not occur after transfer, at least the general timing of the reproductive failure is known. Particularly important is that IVF does not require identification of estrus or prediction of ovulation, two characteristics often difficult to determine in nondomestic species [5]. In our laboratory, IVF has been used to produce viable-appearing embryos of domestic cats (*Felis catus*) [16–18], leopard cats (*F. bengalensis*) [19], and pumas (*F. concolor*) [12]. In each species, eCG stimulates ovarian follicle development consistently, although the number of resulting oocytes varies between and within species [12, 16, 19]. In domestic cats, the sperm/oocyte coculture system routinely results in 65–80% of all mature oocytes cleaving in vitro [17, 18, 20]. In contrast, fewer ovarian follicles are available for aspiration in leopard cats, and oocyte degeneration rate 24 h after insemination is 5-fold higher than in domestic cats [19]. In pumas, eCG is also effective for stimulating follicular growth, but a high proportion of the aspirated oocytes (31%) are immature [12]. Overall cleavage rate in vitro is low (25%), which may be related, in part, to the remarkably high proportion of pleiomorphic spermatozoa (>90%) ejaculated by male pumas [12].

This study was conducted as part of a long-term effort to explore the conservation of fertilization mechanisms among the Felidae family [5]. To extend our comparative observations further, we examined the utility of IVF in the tiger, the largest of all felid species. Our specific objectives were 1) to examine the effectiveness of eCG dose and consecutive treatments at different times of the year on ovarian follicle development and oocyte quality; 2) to assess the IVF ability of laparoscopically aspirated follicular oocytes exposed to homologous, processed spermatozoa; and 3) to determine the in vitro and in vivo developmental competence of these embryos.

MATERIALS AND METHODS

Animals

The study was conducted over a 12-day period in July 1989 (Trial I) and a 10-day period in January 1990 (Trial II) at both the Minnesota Zoological Gardens (Apple Valley, MN) and the Henry Doorly Zoo (Omaha, NE). A total of 11 adult (5–11 yr of age) female tigers (5 *P.t. altaica*; 6 *P.t. tigris*) were used as oocyte donors. Ten females were used in Trial I and 6 in Trial II. Six of the tigers in Trial II also served as oocyte donors in Trial I, so these females provided additional data on the impact of two consecutive gonadotropin treatments on ovarian responsiveness and oocyte recovery and quality. All tigers in both trials had normal reproductive histories (i.e., evidence of reproductive cyclicity), and 5 were mono- or multiparous. As part of an existing management protocol, 2 tigers (#2 and #8) had previously received implants of a progestagen (medroxyprogesterone acetate)-containing contraceptive [21]; these silastic implants were removed 6 wk before the onset of Trial I. Seven adult (5–13 yr of age) males (2 *P.t. altaica*; 5 *P.t. tigris*) served as sperm donors. All males were either proven fertile on basis of live cubs sired or had produced high-quality electroejaculates consistently in previous studies [9, 13]. Each tiger was housed separately with free access to an outdoor area (5.5 × 6.8 m) during daylight hours; during the night, each tiger was confined indoors in a 2.6 × 2.6-m enclosure. Each animal was fed a carnivore diet (Nebraska Brand Feline Diet, North Platte, NE) daily, and water was available ad libitum.

Induction of Ovarian Activity, Laparoscopy and Oocyte Recovery

Female tigers were administered gonadotropins without regard to a specific stage of the reproductive cycle. In Trial I, either 2 500 (n = 5) or 5 000 (n = 5) IU eCG (Equitech Incorporated, Atlanta, GA) were administered i.m. Findings from this trial indicated no effect of eCG dose on follicle or oocyte number or on oocyte quality or fertilization ability. Therefore, all tigers in Trial II were treated with a single 2 500-IU dose of eCG i.m. Females at the Minnesota Zoological Gardens received eCG via blow-dart delivery. The tigers at the Henry Doorly Zoo were placed in a restraint cage, and the eCG was injected by syringe. In both trials, the same injection approaches were used respectively at each institution to give each tiger 2 000 IU hCG i.m. (Sigma Chemical Company, St. Louis, MO) 84 h after eCG.

Oocytes were recovered 24–26 h after hCG treatment by use of a standard laparoscopic procedure for tigers [15] that was modified to allow transabdominal oocyte aspiration [12, 16]. In brief, a surgical plane of anesthesia was induced with xylazine (Rompun®, Mobay Corp., Shawnee, KS; 0.5 mg/kg body weight, i.m.), diazepam (Valium®, Hoffman LaRoche, Nutley, NJ; 0.1 mg/kg, i.m.), and ketamine hydrochloride (Vetalar®, Parke-Davis, Detroit, MI; 5.0 mg/kg, i.m.). To sustain this plane of anesthesia, each female was intubated and maintained on halothane gas/oxygen or given supplemental i.v. injections of ketamine HCl (1.5 mg/kg). Each animal was placed in a supine, head-down position on a surgical table and surgically prepared, and a 10-mm-diam. 180° laparoscope (Richard Wolf Medical Instruments Corporation, Rosemont, IL) was inserted into the abdomi-

nal cavity through a 2-cm-long skin incision made near the umbilicus. Ovaries were evaluated for number of ovarian follicles (clear structures with a well-defined border measuring 2 mm or more in diam.), corpora hemorrhagica (CH), or corpora lutea (CL) according to our previous criteria [15]. Follicle diameter was measured using a 2-mm-diam. Verres needle probe inserted transabdominally [16].

The Verres needle was also used to position and secure the ovary for oocyte aspiration using a procedure described previously for the domestic cat [16], leopard cat [19], and puma [12]. A 22-g, 4-cm-long needle attached to size 100 polyethylene tubing (inner diam., 0.86 mm; Clay Adams, Parsippany, NJ) was rinsed with 2–3 ml of Ham's F10 medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum (Irvine Scientific) and 40 units heparin/ml of medium. A siliconized collection tube (Terumo Medical Corporation, Elkton, MD) was attached to the free end of the polyethylene tubing, and the aspiration system was driven by a vacuum pump (Gast Manufacturing Corporation, Benton Harbor, MI). Distinct follicles ≥2 mm in diam. were perforated with the needle while gentle negative pressure (100 mm Hg) was applied with the vacuum pump. After aspiration of follicles from one ovary, the collection tube and aspiration needle were replaced, and the procedure was repeated for the contralateral ovary. Collection tubes from each animal were emptied into separate plastic culture dishes that were examined by stereomicroscopy. Each oocyte-cumulus cell complex was evaluated for maturational status and classified as 1) mature if corona radiata and cumulus oophorus cells were loosened and expanded (Fig. 1a); 2) immature if the oocyte had a tightly compacted corona radiata; or 3) degenerate if the oocyte appeared ab-

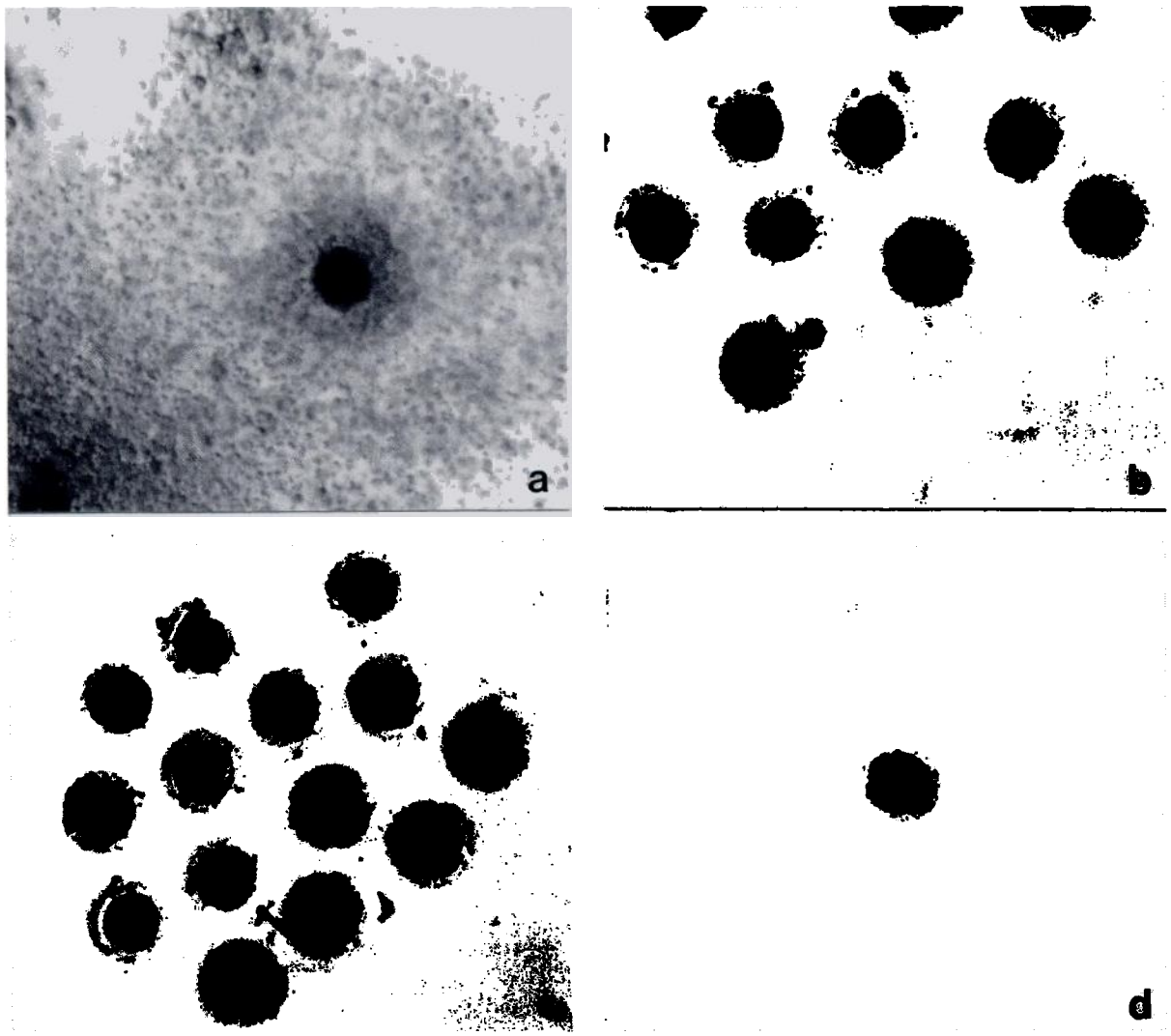

FIG. 1. (a) Mature tiger oocyte with expanded corona radiata and cumulus cell mass. (b) Two- and four-cell stage, in vitro-fertilized tiger embryos after 30 h of culture. (c) Sixteen-cell and morula stage, in vitro-fertilized tiger embryos after 72 h of culture. (d) Early blastocyst, in vitro-fertilized tiger embryo after 96 h of culture. Magnification for a–d: ×300.

normal, pale, and/or lacked an apparent corona radiata. Oocytes were washed 3 times in Ham's F10 under oil and placed in fresh medium (without heparin).

Because neither the Minnesota Zoological Gardens nor Henry Doorly Zoo had tissue-culture facilities, a simplified portable incubation system was developed and tested. A custom culture chamber (5 × 12 × 22 cm) made of 1.2-cm-thick plastic containing 2 air-ports and a removable faceplate was constructed by C.B.S. Scientific Company (Del Mar, CA). Oocytes and/or embryos in culture dishes were placed in the chamber, and one of the air-ports was attached via a plastic hose to a tank of 5% CO_2 in air. To humidify the chamber, the CO_2 gas was bubbled through a water reservoir. After the chamber was gassed for 2 min, both air-ports were occluded, and the chamber was placed inside a drying oven (LabLine, Melrose Park, IL) maintained at 38°C. The chamber was regassed each time the culture dishes were removed for oocyte/embryo examination and/or washing.

Endocrine Activity in Female Tigers

To establish baseline hormonal norms in tigers subjected to eCG/hCG treatment, a 10-ml blood sample was taken from each of the females on the day of oocyte aspiration immediately after a surgical plane of anesthesia was induced. The blood was allowed to clot for 1–3 h, and then the serum was separated by centrifugation and frozen (−80°C). Samples were later thawed and analyzed for estradiol-17β and progesterone by RIAs previously described and validated for the tiger [6]. The estradiol-17β assay has a sensitivity of 2 pg/ml and a coefficient of variation of 20%. The progesterone assay has a sensitivity of 10 pg/ml and a coefficient of variation of 17%.

Electroejaculation and Semen Evaluation and Processing

To obtain spermatozoa for IVF, 8 electroejaculates were collected from the 7 males in Trial I, and 3 electroejaculates were obtained from 3 males in Trial II. Three of the 7 sperm donors in Trial I were used as a source of sperm in Trial II, thus providing consecutive data on ejaculate characteristics and individual male fertilizing capacity. On one occasion in Trial I, Male #1 was electroejaculated twice within 24 h to inseminate oocytes from 4 different females. For the remaining 6 groups of oocytes in Trial I, individual electroejaculates were used to inseminate only oocytes from a single female. In Trial II, each ejaculate from the 3 individual males was split and used to inseminate oocytes from 2 females. The electroejaculation technique was a standardized procedure [8, 10] using a rectal probe with 3 longitudinal electrodes and an AC, 60-Hz sine-wave ejaculator; a total of eighty 3–7-volt electrical stimuli were given over a ~20 min interval. Total ejaculate volume was recorded, and each semen sample was examined immediately (25×) for subjective estimates of sperm percent motility [8, 10]. A 10-μl aliquot of semen was used to calculate sperm concentration by a standard hemocytometer method [10]. Sperm morphology was evaluated in detail after fixing a 25-μl aliquot in 1% glutaraldehyde and examining 300 individual sperm/male at 400× [8, 10, 22].

Semen was processed for IVF by means of a previously described swim-up procedure for recovering a population of highly motile, structurally normal spermatozoa [16, 23]. Electroejaculates were centrifuged (300 × *g*, 8 min) in a 10-ml conical tube, the supernatant was removed, and 100 μl of fresh Ham's F10 was layered gently onto the pellet. The sperm were allowed to migrate from the pellet into the overlying medium during a 1-h interval at 22°C. The supernatant layer was recovered by aspiration, sperm motility was evaluated, and the solution was diluted to provide an IVF concentration of 0.5×10^6 motile sperm/ml for fertilization.

Insemination and Sperm/Oocyte CoCulture

Mature oocytes were inseminated with a 100-μl aliquot of diluted motile sperm (0.05×10^6) under lightweight paraffin oil (Fisher Scientific Company, Fair Lawn, NJ) in a 35 × 10-mm plastic culture dish. Oocytes from individual females were maintained in separate sperm drops (10 oocytes or fewer per drop), and the fertilization dishes were placed into the 5% CO_2-in-air culture chamber at 38°C. To determine the rate of parthenogenesis, control oocytes (one or more representative mature oocyctes from each collection) were cultured under standard conditions in medium containing no spermatozoa. Eighteen–twenty hours after insemination, oocytes were removed from the incubator and washed 3 times in a 0.2% hyaluronidase solution (Type 1-S, from bovine testes; Sigma Chemical Company) for 3 min to remove residual cumulus cells and loosely attached sperm. Oocytes were returned to the incubator in 100-μl drops of fresh, equilibrated Ham's F10 under oil and examined 30 h after insemination.

Assessment of Fertilization

Our fertilization criteria relied on the presence of two polar bodies, two pronuclei, or cleavage to at least the two-cell stage [12, 16, 19]. Oocytes with more than two nuclear structures within the cytoplasm were considered polyspermic. Oocytes failing to cleave were treated with Hoechst #33342 (H342; bisbenzamide; Sigma Chemical Company), a DNA-specific fluorescent stain [16]. Oocytes were counterstained with 0.1% Trypan blue dye (Sigma Chemical Company) for 1–2 min, incubated in H342 (0.09 mg/ml, 15 min, 23°C), and then examined using differential interference contrast and fluorescence optics (250× and 400×) for germinal vesicles, pronuclei, polar bodies, and polyspermic fertilization.

Embryo Transfer/Culture

Embryos cleaving to the two-cell stage or greater were assigned a quality grade [24, 25]. In brief, embryos of good or excellent quality were those that were perfectly symmetrical (or only slightly asymmetrical), spherical, and uniformly dark. Fair- or poor-quality embryos were those that were degenerating partially or severely, were pale, or contained lyzed blastomeres.

A portion of the good/excellent-quality embryos were transferred surgically to the oviducts of five of the original oocyte donors. A sixth recipient was treated with the eCG/hCG regimen described above but not subjected to oocyte aspiration. All recipients were multiparous. At 48 h after initial oocyte recovery, each recipient was anesthetized and surgically prepared (as described above). A mid-ventral abdominal incision was made, and the ovaries and oviducts were exposed bilaterally. Each ovary was examined for number of CL and preovulatory follicles. Two- to four-cell embryos (12–16/female) were aspirated in a total volume of 2 μl of physiological saline in 50 polyethylene tubing, the tip of which was inserted into the fimbriated end of oviduct ipsilateral to the ovary containing the most, normal-appearing CL. The embryos were deposited by gentle injection, and the tubing was re-examined under the stereomicroscope to ensure that the embryos were delivered. Four of six recipients received no further hormone treatment; however, on the day of embryo transfer, two tigers (one from which oocytes had been aspirated and the one non-aspirated female) each received a silastic progesterone implant containing 35 mg crystalline progesterone (Steraloids, Inc., Wilton, MA) [26]. Each recipient was anesthetized (as described above) ~30 and ~60 days later and subjected to abdominal radiography or ultrasonography (Equisonics, Model-LS300S and a 3.5-m-Hz probe). Ultrasound scans were carried out on the abdominal wall along both teat lines and extending laterally to the flank regions. In the event of a positive pregnancy diagnosis, the original implant was replaced immediately with a 50-mg progesterone implant to sustain the pregnancy.

Some of the original embryos also were placed in culture to determine the ability of tiger IVF-produced embryos to advance to later developmental stages in vitro. These embryos were transferred into fresh Ham's F10 medium 30 h after insemination and examined for stage of development at 72 and 96 h after insemination.

Statistics

Mean values are presented as ± standard error of the mean. Differences in follicle number and oocytes collected between eCG treatment groups, tiger subspecies, or time of year were measured by Student's *t*-test [27]. The same statistical approach was used to compare ejaculate characteristics between the *P.t. altaica* and *P.t. tigris* subspecies within trials and over time. In Trial I, Chi-square (X^2) analysis [27] was used to compare the effect of eCG dose on the proportion of oocytes eventually classified as mature, immature, degenerate, or fertilized.

RESULTS

Follicles were observed in 10 of 10 tigers in Trial I (range, 6–52/female) and in 6 of 6 tigers in Trial II (range, 6–52/female) (Table 1). Female #5 in Trial I and females #4 and #5 in Trial II (both of which produced the fewest follicles) had CH on the ovaries at the time of laparoscopy (25 h post-hCG) and thus had begun ovulation before all follicles could be aspirated. Luteal tissue of unknown origin was observed on the ovaries of 2 additional females (#3 and #6, Trial I). These CL were slightly raised above the ovarian surface, and their yellow-orange coloration suggested that they were advanced in age and not formed as a result of the recent gonadotropin injections. Regardless, the presence of CL (and the simultaneous elevation in circulating progesterone, Table 2) failed to affect the ability of these 2 females to produce follicle numbers comparable to those produced by the other tigers (Table 1). Likewise, the luteal status of females #3 and #6 had no detrimental impact on the proportion of mature oocytes recovered (Table 1). On the day of oocyte collection, there was a close relationship between ovarian morphology and circulating steroid hormone concentrations (Tables 1 and 2). Females with no detectable luteal tissue and only preovulatory follicles produced estradiol-17β concentrations greater than 34.5 pg/ml and progesterone levels of 3.7 ng/ml or less. The 5 tigers with luteal tissue (#3, #5, #6 [Trial I] and #4, #5 [Trial II]) produced estradiol-17β concentrations comparable to those in tigers with no CL, even in the presence of progesterone levels ranging from 3.0–40.0 ng/ml. There was no effect ($p > 0.05$) of eCG dose, subspecies, or time of year on ovarian response to treatment. In Trial I, females treated with 2 500 IU eCG produced 29.6 ± 6.2 follicles compared to 27.0 ± 6.2 follicles produced by females treated with 5 000 IU. For both trials, the mean number of follicles in *P.t. altaica* (30.5 ± 3.8) was no different from that observed in *P.t. tigris* (28.2 ± 5.5). Overall, tigers treated with eCG in July (Trial I) produced numbers of follicles (28.9 ± 4.0) comparable to those produced by tigers treated in January (Trial II, 29.8 ± 6.6).

For both trials, a total of 456 oocytes were collected from 468 aspirated follicles (97.4%), providing a mean of 28.5 ± 3.4 oocytes/female. Of the total, 378 (82.9%) oocytes were classified as mature, 48 (10.5%) as immature, and 30 (6.6%) as degenerate. There was no effect ($p > 0.05$) of eCG dose (Trial I), tiger subspecies, or time of year on oocyte recovery efficiency or maturation/quality status. Of the 5 tigers receiving exogenous gonadotropins twice, 3 (#1, #2, #3) demonstrated a remarkable consistency in follicle and oocyte number/quality status between treatment periods (Table 1).

TABLE 1. Ovarian activity, oocyte recovery, and oocyte quality in female tigers treated with eCG/hCG in July (Trial I) and/or January (Trial II).

Trial	Animal number	Ovarian activity on day of aspiration	Number of oocytes			
			Collected	Mature	Immature	Degenerate
I	1[ac]	40 follicles	36	28	0	8
II	1[bc]	48 follicles	48	45	2	1
I	2[ac]	28 follicles	28	24	2	2
II	2[bc]	28 follicles	28	22	4	2
I	3[bd]	32 follicles, 3 CL	32	22	8	2
II	3[bd]	28 follicles	28	26	2	0
I	4[bd]	52 follicles	52	47	4	1
II	4[bd]	6 follicles, 7 CH	6	6	0	0
I	5[ad]	6 follicles, 6 CH	6	6	0	0
II	5[bd]	17 follicles, 7 CH	15	14	0	1
I	6[bc]	32 follicles, 6 CL	32	22	8	2
I	7[bc]	16 follicles	15	10	2	3
I	8[ace]	22 follicles	20	16	3	1
I	9[ad]	39 follicles	39	35	0	4
I	10[bd]	22 follicles	22	13	9	0
II	11[bd]	52 follicles	49	42	4	3

[a]Received 5 000 IU eCG.
[b]Received 2 500 IU eCG.
[c]*P.t. altaica* female.
[d]*P.t. tigris* female.
[e]Served as a nonaspirated, embryo recipient in Trial II.

There was no difference ($p > 0.05$) over time or between subspecies group in any of the ejaculate traits measured. Semen characteristics by individual sperm donor for each trial are provided in Table 3. Overall mean ejaculate characteristics were as follows: ejaculate volume, 7.5 ± 0.7 ml; sperm concentration, 17.3 ± 3.9 × 10^6/ml; sperm percent motility, 85.4 ± 2.0%; motile sperm/ejaculate, 105.9 ± 20.6 × 10^6; and percent morphologically normal sperm/ejaculate, 81.4 ± 2.0%. The most prevalent pleiomorphisms included cells with either a proximal droplet (5.0%), bent

TABLE 2. IVF results and circulating estradiol-17β and progesterone (on day of oocyte aspiration) in female tigers treated with eCG/hCG in July (Trial I) and/or January (Trial II).

Trial	Animal number	No. mature oocytes fertilized (%)		Estradiol-17β pg/ml	Progesterone ng/ml
I	1[ac]	27/28	(96.4%)	103.0	3.7
II	1[bc]	22/41	(53.7%)	109.0	0.5
I	2[ac]	12/22	(54.5%)	95.3	1.8
II	2[bc]	10/22	(45.5%)	94.9	0.7
I	3[bd]	13/20	(65.0%)	58.6	28.0
II	3[bd]	23/25	(92.0%)	105.0	0.4
I	4[bd]	28/43	(65.1%)	162.0	0.4
II	4[bd]	4/6	(66.7%)	40.5	19.0
I	5[ad]	5/6	(83.3%)	96.4	18.3
II	5[bd]	10/13	(76.9%)	126.0	3.0
I	6[bc]	9/20	(45.0%)	79.6	40.0
I	7[bc]	8/9	(88.9%)	34.5	0.7
I	8[ac]	11/15	(73.3%)	145.0	0.5
I	9[ad]	7/33	(21.2%)	140.0	0.5
I	10[bd]	11/12	(91.7%)	85.0	0.2
II	11[bd]	27/41	(65.8%)	87.2	0.4

[a]Received 5 000 IU eCG.
[b]Received 2 500 IU eCG.
[c]*P.t. altaica* female.
[d]*P.t. tigris* female.

TABLE 3. Semen characteristics and IVF results in individual tigers electroejaculated in July (Trial I) and/or January (Trial II).

Trial	Animal number	Ejaculate volume (ml)	Spermatozoa/ml ejaculate ($\times 10^6$)	Sperm motility (%)	Motile sperm/ ejaculate ($\times 10^6$)	Morphologically normal sperm (%)	No. oocytes fertilized/no. inseminated (%)[d]
I	1[ab]	12.0	17	85	173	73.0	35/37 (94.6)
I	1[ab]	13.0	8	80	83	70.5	21/42 (50.0)
II	1[b]	7.0	33	80	184	71.5	32/63 (50.8)
I	2[c]	6.5	10	95	62	83.0	5/6 (83.3)
II	2[c]	5.0	16	90	72	84.5	33/39 (84.6)
I	3[c]	7.0	7	80	39	88.5	13/20 (65.0)
II	3[c]	6.0	8	90	43	87.0	31/47 (66.0)
I	4[b]	7.0	48	75	252	84.0	11/15 (73.3)
I	5[c]	7.0	16	80	90	84.0	28/43 (65.1)
I	6[c]	7.0	21	95	140	88.0	7/33 (21.2)
I	7[c]	5.0	6	90	27	81.5	11/12 (97.7)

[a]Collected on subsequent days.
[b]*P.t. altaica* male.
[c]*P.t. tigris* male.
[d]All oocytes were inseminated with the same number of motile sperm.

midpiece (5.0%), or abnormal acrosome (2.7%). The number of motile sperm/ejaculate in the one male (#1) electroejaculated on consecutive days was reduced by half on the second day of collection, primarily because of a reduction in sperm/ml of ejaculate (Table 3).

Of the 358 mature oocytes inseminated, 227 (63.4%) were fertilized on the basis of the criteria described in *Materials and Methods*. In Trial II at the Minnesota Zoological Gardens, the gamete cocultures for 2 oocyte donors were inexplicably contaminated by 30 h after insemination. These oocytes, which were Hoechst-stained to confirm fertilization, were excluded from subsequent cleavage rate calculations. Of the remaining 195 uncontaminated fertilized oocytes, 187 (96.0%) cleaved to at least the two-cell stage of development (Fig. 1b). All other oocytes failing to cleave (n = 8) contained two pronuclei after Hoechst-staining. Of the cleaved embryos, 165 met the excellent (11.9%) or good (76.1%) quality-rating criteria. Parthogenetic cleavage was not observed in any of the 20 control oocytes. Among individual sperm donors and over both trials, the IVF rate varied more than 3-fold (Table 3). Because all insemination solutions contained similar numbers of structurally normal, motile spermatozoa, the fertilization differences among males could not be attributed to a specific ejaculate characteristic(s).

In Trial I, the mean IVF rate was unaffected ($p > 0.05$) by eCG dose (2 500 IU, 63.5% versus 5 000 IU, 57.7%). Likewise, there was no effect ($p > 0.05$) of subspecies (*P.t. altaica*, 14.1 ± 3.0 versus *P.t. tigris*, 14.2 ± 3.6) or time of year (July, 14.5 ± 2.7 versus January 16.0 ± 3.4) on number of fertilized oocytes/female. The 2 tigers that had contraceptive implants removed 6 wk before the onset of Trial I (#2 and #8) produced numbers of follicles, mature oocytes, and fertilization rates similar to those of nonimplanted counterparts (Tables 1 and 2). Furthermore, the incidence of IVF was comparable between females treated twice with gonadotropins at different times of the year.

By 30 h after insemination, 178 of the 187 (95.2%) cleaving embryos were at the two- or four-cell stage of development (Table 4). The remaining 9 embryos contained 8 blastomeres. Of this total, 45 were used for a parallel embryo cryopreservation study (Donoghue, Johnston, and Wildt, unpublished). Of the remaining 142 cleaved embryos, 12, 12, 16, and 16 two- or four-cell embryos were transferred surgically into oocyte donors #1, #2, #7, and #8 in Trial I; and 15 two- to four-cell embryos were transferred to each oocyte donor #3 and the recipient not subjected to oocyte aspiration (#8), respectively. Pregnancies were not evident on either diagnostic day according to radiography or ultrasound in any nonprogesterone-supplemented recipient in Trial I or in the oocyte-aspirated/progesterone-supplemented recipient in Trial II. However, 61 days from the day of embryo transfer, 2 distinct fetuses and heart-beat activity were observed in the progesterone-supplemented recipi-

TABLE 4. Culture rate and development of in vitro-fertilized tiger embryos.

Culture interval	No. embryos	Stage of development					
		2-cell	4-cell	8-cell	16-cell	Morula	Early blastocyst
30 h	187	73 (39.0%)	105 (56.1%)	9 (4.8%)	—	—	—
72 h	56	5 (8.9%)	13 (23.2%)	9 (16.1%)	14 (25.0%)	15 (26.8%)	—
96 h	46	1 (2.2%)	1 (2.2%)	4 (8.7%)	6 (13.0%)	20 (43.5%)	14 (30.4%)

ent (#8) not subjected to oocyte collection (Fig. 2). The progesterone implant was removed from this female 105 days after embryo transfer. Three live cubs were delivered by Caesarian section 48 h after implant removal. One cub died within 1 h of birth because of respiratory complications.

Totals of 56 and 46 embryos were allowed to culture for 72 and 96 h, respectively. At 72 h, 29 of 56 (51.8%) embryos either contained 16 blastomeres (n = 14) or were at the morula stage of development (n = 15; Fig. 1c). By 96 h, 20 (43.5%) of the embryos were morulae, and 14 (30.4%) were early-stage blastocysts (Table 4; Fig. 1d).

DISCUSSION

These results are the first to demonstrate successful in vitro fertilization, embryo culture, and production of offspring after IVF and embryo transfer in a *Panthera* species representing the largest of the great cats in the family Felidae. However, this study is significant for two other reasons. First, the eventual utility of reproductive biotechnology in the emerging field of conservation biology will depend on a solid database of fundamental biological information. This knowledge, usually obtained from basic research, is critical to making sound decisions about how to conduct the actual artificial breeding attempt. Because by definition, endangered species are available in few numbers, a basic research approach for detailing species biology often is difficult or impossible to implement. One alternative strategy is to rely on common animal models for defining mechanisms and developing concepts and techniques for later application to rarer species [5, 28]. This approach is effective given that reproductive mechanisms are conserved among taxonomically related species. Interestingly, this does not always appear to be the case for certain reproductive characteristics within the Felidae family. For example, males from most felid species vary markedly in ejaculate characteristics [8, 10, 11, 29], pituitary-gonadal hormonal inter-relationships, and adrenal reactivity to specific stressors [7, 8, 29–31]. Likewise, we have observed considerable differences among felid species in ovarian responsiveness to a given eCG/hCG hormone challenge, both in number of follicles produced and resulting oocyte quality [12, 16, 19]. However, the present study is unique because it demonstrates the cross-species utility of a given IVF protocol between two species (cat and tiger) separated considerably on the evolutionary tree [32]. The mean number of oocytes recovered, oocyte quality, and IVF and embryo culture rates in the tiger were similar to recent data using the same system in the domestic cat [18, 20]. Secondly, the positive results of the present study were obtained under less than conventional laboratory conditions. The IVF system was made mobile; that is, all the technology was transferred between laboratories. We did experience contamination in two oocyte cultures during Trial II, no doubt related to working in a clinical laboratory normally used for conducting routine veterinary diagnostics (including fecal parasites). This

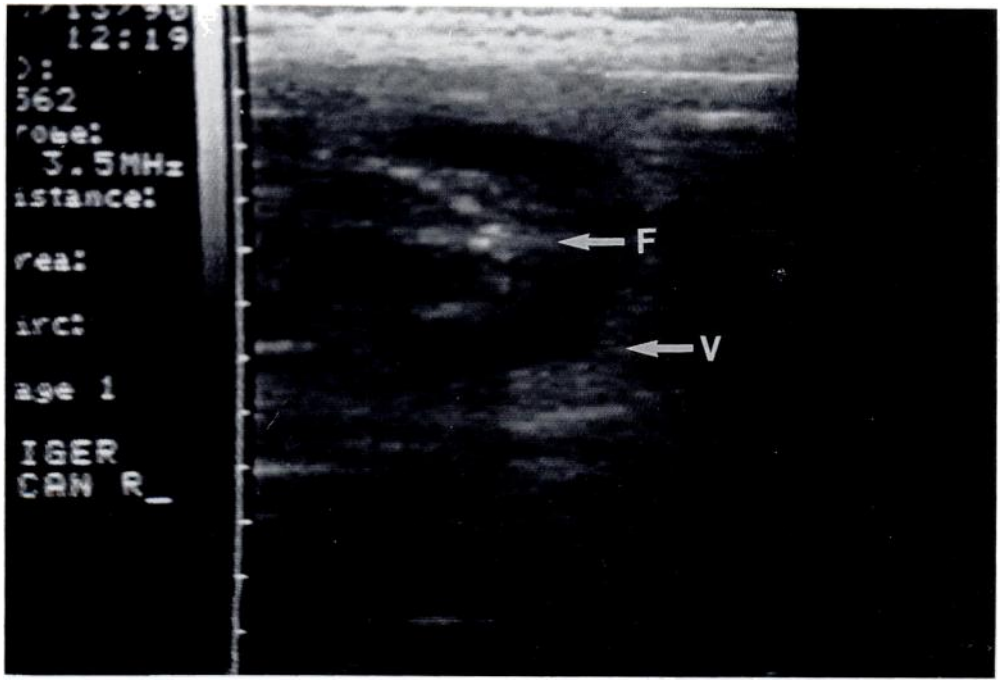

FIG. 2. Ultrasonograph of tiger fetus 63 days after in vitro fertilization and 61 days after surgical transfer of embryos into a hormone-synchronized recipient. V = embryonic vesicle; F = fetus.

experience reinforces the mandate for maintaining high standards of quality control [33].

Single injections of eCG and hCG were at least as effective as or more effective than a similar hormone treatment given to domestic cats [16–18, 20], leopard cats [19], or pumas [12]. In previous studies, the tiger demonstrated a rather striking lack of response to repeated injections of FSH-P [15], a common exogenous gonadotropin used for inducing ovarian activity in farm livestock. The ineffectiveness of FSH-P in tigers is interesting because this preparation stimulates follicular activity efficiently in the cheetah, lion, leopard, puma, and Indian desert cat [14, 15, 28, 34, 35]. There was no evidence that the dosages of eCG tested affected results; follicular recruitment, number of oocytes recovered, and oocyte quality (based on subsequent fertilization/cleavage rates) were similar in the two treatment groups. Within each trial, there was substantial variability in ovarian response between individuals, which was consistent with observations made in the domestic cat [16] and other nondomestic felid species [12, 19]. However, there was surprising uniformity in ovarian responsiveness, including number of mature oocytes recovered, within the individual tigers on two consecutive collection occasions. Of the 5 tigers treated twice, the overall variation among 3 females in the mean number of ovarian follicles counted and oocytes recovered was less than 15 and 18%, respectively. Multiple administrations of eCG and hCG are known to induce an immune response and ovarian refractoriness in rabbits [36] and rhesus monkeys [37]. Similar to recent observations in leopard cats [19], these data also demonstrate that there were no apparent adverse effects of treating tigers on two occasions at a 6-month interval with eCG and hCG. However, because of the previous observations of refractoriness in other species, the impact of repeated eCG injections on the long-term responsiveness of the tiger ovary warrants further study.

There appeared to be no relationship between the extent of ovarian responsiveness (i.e., the number of follicles developing or oocytes recovered) in the tiger and the subsequent ability of these oocytes to fertilize, cleave, and form good-to-excellent-quality embryos. In this context, regardless of the magnitude of the ovarian response to eCG, all recovered oocytes had an equivalent chance of fertilizing and forming cleaved embryos. Likewise, the presence of ovarian luteal tissue and high circulating concentrations of progesterone at the time of follicular recruitment and oocyte aspiration had a negligible impact on oocyte integrity. The ability to collect large numbers of fertilizable oocytes from tiger ovaries under estrogenic or progestogenic influence suggests that embryo production in vitro in this species was largely independent of reproductive status.

Most (>82%) of the 456 tiger oocytes collected during both trials were classified as mature, indicating that the hormonal regimen used was adequate for provoking oocyte maturation in vivo. These data are similar to recent observations in eCG/hCG-challenged leopard cats in which ~12% of aspirated follicular oocytes were categorized as immature [19]. In contrast, a similar study of pumas revealed that 43 of 140 oocytes (30.7%) recovered were immature and required in vitro maturation before insemination [12]. This occurred even though the eCG dose on a per weight basis and the interval from eCG to hCG administration was comparable to that used in the tigers in the present study. These variations in in vivo oocyte maturation may be attributable to species-specific sensitivities to eCG or hCG. The more uniform response of tigers may also be related to the time of year of the trials: females were given gonadotropins during the nonbreeding season (July) or just before the onset of the breeding season (January) when natural ovarian activity was assumed to be minimal. In contrast, the pumas were challenged with exogenous gonadotropins during the midbreeding season [12]. Normal cyclic ovarian activity and hormone production in these animals may have interfered with optimal in vivo oocyte maturation after eCG/hCG administration.

Nevertheless, the IVF and cleavage rate of inseminated tiger oocytes far exceeded our previous experiences with pumas. The overall fertilization rate for tiger oocytes was 63.4%, comparable to recent results in domestic cats [18, 20] and about 20% higher than results achieved in pumas [12]. Likewise, a high proportion (96.0%) of fertilized tiger oocytes underwent cleavage, a considerable increase over the 25% cleavage rate for pumas [12]. There are several possible reasons for the species differences in fertilization and embryo cleavage rates. First, the gonadotropin regimen used in the pumas may have been suboptimal, an assertion supported by the high proportion of immature oocytes recovered. Second, there were differences in the fertilization/culture medium used for the two species (tiger, Ham's F10 versus puma, modified Krebs Ringer bicarbonate [mKRB]). The significance of culture medium on the IVF of puma and tiger oocytes is unknown, but recent evidence indicates that there is little advantage to using Ham's F10 over mKRB for IVF of domestic cat oocytes [18]. It is likely that the quality of the spermatozoa contributed to the improved gamete interaction and embryo formation in the tigers compared to the pumas. Of the nondomestic felid species surveyed to date [8, 9, 11], the tiger consistently produces the fewest pleiomorphic spermatozoa/ejaculate. Of the 11 electroejaculates examined in this study, 81.4% of all spermatozoa were classified as structurally normal, and the predominant abnormal features were cells with a residual cytoplasmic droplet or a bent midpiece. In contrast, puma semen used for IVF attempts has contained more than 90% pleiomorphic sperm forms, and one rare subspecies (*Felis concolor coryi*) produces ejaculates in which more than 50% of the spermatozoa are afflicted with severely deformed acrosomes. Therefore, it seems reasonable to speculate that the marked IVF/cleavage differences between these two species may be related to different degrees of teratospermia. More detailed studies from our laboratory recently

have focused on the in vitro binding and zona-penetrating ability of spermatozoa recovered from normospermic (>60% normal spermatozoa) and teratospermic (<40% normal spermatozoa) domestic cats [23, 38]. In these studies, electroejaculates from both cat populations were swim-up processed so that aliquots used to inseminate oocytes contained comparable numbers of motile, structurally normal spermatozoa. Coculturing immature domestic cat oocytes with normal sperm from teratospermic males resulted in lower binding and zona penetration rates compared to using spermatozoa from the normospermic animals. We have interpreted this to mean that the spermatozoa of teratospermic males are inherently compromised in ability to fertilize in vitro. In the present study, it was impossible to evaluate the impact of teratospermia on IVF because all tigers produced high numbers of structurally normal spermatozoa. However, these results in the context of previous IVF studies in the puma support the idea that a major factor influencing the successful in vitro interaction of homologous gametes from felid species may be the number of pleiomorphic spermatozoa in the insemination solution.

The observation that embryos resulting from IVF readily advance in culture to the morula stage of development was in agreement with recent observations of domestic cat embryos produced by the same approach [18]. In both species, Ham's F10 medium allowed embryos to advance in vitro at a similar rate. Preliminary evidence indicates that, like domestic cat embryos, tiger embryos do not develop readily beyond the morula stage in vitro, supporting our recent assertion that there is a late developmental block for the preimplantation embryos of certain felid species [18]. In this context, the domestic cat is similar to the rabbit, a species in which embryos also arrest in vitro at the morula stage [39]. This observation appears unique, since blocks to embryonic development in other species occur at a much earlier stage of growth: mouse, two-cells [40]; hamster, two- to four-cells [41]; pig, four-cells [42]; sheep, eight- to sixteen-cells [43]; cow, eight- to sixteen-cells [44]. Studies in progress suggest that the ability of domestic cat embryos to overcome the morula-to-blastocyst developmental block in vitro is regulated partially by the presence and type of protein in the culture medium [18].

Previous transfers of domestic cat [16] and Indian desert cat [35] embryos resulting from IVF have resulted in live-born offspring. In this study, the transfer of IVF tiger embryos to a progesterone-supplemented recipient resulted in a single viable pregnancy. The reasons for the pregnancy failures are unknown and are, in part, complicated by our poor understanding of the mechanisms regulating implantation/pregnancy in felids in general (including the domestic cat). However, we observed many unovulated follicles on the ovaries of all recipients on the day of embryo transfer, presumably reflecting a secondary wave of follicular growth after oocyte aspiration. Circulating progesterone concentrations on the day of transfer (2.5–4.5 ng/ml) were in the temporal range for natural estrous, copulating tigers [45]. However, estradiol-17β levels were above normal (38.8–135.0 pg/ml), a finding no doubt related to the concurrent observation of many accessory follicles. We know from a previous study that abnormal endocrine profiles alter embryo transport rate and embryo recovery in gonadotropin-treated domestic cats [46]. Likewise, ovarian hyperstimulation associated with IVF protocols in women is thought to be a common cause of low pregnancy rate after embryo transfer [47, 48]. Although effective for producing many mature, fertilizable oocytes, the gonadotropin treatment for the tiger also may have altered endogenous endocrine patterns in a fashion detrimental to the embryo. If hyperestrogenism indeed occurred, then this condition may have been countered in the progesterone-supplemented recipients, thus at least partially explaining the establishment of the one pregnancy. Because this pregnancy occurred in a tiger in which oocytes were not collected, it also is possible that overly thorough aspiration of individual follicular content may have perturbed normal endogenous hormone patterns by disrupting the formation of CL. Studies are in progress both to assess the utility of exogenous progesterone implants for facilitating IVF pregnancies and to explore the impact of the oocyte collection procedure on subsequent luteal function. Overall, our findings emphasize the need for more fundamental studies directed at the mechanisms controlling early preimplantation embryo survival and implantation in felids.

In summary, this study has demonstrated that an IVF procedure developed for the domestic cat is readily adapted to the tiger. Female tigers responded to an eCG/hCG stimulus by producing large numbers of developing follicles and a high percentage of mature oocytes that were capable of being fertilized and developing in vitro and in vivo. The numbers of mature oocytes being fertilized and cleaving in vitro may have resulted from the successful gonadotropic stimulation of females, optimal in vitro culture conditions, or the naturally high incidence of structurally normal spermatozoa in electroejaculates. Tiger embryos allowed to culture in vitro developed readily to the late morula stage but appeared to experience a partial developmental block to becoming blastocysts. Most importantly, these embryos were biologically competent as demonstrated by the birth of live young. Taken together, these results support the contention that, for certain nondomestic species, an extensive basic research approach may not always be mandatory for generating embryos in vitro given that an adequate database is available in a suitable animal model. Additionally, our findings suggest that, for some species, it may be possible to apply reproductive biotechnology using mobile laboratory research teams. This approach may be more practical for exploiting existing expertise and may use available resources more efficiently. This especially is important in considering the eventual utility of biotechnology for wildlife propagation since funding sources for research/captive

breeding programs are limited and the endangered species list continues to expand relentlessly.

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