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Developmental Competence of Domestic Cat Follicular Oocytes after Fertilization in Vitro[1]

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ABSTRACT

Empirical evaluation of variables affecting oocyte collection, in vitro fertilization, and embryo transfer resulted in establishing a successful procedure for the artificial production of offspring in the domestic cat. Female cats were treated with pregnant mare's serum gonadotropin (PMSG, 150 IU) followed 72 or 80 h later with 100 or 200 IU human chorionic gonadotropin (hCG). After laparoscopic collection, follicular oocytes were inseminated in vitro with ejaculated, processed spermatozoa, cultured (37°C, 5% CO_2), and then examined for evidence of fertilization. Two- to 4-cell stage embryos were transferred to the oviducts of oocyte donors. Oocyte donor cats and naturally mated controls also were subjected to sequential laparoscopic examinations and blood sampling to assess corpora lutea (CL) function.

At 24–30 h of culture, fewer ($p<0.001$) degenerate oocytes were observed in cats receiving 100 IU hCG (8.2%) compared to those receiving 200 IU (20.6%), regardless of the PMSG-hCG interval. Overall fertilization (48.1%) and cleavage (45.2%, at 30 h post-insemination) rates were greatest following an 80-h PMSG-hCG interval combined with the 100 IU hCG dose. Five of the 6 cats receiving 6 to 18 embryos became pregnant and produced from 1 to 4 kittens/litter. Gonadotropin-treated females subjected to follicular aspiration produced morphologically normal CL and circulating progesterone patterns that were qualitatively similar ($p>0.05$) to control cats.

These data indicate that domestic cat follicular oocytes are capable of fertilization in vitro, but success is dependent on both the timing and dose of the hCG stimulus. Follicles subjected to aspiration appear capable of forming normal, functional CL and the birth of live young after embryo transfer unequivocally demonstrates, for the first time, the developmental competence of in vitro-fertilized carnivore oocytes.

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INTRODUCTION

In vitro fertilization (IVF) offers a valuable approach for studying the processes of fertilization and early embryonic development as well as species-specific variables that affect gamete fusion. Although fertilization in vitro has been reported for a number of species (see reviews: Wright and Bondioli, 1981; Brackett, 1985), the birth of offspring following IVF has been accomplished only in rabbits (Chang, 1959), mice (Whittingham, 1968), rats (Toyoda and Chang, 1974), humans (Steptoe and Edwards, 1978), cattle (Brackett et al., 1982), 3 species of nonhuman primates (Balmaceda et al., 1984; Bavister et al., 1984; Clayton and Kuehl, 1984), and pigs and sheep (Cheng et al., 1986). Similar studies in cats could provide a greater understanding of gamete interaction and embryogenesis in carnivores while simultaneously establishing a data base potentially applicable to nondomestic feline species, all of which are classified as threatened or endangered (CITES, 1973).

Few embryo-related studies have been conducted in cats, although successful embryo transfers have been reported between synchronized donor and recipient females experiencing either a natural (Kraemer et al., 1979) or gonadotropin-induced estrus (Goodrowe et al., 1988). In these studies, embryos were recovered surgically, which limits the practicality of the procedure. Because this approach also would be contraindicated in rare species, an alternative strategy is required for obtaining routine access to embryos. Using laparoscopy, cat ovarian follicles can be identified readily (Wildt et al., 1977). Fertilization in vitro using laparoscopic oocyte recovery techniques previously developed in monkeys (Kuehl and Dukelow, 1979; Bavister et al., 1984; Clayton and Kuehl, 1984) and humans (Wood et al., 1981) could have application to felids.

In vitro fertilization has been accomplished with domestic cat oocytes surgically retrieved from the oviducts after gonadotropin treatment and spermatozoa collected from the uterus after mating (Hamner et al., 1970) or from the epididymis and ductus deferens (Bowen, 1977; Niwa et al., 1985). After culture, these oocytes demonstrated morphological evidence of fertilization; however, there have been no attempts to analyze the many variables that can influence oocyte integrity or the ability of the resulting embryos to progress to term in utero.

The present work was conducted to evaluate the ability of laparoscopically recovered follicular oocytes to become fertilized in vitro after culture with homologous spermatozoa collected by electroejaculation. The study was designed to assess the effects of the time interval between pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) administration and hCG dose on fertilization rates in vitro. Additionally, ovarian morphology and endocrine patterns were examined over time to determine if functional corpora lutea (CL) were formed after follicular aspiration. Finally, embryos produced by IVF were transferred to oviducts of mixed autologous and surrogate recipients (oocyte donors) to test their ability to produce live offspring.

MATERIALS AND METHODS

Animals

Random-source, adult female cats with unknown reproductive histories were housed in groups of 2 to 3 and provided with a commercial feline diet (Purina Cat Chow, Ralston-Purina, St. Louis, MO) and water ad libitum for at least 30 days before being designated for treatment. Two chronically maintained, proven breeder males were housed separately and given the same diet. A total of 12 h of artificial fluorescent illumination was provided daily, but because the holding areas contained windows, all animals were exposed to fluctuations in natural daylight.

Induction of Ovarian Activity and Follicular Aspiration

One day before the onset of gonadotropin treatment (Day 0), queens were subjected to laparoscopy for evaluation of ovarian activity (Wildt et al., 1977). A surgical plane of anesthesia was induced and maintained with a combined ketamine HCl (Vetalar®, Parke-Davis, Morris Plains, NJ)-acepromazine maleate (Ayerst Labs, Rouses Pt., NY) mixture (10:1 ratio, 18.0–20.0 mg/kg and 0.2 mg/kg body wt., respectively, i.m.). A pneumoperitoneum was established with 100% CO_2, and the ovaries were observed with a 5 mm diameter, 180° laparoscope assembly placed mid-ventrally through a 1-cm incision. A Verres needle probe (2 mm in diameter) was used to manipulate the reproductive organs into view and to aid in estimating ovarian follicle size. Only females with

follicles <2 mm in diameter or completely inactive ovaries (no follicles or luteal tissue present) were selected for gonadotropin treatment (n = 55). Each cat received a single (150 IU) i.m. injection of PMSG (Sigma Chemical Co., St. Louis, MO) on Day 1 followed by 1 of 4 treatments of hCG (Sigma Chemical Co.) on Day 4 to initiate germinal vesicle breakdown: 1) 100 IU hCG 72 h later, n = 12; 2) 200 IU hCG 72 h later, n = 13; 3) 100 IU hCG 80 h later, n = 19; 4) 200 IU hCG 80 h later, n = 11. Beginning on Day 1, each queen was observed daily for behavioral signs of estrus (Wildt et al., 1981).

All oocyte recovery attempts were made between 25 and 27 h post-hCG (Day 5). Laparoscopic aspirations were accomplished with a 22-g, 4-cm-long needle attached to size 100 polyethylene tubing (inner diameter, 0.86 mm; Clay Adams, Parsippany, NJ), a siliconized collection tube (Terumo Medical Corp., Elkton, MD), and a vacuum pump (Gast Manufacturing Corp., Benton Harbor, MI). Before aspiration, the needle and tubing were rinsed with 2–3 ml of a modified Kreb's Ringer bicarbonate medium (mKRB; Toyoda and Chang, 1974; Niwa et al., 1985; NIH Media Unit, Bethesda, MD) containing 4 mg/ml bovine serum albumin (Fraction V, Miles Laboratories, Inc., Elkhart, IN) and 40 units heparin/ml of medium. After stabilization of the reproductive tract with the Verres probe, the aspiration needle was inserted through the abdominal wall at a site ventromedial to each ovary. Distinct follicles ≥2 mm in diameter were punctured by perforating the follicular apices with the needle, bevel side down, and applying gentle negative pressure (100 mm Hg) with the vacuum pump. The aspiration needle was rotated gently within each follicle to ensure complete suction of the contents. After follicles were aspirated from one ovary, the needle was withdrawn from the body cavity, and, along with the tubing apparatus, was rinsed with 3–4 ml of medium. The collection tube and aspiration needle were replaced and the procedure was repeated for the contralateral ovary. Each collection tube was emptied into a plastic Petri dish (60 × 15 mm) and rinsed with 3 ml of medium. The dishes were searched for oocytes, which were transferred to fresh mKRB medium (without heparin) and placed in a 5% CO_2 in air, humidified environment at 37°C. The morphology of each oocyte and its surrounding cumulus cell complex was examined in detail to assess maturational status. Any oocyte with a tightly compacted corona radiata (see Fig. 1a) was considered immature and not designated for IVF. An oocyte was classified as mature if the corona radiata and cumulus oophorus cells were loosened and expanded (see Fig. 1b). Mature oocytes were washed 3 times in mKRB under oil, placed in fresh medium, and returned to the incubator.

Semen Collection and Preparation

After all oocyte aspirations were completed on a given day, 1 of 2 males (used on a rotating basis to minimize any individual effect) was anesthetized with ketamine HCl (20 mg/kg) and used as a semen donor. Spermatozoa were obtained by electroejaculation using a standardized procedure (Wildt et al., 1983). A 5-μl aliquot of the ejaculate was examined microscopically (25×) to subjectively estimate spermatozoal percent motility and progressive status on a 0–5 scale (0 = no forward progression or movement; 5 = rapid, linear forward progression; Wildt et al., 1983). Only ejaculates containing spermatozoa with at least 70% motility and 3.5 progressive status rating were used. After transfer into a 1.5-ml conical tube (Sarstedt Inc., Princeton, NJ), the ejaculate was subjected to "swim-up" processing (Makler et al., 1984), which initially involved semen dilution with an equivalent volume of mKRB medium (23–25°C) and centrifugation for 8 min at 300 × *g*. The supernatant was aspirated and discarded; subsequently, 150 μl of mKRB medium was slowly layered onto the resulting pellet and the sperm were allowed a 1-h swim-up at room temperature. The layered medium component was aspirated gently from the pellet surface and assessed for spermatozoal concentration/ml as well as sperm motility ratings. The solution was diluted to a final concentration of 2×10^5 sperm cells/ml.

Insemination

A 100-μl drop of the diluted spermatozoal suspension (2×10^4 sperm cells) was placed under lightweight paraffin oil (Fisher Scientific Co., Fair Lawn, NJ) in a 35 × 10 mm Petri dish. Ova were maintained in separate sperm drops (10 oocytes or fewer/drop) according to each of the 4 main treatments (PMSG-hCG interval, 72 h/100 IU hCG, n = 101; PMSG-hCG interval, 72 h/200 IU hCG, n = 101; PMSG-hCG interval, 80 h/100 IU hCG, n = 106; PMSG-hCG interval 80 h/200 IU hCG, n = 108), and the fertilization dishes were placed into a humidified 5% CO_2 in

air incubator at 37°C for culture. To determine the rate of parthenogenetic development, control ova (representative oocytes taken from daily collections, n = 32) were placed under oil in a drop of medium containing no spermatozoa. After 18 to 20 h of culture, the oocytes were removed from the fertilization dishes and washed 3 times in a 0.2% hyaluronidase solution (Type 1-S, from bovine testes; Sigma Chemical Co.) for 3 min to remove residual cumulus cells and loosely attached spermatozoa. Control ova were washed first to avoid possible contamination with spermatozoa from inseminant medium. The oocytes were returned to the incubator in 100-μl drops of fresh, equilibrated mKRB under oil.

Assessment of Fertilization

After insemination, oocytes were examined either at 24 h (28 females, 215 oocytes) or at 30 h (27 females, 201 oocytes) for fertilization and cleavage. Oocytes that were fragmented, distinctly irregular in shape, or that contained numerous, heterogeneously sized cytoplasmic fragments were classified as degenerate. Presumptive evidence for fertilization included the presence of 2 polar bodies or 2 pronuclei, or cleavage to at least the 2-cell stage. Ova with more than 2 nuclear structures within the cytoplasm were classified as polyspermic. Because it was impossible to visualize intracellular structures in this species with conventional light microscopy, oocytes failing to cleave were treated with a DNA-specific fluorescent stain, Hoescht #33342 (H342; bisbenzamide; Sigma Chemical Co.) in a manner similar to that described by Pursel et al. (1985). Oocytes were counterstained with 0.1% Trypan blue (Sigma Chemical Co.) for 1–2 min, then incubated in 0.09 mg/ml H342 for 15 min at 23°C. Staining of oocytes was followed by high-speed centrifugation (15,000 × *g*) for 3 min to displace intracellular lipid (Wall et al., 1985). Ova then were examined for germinal vesicles, pronuclei, polar bodies, and polyspermic fertilization with differential interference contrast (DIC) and fluorescence optics (250 and 400×).

To calculate oocyte dimensions, 17 unfertilized ova were placed in drops (20 μl) of phosphate-buffered saline (PBS) containing 3 mg/ml bovine serum albumin (BSA) (PB1, Whittingham, 1971) on a glass slide, examined with DIC optics, and measured by using an eyepiece micrometer calibrated with a stage micrometer (Schmidt et al., 1987). Measurements included the outer diameter of the zona pellucida, the width of the zona pellucida, and the oocyte diameter (excluding the zona pellucida).

To test the developmental competence of in vitro fertilized cat embryos, some cleaved embryos were selected for culture or transfer. Two-cell embryos designated for culture were placed in 100 μl of mKRB medium under oil in a 5% CO_2 in air, humidified environment at 37°C for 1–3 days. Because the blastomeres of feline embryos also were homogeneously dark in appearance, enumeration of cells beyond the 4-cell stage was difficult. Therefore, embryos were subjected to H342, flattened under a coverslip, and examined by fluorescence microscopy for the presence of nuclei. On 4 separate occasions after IVF, cleaved embryos from 8 females were pooled and designated for transfer into 1 or 2 oocyte donors (n = 6). Two- to 4-cell stage embryos were placed into size 50 PE tubing in 2 μl of PB1 and inserted bilaterally into the fimbriated end of the oviducts at laparotomy. A total of 4 queens from the gonadotropin interval/hCG dose trials (2 receiving 100 IU hCG 72 h after PMSG, 2 receiving 100 IU hCG 80 h after PMSG) received 18, 13, 8, and 6 embryos from 2, 2, 1, and 1 female, respectively, 42–52 h after insemination. An additional 2 females, which received 100 IU hCG 80 h after PMSG (not included in the data tables), received 8 and 7 autologous embryos, respectively. Recipients were monitored for increases in body weight, and pregnant cats were allowed to carry kittens to term.

Assessment of Corpus Luteum Formation and Function after Follicular Aspiration

For evaluation of CL formation and steroidogenic function, each of 11 randomly selected, gonadotropin-treated queens (100 IU hCG 72 h after PMSG, n = 3; 100 IU hCG 80 h after PMSG, n = 3; 200 IU hCG 72 h after hCG, n = 2; 200 IU hCG 80 h after PMSG, n = 3) was subjected to general anesthesia and routine laparoscopy using techniques described earlier. On the day of oocyte aspiration, a record was made of the number and site of each aspirated follicle on the ovarian surface. Each animal then was examined laparoscopically at 7-day intervals for 8 wk (the approximate duration of a nonpregnant luteal phase in a naturally mated queen; Wildt et al., 1981a) and the number and location of each CL were noted and photographed. To evaluate steroidogenic function of resulting CL, blood samples (3 ml) were obtained by jugular venipuncture (between 0800 and 1030 h)

from the same females on Days 0 (day of pretreatment laparoscopy), 1 (day of PMSG injection), 2, 3, 4 (day of hCG injection), 5 (day of follicular aspiration), 6, and 8, and then every 48–72 h for 8 wk. Four naturally estrous cats, mated with a vasectomized male 3 times/day for the first 3 days of estrus (to induce ovulation), served as controls. With this mating regimen, follicular rupture occurs on the fourth or fifth day of estrus (Wildt et al., 1981a); therefore, the expected timing of the natural ovulations was presumed coincident (± 24 h) with the timing of follicular aspiration. Control queens were subjected to the same sequence of laparoscopic evaluations and serial blood sampling as the PMSG/hCG groups.

Blood was centrifuged, and the sera were stored at −20°C and later assayed for estradiol-17β and progesterone with previously validated (Goodrowe et al., 1988), commercially available, ^{125}I double-antibody radioimmunoassay kits (Radioimmunoassay Systems, Inc., Carson, CA). The first and second antibodies for the estradiol-17β assay were rabbit 6-keto-estradiol-17β-6-oxime BSA and goat anti-rabbit gamma globulin, respectively. The cross-reactivities of the first antibody were estradiol-17β, 100%; estrone, 20.0%; estriol, 1.51%; estradiol-17, 0.68%; ethinyl estradiol, testosterone, 5α-dihydrotestosterone, cholesterol, pregnenolone, 17-α-hydroxypregnenolone, progesterone, 17-α-hydroxyprogesterone, 20-α-dihydroprogesterone, 11-desoxycortisol, cortisol, aldosterone, androstendione, dihydroepiandrosterone, and dihydroepiandrosterone sulfate, <0.01%. The minimum and maximum detectable levels were 10.0 and 3000.0 pg/ml, respectively. Inter- and intraassay coefficients of variation were 6.6% (n = 6) and 3.2% (n = 6), respectively.

The first and second antibodies for the progesterone assay were rabbit 11-α-hydroxyprogesterone-11-α-hemisuccinate-human serum albumin and goat anti-rabbit gamma globulin, respectively. The reported cross-reactivites of the first antibody were progesterone, 100%; 20-α-dihydroprogesterone, 6.25%; desoxycorticosterone, 3.20%; corticosterone, 0.42%; 17-α-hydroxyprogesterone, 0.15%; pregnenolone, 0.06%; androstenedione, 0.04%; testosterone, 0.03%; 11-desoxycortisone, pregnenolone sulfate, cholesterol, dihydroepiandrosterone, etiocholanolone, estradiol-17β, estradiol-17α, estrone, estriol, androsterone, aldosterone, and cortisol, <0.01%. The minimum and maximum detectable levels of progesterone for this assay were 0.2 and 40.0 ng/ml, respectively. Inter- and intraassay coefficients of variation were 12.4% (n = 4) and 7.0% (n = 8), respectively.

Statistical Analysis

Values are reported as the mean ± the standard error of the mean (SEM). Differences in the mean number of follicles aspirated and oocytes collected between the two PMSG-hCG time intervals and the two hCG doses were analyzed with a Student's *t*-test evaluation (Steel and Torrie, 1960). Variations within the four time interval/hCG dose treatments were analyzed further with a statistical software package (SYSTAT, Wilkinson, 1987) and two-way analysis of variance. Differences in the proportions of females exhibiting behavioral estrus, the percentage of oocytes recovered, and the proportion of oocytes with germinal vesicles or polyspermy or that degenerated or were fertilized between the two PMSG-hCG time intervals and the 2 hCG doses were evaluated by Chi Square analysis (Steel and Torrie, 1960). Overall variations between the four time interval-hCG dose treatments were evaluated by two-way analysis of variance. Within the 24- and 30-h post-insemination groups, differences in the number of 1- and 2-cell embryos between the 72- and 80-h PMSG-hCG time intervals and the 100 and 200 IU hCG doses were analyzed by Chi Square. Bartlett's test for homogeneity of variance (Armitage, 1971; Wilkinson, 1987) revealed differences ($p<0.05$) between the four time interval/hCG dose treatments. As a consequence, differences in the number of 1-cell and 2-cell embryos between these treatments were evaluated by one-way analysis of variance (Wilkinson, 1987) and a Student-Neuman-Kuehl's multiple range test (Steel and Torrie, 1960).

Individual progesterone profiles in PMSG/hCG-treated domestic cats subjected to follicular aspiration and natural estrus, animals were plotted with a graphics software package (Cricketgraph, Version 1.0, Cricket Software, Philadelphia, PA), and the area under each curve was calculated by using a planimeter (Kueffel and Esser Co., Germany) with a coefficient of variation of 3.7% (n = 5). Mean area under the curve for each treatment group was calculated and differences were analyzed by one-way analysis of variance and the Student-Neuman-Kuehl's multiple range test.

RESULTS

In Vitro Fertilization of Domestic Cat Oocytes

Overall and without regard to specific treatment, slightly more than half (52.7%) of the gonadotropin-injected domestic cats exhibited behavioral estrus, and an average of 11.7 ± 0.7 mature follicles were observed 25–27 h after hCG injection. A high proportion (91.4%) of the oocytes from these follicles was recovered (overall mean, 10.7 ± 0.7 oocytes/cat). When analyzed on the basis of experimental factors, neither the PMSG-hCG interval nor the hCG dose influenced ($p>0.05$) the proportion of cats demonstrating behavioral estrus, the number of ovarian follicles available for aspiration, the number of oocytes collected, or oocyte recovery efficiency (Table 1). Further analysis indicated that these variables were not affected ($p>0.05$) by treatment interactions. Oocytes of varying maturational status based on the degree of cumulus cell expansion, were collected (Fig. 1a,b), but fewer than 10% were considered too immature for an IVF attempt (Table 1).

The morphology of oocytes subjected to fertilization, centrifugation, and H342 staining is presented in Figure 2. The mean diameter of a mature, domestic cat zona pellucida and oocyte combined was 162.4 ± 2.9 μm. The mean zona width and oocyte diameter without the zona pellucida were 17.4 ± 0.5 and 127.5 ± 2.9 μm, respectively. Germinal vesicles, identifiable only after high-speed centrifugation, could be recognized as membrane-bound structures comprising approximately 20% of the intracellular mass. Relative to the entire oocyte, the polar bodies were small (~10 μm in diameter), visible only with DIC optics (Fig. 2a), and could be confirmed by H342 staining (Fig. 2b). The pronuclei were bounded by well-defined membranes but contained no prominent nucleoli (Fig. 2c); thus, distinguishing these organelles from intracellular vacuoles was difficult without DNA-specific staining (Fig. 2d). With fluorescence optics, these chromosomal structures were three-quarter moon-shaped and produced a "softer" brightness than somatic cell nuclei (as would be expected for a haploid structure). Although 2-cell embryos were relatively simple to identify (Fig. 3a), the compactness and opacity of the blastomeres at later developmental stages made it difficult to distinguish the exact number of cells (Fig. 3b). Individual blastomeric nuclei were recognizable when embryos were stained with H342, flattened under a coverslip, and examined by fluorescence microscopy, thereby allowing an accurate count of cell number (Fig. 3c).

Within the two hormone intervals and dose treatments (Table 2) and among the treatment interactions (Table 3), there were no differences ($p>0.05$) in the number of immature oocytes (those with germinal vesicles) or unfertilized oocytes with 1 polar body, 1 pronucleus, or 1 polar body and 1 pronucleus. Approximately 10–20% of all oocytes examined post-insemination had not undergone germinal vesicle breakdown. Overall, the number of polyspermic oocytes was less than 4% and did not vary ($p>0.05$) by treatment (Table 2) or treatment interaction

TABLE 1. Estrous behavior and oocyte aspiration results of domestic cats related to gonadotropin interval and human chorionic gonadotropin (hCG) treatment.

Treatment	No. of females	Estrous behavior		Follicles aspirated		Oocytes collected		% Recovery	No. immature oocytes (%)[b]
		number	(%)	number	mean	number	mean		
Gonadotropin interval[a]									
72 h	31	16	(52)	341	11.0 ± 1.0	335	10.8 ± 1.1	98.2	24 (7.2)
80 h	24	13	(54)	301	12.5 ± 1.1	256	10.7 ± 0.9	85.0	23 (9.0)
hCG dose									
100 IU	25	13	(52)	298	11.9 ± 1.1	295	11.8 ± 1.1	99.0	18 (6.1)
200 IU	30	16	(53)	344	11.5 ± 1.0	296	9.9 ± 0.9	86.0	29 (9.8)

[a]Time interval between injections of pregnant mare's serum gonadotropin and hCG.

[b]Oocytes with tight corona radiata and cumulus cell mass.

(Table 3). Although the PMSG-hCG interval had no influence on the number of degenerate ova, a greater proportion of the oocytes from cats treated with the 200 IU hCG dose appeared morphologically abnormal (Table 2, $p<0.001$), a finding also evident upon examination of the interaction results (Table 3). The overall fertilization rate was particularly sensitive to the PMSG-hCG interval, with the proportion increased by at least 10% ($p<0.001$) in the 80-h compared to the 72-h group (Table 2). The proportion of 1-cell embryos was unaffected by the various treatments at either 24 h (Table 4) or 30 h (Table 5) after insemination; however, the number of cleaved ova at 30 h was influenced by PMSG-hCG interval ($p<0.001$) as well as hCG dose (Table 5, $p<0.025$) when oocytes were examined 30 h post-insemination. Extending the interval between PMSG and hCG by 8 h doubled the cleavage rate, whereas the lower dosage of hCG

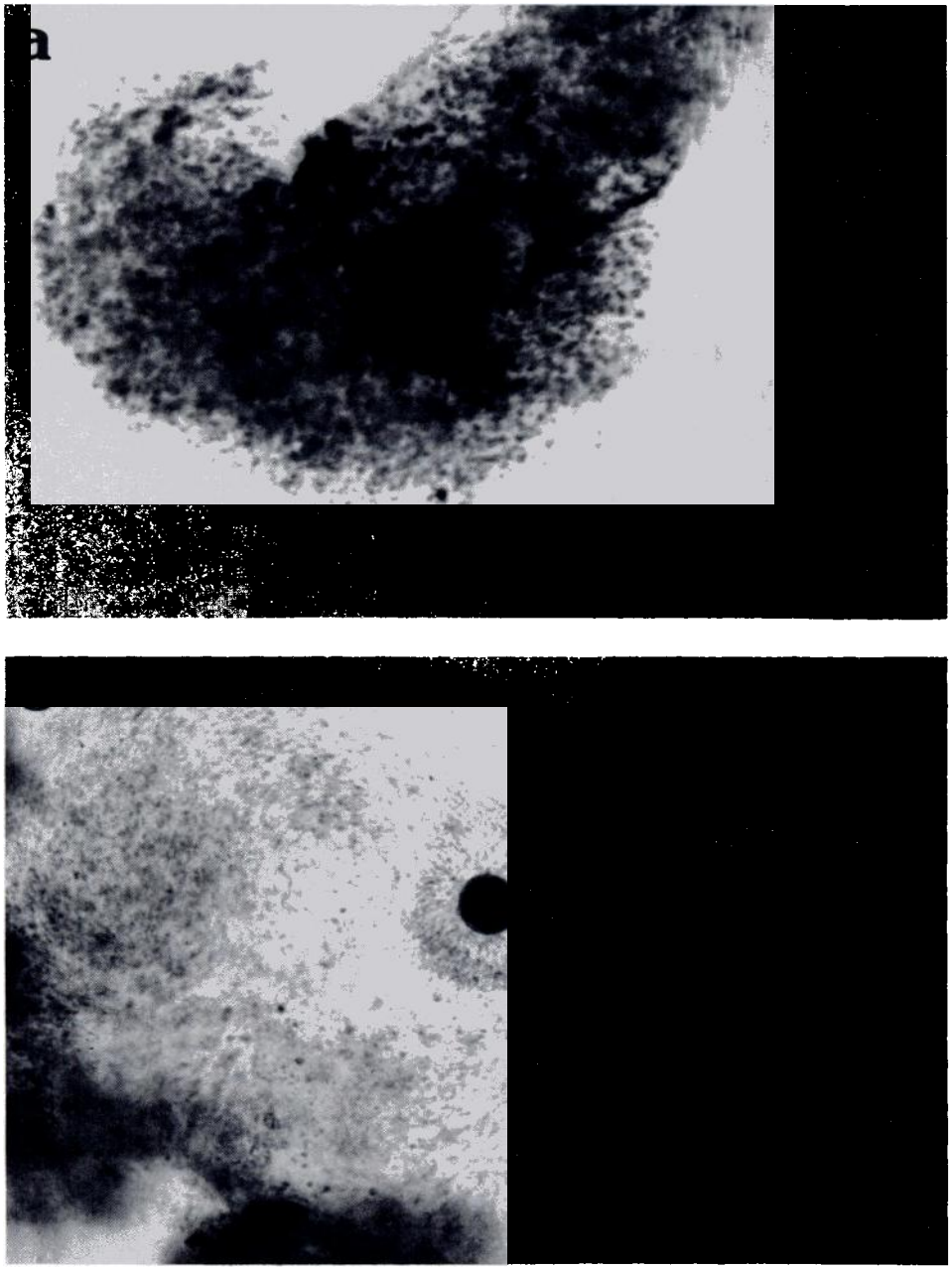
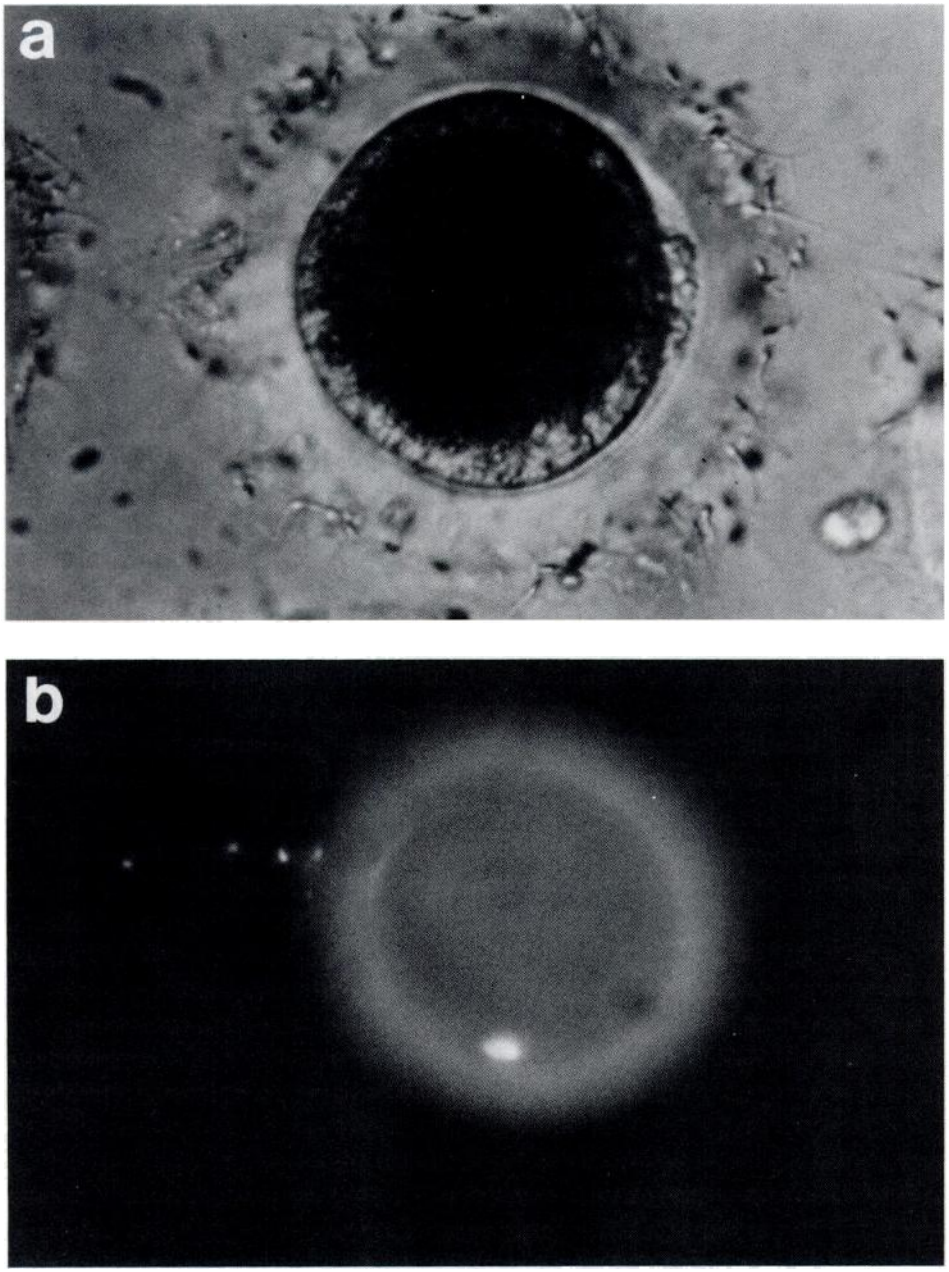


FIG. 1. Domestic cat oocytes: *a)* immature with a tightly compacted corona radiata; *b)* mature with expanded corona radiata and cumulus cell mass (×250.)

increased the cleavage rate by 15% (Table 5). Similarly, based on treatment interaction, the greatest ($p<0.05$) cleavage rate at 30 h post-insemination (45.2%) was observed with a combination of the 80 h PMSG-hCG interval and the 100 IU hCG dose (Table 5). Two of 32 control oocytes (6%) showed morphological evidence of parthenogenetic cleavage.

Fertilized oocytes cultured in mKRB medium for 48 h after insemination developed to the 4-cell stage, while a 72- to 96-h culture interval allowed development to at least the 16-cell stage, as evidence by the presence of numerous blastomeres and nuclei identified with H342 staining (Fig. 3b,c). Five queens diagnosed pregnant by abdominal palpation at 35 days produced normal litters of 4 (4/18 embryos), 1 (1/13 embryos), 3 (3/8 embryos), 1 (1/8 embryos), and 1 (1/7 embryos) kitten (4 males, 6 females), respectively. The sixth queen, which received 8



embryos, did not exhibit obvious signs of pregnancy and was diagnosed as having metastatic mesenteric lymphosarcoma 3.5 mo after embryo transfer.

Assessment of Corpus Luteum Formation and Function after Follicular Aspiration

The mean number of pre-ovulatory follicles (2–5 mm in diameter) before aspiration and the mean number of CL 1 wk after aspiration were similar between the hCG treatment groups, but both were greater ($p<0.01$) than the respective values in the control group (Table 6). In all but a single PMSG/hCG-treated queen, the number of CL exceeded the number of follicles identified and aspirated 1 wk earlier, in one case by 5-fold. In contrast, the number of CL in the control queens corresponded to the number of mature follicles observed before mating.

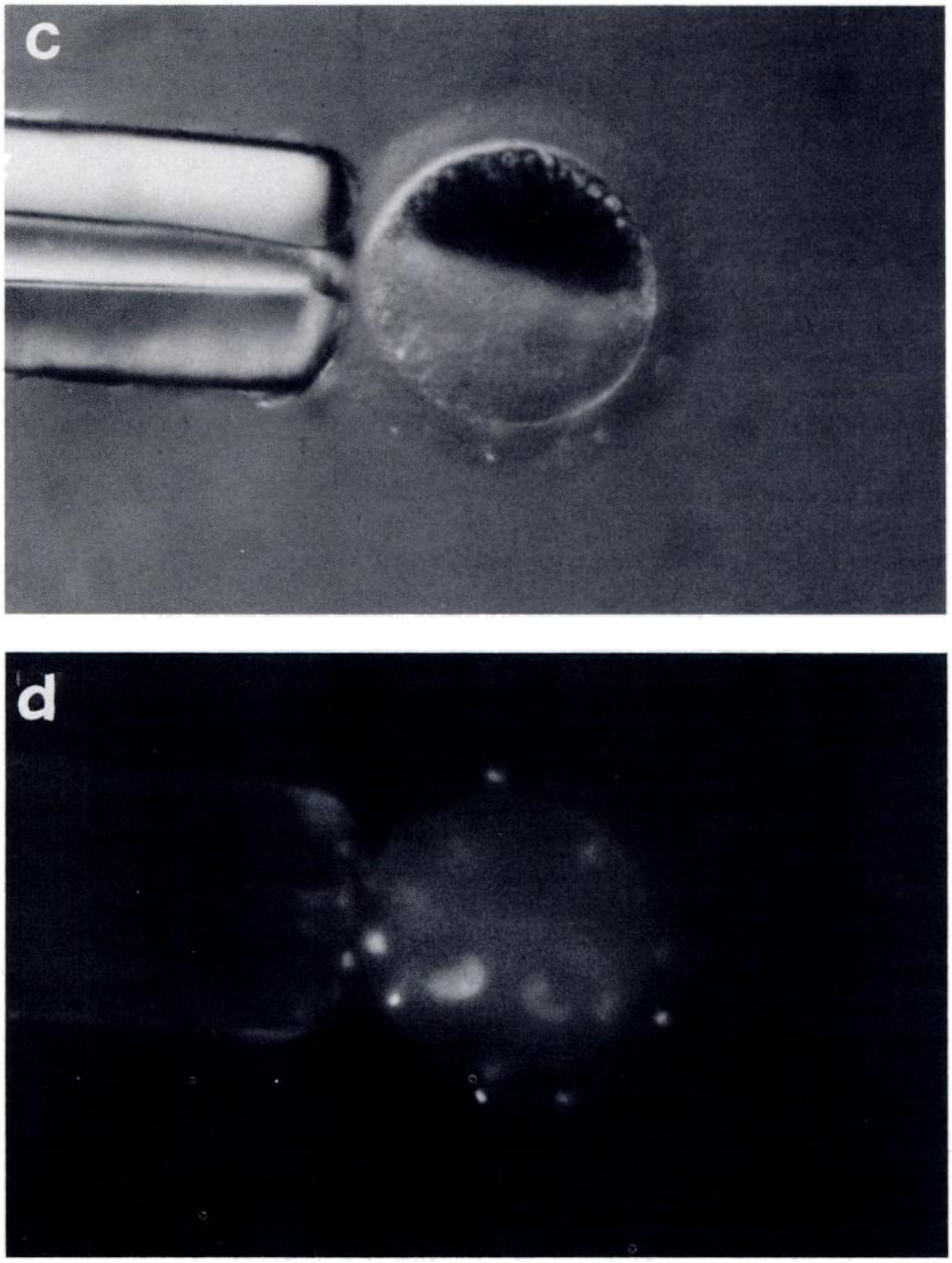


FIG. 2. Domestic cat oocytes after germinal vesicle breakdown, polar body extrusion (*a, b*) and a 1-cell, in vitro-fertilized, and centrifuged embryo (*c, d*) observed with DIC (*a, c*) and fluorescence optics after Hoescht-staining (*b, d*). (×400.)

During the 8-wk post-aspiration interval, CL number within individual queens of all groups remained constant during regression to the corpus albicantia stage.

Although the administration interval between PMSG and hCG had no effect ($p>0.05$) on hormonal profiles, hCG dose did exert an influence. Consequently, temporal estradiol-17β and progesterone profiles are presented for the control animals as well as for PMSG-treated queens receiving either 100 or 200 IU hCG (Figs. 4 and 5, respectively). Overall, estradiol-17β profiles were not different ($p>0.05$) among groups from immediately post-ovulation through Day 44. There was a rise ($p<0.05$) in estradiol-17β in control females coincident with the end of the luteal phase (Fig. 4, Day 46) that was not evident in the PMSG/hCG-treated cats. Among all groups, the area under the progesterone profile curve was positively correlated with the number of CL ($r = 0.78, p<0.001$). Although the mean area under the curves did not differ ($p>0.05$) between the two hCG dosages (100 IU hCG, 14.2 ± 2.1 cm^2; 200 IU hCG, 18.1 ± 2.0

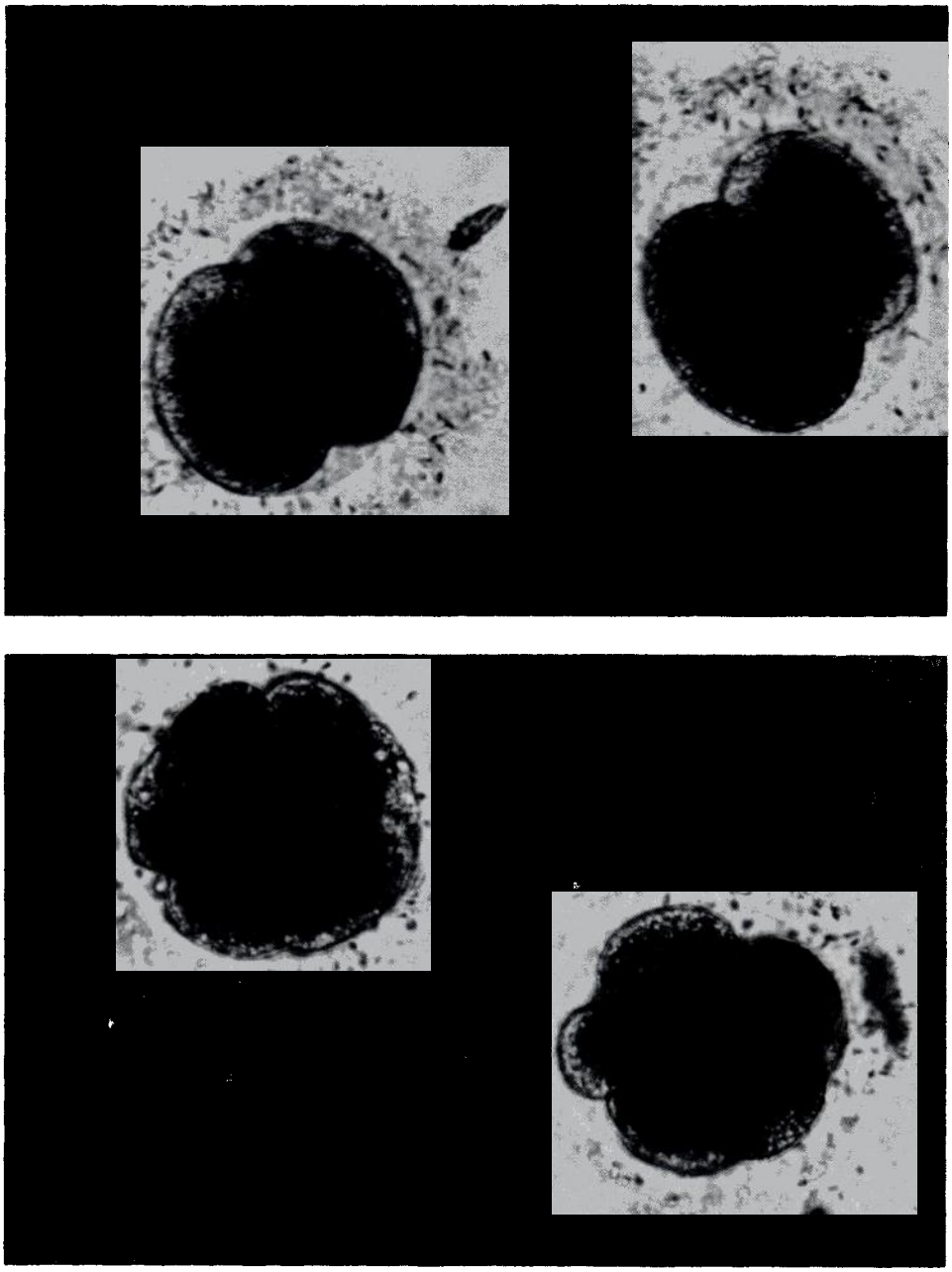


TABLE 2. In vitro fertilization results by time interval or human chorionic gonadotropin (hCG) dosage.

Treatment	Number of females	Total no. of oocytes[a]	No. with germinal vesicles (%)[b]	No. with 1 pb, 1 pn or 1 pb/1 pn (%)[c]	No. polyspermic (%)	No. degenerate (%)[b]	Percentage fertilized[d]
Gonadotropin interval[e]							
72 h	31	202	41 (20.3)	56 (27.7)	4 (2.0)	31 (15.4)	34.6[f]
80 h	24	214	30 (14.0)	51 (23.8)	7 (3.3)	29 (13.6)	45.3[g]
hCG dose							
100 IU	25	207	37 (17.9)	60 (29.0)	4 (1.9)	17 (8.2)[f]	42.0
200 IU	30	209	34 (16.3)	47 (22.5)	7 (3.4)	43 (20.6)[g]	37.3

[a]Initially degenerate and/or oocytes damaged prior to analyses have been excluded (n = 96).

[b]Post-insemination.

[c]pb = polar body, pn = pronucleus.

[d]Pooled fertilization rates from 24- and 30-h observations.

[e]Time interval between injections of pregnant mare's serum gonadotropin and hCG.

[f,g]Values within columns and treatment with different superscripts differ, $p<0.001$.

cm^2), the latter value was greater ($p<0.05$) than the mean area under the control curve (8.5 ± 1.0 cm^2). Mean peak progesterone levels were similar (Fig. 5, $p>0.05$) between the 100 IU (55.1 ± 10.6 ng/ml) and 200 IU (72.4 ± 11.7 ng/ml) hCG groups and were not different than mean progesterone concentrations in the control group (30.8 ± 6.7 ng/ml; $p>0.05$). The duration of the luteal phase, when circulating progesterone was greater than 1 ng/ml (Fig. 5), was similar ($p>0.05$) among the three groups.

DISCUSSION

Although fertilization in vitro has been reported previously for the domestic cat (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985), the present study

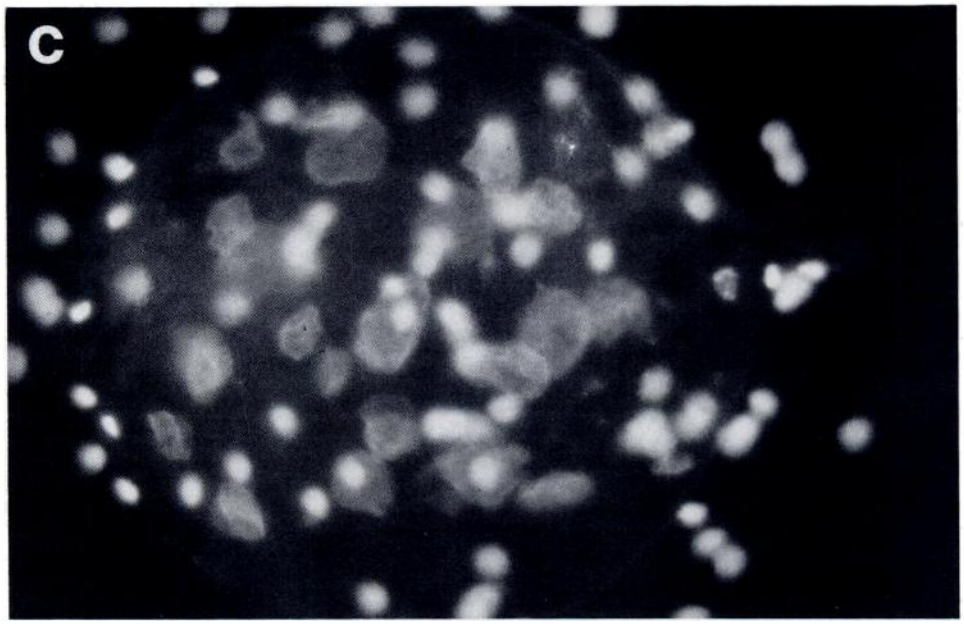


FIG. 3. *a)* Two-cell stage, in vitro fertilized cat embryos observed with phase contrast optics. *b)* Morula-stage in vitro fertilized cat embryos. *c)* Morula-stage embryo flattened and Hoescht-stained to observe individual blastomeric nuclei (smaller fluorescent areas are nuclei of spermatozoa attached to the zona pellucida). (×400.)

TABLE 3. In vitro fertilization of feline follicular oocytes.

Gonadotropin interval/ hCG dose	Number of females	Total no. of oocytes[b]	No. with germinal vesicles (%)[c]	No. with 1 pb, 1 pn or 1 pb/1 pn (%)[d]	No. polyspermic (%)[c]	No. degenerate (%)[c]	Percentage fertilized[e]
72 h/100 IU	12	101	19 (18.8)	35 (34.6)	1 (1.0)	8 (7.9)[f]	37.6
72 h/200 IU	19	101	22 (21.8)	21 (20.8)	3 (3.0)	23 (22.8)[g]	31.7
80 h/100 IU	13	106	18 (17.0)	25 (23.6)	3 (2.8)	9 (8.5)[f]	48.1
80 h/200 IU	11	108	12 (11.1)	26 (24.1)	4 (3.7)	20 (18.5)[g]	42.6

[a]Time interval between injections of pregnant mare's serum gonadotropin and human chorionic gonadotropin, (hCG).

[b]Initially degenerate and/or oocytes damaged prior to analyses have been excluded (n = 96).

[c]Post-insemination.

[d]pb = polar body, pn = pronucleus.

[e]Pooled fertilization rates from 24 and 30 h observations.

[f,g]Values within columns with different superscripts differ, $p<0.005$.

was the first to use follicular oocytes collected by a relatively atraumatic approach and to unequivocally demonstrate the developmental competence of the resulting embryos in vivo. Hamner et al. (1970), using ovulated oocytes and spermatozoa capacitated in utero, reported that 44 of 92 oocytes (47.8%) appeared fertilized in vitro (range, 0–90%). Bowen (1977) also tested ovulated oocytes, but with ductus deferens spermatozoa and two different media (mBSW, Ham's F-10) and reported fertilization rates of 77.8 and 80.1%, respectively ($p>0.05$). The first evidence of embryo cleavage was observed 20–28 h post-insemination, comparable to cleavage onset in the present study. To study the events associated with early embryonic development, Niwa et al. (1985) examined spermatozoal penetration and pronuclear formation of in vitro-fertilized cat oocytes. Fifty-nine oviductal ova were observed from 15 min to 5 h following insemination with epididymal spermatozoa. Penetration rates ranged from 0 to 100%, with decondensation of sperm heads and male pronuclear formation observed 3–4 h after insemination. In contrast, in the present study, pronuclear formation within follicular oocytes was observed 24 h after the addition of spermatozoa. This difference may be attributable to natural variations in the maturational status of ovulated versus follicular oocytes as well as to variations in the capacitation requirements of epididymal versus ejaculated spermatozoa.

Ejaculated spermatozoa from laboratory and farm animals apparently are more difficult to capacitate than epididymal or ductus deferens spermatozoa (see reviews: Brackett, 1981; Yanagimachi, 1981). Hamner et al. (1970) reported that ejaculated cat spermatozoa

TABLE 4. Results of in vitro fertilization 24 h after insemination.

Treatment	Number of females	Total no. of oocytes	No. of 1-cell embryos (%)	No. of cleaved embryos (%)
Gonadotropin interval[a]				
72 h	16	105	13 (12.4)	11 (10.5)
80 h	12	110	12 (10.9)	17 (15.4)
hCG dose				
100 IU	13	105	8 (7.6)	16 (15.2)
200 IU	15	110	17 (15.4)	12 (10.9)
Gonadotropin interval[a]/hCG dose				
72 h/100 IU	7	61	6 (9.8)	6 (9.8)
72 h/200 IU	9	44	7 (15.9)	5 (11.4)
80 h/100 IU	6	44	2 (4.5)	10 (22.7)
80 h/200 IU	6	66	10 (15.2)	7 (10.6)

[a]Time interval between injections of pregnant mare's serum gonadotropin and human chorionic gonadotropin, (hCG).

TABLE 5. Results of in vitro fertilization 30 h after insemination.

Treatment	Number of females	Total no. of oocytes	No. of 1-cell embryos (%)	No. of cleaved embryos (%)
Gonadotropin interval[a]				
72 h	15	97	27 (27.8)	19 (19.6)[b]
80 h	12	104	25 (24.0)	43 (41.3)[c]
hCG dose				
100 IU	12	102	26 (25.5)	39 (38.2)[d]
200 IU	15	99	26 (26.3)	23 (23.2)[e]
Gonadotropin interval[a]/hCG dose				
72 h/100 IU	5	40	15 (37.5)	11 (27.5)[f]
72 h/200 IU	10	57	12 (21.1)	8 (14.0)[f]
80 h/100 IU	7	62	11 (17.7)	28 (45.2)[g]
80 h/200 IU	5	42	14 (33.3)	15 (35.7)[f]

[a]Time interval between injections of pregnant mare's serum gonadotropin and human chorionic gonadotropin (hCG).

[b,c]Values within columns with different superscripts differ, $p < 0.001$.

[d,e]Values within columns with different superscripts differ, $p < 0.025$.

[f,g]Values within columns with different superscripts differ, $p < 0.05$.

were incapable of fertilizing oocytes in vitro without prior in utero incubation. The present data demonstrate that electoejaculated cat spermatozoa subjected to mKRB medium and swim-up processing become capacitated in vitro without in utero exposure, a finding that considerably increases the practicality of IVF as a potential tool for improving reproductive efficiency in felids. In vitro spermatozoal capacitation varies markedly among species and among individuals within species (Brackett, 1981, 1985); in certain animals, successful capacitation requires specialized conditions such as a high ionic strength medium (rabbit and bull, Brackett and Oliphant, 1975; Brackett, 1981; Brackett et al., 1982), the addition of caffeine or cyclic adenosine 3', 5'-monophosphate (rhesus monkey, Boatman and Bavister, 1982), or simply slight variations in medium constituents (dog, Mahi and Yanagimachi, 1978). However, for the cat, the high rates of IVF in three independent laboratories using four biologically different capacitation preparations (Hamner et al., 1970; Bowen, 1977; Niwa et al., 1985) indicate that these spermatozoa appear relatively insensitive to variations in capacitation media.

Laparoscopy provided a consistently reliable and minimally invasive approach for retrieving feline follicular oocytes, similar to that previously found in humans (Wood et al., 1981), nonhuman primates (Kuehl and Dukelow, 1979; Bavister et al., 1984; Clayton and Kuehl, 1984), and cattle (Brackett et al., 1982). In most instances, follicular oocytes are less capable of fertilization and cleavage than are ovulated ova collected from the ovarian surface or oviducts (Brackett, 1981; Moor et al., 1983). The intracellular protein and RNA content of follicular oocytes probably is insufficient for self-maintenance (Brackett, 1981) and, compared to ovulated ova, fewer of these cells have undergone nuclear maturation. In humans, pigs, and mice, the degree of corona radiata/cumulus mass expansion is the most convenient indicator of oocyte maturity and is correlated positively with rates of fertilization in vitro (Gilula et al., 1978; Brackett, 1985; Motlik et al., 1986). Because polar body extrusion was difficult to identify in cat oocytes, cumulus cell mass expansion appeared to be the most consistently reliable index of maturation.

One distinct feature of all carnivore oocytes studied to date (ferret, dog, mink, cat) is the uniformly dark appearance of the oocyte and embryo (Chang, 1968; Bowen, 1977; Mahi and Yanagimachi, 1978), which likely is attributable to a high intracellular

TABLE 6. Influence of human chorionic gonadotropin (hCG) on the number of mature follicles and formation of corpora lutea (CL) following natural mating or follicular aspiration.

Treatment	No. of females	Preovulatory follicles[a]		CL formed[b]	
		Mean	Range	Mean	Range
Control	4	4.2 ± 0.5[c]	3–5	4.2 ± 0.5[c]	3–5
100 IU hCG	6	10.3 ± 2.5[d]	2–16	17.0 ± 3.3[d]	7–29
200 IU hCG	5	14.2 ± 2.5[d]	8–20	23.6 ± 5.4[d]	14–43

[a]Follicles detected at laparoscopy prior to mating for natural estrus (control) females and prior to aspiration for hCG-treated females.

[b]Detected at laparoscopy 1 wk after mating or aspiration.

[c,d]Values within columns with different superscripts differ ($p < 0.01$).

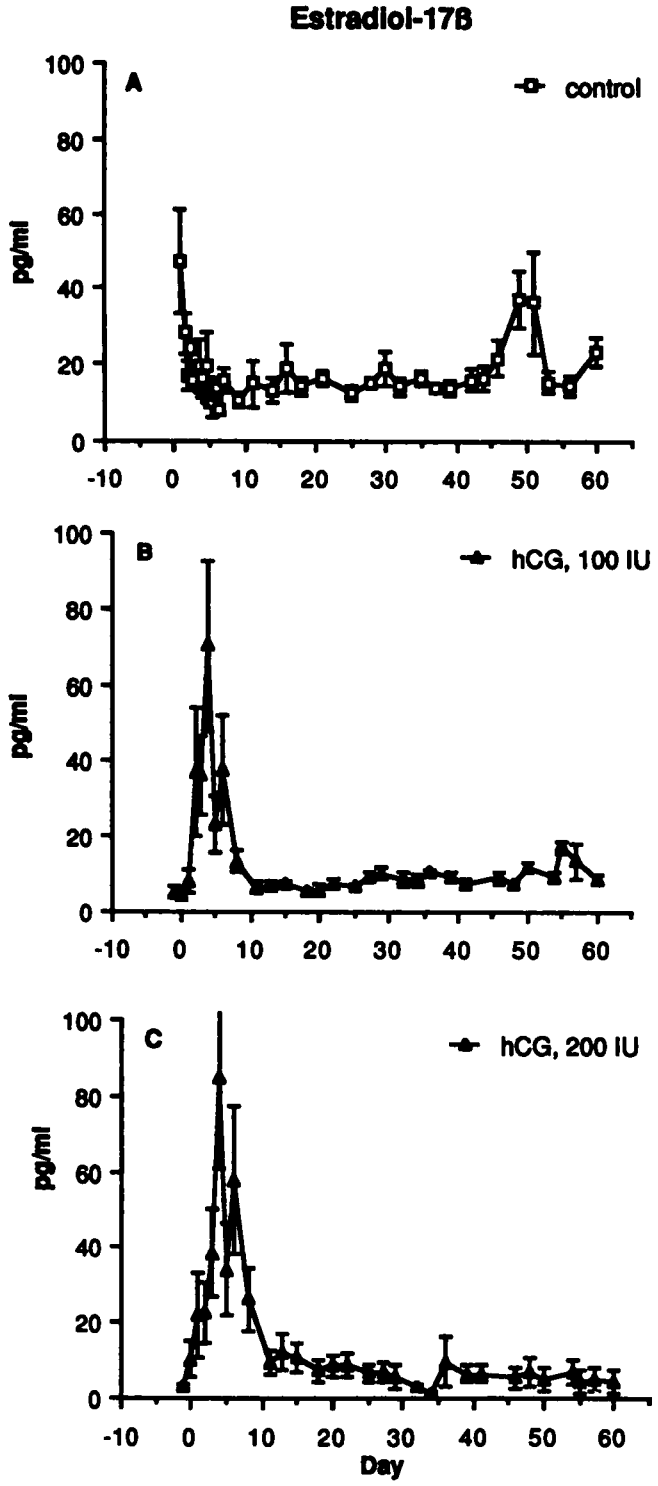


FIG. 4. Mean (± SEM) estradiol-17β profiles during an 8-wk sampling interval from natural estrus (control, *Panel A*, n = 4) cats that ovulated after mating versus those treated with pregnant mare's serum gonadotropin (PMSG) and either 100 (*Panel B*, n = 6) or 200 (*Panel C*, n = 5) IU hCG followed by follicular aspiration. For *Panel A*, Day 0 represents samples collected 1 day prior to mating; for *Panels B* and *C*, Day 0 represents samples collected 1 day prior to onset of PMSG treatment.

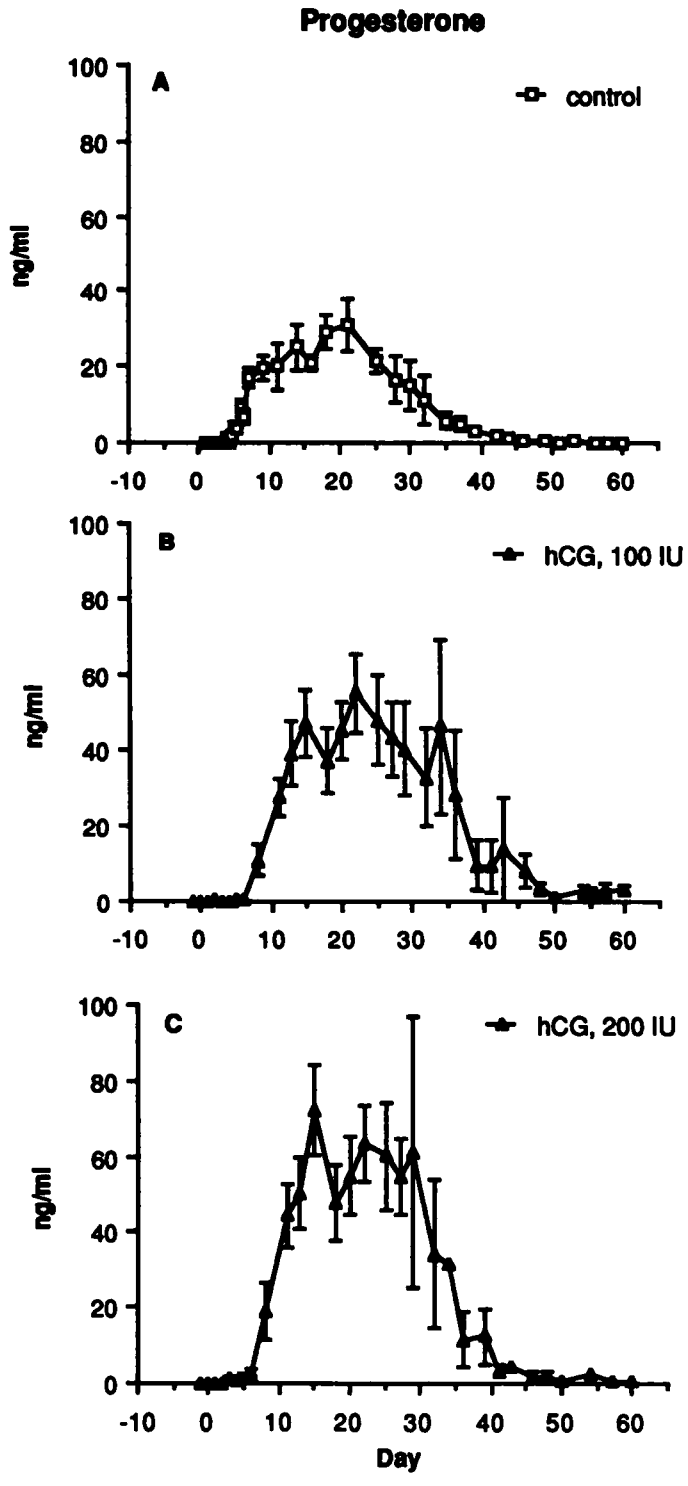


FIG. 5. Mean (± SEM) progesterone profiles during an 8-wk sampling interval from natural estrus (control, *Panel A*, n = 4) cats that ovulated after mating versus those treated with pregnant mare's serum gonadotropin and either 100 (*Panel B*, n = 6) or 200 (*Panel C*, n = 5) IU human chorionic gonadotropin (hCG) followed by follicular aspiration. See *Figure 4 legend* for explanation of Day 0.

concentration of lipid (Guraya, 1965). This characteristic, which prevents determining meiotic status, also has been observed in oocytes and embryos of pigs (Wall et al., 1985). The high-speed centrifugation technique used by the latter investigators to displace intracellular lipid also was effective with domestic cat oocytes, readily allowing identification of germinal vesicles and/or pronuclei.

The success rate of IVF in the cat was highly dependent on the time interval between PMSG and hCG administration as well as hCG dose. Maintaining the oocytes intrafollicularly for an additional 8 h before the hCG challenge consistently facilitated fertilization and cleavage. The timing of gonadotropin therapy also has been shown to influence oocyte retrieval, maturity, and fertilizability in vitro for a variety of other species (Mizoguchi and Dukelow, 1980; Jones et al., 1983; Hillier et al., 1985). An increase in the administration interval between gonadotropins from 56 to 72 h in hamsters (Mizoguchi and Dukelow, 1980) and from 40 to 50 h in mice (Hillier et al., 1985) increases fertilization rates significantly, whereas decreasing this interval from 60 to 48 h in mice adversely affects IVF (Edgar et al., 1987). Although increasing the hCG dosage has been shown to increase the ovulation rate in cats (Wildt and Seager, 1978), the two dosages tested in this study had no apparent affect on the number of mature follicles available for aspiration. The effect of the therapeutic level of hCG on ovum integrity has not been addressed extensively in the literature, although there appears to be no detrimental effects of increasing hCG dosage on embryonic development in the mouse (Spindle and Goldstein, 1975) and the Chinese hamster (Roldan et al., 1987). In contrast, results from the present study indicated that a greater hCG dose contributed to an increased incidence of oocyte degeneration and lower fertilization rates in culture. Because the oocyte is incapable of independent metabolism, maturation is dependent on cell-to-cell communication with the surrounding granulosa cells (Gilula et al., 1978; Motlik et al., 1986). The initiation of the final biochemical alterations for oocyte maturation are gonadotropin-stimulated; a follicle stimulating-type signal is responsible for the final stages of oocyte maturation, while a luteinizing hormone (LH) or hCG stimulus initiates nuclear maturation (Dekel et al., 1981). Germinal vesicle breakdown (GVBD) and the metabolic events associated with cytoplasmic maturation are independent, as evidenced by GVBD prior to cumulus cell mass expansion, thereby allowing nuclear maturation to occur within a metabolically immature oocyte (Moor et al., 1981). Therefore, in the domestic cat, an excessive ovulatory signal (hCG) or a poorly timed gonadotropin interval may have resulted in premature granulosa cell uncoupling, thus interfering with final oocyte development and disrupting oocyte integrity and fertilizability. This hypothesis is supported by the data of Williams and Hodgen (1980) who reported that a premature hCG stimulus prohibits the granulosa cells from completing oocyte maturation in rhesus and cynomolgous monkeys. In contrast, prolonging the interval between sequential gonadotropin challenges can result in ova that fail to fertilize or that are vulnerable to polyspermia (Chang and Fernandez-Cano, 1958; Laufer et al., 1984). Because the cat is an induced ovulator, maturing oocytes must be maintained within the follicle for unpredictable intervals following estrus onset, in contrast to a fixed interval for most spontaneously ovulating species. Therefore, it is logical that an intrafollicular mechanism exists to maintain feline oocytes in an "ovulation-ready" state for a period of 6 days or longer. Even so, the oocytes of this species remain highly sensitive to gonadotropic stimuli and apparently continue to experience critical maturational events up to the time of ovulation.

Punctured and aspirated cat follicles underwent normal luteal development as attested to by the time course of the progesterone rise, which demonstrated apparently normal steroidogenic changes. The progesterone profiles and luteal phase duration were qualitatively similar to those of cats in natural estrus that were mated with a vasectomized male and experienced a nonpregnant luteal phase (Wildt et al., 1981a). Kerin et al. (1981) and Oskowitz et al. (1986) reported that in spontaneous human cycles, aspiration of the single pre-ovulatory follicle following the LH surge resulted in progesterone profiles and luteal phase durations comparable to nonaspirated controls. Compared to untreated controls, domestic cats injected with PMSG/hCG demonstrated quantitatively greater progesterone levels, which were correlated positively with CL number. In humans, treatment with clomiphine citrate or human menopausal gonadotropin in combination with hCG results in greater than normal circulating progesterone concentrations (Kemeter et al., 1982; Dlugi et al., 1984; Huang et al., 1986; Vargyas et al., 1986), which

has been attributed to multiple CL formation resulting from the gonadotropin therapy. In the cat, although the aspirated follicles formed functionally competent CL and subsequently secreted progesterone, it is probable that some of the circulating progesterone was being contributed by "ancillary" CL that were noted at laparoscopy 1 wk after aspiration. The origin of these CL is unknown; however, the combined use of pituitary-derived follicle-stimulating hormone (FSH-P) and hCG in the domestic cat is known to result in delayed secondary follicle development and CL formation several days after ovulation (Wildt et al., 1978; Goodrowe and Wildt, 1987). Because of the long half-life of PMSG and hCG, it is possible that a prolonged effect of these gonadotropins occurred, resulting in secondary ovulations following oocyte recovery.

The developmental competence of oocytes fertilized in vitro can be affected by a number of factors including normalcy of the recipient's luteal phase. Accelerated and extreme elevations in circulating progesterone as a result of hormonal therapy have been postulated to adversely affect the intrauterine environment in humans, potentially contributing to failed implantation after embryo transfer (Garcia et al., 1984; Vargyas et al., 1986). Data also are available indicating that the IVF procedure is somehow detrimental to subsequent embryo development in vivo. Vanderhyden et al. (1986) demonstrated that rat embryos resulting from IVF were comparable to embryos resulting from in vivo fertilization, but only to the 2-cell stage of development. Embryo mortality was accelerated after this stage, indicating that, although there is no deficiency in the ability of in vitro fertilized oocytes to undergo the first cleavage division, IVF eventually leads to defects caused by retardation in early embryo development and increased implantation losses. The high proportion of cats becoming pregnant after embryo transfer (5 of 6) suggested that excessive progesterone production is not inhibitory to establishing a pregnancy. It is possible, however, that the relatively small litter sizes compared to the number of embryos transferred could be explained on the basis of an altered endocrine milieu adversely affecting the oviductal or uterine environment, not unlike the situation recently observed in FSH-P-treated/mated cats subjected to embryo recovery (Goodrowe et al., 1988). Likewise, the high rate of embryonic mortality following IVF and embryo transfer in the cat may, in part, be due to inherent anomalies in embryo development or functional integrity, causes which have not been identified by methods used currently.

The routine production of in vitro-fertilized domestic cat embryos in conjunction with the birth of live offspring suggests that this approach potentially may be useful for basic studies of gamete interaction as well as applied efforts to artificially propagate selected species of endangered Felidae. Laparoscopy (for ovarian examination) and electroejaculation (for semen collection) are procedures used commonly in exotic cats; likewise, the ovaries of several of these species are known to respond to exogenous gonadotropins (Wildt et al., 1981b; 1983; Phillips et al., 1982). It is likely that the success of IVF in these species will be limited initially by a meager data base on basic reproductive/endocrine norms. Among the Felidae, species specificity does exist in terms of ovarian response to exogenous gonadotropins (Wildt et al., 1981b; Phillips et al., 1982), endocrine profiles (Wildt et al., 1984; 1987b; 1988; Brown et al., 1988), and electroejaculate characteristics (Wildt et al., 1983; 1987a; Howard et al., 1984). Efforts must now determine if the basic laboratory procedures standardized for the domestic cat can be adapted directly to nondomestic felids or whether species-specific differences will dictate the need for an extensive research and development program for each endangered species of interest.

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Names of commercial manufacturers are provided for identification purposes only. Inclusion does not imply endorsement by the National Institutes of Health, U.S. Public Health Service, or the U. S. Department of Health and Human Services.

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