Journal of Assisted Reproduction and Genetics, Vol. 10, No. 8, 1993

SHORT COMMUNICATIONS

WASHINGTON, D.C.

Influence of Oviductal Cell Monolayer Coculture and the Presence of Corpora Hemorrhagica at the Time of Oocyte Aspiration on Gamete Interaction in Vitro in the Domestic Cat

Submitted: March 7, 1993
Accepted: June 6, 1993

INTRODUCTION

Assisted reproduction techniques, including in vitro fertilization (IVF) and embryo transfer, will become important for maintaining genetic diversity in both captive and natural populations of nondomesticated, felid species (1). In our laboratories, the domestic cat serves as a useful model (2–5) for studying the many factors potentially affecting sperm–oocyte interaction, fertilization, and embryo development in a host of wild felids including the leopard cat (*Felis bengalensis*) (6), puma (*F. concolor*) (7), tiger (*Panthera tigris*) (8), and cheetah (*Acinonyx jubatus*) (9). Although the incidence of IVF and embryo growth to the morula stage is high in the domestic cat, most morulae experience a developmental block in vitro, failing to achieve blastocyst formation (3,4). Tiger and leopard cat IVF embryos experience a similar morula-to-blastocyst developmental block in vitro (8; Donoghue and Wildt, unpublished data), suggesting that this phenomenon may occur throughout the Felidae family. Also of interest are species-specific differences in overall IVF efficiency. For example, although tiger oocytes fertilize in vitro at rates similar to those of the domestic cat, cleavage success, using the same IVF system, is significantly poorer in the puma and cheetah (see Review in Ref. 10).

Growth blocks in vitro are not unique to felids, but usually occur earlier in development in mouse (11), pig (12), cow (13), sheep (14), and goat (15) embryos. In these same studies, the block to development was circumvented by coculturing embryos with oviductal and/or uterine cells. Several comparative studies have demonstrated the superiority of oviductal cells over other cell types (uterine epithelial or fibroblast and fetal fibroblast cells) in promoting embryo growth through the in vitro block (14,16,17). The mechanism of enhancement may be related to the production of stimulatory components and/or the removal of inhibitory compounds in the culture medium (18). Oviductal cells also may support sperm function by enhancing motility longevity and increasing the proportion of hyperactivated cells (19).

The present study had a prospective and retrospective objective. The former involved investigating the potential of gamete coculture on conspecific oviductal cell monolayers (OCM) in 96-well plates for (i) supporting IVF in the cat and (ii) enhancing development by overcoming the partial morula-to-blastocyst developmental block. Our logic was that if either of these objectives could be achieved in the cat, then perhaps a similar IVF system would prove even more beneficial in parallel studies with "less-efficient" felid species. The retrospective purpose involved analyzing the overall data set on the basis of ovarian activity at the time of follicular oocyte aspiration. Our interest in this factor was derived from recent work indicating that the type of ovarian activity (follicular or luteal) influences the ability of immature antral cat oocytes to achieve metaphase II in vitro (20). Because some oocyte donors already had initiated ovulation by the time of aspiration, this study provided a prime opportunity to examine the influence of presence or absence of luteal tissue on the subsequent ability of oocytes to fertilize and develop normally in vitro.

1058-0468/93/1100-0523$07.00/0

MATERIALS AND METHODS

Oviductal Cells

An oviductal epithelial cell line was generated from primary cultures established from the oviducts of a domestic cat following ovariohysterectomy. Oviducts were dissected from the uterus and supporting ligaments, rinsed with saline, and blotted dry. To recover epithelial cells, oviducts were flushed by inserting a 22-gauge needle at the isthmus and slowly forcing 3–5 ml of Dulbecco's minimum essential medium (DMEM/F12; GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS), 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (Collaborative Research, Inc., Bedford, MA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (GIBCO) through each oviduct into a Petri dish. The resulting cell suspension was transferred from the Petri dish into a 25-cm^2 tissue culture flask and incubated (5% CO_2 at 37°C) undisturbed for 72 hr.

Nonadhered cells were removed, and the medium was replaced every 72 hr until the cells formed a confluent monolayer. Cells were subcultured three to five times using 0.25% trypsin, and then DMEM was replaced with Ham's F10 medium (Irvine Scientific, Irvine, CA) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After two additional subcultures in Ham's F10, cells were resuspended at 1×10^6 cells/ml in Ham's F10 containing 20% FCS and 10% dimethyl sulfoxide and frozen in cryovials (1 ml/vial) at −70°C overnight. Cryovials were plunged into and stored in liquid nitrogen 24 hr later.

To verify the epithelial lineage of the established monolayers, the intermediate filament content was determined. Cells were subcultured in LabTek eight-chambered slides (Miles Scientific, Naperville, IL) by diluting cells to 1×10^4/ml and placing 250 μl of the cell suspension in each chamber. Cells were incubated for 24 hr, then fixed in cold absolute methanol for 10 min and air-dried. Slides were rinsed with phosphate-buffered saline (PBS) and incubated with mouse antipancytokeratin (BioGenex Laboratory, Dublin, CA) for 1 hr at 37°C. After rinsing with PBS, antibody binding was visualized using a Supersensitive Strepavidin-Biotin Kit (BioGenex Laboratory). Diaminobenzidine was used as a chromagen, and slides were counterstained with hematoxylin. All cells stained positive for cytokeratin.

Embryo Culture Preparation

Four days before oocyte collection, 1-ml aliquots of oviduct epithelial cells were thawed rapidly in a 37°C water bath. The freezing solution was diluted by slowly (over a period of 7–10 min) adding Ham's F10 culture medium to the cell suspension while mixing vigorously until cells were diluted to a concentration of 2×10^5 cells/ml. Epithelial cells (2×10^4) were pipetted into four wells (100 μl/well) of a 96-well, round-bottom plate (two wells for IVF and two for embryo culture). Medium was replaced 24 hr before oocyte collection, and 100 μl of medium was added to four control wells. At this time, wells were overlaid with 40 μl of paraffin oil (Fisher Scientific Company, Fairlawn, NJ). Because we routinely use Petri dishes in our IVF system, two such "control" dishes were prepared, each containing two 100-μl drops of Ham's F10 overlaid with oil.

Oocyte Collection and IVF

Oocytes were recovered from nine adult female cats stimulated with 150 IU equine chorionic gonadotropin i.m. (eCG; Sigma Chemical Co., St. Louis, MO), followed 84 hr later with 100 IU human chorionic gonadotropin i.m. (hCG; Sigma Chemical Co.) (5). Twenty-four to twenty-seven hours later, each cat was anesthetized and subjected to a laparoscopic ovarian examination, followed immediately by oocyte aspiration (2). For the former, all aspects of each ovary were observed using an ancillary manipulatory probe, and the presence and number of fresh ovulation sites (CH determined). Preovulatory ovarian follicles (≥2 mm in diameter) then were aspirated using a 22-gauge needle attached to polyethylene tubing, a collection tube, and a vacuum pump. The follicular contents were collected into tubes containing Ham's F10 culture medium supplemented with heparin (40 IU/ml) and maintained at 37°C. Oocytes were retrieved from collection tubes and transferred to a Petri dish containing 3 ml of Ham's F10. After rinsing oocytes through two additional dishes of Ham's F10, good- to excellent-quality, mature oocytes (darkly pigmented, uniform oocytes with an expanded cumulus) from each donor were distributed evenly among three treatment conditions: (i) a well with a confluent OCM, (ii) a well with control Ham's F10 medium (CON), and (iii) a 100-μl drop of Ham's F10 in a Petri dish (PET). For each replicate, 10 to 29 oocytes were assigned per treatment.

Semen was collected by electroejaculation (21) from a single normospermic (>60% morphologically normal sperm) domestic cat, diluted with an equal volume of Ham's F10 medium, and centrifuged (100*g*, 10 min). The supernatant was removed and the sperm pellet overlaid with 200 μl of Ham's F10 and allowed to swim up for 1 hr at 25°C. Because Bongso *et al.* (19) reported that monolayers impede sperm motility, we compensated for a potential decrease in sperm motility by inseminating oocytes in all groups with a greater number of sperm (5×10^4) than normally used (2×10^4) in our domestic cat IVF system (3–5,20). At 7 hr post-insemination, oocytes were removed from IVF wells and drops, and cumulus cells were dislodged by gently pipetting oocytes through a small-bore pipette. Cumulus-free oocytes then were transferred to their respective wells and microdrops for subsequent culture. All oocytes were assessed for fertilization (based upon evidence of cleavage to at least the two-cell stage) at 30 hr and, again, at 54 hr post-insemination.

Evaluation of Embryo Development

Developmental progress of embryos cleaving to at least two cells by 54 hr was recorded microscopically (100×) every 24 hr for the next 72 hr. Embryos that stopped developing or became degenerate were removed from the wells and fixed in 2% glutaraldehyde. At 126 hr post-insemination, all embryos were evaluated a final time, fixed in 2% glutaraldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) and examined using fluorescent microscopy to verify developmental stage.

Statistical Analysis

The study was replicated three times with three oocyte donors per replicate (77–157 oocytes/replicate). Differences in fertilization and the proportion of cleaved embryos achieving morula formation among the three treatments were evaluated by chi-square analysis. Similarly, chi-square analysis was used to determine differences in the proportions of embryos at each stage of development for each evaluation interval (30, 54, 78, 102, and 126 hr).

RESULTS

Cat oocytes and sperm cocultured on conspecific oviductal cell monolayers were capable of forming cleaved embryos at a rate equivalent ($P > 0.05$) to that of gametes not exposed to oviductal cells or those placed into our standard IVF system (Table I). The OCM treatment also supported embryo development to the morula stage, but at a rate no different ($P > 0.05$) from that of the CON treatment; however, the proportion of embryos developing to morulae was substantially less ($P < 0.05$) in microdrops than in wells (Table I). No blastocysts were observed in any treatment group by 126 hr of culture.

The development rate of cleaved embryos in OCM and CON wells was similar ($P > 0.05$; data not shown), so these data were pooled for further analysis (Table II). The rate of embryo development was similar between the well and the Petri dish treatments except at 54 hr when a greater proportion of cleaved embryos in wells was five to eight cells ($P < 0.01$) and a smaller proportion ($P < 0.01$) was two cells. However, thereafter, embryos developed at a similar rate ($P > 0.05$) regardless of treatment.

The mean (±SE) number of oocytes recovered from cats without CH ($n = 4$; 48.7 ± 12.5) did not differ ($P > 0.05$) from that of cats with CH ($n = 5$; 27.2 ± 2.9), in part, because of the wide range in oocyte number in the former group (34 to 86 among

Table I. Fertilization of Domestic Cat Oocytes and Embryo Development to Morulae in 96-Well Plates with Oviductal Cell Monolayers (OCM) and Control Medium (CON) and in Petri (PET) Dish Microdrops[a]

	Number of oocytes		Number of morulae (% of embryos)
	Inseminated	Fertilized (%)	
OCM wells	91	59 (64.8)[a,*]	42/59 (71.2)[a]
CON wells	98	58 (59.2)[a]	45/58 (77.6)[a]
PET dish	98	52 (53.1)[a]	20/52 (38.5)[b]

[a] No blastocysts were observed at 126 hr postinsemination.
* Values within columns with different superscripts differed ($P < 0.01$).

Table II. Developmental Rate of Embryos in Wells of 96-Well Plates and in Microdrops in Petri Dishes[a]

Culture interval (hr)	Treatment	Number of embryos	Stage of development (number of cells)				
			2	4	5–8	9–16	Morula
30	Wells	104	35.6[a,*]	43.3[a]	21.1[a]	0.0	0.0
	Petri	52	48.1[a]	44.2[a]	7.7[a]	0.0	0.0
54	Wells	117	8.6[a]	17.9[a]	45.3[a]	28.2[a]	0.0
	Petri	52	32.7[b]	28.9[a]	17.3[b]	21.1[a]	0.0
78	Wells	100	—	—	28.0[a]	33.0[a]	39.0[a]
	Petri	25	—	—	32.0[a]	48.0[a]	20.0[a]
102	Wells	99	—	—	—	31.2[a]	68.8[a]
	Petri	25	—	—	—	36.0[a]	64.0[a]
126	Wells	99	—	—	—	12.1[a]	87.9[a]
	Petri	20	—	—	—	11.8[a]	88.2[a]

[a] Embryo developmental rate in control and coculture wells did not differ ($P > 0.05$), so data are combined under the heading "wells."
* For each culture interval, values within columns with different superscripts differed ($P < 0.01$).

donors). However, the proportion of all oocytes that were rated good- to excellent-quality was greater ($P < 0.01$) for cats without CH (97.4%), than for those with premature ovulation (75.0%). Furthermore, fertilization success was increased ($P < 0.01$) 17.8% and development to morulae enhanced ($P < 0.01$) 2.5-fold for the oocytes recovered from the cats without CH (Table III).

DISCUSSION

Gamete coculture on conspecific oviductal cells supported cat IVF but provided no clear advantage for promoting fertilization over our existing IVF system. Whether these oviductal cells or their secretory products influence other aspects of gamete interaction in this species remains to be determined. However, it is important that oviductal cell coculture at least supported IVF given earlier speculation that one of the primary advantages of this system is the promotion of sperm function. For example, when detached oviductal cells are cocultured with human gametes, sperm longevity and the proportion of sperm exhibiting hyperactivated motility are increased, which appears to be related to as much as an 18% improvement in IVF (19). Furthermore, bovine oviductal cell monolayers induce capacitation in bull spermatozoa at an efficiency equaling that observed in vivo (22). Both poor sperm morphology and poor sperm motility have been associated with compromised sperm–oocyte interaction in vitro in an array of felid species, some of which are endangered (7,9,10). Especially important is a recent report on the cheetah indicating a high correlation between IVF failure and the inability of sperm to remain motile in vitro for 6 hr (9). Therefore, now that it is known that oviductal cell coculture supports IVF in the normospermic cat, studies are planned to examine systematically the effects of this coculture system on sperm viability and IVF in conspecific and heterospecific teratospermic felids. Although this was not an objective of the present study, we noted that cat spermatozoa immediately interacted with the oviductal cell monolayer by momentarily contacting and attempting to penetrate, as if the cells were cumulus. Bongso *et al.* (23) reported that human sperm actually bound to the cellular monolayers, thereby impeding sperm motility. Our observation of equivalent IVF success in control versus oviductal cell coculture wells indicated that the cat sperm–monolayer interaction did not interfere with the sperm–oocyte interaction.

Table III. IVF and Subsequent in Vitro Embryo Development Using Oocytes Collected from Cats With and Without Fresh Corpora Hemorrhagica (CH)

	Number of oocyte donors	Range in number of CH/donor	Number of oocytes		Number of morulae (% of embryos)
			Inseminated	Fertilized (%)	
Fresh CH	5	4–8	133	67 (50.4)[a,*]	31 (46.3)[a]
No CH	4	—	154	105 (68.2)[b]	76 (72.4)[b]

* Values within columns with different superscripts differed ($P < 0.01$).

The cellular coculture system also neither inhibited nor enhanced the overall proportion of embryos developing in vitro. A high proportion of the fertilized oocytes cultured in microwells containing either oviductal cells or control medium achieved morula status (>70%), similar to values reported earlier in our laboratory (3,4). However, these previous studies relied upon the Petri dish method, so we were surprised by the unusually low proportion (<40%) of embryos developing to morulae in the present study using this treatment. Our most reasonable explanation was that the mean value was markedly decreased by a single lot of 18 two- to four-cell embryos in Replicate II that unexplicably stopped developing. Therefore, we are not suggesting that microwells are more appropriate than Petri dishes for the in vitro fertilization and culture of cat oocytes.

The proportion of two-cell embryos developing to early-stage blastocysts is low in the domestic cat (13–35%) (4) and in the tiger (30%) (8). In this study, no blastocysts were observed by 126 hr in any treatment group, indicating that oviductal cell coculture was ineffective for overcoming the block. This developmental problem in the cat is fundamentally different from that observed in other commonly studied species. For example, cow and sheep embryos arrest in vitro at 8–16 cells (24,14), pig embryos at 4 cells (25), hamster embryos at 2 and 4 cells (26,27), and mouse embryos at 2 cells (28). The only species that remotely mimics the cat is the rabbit (another induced ovulator), in which embryos also cease developing in culture at the morula stage (29). Beyond this, there is little similarity because the rabbit embryo block is overcome simply by adding amino acids to the culture medium (30). In contrast, cat embryos routinely are cultured in Ham's F10, a complex medium containing all 20 amino acids, and yet at least 65% of these embryos fail to become blastocysts.

For some species, in vitro arrest in embryo development has been purported to be associated with activation of the embryonic genome (31). The precise time at which the cat embryo becomes activated is unknown, but it seems unlikely that this is contributing to arrest because the block occurs so late (morula stage) in development. We also have eliminated several other factors including culture temperature, gas atmosphere (4), type of protein supplement in the culture medium (3), and interval between eCG and hCG treatment in oocyte donors (5), none of which appears to influence blastocyst formation in vitro. Furthermore, although oviductal cell coculture circumvents the developmental block in the cow (13), pig (12,17), sheep (14), goat (15), and mouse (11) embryo, we determined no such advantage for the cat. Perhaps of the most relevance and interest are recent data collected in our laboratory on in vitro development of embryos produced in vivo. Our results indicate that 78.32% of embryos fertilized in vivo, recovered, and then cultured in Ham's F10 develop into blastocysts (32). This suggests that the partial morula-to-blastocyst developmental block originates as a result of some event associated with fertilization or very early development in vitro. Obviously, further studies are required to identify the absent promoter/enhancer (or perhaps the detrimental inhibitor) within the culture environment that prevents cat blastocyst formation.

Another factor known to influence oocyte maturation and sperm–oocyte interaction in the cat is the ovarian/hormonal status of the donor at the time of oocyte recovery. Johnston *et al.* (20) determined that the proportion of immature oocytes subsequently able to achieve metaphase II in vitro was lower when the donor was in a luteal phase (29%) compared to a follicular phase (56%) of the cycle. The present findings both support and extend these earlier observations because donors already experiencing premature ovulation (an early luteal phase) produced oocytes severely compromised in fertilization and developmental ability compared to their preovulatory counterparts. Hormonal status was not measured in these females, but it is well established that circulating progesterone concentrations rise coincident with the onset of ovulation after natural mating (33) or eCG/hCG treatment (5). Therefore, we expect that the cat may express a strong hormone–gamete interdependency. Acute changes in systematic and/or local hormone concentrations associated with ovulation appear to affect the quality and fertilizability of the remaining follicular oocytes. Perhaps this serves as a mechanism for ensuring that only viable oocytes are ovulated and fertilized during a short interval, thereby promoting compatibility with the rapidly changing milieu of the reproductive tract. Nonetheless, this finding also provides important practical information, because it now is apparent that follicular oocytes from donors already initiating ovulation are vastly different in biological competency from those collected from nonovulating females. This finding must be considered in designing future studies that examine the

factors dictating successful in vitro sperm–oocyte interaction and preimplantation embryo development.

Finally, the significant differences in fertilizability and development between oocyte donors with a different ovarian status (pre- versus post-ovulation) also may have relevance to the morula-to-blastocyst block in this species. We recently demonstrated that a high proportion of cat oocytes (>60%) aspirated from ovarian follicles 24–26 hr post-hCG (immediately preovulation) has not yet achieved metaphase II (34). This finding was unexpected because cat oocytes supposedly are ovulated after meiosis is complete. Perhaps this surprising observation also is related to a local (ovarian) endocrine effect, suggesting that more attention be placed on considering hormonal factors in the cat oocyte culture system. Certainly this reasoning agrees with our recent speculation, based on embryogenesis in vivo, that the cat morula-to-blastocyst block originates coincidentally with fertilization and/or very early developmental events. This hypothesis is being tested by examining the significance of hormone supplementation of culture media and hormonal priming of oviductal cells prior to gamete coculture.

ACKNOWLEDGMENTS

This research was funded, in part, by grants from the Phillip Reed Foundation to NOAHS Center, the National Institute of Child Health and Human Development (HD 23853), the Ralston Purina Company/American Association of Zoological Parks and Aquariums Conservation Endowment Fund, and the Scholarly Studies Program of the Smithsonian Institution.

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Induction of Ovulation and Systemic Sclerosis: A Case for Surrogacy

Submitted: October 26, 1993
Accepted: February 1, 1994

INTRODUCTION

With the advent of assisted reproduction, surrogacy has become an established practice, and a real alternative to those patients who, for medical or other reasons, are unable to achieve a pregnancy (e.g., cases of hysterectomy for benign or malignant disease, congenital Müllerian duct defects, recurrent spontaneous abortions, and systemic illness). Therefore, it is not surprising that more couples are now coming forward requesting assisted conception with controlled ovarian hyperstimulation (COH), oocyte retrieval, in vitro fertilization (IVF), and cryopreservation of the embryos with a view to surrogacy. The question has to be asked, "Should treatment be offered to patients with systemic disease when the effects of the drugs administered are unknown and the long-term outcome of the medical condition is doubtful?" Apart from the medical implications and risks involved in treating women with systemic sclerosis (SSc), there are other aspects which require serious consideration. These include legal and social issues. The case history and treatment are outlined below.

CASE PRESENTATION

A 26-year-old woman, para 0 + 2, presented to the London Gynaecology and Fertility Centre in February 1993 requesting IVF and freezing of the embryos. She had normal menstrual cycles and a cervical smear in 1992 was normal. Both her previous pregnancies ended in spontaneous first-trimester miscarriages. She had two laparoscopies, one in 1987 due to a suspected ectopic pregnancy and one in 1992 for dyspareunia, when she was found to have mild endometriosis without any other