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Developmental Competence of Domestic Cat Embryos Fertilized In Vivo Versus In Vitro[1]

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ABSTRACT

Development of in vitro-fertilized (IVF) cat embryos was compared to that of naturally produced cat embryos in vivo and in vitro. To obtain in vivo-fertilized embryos, queens were mated three times daily on the second and third days of natural estrus and ovariohysterectomized at 64, 76, 100, 124, or 148 h after the first copulation. Embryos were flushed from the reproductive tract, evaluated for developmental stage, and cultured. For IVF, oocytes from gonadotropin-stimulated queens were inseminated with electroejaculated cat sperm in Ham's F-10 and evaluated for fertilization (cleavage to ≥ 2 cells) at 30 h. In vitro development of embryos fertilized in vivo (n = 109) and in vitro (n = 46) was evaluated every 24 h for up to 10 days. High-quality embryos recovered at 64, 76, 100, 124, and 148 h after the first copulation were typically 1 to 2 cells (13 of 20), 5 to 8 cells (18 of 28), 9 to 16 cells (14 of 24), morulae (15 of 21), and compact morulae (11 of 18), respectively, suggesting blastomere cleavage once per day in vivo after the first three rapid cell divisions. A similar developmental rate to the morula stage ($p \geq 0.05$) was achieved in vitro by embryos derived from both in vitro and in vivo fertilization. Additionally, the proportion ($p \geq 0.05$) of in vivo-generated embryos (2 to 16 cells) that developed to morulae (64 of 83; 77.1%) was similar to that of IVF embryos (28 of 46; 60.9%). However, none of the IVF embryos (0/46), but 70.6% (77 of 109) of the in vivo-produced embryos, achieved blastocyst formation in culture ($p \leq 0.05$). Furthermore, 66.2% (51 of 77) of these blastocysts exhibited zona hatching. Incidence of morula and blastocyst formation in the in vivo group was influenced by stage of the embryo at collection. Embryos that were at the 9- to 16-cell stage at recovery were more likely ($p \leq 0.05$) to achieve morula or blastocyst status and emerge from the zona pellucida than younger-stage counterparts. In summary, the in vivo and in vitro growth rate of cat embryos produced after natural mating was comparable to that of embryos fertilized and cultured in vitro. However, developmental ability to the blastocyst stage was superior for embryos produced in vivo after natural mating. In vitro conditions during fertilization or early embryogenesis appeared to compromise developmental competence from the morula to the blastocyst stage of IVF or early-stage in vivo-produced cat embryos.

INTRODUCTION

Domestic cat embryos are readily produced by in vitro fertilization (IVF) using several different culture systems [1–4]. Domestic cat IVF technology also has been successful when applied to nondomestic felid species including the leopard cat (*Felis bengalensis*) [5], Indian desert cat (*F. silvestris ornata*) [6], tiger (*Panthera tigris*) [7], and cheetah (*Acinonyx jubatus*) [8]. Although the competency of IVF felid embryos has been demonstrated by the production of live offspring, pregnancy and embryo survival rates are low [2, 4, 7, 9]. Poor embryo survival may be a consequence of adverse maternal or embryonic factors. The maternal support provided by gonadotropin-stimulated embryo transfer recipients may be unsuitable because of asynchrony between the recipient and the IVF embryos [4], luteal dysfunction, or an abnormal endocrine milieu [7], all of which could contribute to an inappropriate oviductal/uterine environment and implantation failure. Embryonic factors also are a major concern, especially the possibility that a high proportion of IVF-generated embryos are developmentally incompetent.

Fertilization success in vitro of in vivo-matured cat oocytes generally is high (70–80%), and most of these embryos (~80%) achieve the morula stage of development by 96 h post-insemination [3, 10, 11]. However, most IVF cat embryos developmentally arrest in vitro at the morula stage with only a few morulae (10–30%) forming blastocysts [3, 4, 10, 11]. Although not unique to the cat, this in vitro embryo developmental block appears fundamentally different from that observed in other commonly studied species. For example, cow, sheep, pig, and mouse embryos arrest in vitro at much earlier stages of development [12–15]. Growth blocks in these species can be overcome by culturing embryos on oviductal cell monolayers [13, 15–17], a technique that fails to enhance blastocyst formation in the cat [18]. In the rabbit, embryos also fail to develop in culture beyond the morula stage [19], but the block is circumvented simply by adding amino acids to the culture medium [20]. In contrast, although routinely cultured in a complex medium (Ham's F-10), at least 65% of cat embryos fail to become blastocysts. Factors that have been eliminated as potential causes for the growth arrest include culture temperature, gas atmosphere [10], and type of protein supplement in the culture medium [3]. Therefore, it seems logical to question the developmental competency of IVF-generated cat embryos.

Although cat IVF embryos arrest late in development, the block actually may be a consequence of inappropriate culture conditions yet to be determined during final oocyte maturation, fertilization, or initial cleavage. To identify the

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cause of this embryo developmental block, the first step is to distinguish between intrinsic embryo quality and culture system inadequacy as potential responsible factors. Until now, no data have been available on the developmental ability in vitro of naturally produced cat embryos. Therefore, the objective of the present study was to determine the normality of IVF cat embryos by comparing 1) the developmental rate in vivo and in vitro of naturally produced cat embryos (i.e., after natural estrus and mating) to that of IVF-generated embryos cultured in vitro and 2) the ability of in vitro- and in vivo-fertilized embryos to achieve morula and blastocyst formation in our standard culture system.

MATERIALS AND METHODS

Oocyte Collection and IVF

Oocytes were recovered from four adult female cats stimulated with eCG (150 IU, i.m.; Sigma Chemical Co., St. Louis, MO) and 84 h later with hCG (100 IU, i.m.; Sigma) [21]. Twenty-four to 27 h after the hCG, each cat was induced into a surgical plane of anesthesia with a ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, NJ) plus acepromazine maleate (Ayerst Laboratories, Rouses Pt., NY) mixture (10:1 ratio, 18.0–20.0 mg/kg BW and 0.2 mg/kg BW, respectively, i.m.); anesthesia was maintained by delivering isoflurane gas/oxygen via a face mask. Laparoscopy was performed, and preovulatory ovarian follicles (≥ 2 mm in diameter) were aspirated using a 22-gauge needle attached via polyethylene tubing to a collection tube under gentle suction [2]. Follicular contents were collected into pre-warmed (37°C) tubes containing Ham's F-10 medium (HF10; supplemented with 0.011 mg/ml pyruvate, 0.284 mg/ml glutamine, and 5% fetal calf serum; Irvine Scientific, Princeton, NJ) with 40 IU/ml heparin. Oocytes were recovered from collection tubes and transferred to a petri dish containing 3 ml HF10, where they were rinsed further by pipetting through three consecutive 100-μl drops of HF10 overlaid with oil. Good-to-excellent quality, mature-appearing oocytes (dark, uniformly pigmented oocytes with an expanded cumulus and distinct corona radiata) [22] from each donor were placed (5–10 oocytes per drop) in IVF drops (90 μl HF10 overlaid with oil in a petri dish) in a humidified incubator (37°C; 5% CO_2 and air).

Two normospermic (> 60% morphologically normal sperm per ejaculate) domestic cats were induced into a surgical plane of anesthesia with Telazol (A.H. Robins Company, Richmond, VA; ~4 mg/kg BW, i.m.) for semen collection by electroejaculation [23, 24]. Semen was diluted with an equal volume of HF10 and centrifuged (150 × *g*; 8 min). After removal of the supernatant, the sperm pellet was overlaid with 200 μl of HF10 and sperm were allowed to swim up for 1 h at 22°C [2]. Spermatozoa in the swim-up fraction were assessed subjectively for percentage motility and rate of progressive motility (0 = no forward movement to 5 = rapid, linear, forward movement) [24]. Sperm concentration was determined with a hemacytometer, and the sample then was diluted with HF10 to 2×10^6 motile sperm/ml. Ten microliters of this sample was used to inseminate each 90-μl IVF drop (final IVF concentration, 2×10^5 motile sperm/ml). All oocytes were inseminated 1 to 4 h after recovery.

Eight hours after insemination, oocytes were removed from IVF drops; cumulus cells were dislodged by gently pipetting oocytes through a small-bore pipette; and cumulus-free oocytes were transferred into fresh 100-μl drops of HF10 under oil for continued culture. All oocytes were assessed for fertilization (on the basis of evidence of cleavage to at least the 2-cell stage) at 30 h post-insemination.

Collection of In Vivo-Fertilized Embryos

This study was conducted over a ~1-yr period. Queens were checked daily for behavioral signs of estrus (treading of the hind feet, tail deflection, and lordosis posturing). Naturally estrual females were mated three times per day at 3-h intervals on the second and third days of estrus [25]. The same two normospermic domestic cats that served as semen donors for IVF also were used on a rotating basis to naturally breed the in vivo embryo donors. Mated queens were assigned randomly to one of five time interval groups: 64, 76, 100, 124, or 148 h after the first copulation. These times were chosen to simulate the schedule for evaluating IVF embryo development (once every 24 h) and in an effort to obtain in vivo-produced embryos at all cleavage stages given the following known information: 1) in a naturally estrual cat, mating three times daily causes an LH surge that elicits ovulation 48–64 h after the first copulation [25, 26], and 2) because sperm capacitation occurs readily in the cat [27], fertilization and first cleavage division in vivo can be expected to occur by 64 h after the first mating.

To confirm ovulation, all aspects of both ovaries were visualized laparoscopically for the presence of fresh CL. All queens with fresh CL were ovariohysterectomized immediately via laparotomy under a surgical plane of anesthesia as described for oocyte recovery (64 h, n = 5; 76 h, n = 11; 100 h, n = 8; 124 h, n = 7; 148 h, n = 5). Initially, five females were assigned to each group, but if no "good"-quality embryos were recovered from one or more of these cats, additional females were assigned to the group until good-quality embryos were recovered from a total of five queens per interval. Within 30 min of excision, oviducts and uterine horns were flushed repeatedly with 1–5 ml of warm (37°C) HF10 and the recovered fluid was examined for embryos. Embryos were washed through three drops of HF10 (100 μl/drop) and evaluated for developmental stage and quality grade (grade 1 = dark, homogenous coloration and uniformly shaped blastomeres; grade 2 = lighter in color, some abnormally shaped blastomeres, slight vacuolation; grade 3 = degenerate, pale, fragmenting blasto-

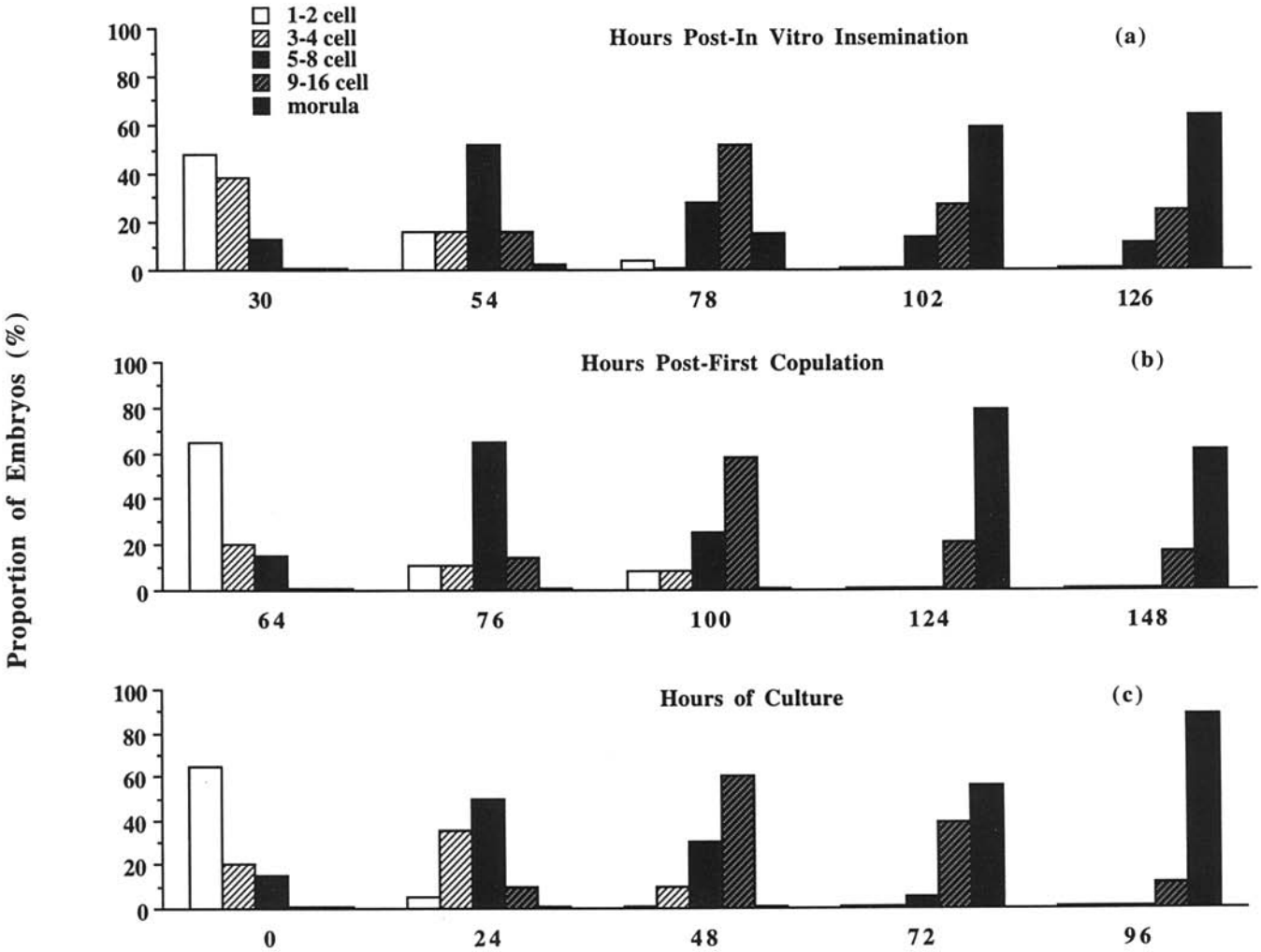


FIG. 1. Developmental stages of preimplantation cat embryos. a) After in vitro insemination of in vivo-matured oocytes (n = 46 cleaved embryos); (b) collected from the reproductive tracts at specified intervals after first copulation (n = 109 total embryos) of naturally estrual and mated queens; and (c) cultured in vitro after collection from naturally estrual and mated queens at 64 h after first copulation (n = 20 embryos).

meres). They were then cultured in 100-μl HF10 drops under conditions identical to those described for IVF embryos.

Evaluation of Embryo Development

In vitro development of embryos fertilized in vitro (n = 46) and in vivo (n = 109) was evaluated every 24 h for up to 10 days or until embryos degenerated. Embryos were assessed at each time for cleavage stage, assigned a quality grade, and examined for compaction, blastocyst formation, and zona hatching.

Statistical Analysis

Developmental rates of in vitro- and in vivo-fertilized embryos were compared by regression analysis according to the Statistical Analysis System [28]. The proportions of in vitro- and in vivo-fertilized embryos achieving morula and blastocyst formation and hatching from the zona were compared by chi-square (X^2) analysis.

RESULTS

Oocyte and Embryo Recovery and IVF Success

Ninety-three oocytes were aspirated from the four gonadotropin-stimulated donors (23.3 ± 2.3 oocytes per queen). Of these, 88 (94.6%) were grade 1 quality and were inseminated. Oocytes from two queens were inseminated with sperm from one male, and oocytes from the other two queens were inseminated with sperm from the second male. Both ejaculates contained a high proportion of motile and morphologically normal (≥ 70%) sperm. Oocytes from all four females were fertilized (range, 41–87%), and a total of 46 oocytes (52.3%) cleaved to 2 cells. From queens ovariohysterectomized at 64, 76, 100, 124, and 148 h post-copulation, 20, 28, 24, 19, and 18 good-quality (grade 1 or 2) embryos were recovered, respectively. Characteristics of embryos, distribution within the reproductive tract, and the incidence of embryo mortality in vivo in this population of

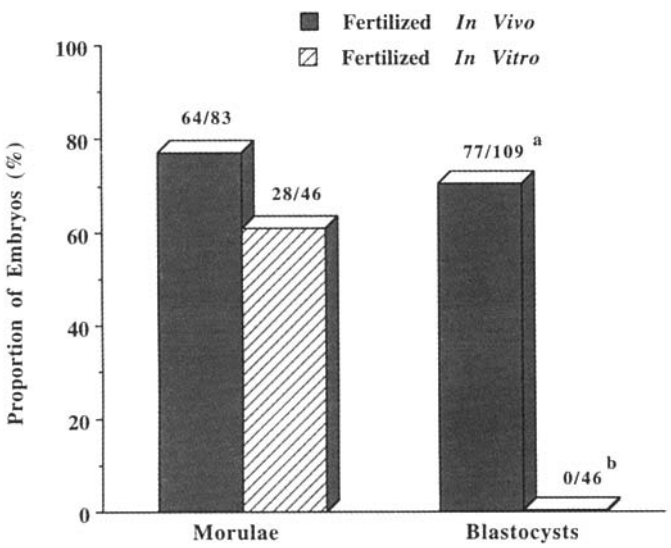


FIG. 2. Proportion of embryos developing in culture to morulae or blastocysts after fertilization in vivo and in vitro. Different superscripts indicate differences ($p \leq 0.05$) within developmental stage.

naturally mated cats are the subjects of a companion paper [29].

Embryo Developmental Rate

By 30 h post-insemination, most IVF embryos (27 of 46; 58.7%) had cleaved to the 2-, 3- or 4-cell stage of development (Fig. 1a). These embryos continued to cleave at the rate of approximately one cell division every 24 h, with most (26 of 46; 56.5%) achieving morula formation (> 16 cells) by 102 h. In vivo-fertilized embryos collected at 64 and 76 h after the first copulation were primarily 1 to 2 cells (13 of 20; 65.0%) and 5 to 8 cells (18 of 28; 64.3%), respectively, indicating a rapid rate of cell division early in development (Fig. 1b). After reaching the 5- to 8-cell stage, embryos continued developing in vivo at the rate of one cleavage division every 24 h. Embryos produced in vivo and collected at 64 h after first copulation (primarily 1 to 2 cells) continued to develop in vitro, achieving the 3- to 4-cell (7 of 20; 35.0%) or 5- to 8-cell (10 of 20; 50.0%) stage of development at 24 h and then cleaving approximately once every 24 h for the next 72 h (Fig. 1c).

Morula and Blastocyst Formation

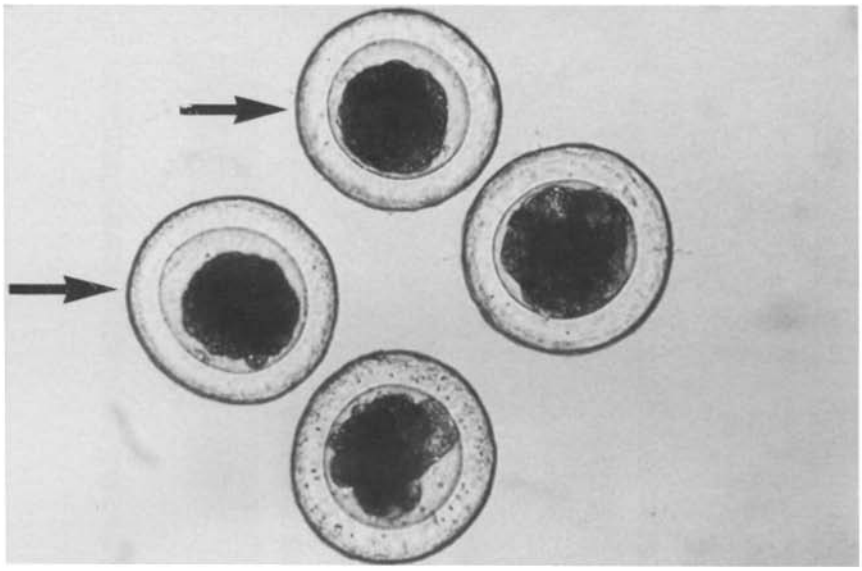
A high proportion (> 60%) of both in vitro- and in vivo-generated embryos (≤ 16 cells at collection) achieved morula formation in culture (Fig. 2). However, only embryos fertilized in vivo underwent compaction after reaching the morula stage (Fig. 3a), whereas IVF embryos maintained distinct blastomeres after developing into morulae (Fig. 3b). None of the IVF-generated embryos, but ~70% of the in vivo-fertilized embryos, developed to blastocysts in culture (Fig. 2). Developmental stage of the embryo at the time of collection from the reproductive tract affected subsequent morula and blastocyst formation in culture (Fig. 4). Although ~54% of the 1- to 2-cell embryos developed to morulae, an even greater ($p \leq 0.05$) proportion of 5- to 8-cell and 9- to 16-cell embryos achieved morula status (85.0% vs. 92.0%, respectively). Likewise, more ($p \leq 0.05$) 9- to 16-cell than 1- to 8-cell embryos achieved blastocyst formation (92.0% vs. 33.3–66.7%, respectively).

Quality grade of the embryo at collection also was related to ability to develop into a morula or blastocyst. The proportion of grade 1 embryos developing to morulae (76 of 84; 90.5%) was greater ($p \leq 0.05$) than for grade 2 embryos (18 of 29; 62.1%), which in turn was greater ($p \leq 0.05$) than for grade 3 embryos (2 of 12; 16.7%). Additionally, grade 1 embryos were more likely ($p \leq 0.05$) to develop to blastocysts than grade 2 or 3 embryos (63 of 84, 75%; 13 of 29, 44.8%; and 2 of 12, 16.7%, respectively). Finally, a greater proportion ($p \leq 0.05$) of grade 3 embryos degenerated (10 of 12; 83.3%) compared to their grade 2 (11 of 29; 37.9%) or grade 1 (8 of 84; 9.5%) counterparts.

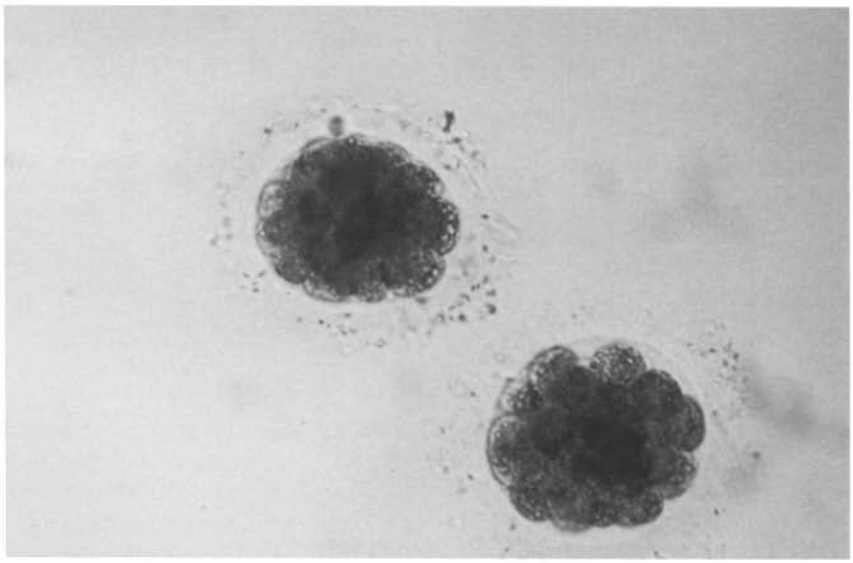
Blastocyst Hatching

In vivo-fertilized embryos achieving blastocyst formation in culture frequently initiated zona hatching. Stage of development at collection had a significant ($p \leq 0.05$) impact on zona hatching efficiency (Fig. 5). None of the 1- to 2-cell embryos, but almost 90% of the 9- to 16-cell embryos, exhibited some degree of zona hatching. Furthermore, the morphological characteristics of the hatching process differed depending on the length of time embryos were maintained in culture before blastocyst formation. Embryos collected at the morula stage typically demonstrated uniform expansion and thinning of the characteristically thick zona pellucida with eventual dissolution of the zona over an entire pole allowing the expanded blastocyst to emerge (Fig. 6a). In contrast, 5- to 8-cell stage embryos developing to blastocysts in culture demonstrated "zona escaping," in which the zona pellucida remains primarily intact while rupturing at a small, single site to allow extrusion of trophoblast cells (Fig. 6b). Although more than half (56 of 109; 51.4%) of the in vivo-fertilized embryos exhibited zona hatching, only 8.9% (5 of 56) completed the process (as defined by the complete escape of the embryo from the zona pellucida).

The chronological sequence of cat embryo development in vivo vs. in vitro is summarized in Figure 7; the most common embryo developmental stage is plotted for each group at each evaluation time. Regression analysis indicated no differences ($p \geq 0.05$) in developmental rates to the morula stage for IVF- vs. in vivo-generated embryos developing in culture. However, embryos fertilized and developing in vivo cleaved more rapidly ($p \leq 0.05$) to the 5- to 8-cell stage than IVF-generated embryos did. Additionally, IVF embryos failed to advance beyond the morula



(a)



(b)

FIG. 3. a) In vivo-fertilized cat embryos undergoing compaction at the morula stage of development. Two of these morulae (arrows) had completed the process at the time of collection, 148 h after the first copulation (×200). b) IVF cat embryos demonstrating the typical morphology of non-compacting cat morulae with easily identified individual blastomeres at 126 h post-insemination (×240).

stage, but in vivo-produced embryos formed blastocysts after 48 h at the morula stage. Finally, onset of zona hatching of in vivo-fertilized embryos occurred ~48 h after initial blastocyst formation.

DISCUSSION

The developmental rate and competency of IVF-generated embryos in vitro were compared with those of naturally produced embryos in the domestic cat. These are the first data describing the chronological staging of embryonic development after mating and the ability of in vivo-fertilized cat embryos to grow in vitro—information essential to assessing the normality of IVF embryos in this species. Surprisingly, although IVF in the cat dates back more than 20 years [30], until now a comprehensive, comparative database for natural cat embryo development has been unavailable [29]. This basic information is critical for enhancing our understanding of reproductive processes in the

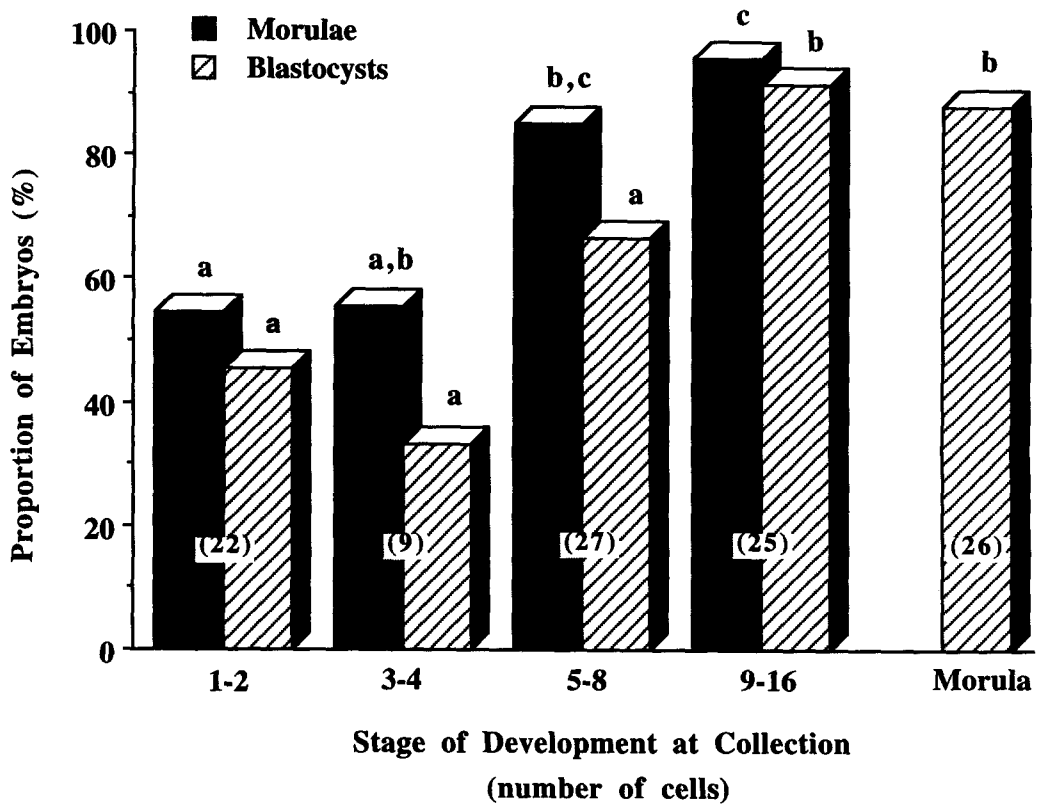


FIG. 4. Proportion of in vivo-fertilized embryos achieving morula and blastocyst formation in culture after collection at different developmental stages. Different superscripts indicate differences ($p \leq 0.05$) in morula or blastocyst formation among developmental stages. Number of embryos collected at each developmental stage is in parentheses.

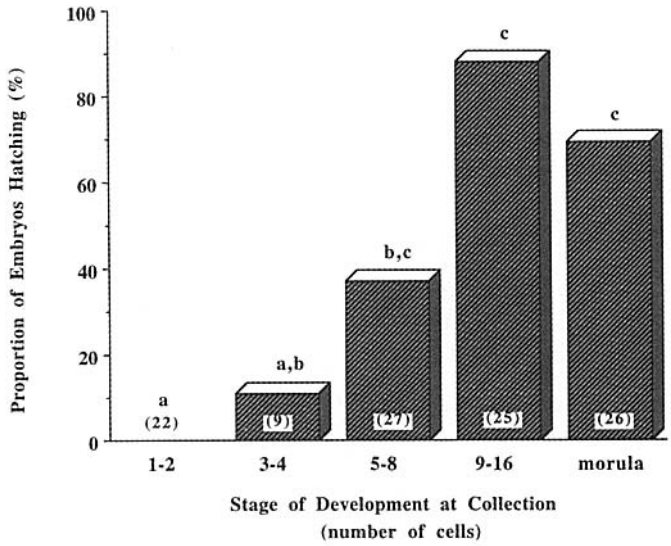
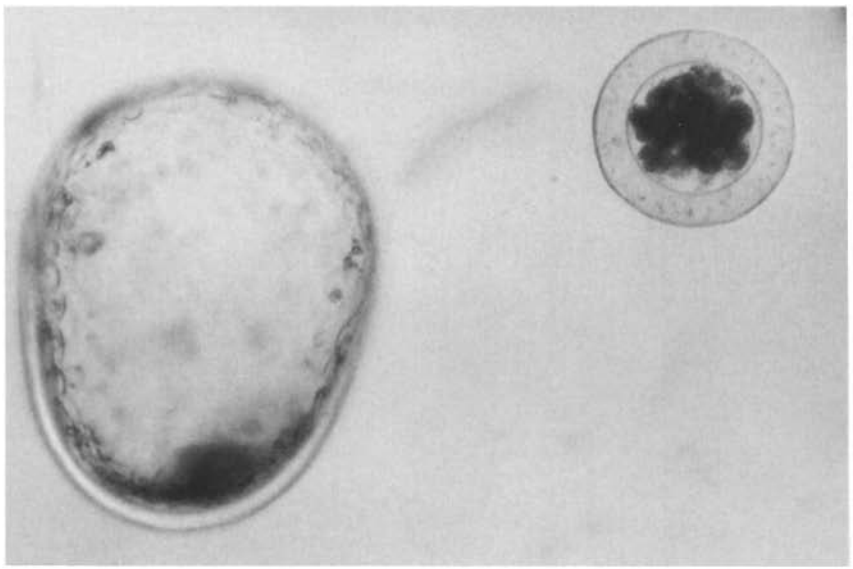


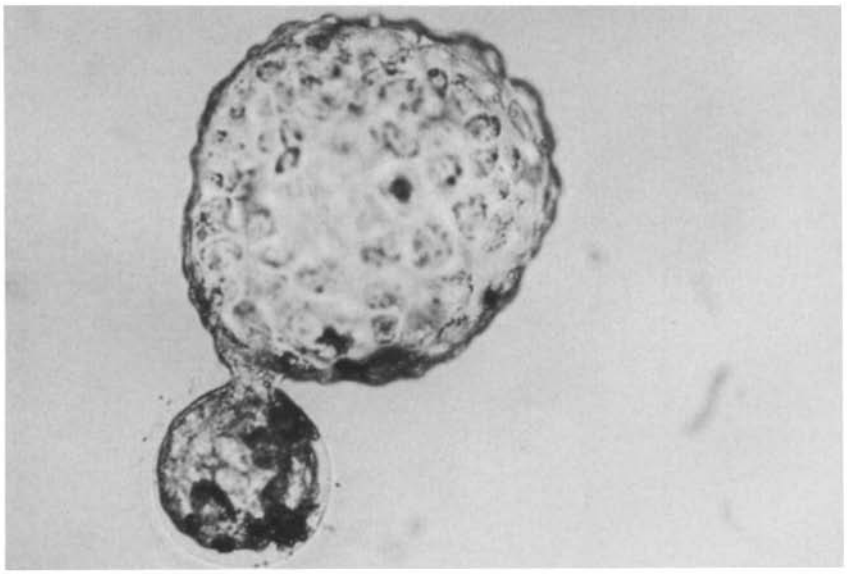
FIG. 5. Proportion of in vivo-produced embryos demonstrating zona hatching in culture after collection at different developmental stages. Different superscripts indicate differences ($p \leq 0.05$) among developmental stages. Number of embryos collected at each developmental stage is in parentheses.

domestic cat. Likewise, the information will be useful as "model" data in parallel efforts to assist reproduction in nondomestic felid species, most of which are endangered.

Growth rate is one important component in the assessment of embryo normality. We previously reported that IVF cat embryos cleave approximately once every 24 h while maintained in culture [10, 11]. In the present study, IVF embryos completed the first three cell cycles relatively quickly. First cleavage division usually occurs within 24–30 h of sperm exposure with embryos reaching the 5- to 8-cell stage by 54 h after insemination [10, 11]. In vivo-fertilized embryos also exhibited rapid early development, achieving the 5- to 8-cell stage within 76 h of first copulation (≤ 48 h postovulation). Although these data appear to suggest that early cleavage of IVF-generated embryos may be slightly retarded compared to that of embryos developing in vivo, the difference more likely is a consequence of variation in the timing of ovulation. Cats are induced ovulators, and when mated in a similar fashion, queens ovulate over a wide time range but usually within 28–52 h of the first copulation [25, 26, 31–33]. Therefore, onset of ovulation and time of



(a)



(b)

FIG. 6. a) Expanded cat blastocyst emerging from the zona pellucida after 48 h in culture. The embryo was fertilized in vivo and collected as an early morula. The thinning of the zona pellucida relative to the characteristically thick zona of the adjacent, degenerating embryo is apparent (×200). b) Cat blastocyst exhibiting the "zona escape" process typical of embryos placed in culture at early stages (< 9 cells) of development. This embryo was fertilized in vivo and collected at the 4-cell stage (×200).

first sperm exposure for the naturally released oocytes were impossible to predict precisely. However, even when the most conservative estimate (28 h) is used as the time of first sperm exposure within the oviduct, it is apparent that these oocytes cleaved three times within the next 48 h. This early rapid development rate is similar to that reported for cow, mare, sheep, pig, and rabbit embryos [34].

Even 1- to 2-cell embryos placed in culture after being produced originally in vivo demonstrated rapid early cleavage, albeit slightly (nonsignificantly) retarded as compared to that in the in vivo embryos recovered at later intervals. This difference was probably due to abrupt change in the nurturing environment. After reaching the 5- to 8-cell stage, growth of embryos in all groups slowed to approximately one cell division every 24 h. Similar developmental patterns have been described for preimplantation embryos of several species, including the cow [35] and human [36]. In our study, embryo growth profiles to the morula stage for

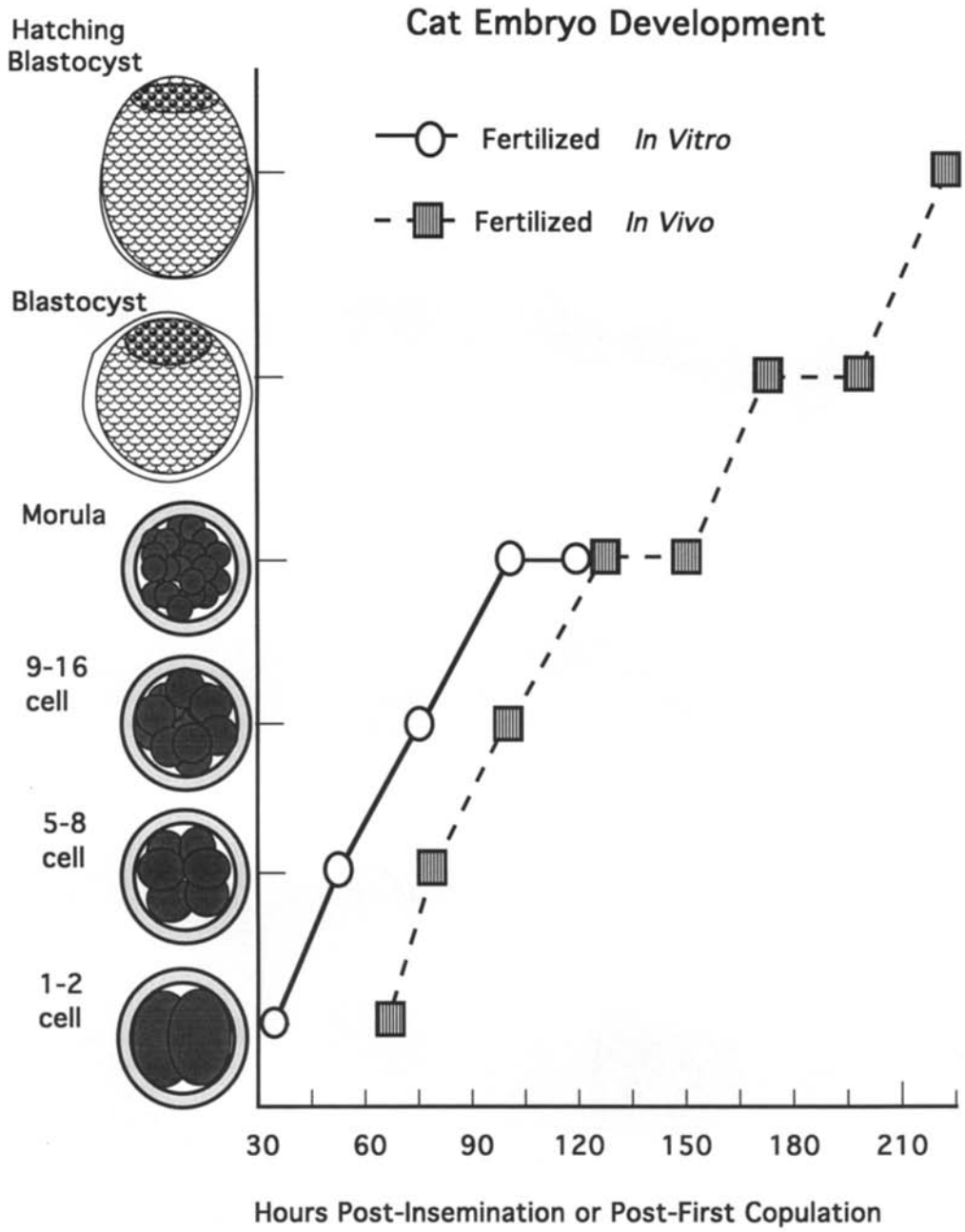


FIG. 7. A summary of the kinetics of cat embryo development after fertilization in vitro vs. in vivo. Developmental rates to the morula stage were similar ($p > 0.05$), but IVF embryos failed to advance beyond the morula stage, and only in vivo-fertilized embryos formed blastocysts and hatched from the zona pellucida.

the IVF and in vivo-fertilized embryos were similar, but this is not always the case for other species [37, 38]. Retarded development for cow IVF embryos, possibly explained by changing conditions of gas and temperature [39], has been reported.

The ability to develop fully to the blastocyst stage also must be considered in the assessment of embryo normality. Despite a high rate of fertilization and successful growth to morulae, IVF cat embryos express a partial but highly significant in vitro developmental block at the morula-to-blastocyst stage [3, 4, 10, 11]. The cat embryo developmental block has not been resolved by making conventional changes in the culture system. Incubation temperature, gas atmosphere, culture medium, protein supplements in the medium, and oviductal cell monolayer co-culture have all failed to circumvent the block [3, 4, 10, 18]. This resistance to conditions that have proven useful for most other species [13, 15–17, 20] suggests that the cause of in vitro developmental arrest in the cat is somewhat unique. The present study provided an excellent opportunity to begin determining

whether the block is related to culture conditions or to an inherent deficiency in the embryo as a result of its IVF origin. Because a high proportion of grade 1 and grade 2 in vivo-fertilized embryos readily developed to blastocysts in vitro, it is likely that the morula-to-blastocyst problem is originating with the oocyte (or with the effect of the culture system on the oocyte), at fertilization or during very early embryo development. Alternatively, inherent deficiencies may be associated with mature oocytes resulting from exogenous hormone treatment. Furthermore, the present results provided even more evidence for the novelty of the cat embryo block because in vivo-produced embryos from other species that are placed in culture typically arrest at the same stage as their IVF counterparts [15–17, 20].

A more detailed analysis of the data indicated that indeed our culture environment was influencing embryonic growth rate and potential. Specifically, successful blastocyst formation was related inversely to the number of days embryos were maintained in vitro. If embryos were placed in culture at the 9- to 16-cell stage, > 90% formed blastocysts; in contrast, if embryos were placed in culture before reaching the 5- to 8-cell stage, only ~40% formed blastocysts. Oocytes that were placed in culture before fertilization and then became IVF embryos failed completely to achieve blastocyst formation. Therefore, this culture system either lacks an important component or contains a detrimental or suppressive factor(s). The result is a failure to support maximal development (beyond the morula stage) of IVF embryos or in vivo-fertilized embryos recovered before the fourth cleavage division (2 to 8 cells).

In vivo-fertilized cat embryos also exhibited distinct morphological changes at the morula stage that were not observed in IVF embryos. Specifically, the former underwent compaction at the morula stage whereas IVF morulae maintained distinct, spherical blastomeres (Fig. 3). Compaction is a physiological process involving the flattening of blastomeres to obliterate intercellular space by formation of tight cell-to-cell junctions; it can occur at the 8-cell [40] or morula [35] stage, depending on the species. Interestingly, because compaction is considered a prerequisite for blastocoele formation [40, 41], its failure may in part be responsible for failed blastocyst formation and may be the first noticeable developmental deficiency in IVF cat embryos. Therefore, our future studies concerning the morula-to-blastocyst block in the cat will include assessing the role of blastomeric compaction in subsequent development.

There also were developmental differences among in vivo-fertilized embryos depending on the cleavage stage at initial placement in culture. Development to blastocysts was superior for embryos collected at later (9 to 16 cell) in contrast to earlier (1 to 2 cell) stages. The hatching of these blastocysts from the zona pellucida also was affected by embryo stage at recovery and the time at which the embryo was first subjected to culture. Not only did a higher proportion of later-stage embryos hatch, but the hatching process itself also differed among groups (Fig. 6). For embryos collected at the morula stage, there was gradual and severe thinning of the zona pellucida during blastocyst expansion. The thinning process was more pronounced over one pole with local zona dissolution occurring to allow the expanded blastocyst to emerge from this site. In contrast, there was little zona pellucida thinning for the embryos originally collected at the 1- to 2- through 5- to 8-cell stages, and these zonae largely remained intact. In these embryos, the trophoblast of the expanded blastocyst eventually extruded from a small hole formed in the zona pellucida. These embryos rarely were capable of complete hatching in our in vitro conditions, but they rather frequently became entrapped with embryonic cells located both internal and external to the zona pellucida. Interestingly, these same differences in zona hatching of in vivo-produced embryos placed in culture have recently been revealed in a preliminary study of the hamster [42].

Our data indicated that in vitro culture conditions inhibited the zona hatching process in the cat embryo. For some species, blastocyst release from the zona pellucida is inhibited by substandard culture conditions induced by a lack of protein supplementation to the medium [43]. However, as in the cat, embryo hatching in the human [44] and hamster is impaired [42] even in the presence of protein-rich culture medium. Zona pellucida breakdown and blastocyst emergence have been attributed both to lytic peptides produced by embryos and the uterus [45] and to repeated expansion/contraction of the blastocyst itself [46]. We saw no evidence that cat blastocysts—unlike the rather dynamic mouse blastocyst [46]—frequently contracted or re-expanded in vitro. However, these embryos generally were examined only once daily and may have collapsed and expanded more often than noted with our observation schedule. In any case, we suspect that the culture conditions, and not embryo inertia, were responsible for the differences and impairment in hatching ability for cat embryos cultured early during development.

In summary, cat embryos produced in vivo (natural estrus followed by mating) developed at a similar rate to the morula stage in vitro and in vivo, and this rate was comparable to that of IVF embryos cultured in vitro. This strongly suggests that current culture conditions support early cat embryo development and that growth rates to the morula stage are "normal" for IVF embryos. The first three cleavage divisions of cat embryos occur more rapidly than subsequent cell divisions; after the first three divisions, individual blastomeres divide once daily. In the present study, IVF embryos ceased developing at the morula stage, but in vivo-fertilized embryos underwent morula compaction followed by blastocyst formation and hatching from the zona pellucida. Compaction and blastocyst formation occur both in vivo and in vitro; however, the probability that in vivo-fertilized embryos will achieve blastocyst formation and

hatching in culture increases markedly if embryos are allowed to develop to the 9- to 16-cell stage in vivo. Although the in vitro culture system described here supports IVF embryo development to the morula stage, it inhibits further development in vitro. Cat embryos subjected to in vitro conditions during initial cleavage (or before/during oocyte fertilization) are compromised in their ability to undergo compaction, form blastocysts, and hatch from the zona pellucida. These in vitro-induced developmental deficiencies may, in part, explain low embryo survivability and pregnancy rates after IVF and embryo transfer in the domestic cat and related felid species.

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