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Quality and Age of Companion Felid Embryos Modulate Enhanced Development by Group Culture[1]

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ABSTRACT

For some species, embryos cultured with conspecific companions may have enhanced in vitro development compared with singletons. The objective of this study was to determine the effect of quality and age of companion embryos on single felid embryos produced by in vitro maturation or in vitro fertilization. Test oocytes (intermediate quality) were inseminated and incubated alone or with 10 embryos derived from oocytes with a high, intermediate, or low glucose uptake. The effect of relative age of companion embryos on test embryo development was also examined by insemination and incubation of test oocytes alone or with 10 conspecific embryos that were older, younger, or the same age. Test embryos coincubated with better- or equal-quality companions had better development and more cells per embryo (mean ± SEM number, 74.9 ± 16.9 and 40.6 ± 8.8, respectively, Day 7; $P < 0.05$) than test embryos coincubated with lesser-quality companions (5.1 ± 1.4) or alone (8.4 ± 3.7). Intermediate-quality embryos incubated with older companions had more cells per embryo (88.3 ± 17.0; $P < 0.01$) than those incubated with synchronous (49.3 ± 12.1) or younger (29.4 ± 6.1) embryos. The cell number of solitary embryos (9.8 ± 3.1) was less ($P < 0.05$) than that of every group of test embryos incubated with companions, regardless of age. In vitro development of solitary cat embryos is improved by culture with excellent-quality conspecific companions, particularly companions of an advanced age.

early development, embryo, gamete biology, in vitro fertilization, reproductive technology

INTRODUCTION

Of 37 species in the Felidae family, virtually all are threatened by extinction due to human pressures [1]. For some populations or entire species, there is a quantifiable loss in genetic heterozygosity [2–5] causing a reduced vigor [6]. Preserving extant genetic diversity by all means (including in vitro maturation [IVM]) can ensure viable populations for the future. One of these means is ''gamete rescue,'' whereby sperm or oocytes are recovered postmortem or after excision of gonads for medical reasons [7, 8]. The domestic cat has been used extensively as a model for understanding reproductive mechanisms and even for propagating rare felid species. For example, knowledge of the domestic cat allowed the development of artificial insemination or in vitro fertilization (IVF) for the puma [9], clouded leopard [10, 11], snow leopard [12], ocelot [13], cheetah [11, 14], and tiger [15, 16], among others. A similar approach is being used to develop IVM for rescuing oocytes from such species.

Cat oocytes are easily harvested from freshly excised ovaries by mincing or follicular aspiration [17, 18]. Most oocytes complete nuclear maturation in vitro within 24–32 h of maturation [19]. Excellent-grade oocytes [20] mature and fertilize in vitro [17–25], develop to morulae or blastocysts [19, 20], and are biologically competent [24]. However, even IVM oocytes selected for the best morphology (uniformly dark cytoplasm and an intact cumulus cell investment [20]) develop poorly after IVF [17, 19, 25, 26] compared with counterparts that mature in vivo [27–29], possibly due to deficient cytoplasmic maturation [30].

It is possible that the post-IVF development of these suboptimal oocytes could be improved by culture with ''companion'' cells. However, culturing embryos with somatic cells has an equivocal impact on development [31–36]. Bavister [37] has discussed the diverse species- and tissue-specific results related to somatic cell culture and has questioned the validity of results showing embryotropic effects of somatic cells. Additionally, coincubating embryos with somatic cells prevents the use of media designed specifically for embryos because the in vitro environment must cater to two quite different cell types [32]. In contrast, when embryos are used as the companion cells, the medium can be embryo specific. Group embryo culture has been shown to enhance overall development in vitro [38–41], possibly because the companion embryos produce autocrine or paracrine factors or remove factors that are detrimental to embryo development, such as reactive oxygen. However, the group embryo culture effect is also inconsistent [42–44], and the benefits derived from coembryo culture may be masked by suboptimal culture conditions [45] or, we suggest, embryo quality.

Random culture of embryos together risks placing inherently good embryos adjacent to poor-quality companions. Marginal or degenerating embryos may fail to secrete beneficial factors or may even produce development-inhibiting factors. For example, reactive oxygen can be secreted by infiltrating leukocytes [46] and unhealthy gametes [47, 48] and is detrimental to gamete [49] and embryo [50] survival. Additionally, certain enzyme transcriptions that may protect against reactive oxygen appear to be an important component of oocyte cytoplasmic maturation [51]. Oocytes that do not complete this process are more susceptible to damage and, therefore, are less likely to develop [51]. Best-quality embryos derived from oocytes with this capacity may be able to ''condition'' medium, improving the environment for embryos derived from intermediate oocytes with excellent morphology but suboptimal development (i.e., marginal embryos). Incubation with degenerating oocytes is known to decrease embryo development significantly in the mouse, whereas group culture with healthier embryos improves development in the same embryo population [41]. So, it is possible that poor-quality companions

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have masked any beneficial effects imparted by healthy embryos and may explain the conflicting results regarding coembryo culture.

In the cat, metabolism is a useful index for distinguishing oocytes with varying capacities for maturation, fertilization, and development in vitro [52]. Furthermore, in other species it is known that embryos of differing ages have different energy substrate requirements [53], so it is plausible that a companion effect may be age- or developmental stage-specific. Given this, the general aim of our studies was to determine the impact of companion embryo quality and age on single embryo development. Our hypothesis was that single embryos derive greater benefit from companions of better quality and are not influenced by companion embryo age.

MATERIALS AND METHODS

Gonadal Collection and Storage and Oocyte Recovery and Maturation

Domestic cat ovaries were recovered after ovariohysterectomies performed at local veterinary clinics throughout each week from November through July [30]. During transport, ovarian pairs were stored in Dulbecco PBS (DPBS, pH 7.4, containing 100 IU/ml penicillin and 100 mg/ml streptomycin; Sigma Chemical Co., St. Louis, MO) at 4°C with ice packs and paper padding. Container temperature was monitored with a thermocouple (Omega Engineering Inc., Stanford, CT). Oocytes were recovered within 6 h of excision by mincing with a scalpel blade to liberate oocytes into 38°C modified Hepes-buffered minimum essential medium (H-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 1.0 mM pyruvate, 2.0 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 4 mg/ml bovine serum albumin (BSA; Miles Pentex, Bayer Diagnostics, Kankakee, IL). Only grade I immature oocytes with excellent morphology (defined by uniform, dark cytoplasm and an intact cumulus cell investment [20]) were selected for maturation and were rinsed three times in H-MEM. Oocytes were incubated (10 oocytes/50 μl) in maturation medium (MEM) containing 1.0 mM glutamine, 1.0 mM pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, 4 mg/ml BSA, 1 μg/ml FSH (1.64 IU/ml; NIDDK-oFSH-17, lot 3082; National Hormone and Pituitary Program, Rockville, MD), 1 μg/ml LH (1.06 IU/ml; NIDDK-oLH-25, lot 3502; National Hormone and Pituitary Program), and 1 μg/ml estradiol (Sigma Chemical Co.). Oocytes were cultured in this medium under mineral oil for 32 h at 38°C in a humidified environment of 5% CO_2 in air [19].

Oocyte Glucose Uptake Analysis

All oocytes were denuded of cumulus cells by gentle pipetting and thrice washing in Hepes-buffered modified mouse tubal fluid medium (mMTF [54]) containing 1 mM each of glucose, glutamine, and pyruvate. Each oocyte was then placed individually in a microdrop (40–80 nl) of this medium under oil on a siliconized microscope slide. The concentration of energy substrates was determined by microfluorescence [55]. Briefly, reagent solutions were used to link one energy substrate (e.g., glucose) to NAD(P)H reduction or oxidation. Reagent droplets were assessed for initial fluorescence with a fluorescent microscope (Leica, Germany) with photometer and photomultiplier attachments (Olympus, Melville, NY). Serial nanoliter samples were removed from the embryo incubation medium and added to these reagent droplets, and the fluorescence was reassessed. Nucleotides NADPH and NADH fluoresce when excited by 340 nm wavelength, whereas the oxidized forms NAD^+ and $NADP^+$ do not. Formation of NAD(P)H (caused by adding a specific substrate) increased droplet fluorescence, whereas oxidation of these nucleotides reduced fluorescence. Thus, a change in fluorescence was directly related to the amount of added substrate. A standard curve was used to determine the actual substrate concentration in the oocyte droplet. The concentration of each substrate was determined at different times. The rate of oocyte glucose uptake was defined by the rate of decrease or increase in substrate concentrations over time.

Oocytes were categorized on the basis of high (≥2.0 pmol oocyte^{-1} h^{-1}), intermediate (≥0.1 and <2.0 pmol oocyte^{-1} h^{-1}), or low (<0.1 pmol oocyte^{-1} h^{-1}) glucose uptake. Cat oocytes with greater glucose uptake have a higher incidence of blastocyst development in vitro after IVF [52]. Thus, oocytes with greatest glucose uptake are predicted to produce best-quality embryos, oocytes with intermediate glucose uptake are most likely to produce intermediate-quality embryos, and oocytes with the least glucose uptake are precursors of poorest-quality embryos. At the termination of culture, companion embryo quality was assessed on an individual and group basis, and predicted development was confirmed by examining developmental stage (using light microscopy) and cell number (using fluorescent nuclear stains). Embryos derived from best-quality oocytes were predicted to have ≥32 cells, and each best-quality companion group was predicted to have an average of ≥50 cells per embryo. Embryos derived from intermediate-quality oocytes were predicted to have 8–64 cells, with a group average of 10–50 cells per embryo. Embryos derived from poor-quality oocytes were predicted to have ≤16 cells, with a group average of ≤10 cells per embryo (unpublished data and [52]). Companion embryo groups that achieved developmental stages other than those predicted on the basis of original glucose uptake were excluded from further analysis.

Oocyte Insemination

After metabolic assessment, each denuded oocyte was placed in a 10 μl droplet of Ham F10 complete [56] that was composed of Ham F10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 4.0 mM pyruvate, 2.0 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). Sperm recovered by electroejaculation (protocol approved by the Institutional Animal Care and Use Committee) on the day of insemination and with ratings of ≥80% motility and at least 3.5 progressive status [56] were swim-up processed [17, 56] and reassessed for motility and status. Sperm concentration was determined with a hemocytometer, and each oocyte was inseminated at a final concentration of 2×10^5 motile sperm/ml (2000 sperm in a 10-μl drop) under mineral oil at 38°C in 5% CO_2 in air.

Embryo Culture

A ''test'' embryo was any embryo produced from an oocyte with excellent morphology but intermediate glucose uptake. Individual test embryos were incubated in 20 μl Ham F10 complete with 10 companions derived from oocytes with excellent morphology but of various qualities (based on glucose uptake) and ages. Solitary test embryos served as controls. An equal number of test embryos was incubated with companion embryos from each category on each day of experimentation to eliminate any variability due to the sperm used for insemination and the seasonality of oocytes. Each test embryo was separated physically, but not chemically, from its neighbors with a specially designed coincubation chamber (patent pending). Essentially, this chamber is composed of an embryo safe culture dish bisected by a fine nylon mesh barrier with 105-μm perforations that prevent embryo passage but allow free chemical exchange (Fig. 1). Embryos were transferred to fresh medium (each test embryo and companions remained as a unit through culture) on Days 3 and 5 (Day 0 = day of insemination). Each test and companion embryo was examined for stage and cell number by Hoechst 33342 staining on Day 7 [30].

Study I: Influence of Companion Embryo Quality

To determine the influence of companion embryo quality, single intermediate-quality test embryos (n = 12–14 per companion group) were incubated (as described previously) alone or with 10 companions that were derived from oocytes with a 1) high (≥2.0 pmol/h, best-quality companions), 2) intermediate (≥0.1 and <2.0 pmol/h, intermediate-quality companions; identical to test embryos), or 3) low (<0.1 pmol/h, poor-quality companions) rate of glucose uptake.

Study II: Influence of Relative Companion Embryo Age

To determine the impact of relative companion embryo age, single test (intermediate-quality) embryos (n = 15 per treatment group) were incubated (as described previously) alone or with 10 intermediate-quality companions that were 1) 2 days older, 2) the same age, or 3) the same age for 2 days and then with embryos 2 days younger for the remaining culture period. The original group of companion embryos was cultured under identical conditions to verify quality.

Statistical Analysis

All percentage data were arcsine transformed before they underwent further analysis. Differences in cell number and stage of development among embryos incubated under varying conditions were assessed by ANOVA, and multiple comparisons were assessed with the Dunnett test. All

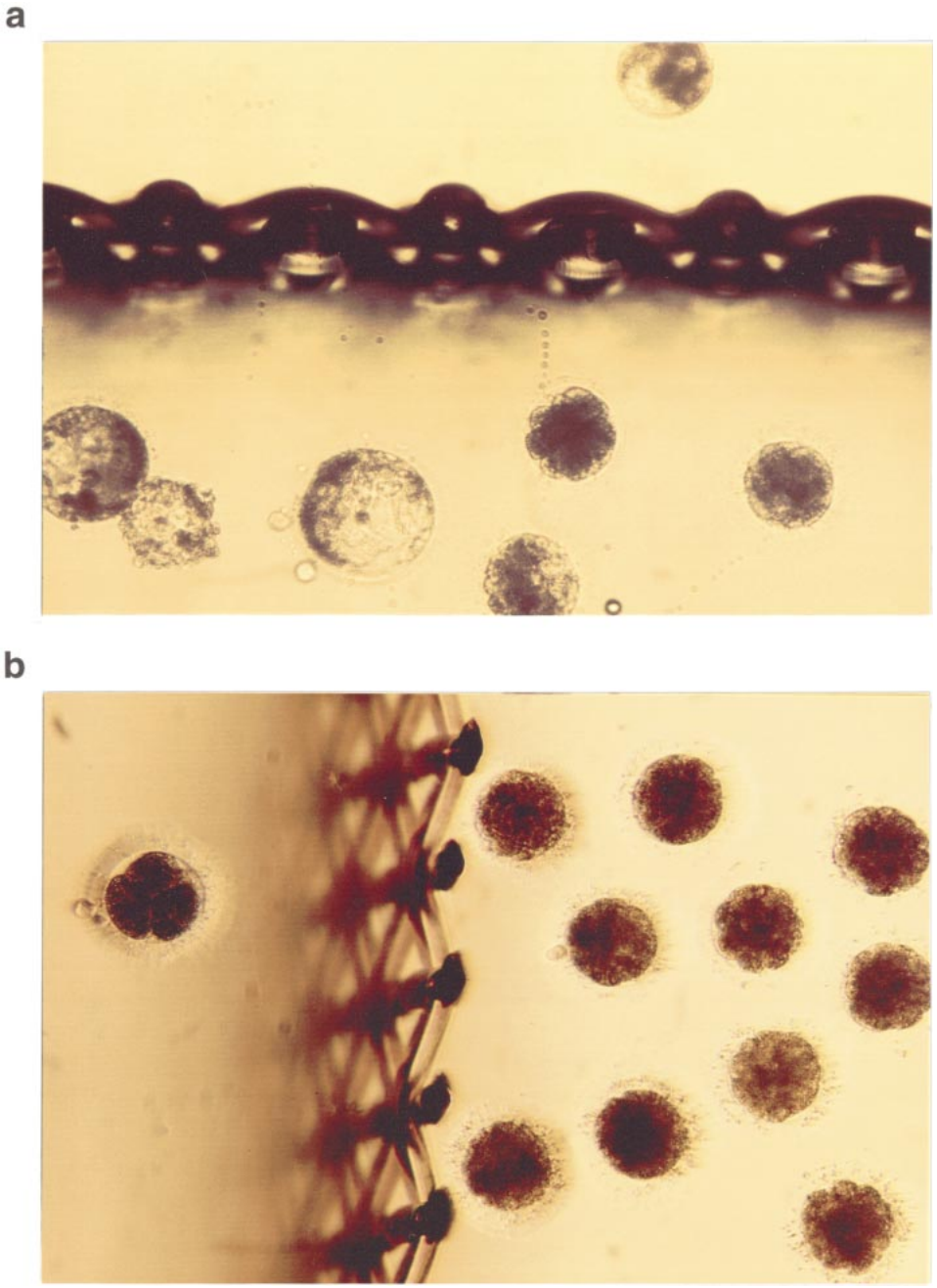

FIG. 1. A mesh barrier prevents mixing of test and companion embryos incubated in the same drop. For example, intermediate test quality embryos are incubated with companions of greater (**a**), equal, or lesser quality or with younger, synchronous, or older (**b**) companions.

data were expressed as mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

A total of 2367 cat oocytes was assessed noninvasively for glucose uptake as an index of oocyte quality, and 942 of these were used as test or companion embryos. As observed in a previous study in our laboratory [52], there was a strong positive relationship between original oocyte quality (on the basis of glucose uptake) and subsequent morulae and blastocyst formation and embryo cell number by Day 7 of incubation. Companion embryos categorized metabolically as best quality averaged >100 cells by Day 7, with the majority reaching the morula (21.4% ± 2.8%) or blastocyst (53.2% ± 12.9%; Table 1) stage. More than half of the intermediate-quality embryos completed compaction, but only 10% completed blastulation (Table 1). Poor-quality oocytes resulted in embryos with a minimal compaction and no blastocyst formation (Table 1). All but 13 embryos developed as predicted with respect to cell number and stage of development. This affected a total of two best-quality and two intermediate-quality companion groups, thus excluding four test embryos from further analysis in study I, each on different days of experimentation. No test embryos were excluded from study II analysis.

Study I: Influence of Companion Embryo Quality

Single test embryos derived from intermediate-quality oocytes incubated with equal-quality companions completed compaction (8/12, 66.7%) and blastulation (5/12, 41.7%). However, blastocyst expansion by Day 7 occurred only in the test embryos incubated with best-quality companions (3/12, 25.0%). Test embryos coincubated without companions or with poor-quality companions failed to

TABLE 1. Stage of development and cell number of all companion embryos derived from oocytes categorized on the basis of glucose uptake.

Oocyte category	n	Average glucose uptake (pmol oocyte^{-1} h^{-1})	% Morula on Day 7[a]	% Blastocyst on Day 7[a]	Cell number on Day 7[a]
Best	120	2.7 ± 0.3[b]	21.4 ± 2.8[b]	53.2 ± 12.9[b]	105.6 ± 19.3[b]
Intermediate	720	0.4 ± 0.0[c]	53.0 ± 4.9[c]	10.0 ± 3.5[c]	38.2 ± 9.0[c]
Poor	140	0.01 ± 0.0[d]	9.0 ± 1.8[d]	0.0 ± 0.0[d]	3.1 ± 1.7[d]

[a] Day 0 = day of in vitro insemination.
[b,c,d] Values with different superscripts within columns differ ($P < 0.05$).

achieve compaction, and approximately one quarter (3/14, 21.4% and 4/14, 28.6%, respectively) did not cleave at all compared with <10% for uncleaved oocytes that were incubated with companions of best (1/12, 8.3%) or intermediate (1/12, 8.3%) quality. In all cases, test embryos incubated with best- or intermediate-quality companions had more ($P < 0.05$) cells (74.9 ± 16.9 and 40.6 ± 8.8 cells per embryo, respectively) than test embryos coincubated with poor-quality companions (5.1 ± 1.4) or alone (8.4 ± 3.7; Fig. 2).

Study II: Influence of Relative Companion Embryo Age

Test embryos incubated with older companions (derived from equal-quality oocytes) first completed blastulation (2/15, 13.3%) within 5 days of incubation, although blastocyst formation continued through Day 7 (total, 7/15; 46.7%). Blastulation did not occur until Day 7 in test embryos incubated with same-age (3/15, 20.0%) or younger (1/15, 6.7%) companions. Solitary embryos did not form morulae or blastocysts. Embryos incubated with older companions had ~2-fold more ($P < 0.05$) cells (88.3 ± 17.0 cells per embryo) than those incubated with synchronous embryos (49.3 ± 12.1) and a 3-fold increase relative to those incubated with younger companions (29.4 ± 6.1) (Fig. 3). The cell number of solitary embryos (9.8 ± 3.1) was less ($P < 0.05$) than that of any group of test embryos incubated with companions, regardless of age (Fig. 3).

DISCUSSION

The results of these studies reaffirm previous findings [52] that rapid glucose uptake by individual oocytes predicts greater percentages of morula and blastocyst development in vitro (Table 1). More importantly, for the first time, it was possible to examine the impact of companion embryo culture knowing the quality of the companions. Additionally, test embryos used in this study were of intermediate quality, and such embryos are known [52] to have a marginal ability to fertilize and develop in vitro. The overall results revealed three important new findings. First, the quality of the companions, based on glucose uptake of the original oocyte, indeed influenced the ability of test embryos to develop in culture. Second, there was an age effect, with older companions accelerating test embryo development more than age-synchronous or younger embryos. Using companions of best quality and advanced age enhanced the developmental capacity of these marginal (intermediate-uptake) embryos, indicating a likelihood of a ''rescue'' benefit. And third, we noted that there was an advantage to companion embryo culture in this cat system with every category of embryo except for poor-quality embryos. Even intermediate-quality test embryos cocultured with companions of disparate age experienced improved development compared with solitary controls.

Compared with cocultured counterparts, each singleton embryo was at a disadvantage, a finding that could not be explained solely by the lack of companions, because this same culture system has been used to grow singletons to blastocysts in vitro [19, 20]. This finding was likely due to the fact that all of the test embryos were of intermediate quality. Additionally, the test embryos used here underwent some additional manipulation. For example, the metabolic assessments required incubation in Hepes-buffered medi-

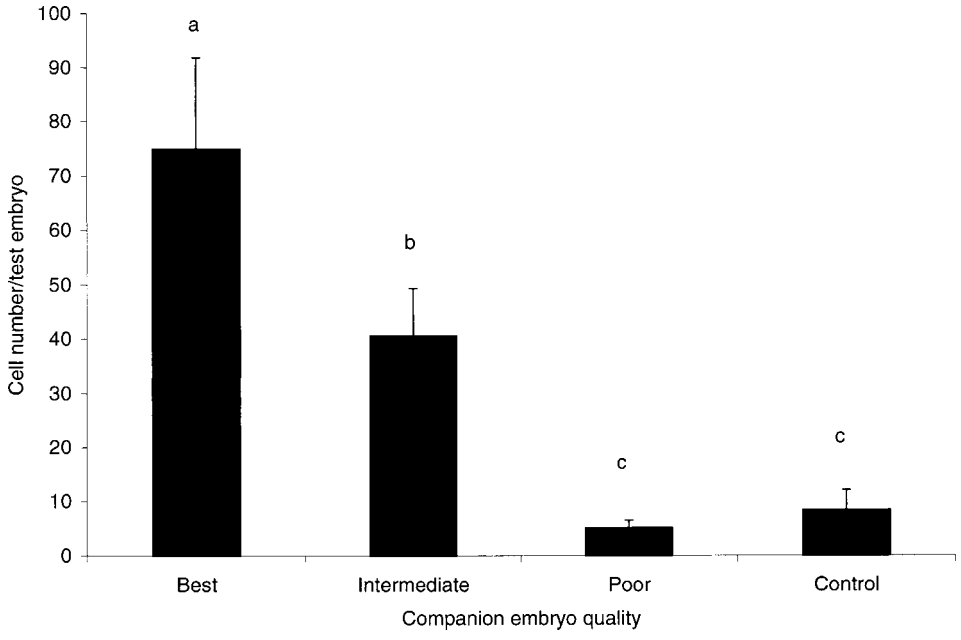

FIG. 2. Test embryo (intermediate quality) cell number (mean ± SEM) on Day 7 (Day 0 = day of insemination) of culture with best-, intermediate-, or poor-quality companions, or alone (control). [a]Different superscripts denote difference among treatments ($P < 0.05$).

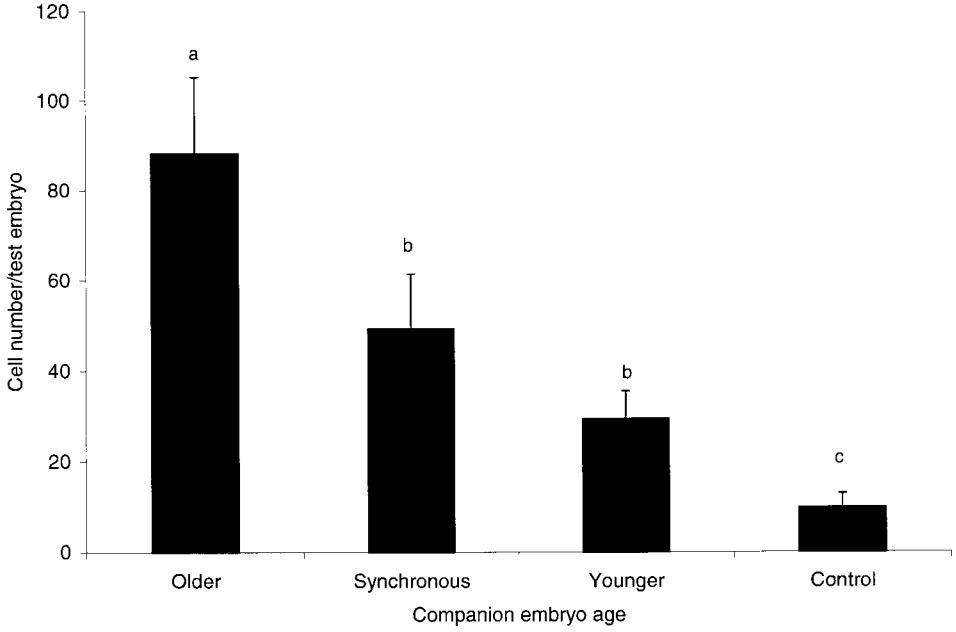

FIG. 3. Test embryo (intermediate quality) cell number (mean ± SEM) on Day 7 (Day 0 = day of insemination) of culture with older, synchronous, or younger intermediate quality companions, or alone (control). [a]Different superscripts denote difference among treatments ($P < 0.05$).

um, known to be detrimental to development [57]. The oocytes also were denuded of cumulus cells before insemination, which can reduce fertilization success [58]. Thus, it is worth speculating that the companion effect may have been even more profound if these handling procedures had been avoided.

To date, the cause of the benefit of companion embryo culture has not been elucidated, although obvious candidates are 1) beneficial macromolecular factors produced by the embryos themselves or 2) the removal of detrimental factors such as oxygen radicals (i.e., an absorptive effect). The level of benefit from this coculture strategy has also been controversial. Although improved in vitro development has been observed with group culture of mouse [39–41, 59], cattle [38], and rabbit [60] embryos, no such benefits were measured in hamster embryos [45], and results have been equivocal for human embryos [43, 44]. Based on the present results, it is possible that some of the ambiguity from the previous work may have been due to the quality of the companion embryos used. Certainly, this may have been the case in the human studies, in which it is likely that the companions were derived from questionable-quality oocytes from infertile patients. Additionally, it has been established that mouse embryos incubated with unfertilized oocytes experience compromized development [41]. It is possible that these oocytes simply did not secrete the beneficial factors required for development, but perhaps it is more likely that detrimental factors were secreted into the medium during oocyte degeneration. We have begun experiments to determine if the source of the effect is embryotrophic or related to a neutralization of detrimental factors. Such elucidation may be important to the improved development of rare embryos to the blastocyst stage as was observed in the present experiments. In turn, increased blastocyst development and embryo quality generally increase the opportunity for successful intrauterine embryo transfer of few, or even single, genetically valuable embryos, as in humans [61]. This method may also reduce the number of procedures required to produce valuable offspring, while reducing the risk to recipient females that must gestate more than the usual number of fetuses.

The companion effect also was influenced significantly by embryo age, which may reflect a direct influence of the specific stage of embryo development or merely embryo size and/or cell number. From an applied perspective, when the objective is to promote growth of singular test embryos, the companions should be older rather than the same age or younger. This finding provokes an intriguing question: are the presumed secretory or absorptive benefits of companions due to 1) the need for a certain cell number or 2) a specific mechanism related to functions of the growing embryos, such as compaction or blastulation? For example, potential target mechanisms may include best-quality embryos secreting factor(s) that promote blastocyst formation, whereas poor-quality counterparts may produce factor(s) that interfere with the fluid accumulation that is necessary for blastocyst formation. Although our study indicated that there was a definitive age effect, the positive influence of companion embryo culture occurred regardless of the age of these neighboring embryos. Thus, companion embryos of different types influence embryos by degrees with two of the modulating factors: 1) the quality of the companions and 2) the age, which likely reflects stage of development, including cell number and/or function.

The benefits of companion culture are also likely influenced by the culture conditions themselves. Bavister [37] has argued that companion culture may simply be a means of masking the use of suboptimal culture media. Knowing embryo-specific needs allows the development of species-specific media that will more likely maximize target embryo development in vitro. Certainly, it would be beneficial to develop a cat-specific culture medium for IVM/IVF, and it would be especially interesting to determine if the distinctive benefits of companion culture are sustained. In the present studies, we relied on Ham F10, which allowed comparative assessments with years of previous developmental and metabolic data obtained with the same medium. There is, however, another advantage to the companion embryo culture system. In our case, the domestic cat system is largely used as a model for similar studies in other Felidae species. Although a domestic cat-specific culture medium may be applicable to species such as the cheetah, clouded leopard, and ocelot, there is clear evidence of remarkable differences in reproductive mechanisms among the felids,

including gamete interaction and embryo culture [62, 63]. We have recently begun to explore the benefits of heterospecific companion embryo culture [64], with some early evidence that good-quality companions of one species may be advantageous in culture for another. Thus, coembryo culture has promising application in cases in which it will be impossible to elucidate every species-specific culture medium and when embryos from rare species require rescue and in vitro development.

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