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Improved felid embryo development by group culture is maintained with heterospecific companions

Rebecca E. Spindler [a,*], Elizabeth G. Crichton [b], Yuksel Agca [c], Naida Loskutoff [b], John Critser [c], David K. Gardner [d], David E. Wildt [a]

[a] *Department of Reproductive Sciences, Conservation and Research Center, Smithsonian's National Zoological Park, 1500 Remount Road, Front Royal, VA 22630, USA*
[b] *Center for Conservation and Research, Henry Doorly Zoo, Omaha, NE 68107, USA*
[c] *University of Missouri, Comparative Medicine Center, College of Veterinary Medicine, Columbia, MO 65211, USA*
[d] *Colorado Center for Reproductive Medicine, Englewood, CO 80110, USA*

Abstract

Domestic cat embryos of excellent quality appear to improve development of conspecific embryos when cultured together, providing an avenue for improving development of embryos from valuable species or individuals. To have relevance to rare species, it would be useful to understand if this advantage could be conferred by heterospecific companions because there usually are severely limited numbers of conspecific embryos available from wildlife donors. In the first study, we incubated single test cat embryos alone (controls) or with 10 cat embryos or 10 or 20 mouse embryos under similar regimented conditions (each group shared 20 μl medium). In the second study, single test cat embryos were cultured alone, with 10 conspecific or 20 mouse embryos or 10 cattle embryos (each group shared 20 μl medium). Single test embryos in all treatment groups achieved similar ($P > 0.05$) stages of compaction and blastocyst development. In the first study, only the test embryos incubated with 10 cat or 20 mouse companion embryos achieved blastocyst expansion. The average total cell number within test embryos incubated with 10 cat or 20 mouse companions was greater ($P < 0.05$) than controls or those placed with 10 mouse embryos. In the second study, test embryos in all groups achieved blastocyst expansion and had more ($P < 0.05$) total cells per embryo than the solitary controls. Inner cell mass to trophoblast cell ratio did not differ among treatments in either study. Thus, companion mouse and cattle embryos selected for excellent quality confer a benefit to singleton cat embryos, although the number of companions necessary to grant an advantage may be species dependent. If this phenomenon can be extrapolated across species, this may be an avenue for 'common animal embryos' to improve developmental potential of embryos from rare, unrelated taxa.

Keywords: Blastocyst; Autocrine; Paracrine; Reactive oxygen; Embryo group culture; Heterospecific

1. Introduction

Ex situ (captive) wildlife populations serve as species ambassadors, providing education and motivation opportunities for the public about priority conservation issues [1,2]. These collections also are a safe haven for rare species that deserve research attention or that are being bred for reintroduction, examples being the black footed ferret [3], Puerto Rican crested toad [4,5] and Californian condor [6], among others. The success of such programs was founded on performing basic research that eventually included intensive genetic and reproductive

* Corresponding author at: Toronto Zoo, 361A Old Finch Ave, Scarborough, Ont. M1B 5K7, Canada. Fax: +1 416 392 4979.
E-mail address: spindler@uoguelph.ca (R.E. Spindler).

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management culminating in self-sustaining populations, viable for reintroduction.

While artificial insemination and in vitro fertilization (IVF) are now commonly employed tools in captive breeding, ensuring procreation of all individuals to avoid the loss of genetic diversity often requires more heroic measures. However, techniques such as the rescue of gametes from genetically valuable individuals that unexpectedly die have, thus far, been limited in improving reproduction in small populations. Success is poor partly due to the low gamete numbers and because age and health status of most candidates for this technology often compromise the quality of gametes recovered. Fundamental scholarly knowledge about requirements for oocyte maturation, fertilization and embryo development in vitro, particularly with respect to these compromised and singleton gametes is essential to the routine use of this technology for genetic management.

Artificial insemination has been achieved using epididymal sperm [7–9], but low numbers of sperm recovered, poor motility and damage caused by cold storage [10] and cryopreservation [11] result in few pregnancies. Techniques such as zona piercing [12] and intracytoplasmic sperm injection [13,14] can be used to circumvent fertilization barriers of sub-optimal ejaculated or rescued sperm. Domestic cat embryos can be readily generated by IVF using oocytes recovered from ovarian follicles and matured in vitro [15–20]. In contrast, oocytes from the excised ovaries of wild felid counterparts generally experience lower in vitro maturation/fertilization success [21] possibly due to the age and health status of the donors.

When working with laboratory and domestic species, oocytes can often be retrieved from young healthy females in large numbers. However, non-domestic females that die without reproducing are candidates for gamete rescue and are often of advanced age, have reproductive dysfunction or are generally in poor health. These issues are also of primary concern in domestic animal and even human assisted reproduction clinics as these conditions reduce gamete viability [22–27]. Further, when attempting gamete rescue from non-domestic species and assisted reproduction in humans, the approach of combining oocytes from several different donors is not available as each oocyte must be traceable back to the donor. This places additional pressure on these valuable oocytes as it is thought that embryos grown in groups develop better than those grown alone and few options are available for improving viability of lone oocytes of compromised quality.

Co-incubation has often been used as a tool to beneficially modify embryo culture medium and improve embryo development, albeit with varying success [28–34]. These co-culture experiments all involved somatic cells as the supportive element for embryos. In turn, this has led to criticisms that: (1) the chosen culture medium could not adequately provide the requirements for two such diverse cell types [35–37]; (2) somatic cell layers (and the serum required to support these cells) can cause fetal abnormalities [38–41]; (3) the more vigorous, numerous somatic cells may too rapidly deplete the medium of necessary substrates at critical times for the embryo [42].

Co-incubation using companion embryos (in contrast to somatic cells) permits using defined embryo-specific culture media while eliminating the above concerns [43]. However, results have been equivocal with some studies reporting benefits of embryos grown in groups [44–48] and others finding no significant impact [49–51]. It has been suggested that the advantages measured with group embryo culture occur only when sub-optimal medium is used [36]. Others assert that the benefits arise from the secretion of favorable autocrine and paracrine factors [47,52].

Recently, we have demonstrated that the quality of cat embryos can be predicted from the metabolic pattern of the source oocytes [53] as in cattle [54]. Metabolic selection of individual companion embryos is labor intensive and may not be available at the site of gamete rescue. As oocyte metabolism is linked to overall developmental potential [53,54], including time to first cleavage [55], we suggest that selection of high quality cat embryos can also be performed by assessing time to first cleavage as in cattle [56] and humans [57].

Singleton 'test' cat embryos benefited from group culture with better versus poorer quality conspecific companion embryos [55]. Thus, we suspect that some of the diversity in earlier published results about the value of group culture could be explained on the basis of companion embryo quality. That is, those studies showing no benefit may have used sub-optimal companions. Thus, selection of oocytes using either metabolism or cleavage rate post-fertilization may reduce any detrimental effects by removing sub-optimal embryos, but further limits the availability of conspecific companions. Direct application of this technique to wildlife only is likely if embryos from common, heterologous species confer advantages to the singleton embryos from rare species. There is evidence that both cat and bovine embryo growth is enhanced by co-culture with mouse embryos [58,59]. Thus, the objective of the present studies was to determine if the benefit of "test" domestic cat embryos (observed with conspecific companions) would be maintained

when using either high quality bovine or murine companions in vitro.

2. Materials and methods

2.1. Chemical reagents

All chemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, except where noted.

2.2. Cat ovary collection, oocyte recovery and maturation

Post-pubertal domestic cat ovaries were recovered after ovariohysterectomy from local veterinary clinics throughout the most reproductively active periods of the year [53,55], from November through January (Study I) and January through April (Study II). During transport, ovarian pairs were stored in Dulbecco's phosphate buffered saline (D-PBS, #D1283; pH 7.4, containing 100 IU/ml penicillin and 100 mg/ml streptomycin) at 4 °C using ice-packs and paper padding. Container temperature was monitored using a thermocouple (Omega Engineering Inc., Stanford, CT). Within 6 h of excision, ovaries were minced, and oocytes were liberated into 38 °C minimum essential medium (MEM, #M2279) with 25 mM HEPES (H-MEM, #H0887) supplemented with 4 mg/ml bovine serum albumin (BSA; Miles Pentex, Bayer Diagnostics, Kankakee, IL). Only Grade I (best quality) immature oocytes with excellent morphology—defined by uniform, dark cytoplasm and an intact cumulus cell investment [17], were selected for maturation and were rinsed three times in H-MEM. Oocytes (10/50 μl drop) were placed in maturation medium (MEM) containing 1.0 mM glutamine (#G6392), 1.0 mM pyruvate (#P4562), 100 IU/ml penicillin (#P3032), 100 mg/ml streptomycin (#S1277), 4 mg/ml BSA (Miles Pentex), 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 (#E2758) and cultured under mineral oil for 32 h at 38 °C in a humidified environment of 5% CO_2 in air [15].

2.3. Cat oocyte insemination and embryo selection

After in vitro maturation (IVM), cat oocytes were denuded and placed in 20 μl droplets of medium G1.2 [60] supplemented with 5% fetal bovine serum (FBS, #10438-026, Gibco, Grand Island, NY). 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 [61] were centrifuged at 200 × *g* for 8 min and the supernatant replaced with 100 ml of G1.2 with 5% FBS. Sperm were allowed to 'swim-up' into the medium for 30 min and then were reassessed for motility and status. Sperm concentration was determined using a hemocytometer, and each oocyte was inseminated at a final concentration of 2×10^5 motile sperm/ml (10,000 sperm in a 5 μl drop) under mineral oil (#M8410) at 38 °C in 5% CO_2 in air for 16 h.

Cat embryos that had achieved one cleavage (to the two-cell stage) at 24 h post-insemination (h.p.i.) were indicated to be intermediate quality and were chosen as 'test' embryos for all treatments [55]. Intermediate embryos were chosen as test embryos to allow greatest latitude in demonstrating a beneficial or detrimental effect of incubation with companions. Embryos that had cleaved twice (to four-cells) by 24 h.p.i. were judged to be excellent quality and were chosen as conspecific companions.

2.4. Mouse embryo production, cryopreservation, thawing and selection

Mice (ICR type; Harlan Sprague Dawley Inc., Indianapolis, IN) were maintained according to policies of the Purdue University Animal Care and Use Committee and the 'Guide for the Care and Use of Laboratory Animals'. Mice were fed ad libitum with a standard laboratory diet and held in a temperature and light-controlled room (22 °C, 14L:10D; light starting at 07:00 h). Five to 6-week-old females were treated i.p. with 7.5 IU PMSG (#G4527) followed 48 h later by 7.5 IU hCG (#C8554). Females were placed with ICR males immediately after hCG administration, and two-cell stage mouse embryos were collected from excised oviducts ~36 h later.

Embryos were washed three times in TL-HEPES (BioWhitaker Inc., Walkersville, MD) before being frozen using a standard mouse protocol [62]. Embryos were equilibrated in 1.5 M DMSO in TL-HEPES for 10 min before loading into 0.25 ml French straws containing a separate column of 0.5 M sucrose (#S9031) solution of equal volume. Straws were placed into a programmable freezer (Planer, Model Kryo 10 Series II; Perkasie, PA), cooled to −7 °C at 2 °C/min and held for 10 min. Ice nucleation was induced by touching the straws with liquid nitrogen (LN_2)-cooled forceps at −7 °C (seeding). Ten minutes later, cooling

resumed at 0.5 °C/min until −40 °C, at which time the straws were quickly removed from the freezing apparatus and immediately submerged in LN_2.

Each straw containing embryos was thawed in air for 10 s and then plunged into a 20 °C water bath for 10 s. Straw content was emptied into a Petri dish with the embryos maintained for ~5 min in the thawed DMSO-sucrose–TL-HEPES solution before being transferred into fresh TL-HEPES solution at room temperature. Embryos were washed and incubated in 20 μl of medium G1.2 under mineral oil for 6 h at 37 °C before selection. Time to first cleavage is an effective predictor of both cryosurvival [63] and developmental potential [56,57], indicating that the two are linked. Thus, we have used embryo cryosurvival based on normal cell morphology post-thaw to select embryos with good developmental potential.

2.5. Cattle embryo production, transport and selection

Cumulus–oocyte complexes (COCs) were aspirated from slaughterhouse ovaries in Omaha, NE and washed in TL-HEPES. Good-to-excellent quality oocytes were transferred to Nunc wells (#P8116) containing 500 μl of warmed maturation medium: TCM 199 (#11150-59, Gibco) supplemented with 10% FBS, 0.01 unit/ml each of bovine FSH and LH and 1% penicillin, 1% streptomycin, and incubated for 18–24 h at 38.5 °C in humidified 5% CO_2 in air. The following day, COCs were removed and washed in TL-HEPES, then transferred to 500 μl warm fertilization medium: Tyrode's solution containing pyruvate (#T1788) with 0.3% BSA, fraction V (#A3059), and 1% penicillin, 1% streptomycin to which 10 μg/ml each of sodium heparin (#H4784), penicillamine (#P4875), hypotaurine (#H1384) and epinephrine (#E4250) were added. Thawed bull sperm were added at a final concentration of 1×10^6 ml^{-1} and the cultures incubated for 21 h as above. Presumptive zygotes were removed the next day, freed of excess sperm and cumulus cells by repeated pipetting, transferred into culture tubes containing 1 ml of G1.2 and gassed with 5% O_2, 5% CO_2 and 90% N_2. Falcon tubes (#352001, Becton Dickinson, Franklin Lakes, NJ) were tightly capped and covered with Parafilm®, then placed in a portable incubator (Minitube, Verona, WI) which maintained the temperature at 38.5 °C (lowest temperature at arrival was 35.5 °C) during express delivery to the Conservation and Research Center. Cattle embryos that had cleaved at least once after 14 h of culture were selected as companions.

2.6. Embryo co-incubation and test embryo assessment

All embryos of each of the three species were incubated throughout the duration of the two studies at 38 °C in medium (as described below) under mineral oil in 5% CO_2 (the balance being 6% O_2 and 89% N_2). Test embryos were single cat embryos at the two-cell stage 24 h.p.i. Numbers of domestic cat test embryos were equally divided across each day of experimentation to reduce variability due to spermatozoa used for insemination or season of oocyte collection. Each test embryo was separated physically, but not chemically, from its companion embryos (treatments described below) using a specially designed co-incubation chamber [55]. In brief, this device is comprised of a Falcon embryo-safe culture dish (#3801, Becton Dickinson) bisected by a fine nylon mesh barrier (pore size = 105 μm) that prevents embryo passage, but allows free chemical exchange. Selected embryos were incubated in 20 μl of medium G1.2 for 72 h before being transferred to 20 μl of medium G2.2 [60]. After 24 h all (test and companions as a unit) embryos were transferred into fresh G2.2 for an additional 72 h. The purpose of this additional embryo change was to emulate conditions of test cat embryos matched with mouse embryos that were replaced after 96 h. Embryo development was evaluated after 168 h of culture by examining each test embryo under light microscopy (200X, Olympus BX40, Melville, NY) and rolling embryos to estimate cell numbers or stage of development. The total number of cells was determined for each embryo (with the exception of blastocysts) between 168 and 170 h of culture after staining with Hoechst 33342 [64]. For blastocysts, number of trophoblast and inner cell mass cells was evaluated using a simple differential cell staining procedure [65]. Briefly, cat blastocysts were incubated at 22 °C in 200 μl of protein free HEPES buffered G1.2 with 1% Triton X-100 and 100 μg/ml propidium iodide for 15 s. Embryos were rinsed in medium G1.2, incubated in 100% ethanol with 25 μg/ml bisbenzimide (Hoechst 33258, #B1155) and then placed in a 4 °C cold room for 16 h. Then, each embryo was placed on a glass microscope slide in a drop of glycerol and gently flattened under a cover slip. Cells then were viewed and counted using fluorescent microscopy (200X, Olympus BX40) with a 460 nm excitation filter.

2.7. Experimental design

Study I was designed to examine the influence of mouse companions on cat 'test' embryos. In addition to

examining the species heterologous effect, this study evaluated the impact of companion group size. In a preliminary analysis, we determined that the surface area of the two-cell mouse embryo was only 42 ± 4% of that of a similarly staged cat embryo (mouse:cat surface area ratio = 1:1.59 based on overall surface area at the outer layer of the zona pellucida; $n = 35$ and 30 measurements for the mouse and cat, respectively). Study I treatments, therefore, were designed to examine similar embryo number versus similar surface area across the companion group. Thus, the treatment groups included a control (solitary cat embryos; $n = 20$) versus cat test embryos cultured with 10 cat embryos (Fig. 1a; $n = 18$ companion groups) or 10 ($n = 21$ companion groups) or 20 ($n = 21$ companion groups) mouse embryos. The latter two companion groups approximated 40% and 79% of the embryo surface area provided by the 10 cat companions.

Because the normal period of mouse development to blastocyst is only 4 days [66] compared with 7 days (168 h) for the cat [19], it was necessary to remove the mouse blastocysts and morulae from culture after 96 h and replace them immediately with an equal number of fresh eight-cell stage embryos (analogous to this timing stage for the cat). No other embryo exchanges were made throughout the remainder of incubation.

Study II was designed to expand the breadth of species from which companion embryos could be utilized. Thus, in addition to the singleton controls ($n = 12$), other cat test embryos were incubated with 10 cat embryos ($n = 12$ tests), 20 mouse embryos (Fig. 1b; $n = 12$ tests) or 10 cattle embryos (Fig. 1c and d; $n = 12$ tests).

Any treatment with more than two companions that ceased development or became degenerate at any time during the 168 h incubation period were deleted from the study. This resulted in the withdrawal of a total of 14 test companion embryo units throughout the two studies. On three occasions, this eliminated an entire treatment group and thus, the entire experiment on that day. When only one or two companion embryos per group degenerated or appeared retarded in development, they were removed from culture, but the remaining experimental group was retained and the results were analyzed.

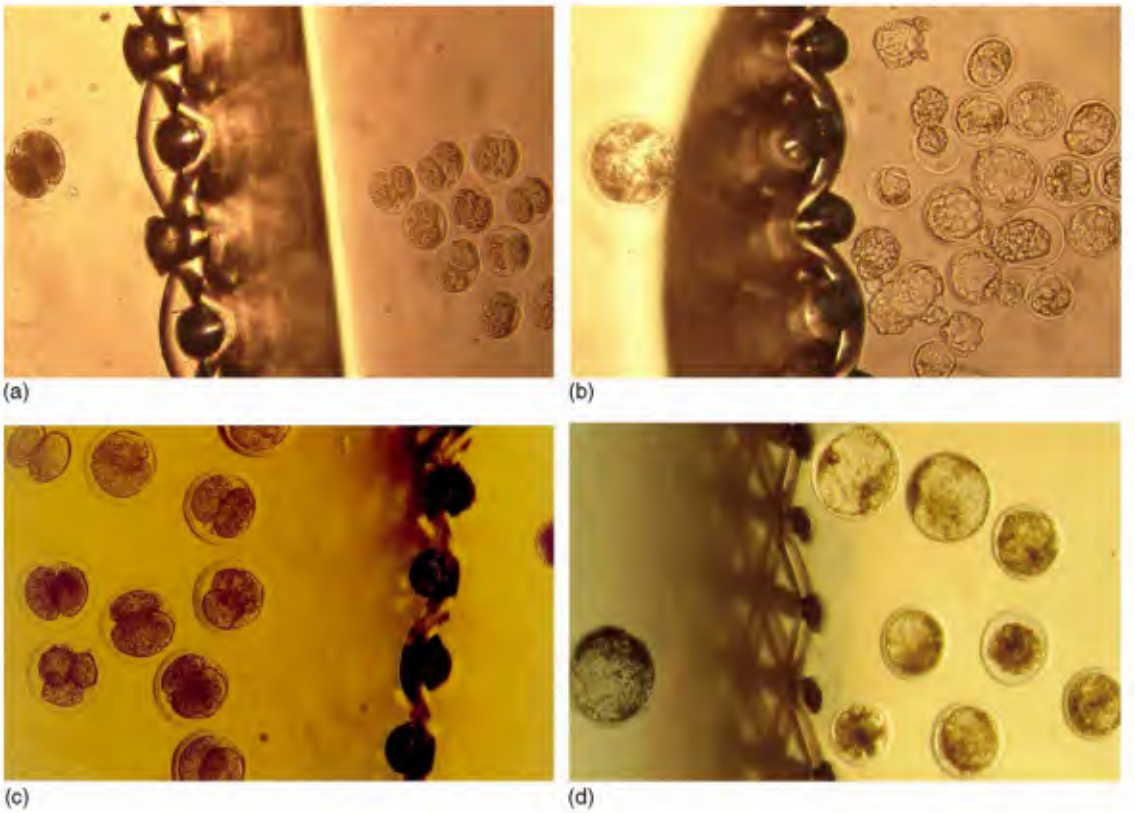

Fig. 1. Single test domestic cat embryos were incubated on opposite sides of a mesh barrier that physically separated, but allowed chemical communication with companion embryos from the cat, mouse and cow. Embryos were placed in culture at the two to four-cell stage (a and c) and incubated for 168 h, to the blastocyst stage (b and d).

2.8. Statistical analysis

All percentage data were arcsine-transformed before undergoing further analysis. Differences in cell number and stage of development among embryos incubated under varying conditions were assessed by ANOVA and multiple comparisons via Dunnett's test. All other data are expressed as mean ± S.E.M. P values less than 0.05 were considered significant.

3. Results

3.1. Development and success of companion embryos

At the time of selection (24 h.p.i.), 11.3 ± 4.1% of all inseminated cat oocytes were four-cells, 37.3 ± 8.4% were two-cells and 51.5 ± 5.6% had not cleaved or were degenerate. Of the 350 four-cell cat embryos selected as companions, 25.2% reached the morula stage at the end of the 168 h culture period, whereas more than half (54.1%) had become blastocysts by this same time. Sixty-eight (5.2%) of the companion cat embryos selected for excellent quality based on time to first and second cleavage did not develop as predicted, and were found to be degenerate or ceased development at some point during the 168 h culture. These embryos were removed individually (up to two within a group; $n = 41$), or, in the case of three groups, brought about the removal of the entire test/companion embryo group.

Of the 1120 mouse embryos thawed, 940 (83.9%) had normal morphology for the two-cell or eight-cell stage. Of these, 12.1% completed compaction and 76.2% underwent blastulation after 96 h (time of embryo replacement) or at the end of the experiment. Only 101 embryos (9.3%) displayed retarded development or were degenerate at the time of replacement or by the end of the experiment. After 96 h, 61 embryos were removed individually and in six cases, the total number of degenerate mouse embryos eliminated the entire test/companion embryo group. Of the 150 cattle embryos that were selected as companions at the two-cell stage, 53.9% developed into morulae and 29.8% into blastocysts. Retarded development or degeneration was seen in 37 embryos (24.6%) that were removed individually ($n = 9$) or necessitated the removal of two entire test/companion groups.

3.2. Study I

Single test embryos in all treatment groups achieved a similar ($P > 0.05$) level of compaction (control: 25.0%, $n = 20$; 10 cat: 27.8%, $n = 18$; 10 mouse: 38.0%, $n = 21$; 20 mouse: 38.1%, $n = 21$) and blastocyst development (20.0% for controls; 22.2% for 10 cat companions; 19.0% for 10 or 20 mouse companions). However, only test embryos incubated with 10 cat (27.7%) or 20 mouse (19%) companions achieved blastocyst expansion while the other two groups did not. Average total cell numbers of test embryos after 168 h incubation with 10 cat or 20 mouse embryos were greater ($P < 0.05$) than counterpart treatment values from controls or with 10 mouse companions (Fig. 2a). Differential cell staining revealed that test embryos that developed to blastocysts after incubation with 10 cat or 20 mouse companions had more ($P < 0.05$) trophoblast and inner cell mass cells than controls or those incubated with only 10 mouse embryos (Fig. 2b). The ratio of trophoblast cells to inner cell mass cells of all blastocysts did not differ ($P > 0.05$) among groups,

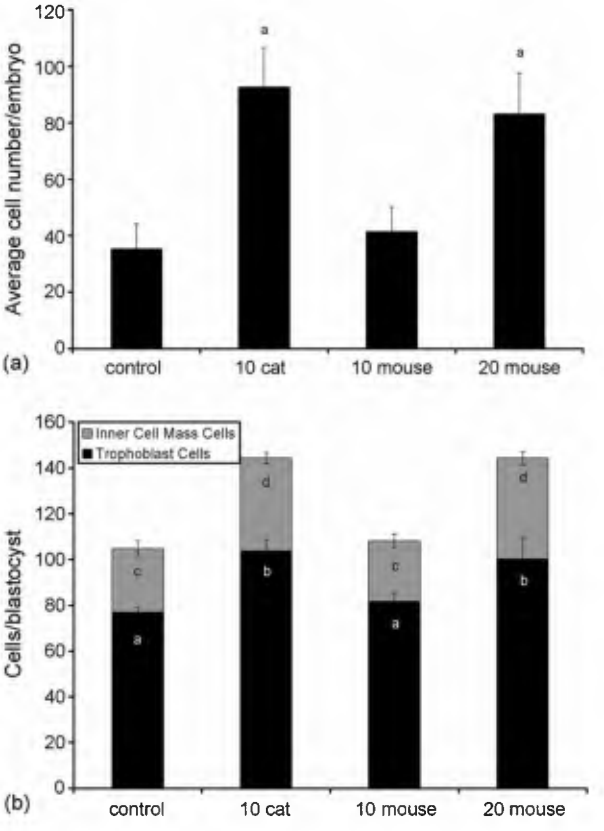

Fig. 2. (a) Average (±S.E.M.) cell number of test cat embryos at 168 h of culture after incubation with 10 conspecific or 10 or 20 mouse companions. Controls are solitary embryos with no companions. The letter (a) denote treatments different from control ($P < 0.05$). (b) Average (±S.E.M.) test cat blastocyst trophoblast and inner cell mass cell numbers after 168 h of culture in the same treatment groups. Different letters (a, b, c, d) denote differences ($P < 0.05$) among treatments.

although there was a decreased ratio trend in blastocysts cultured with 10 cat or 20 mouse embryos (control, 3.3 ± 0.3; for 10 cat embryos, 2.6 ± 0.1; for 10 mouse embryos, 3.9 ± 0.4; for 20 mouse embryos, 2.2 ± 0.1; $P = 0.08$).

3.3. Study II

There was no difference ($P > 0.05$) in the incidence of compaction (controls: 33.3%, $n = 12$; 10 cat companions: 50.0%, $n = 12$; 20 mouse companions: 41.7%, $n = 12$; 10 cattle companions: 41.7%, $n = 12$) or blastulation (16.7% for controls; 25.0% for 10 cat companions; 33.3% for 10 cattle companions) among the treatment groups. The proportion of test embryos achieving blastocyst expansion was 16.7%, 16.7% and 33.3% ($P > 0.05$) for test embryos co-cultured with cat, mouse or cattle companions, respectively, compared to 0% for controls ($P < 0.05$ when compared to all group cultures). Additionally, average total cell numbers within test embryos incubated without neighbors were lower (27.4 ± 7.2; $P < 0.05$) than those of the companion groups (10 cat: 79.4 ± 9.0 cells/embryo; 20 mouse embryos 68.2 ± 8.4 cells/embryo; 10 cattle embryos: 86.8 ± 13.0 cells/embryo; Fig. 3a). Cat blastocysts resulting from all treatments had similar ($P > 0.05$) numbers of trophoblast and inner cell mass cells (Fig. 3b). The ratio of trophoblast cells to inner cell mass cells of all blastocysts did not differ ($P > 0.05$) among groups (control, 2.3 ± 0.0; for 10 cat embryos, 2.7 ± 0.1; for 20 mouse embryos, 2.8 ± 0.4; for 10 cattle embryos, 2.7 ± 0.5).

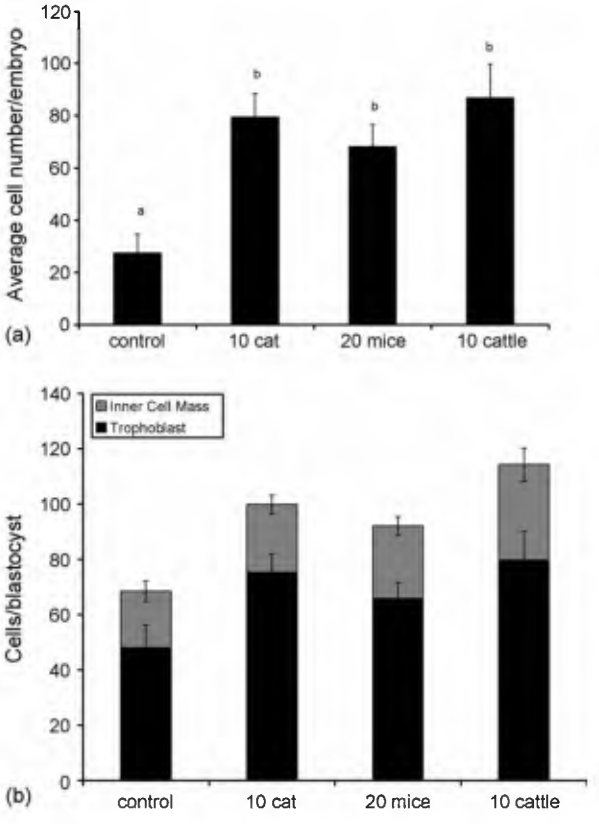

Fig. 3. (a) Average cell numbers of test cat embryos (mean ± S.E.M.) at 168 h (day 0 = day of insemination) of culture with 10 conspecific, 20 mouse or 10 cattle embryo companions, or alone. Controls are solitary embryos with no companions. Different letters (a and b) denote differences ($P < 0.05$) among treatments. (b) Average (±S.E.M.) test cat blastocyst trophoblast and inner cell mass cell numbers in the same treatment groups.

4. Discussion

These findings confirmed recent observations [55] that companion cat embryos can benefit the in vitro development of a single targeted cat embryo, allowing improved development to advanced stages in culture. More importantly, present investigations demonstrated, for the first time, that enhanced development was also promoted by heterospecific (in this case mouse and cattle) companion embryos. Thus, the mechanisms whereby neighboring embryos confer developmental advantages appeared conserved, at least within the constraints of the three species examined in the present study. This knowledge has potentially important applications for the rescue of oocytes and embryos from rare species, where frequently only one or two embryos are recovered or produced on short notice and where generally there are no conspecific companions available. For example, a logical next step in this research would be testing in vitro developmental competence of embryos from any of the extant 36 felid species during co-incubation with readily available domestic cat, mouse or cattle embryos. The companions could be stored in liquid nitrogen and thawed as needed when rescue opportunities arise. Additionally, the benefits of heterospecific embryo culture warrant attention across other taxa, including non-carnivores.

Further, cat embryos selected on the basis of time to first cleavage were developmentally similar to those selected on the basis of metabolic rates [53] and cattle companion embryos that were also selected on the basis of cleavage rate were found to have highly predictable development. These findings tend to support previous work indicating that the number of cleavage events undergone by individual cat zygotes by 24 h.p.i. appears to predict development to the blastocyst, as in other species [56,57]. The ability to select cat embryos on the basis of cleavage rate, as well as metabolism, provides

greater scope for companion embryo selection where metabolic analysis may be unavailable.

It is clear from these and previous studies that target and companion embryos separated physically do communicate chemically, although it is not clear how. Elucidating the mechanisms whereby companion embryos, even those of diverse species, provide a beneficial impact in culture is of high priority. To date, growth factors, including platelet activating factor [52], insulin-like growth factor II [52], leukemia inhibitory factor [67] and interleukin-6 [67] have been suggested as stimulatory autocrine/paracrine candidates, in part, because these are known to be secreted by embryos. The temporal pattern of secretion and uptake of these growth factors from different numbers and species of companion embryos could be determined as a step towards the development of a defined medium that would avoid the need for companions. However, we suspect that only a few of the natural growth factors have yet been identified, so it is unlikely that a medium could be formulated that wholly emulates the entire constellation of factors needed to maximize early embryo development.

The observed benefit was likely not due exclusively to growth factors. For example, low quality cat embryos reduce the developmental potential of co-cultured conspecifics [55]. Thus, it is possible that sub-par embryos secrete development-inhibiting factors, such as radical oxygen species [68,69] known to be detrimental to gamete [70] and embryo [71,72] survival. An important component of oocyte cytoplasmic maturation appears to be certain enzyme transcripts that may protect against reactive oxygen [73]. In fact, embryos failing to express genes encoding antioxidant enzymes may be less tolerant of the in vitro environment [74], more susceptible to damage and, therefore, less likely to develop [73]. As suggested previously [55], embryos derived from oocytes capable of neutralizing reactive oxygen species also may have the capacity to 'condition' medium, improving the environment for neighboring embryos. Ammonium production from neighboring embryos is also known to be detrimental to development in vitro [75]. Ammonium removal improves embryo survival [76] but it is not clear which embryos are more or less susceptible to ammonium toxicity.

The number of heterologous companions was important. Although 10 mouse embryos had no effect, doubling this number provided a benefit comparable to that measured with 10 cat companion embryos. This may be due to species differences in the type or amount of factors that promote growth or impede reactive oxygen effects. However, the efficacy of growth factors derived from various species that have been used successfully as media supplements indicates that species differences are not significant [77–79]. Similarly, ammonium production and susceptibility is likely equivalent among species [76,80]. There may be a difference in the species sensitivity to reactive oxygen, with mouse embryos expressing a wider array of antioxidant genes than cattle embryos [74]. In contrast, our findings suggest that mouse embryos were less beneficial to development of cat embryos as compared to cattle companions until their numbers were increased.

In the present study, cryopreservation may have diminished the ability of mouse embryos to modify the in vitro environment, or, perhaps, the mouse embryos were more sensitive to the incubation temperature (38 °C; 1 °C above body temperature) than bovine embryos (1 °C below average cattle body temperature). Although the altered culture temperatures might adversely influence development, in vitro growth was comparable to that reported previously [28,46,60,78,81–83]. More likely, the beneficial effects exerted by the increased number of mouse companions were due to the increased collective surface area of the embryos. While cat and cattle embryos have similar surface areas, the number of mouse embryos must be doubled to achieve a similar surface area. The ability of the embryo to (1) produce macromolecules and (2) take up and neutralize negative factors, such as reactive oxygen or ammonium, likely are affected by both surface area and cell number and size.

Once the number of mouse embryo companions was increased, both mouse and cattle companion embryos were comparable to cat embryos in improving blastocyst formation in test embryos, as well as increasing the inner cell mass and trophoblast cell numbers of each blastocyst (compared to controls). This pattern was mimicked in the ratio of inner cell mass to trophoblast cells in individual test blastocysts, although the differences were not significant, likely due to the low power of analysis between groups where few blastocysts were produced. Interestingly, embryos cultured without companions developed to more advanced stages in these studies than previously reported using this same co-incubation system [53]. We expect that this resulted from the less stringent selection of intermediate quality test embryos resulting from cleavage rate rather than metabolism. While cat embryos of intermediate quality, as determined by metabolism, are likely to have cleaved within 24 h, approximately 11% were still at the zygote stage at this time point [55]. Thus, in earlier experiments, a mix of zygotes and two-cell embryos were included as test embryos, whereas, in the present study, only two-cell

embryos were selected. Finally, a change of medium from Ham's F10, which is capable of supporting culture of embryos as well as somatic cells [84,85], to sequential media G2.1 and G2.2, specifically designed for culture of embryos, was necessary to support development of the mouse and bovine embryos and may improve development of cat embryos. Neither change eliminated the beneficial effect of excellent quality companion embryos.

In conclusion, the results of the present study suggest that embryo co-incubation may provide an avenue for supporting development of single valuable embryos of rare and endangered species or individuals. This idea is further enhanced by the discovery that companion embryos need not be conspecific, and may be previously cryopreserved. This information permits the development of a cryo-bank of excellent quality embryos from common species, ready to be thawed when required. Determination of the specific mechanisms involved in group culture with embryos of varying quality will further broaden the application of these techniques to gamete rescue and the culture of singleton embryos.

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