

Conditions for In Vitro Maturation and Artificial Activation of Ferret Oocytes¹

Ziyi Li,³ Qinshi Jiang,³ Maryam Rezaei Sabet,³ Yulong Zhang,^{3,5} Teresa C. Ritchie,^{3,5}
and John F. Engelhardt^{2,3,4,5}

Departments of Anatomy & Cell Biology³ and Internal Medicine⁴ and the Center for Gene Therapy of Cystic Fibrosis and Other Genetic Diseases,⁵ College of Medicine, University of Iowa, Iowa City, Iowa 52242

ABSTRACT

The ferret represents an attractive species for animal modeling of lung diseases because of the similarity between ferret and human lung biology and its relatively small size and short gestation time. In an effort to establish experimental protocols necessary for cloning ferrets, optimized conditions for in vitro maturation and artificial activation of ferret oocytes were examined. Cumulus-oocyte complexes were harvested from ovaries of superovulated ferrets, and in vitro maturation was evaluated in three different culture media: medium 1 (TCM-199 + 10% FBS), medium 2 (TCM-199 + 10% FBS with eCG [10 IU/ml] and hCG [5 IU/ml]), or medium 3 (TCM-199 + 10% FBS with eCG, hCG, and 17beta-estradiol [2 µg/ml]). After 24 h of maturation in vitro, the maturation rate of oocytes cultured in medium 2 (70%, n = 79) was significantly greater ($P < 0.01$) than those of oocytes cultured in the other two media (27%–36%, n = 67–73). At 48 h, similar maturation rates (56%–69%, n = 76–87) were observed for all three types of media. For activation experiments, oocytes cultured in medium 2 were stimulated with electrical and chemical stimuli either individually or in combination. Treatment with cycloheximide and 6-dimethylaminopurine (6-DMAP) following electrical stimulation resulted in 43% (n = 58) of the oocytes developing to the blastocyst stage. Such an activation rate represented a significant improvement over those obtainable under other tested conditions, including individual treatment with electrical pulses (10%, n = 41), cycloheximide (3%, n = 58), or 6-DMAP (5%, n = 59). Blastocysts derived from in vitro activation appeared to be normal morphologically and were composed of an appropriate number of both inner cell mass (mean \pm SEM, 10.3 ± 1.1 ; n = 11) and trophectoderm (60.8 ± 2.9 , n = 11) cells. These results have begun to elucidate parameters important for animal modeling and cloning with ferrets.

assisted reproductive technology, early development, embryo, mechanisms of hormone action, oocyte development

INTRODUCTION

The domestic ferret, *Mustela putorius furo*, has proven to be a useful model for investigating airway physiology and pathophysiology because of the remarkable similarity of ferret and human lung cytoarchitecture [1, 2]. Previous studies have demonstrated that ferrets and humans have identical airway cell types and a similar distribution of sub-

mucosal glands (SGs) [3, 4]. Distribution of the cystic fibrosis transmembrane conductance regulator (CFTR) in airway cell types is also similar in humans and ferrets, including a high density in SG serous cells [5]. This similarity has been exploited in the development of a ferret airway xenograft model to study, in relation to the lung pathology associated with cystic fibrosis (CF), various features of the airway, such as contribution of SGs to surface fluid ionic composition, antibacterial activity, and delivery of viral vectors for gene therapy [6, 7]. An appropriate animal model for CF lung disease is urgently needed, in particular because the CFTR knock-out mouse does not display CF phenotype in the lung. With all the comparative advantages regarding human airway and lung structure that the ferret has over the mouse, the advancement of effective therapies for CF will benefit considerably if a ferret CF model is developed.

Of small animal species, the ferret, with periods of 42 days for gestation and 6 mo to sexual maturity, has obvious advantages over larger species, such as sheep and nonhuman primates, for animal modeling. Recent successes in creating cloned animals by somatic nuclear transfer have made animal modeling in less-studied species, such as the ferret, more feasible. Previously, we reported conditions for superovulation in ferrets and in vitro culture of ferret embryos [8]. These protocols were successfully used to produce live offspring following embryo transfer [8], and they also paved the way for genetic manipulation of ferret oocytes and embryos. However, parameters critical to the success of somatic cell nuclear transfer of in vitro maturation (IVM) and parthenogenetic activation of ferret oocytes are yet to be determined. With the ultimate goal of generating a ferret CF model using nuclear transfer technologies, we performed experiments to establish the optimal conditions for IVM and artificial activation of ferret oocytes. Our results indicate that ferret oocytes matured in vitro are good candidates for parthenogenetic activation, and that both electrical stimulation and chemical treatment are needed for relatively efficient parthenogenetic activation of ferret oocytes.

MATERIALS AND METHODS

Reagents

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Animals and Housing Conditions

Female ferrets aged 6–12 mo were purchased from Marshall Farms (North Rose, NY) and were in the estrous cycle when delivered to the animal facility. All ferrets were housed in separate cages under controlled temperature (20–22°C) and a long daylight cycle (16L:8D). The use of animals in this study was carried out according to a protocol approved by the University of Iowa animal care review committee and conformed to or exceeded National Institutes of Health standards.

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²Correspondence: John F. Engelhardt, Department of Anatomy and Cell Biology, College of Medicine, University of Iowa, 51 Newton Road, Room 1-111 BSB, Iowa City, IA 52242. FAX: 319 335 6581; e-mail: john-engelhardt@uiowa.edu

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Collection of Oocytes

Immature ferret oocytes were harvested from small vesicular follicles on ovaries from superovulated ferrets. For superovulation, ferrets were given an i.p. injection of 100 IU/kg of eCG (Sigma G-4877), followed by an i.p. injection of 100–150 IU/kg of hCG (Sigma C-1063) at an interval of 72 h [8]. To retrieve oocytes, ferrets were killed by i.p. administration of sodium pentobarbital (50–100 mg/kg) 24–32 h after hCG injection. Ovaries, oviducts, and parts of uterine horns were excised and washed three times with 0.9% (w/v) saline supplemented with 1% (v/v) penicillin and streptomycin at 37–38.5°C. The fat around the ovaries was removed using tweezers and a scalpel after *in vivo*-matured oocytes were flushed from the oviducts with mPBS (Dulbecco PBS supplemented with 0.1% [w/v] D-glucose, 36 mg/L of pyruvate, and 0.4% [w/v] BSA) for structural comparison with *in vitro*-matured oocytes. The ovaries were then washed again. The small vesicular follicles (diameter, 0.5–2.0 mm) on the ovarian surface were incised with a scalpel in a Petri dish containing mPBS to release the cumulus-oocyte complexes (COCs). The number of COCs collected from each animal ranged from 10 to 35 (mean \pm SEM, 19.4 \pm 1.6).

IVM of Oocytes

It has been reported that FSH/eCG, LH/hCG, and 17 β -estradiol help to facilitate oocyte maturation *in vitro* following culturing times of 22 h for cattle [9, 10], 27 h for goats [11], and 44–50 h for pigs [12, 13]. To our knowledge, however, the effects of these hormones on IVM of ferret oocytes has not been previously reported. Based on the above reports in other large animal species, we chose two time points (24 and 48 h) for IVM of ferret oocytes. All culture media were based on TCM-199 (catalog no. 12340-030; Life Technologies, Inc., Rockville, MD) with 10% (v/v) FBS (Sigma F-4135). The control medium (medium 1) contained no added hormones. Medium 2 contained 10 IU/ml of eCG (Sigma G-4527) + 5 IU/ml of hCG (Sigma C-8554), and medium 3 contained 10 IU/ml of eCG + 5 IU/ml of hCG + 2 μ g/ml of 17 β -estradiol (Sigma E-2257). The COCs with uniform cytoplasm and several layers of cumulus cells were selected, washed three times with mPBS, and cultured in three types of media with or without hormones. In general, 20–40 COCs harvested from two to three ferrets were pooled, divided into different groups, and cultured under 500 μ l of appropriate medium in four-well Petri dishes (catalog no. 176740; Nunc, Roskilde, Denmark). The COCs cultures were covered with mineral oil (Sigma M-8410) and incubated at 38.5°C in 5% CO₂/95% air for 24 and 48 h.

Assessment of Oocyte Maturity

On completion of *in vitro* culture for 24 or 48 h in different media, COCs were removed and placed in mPBS containing 0.2% (w/v) hyaluronidase (Sigma H-4272) for 1–3 min. Cumulus cells were removed from the oocytes by pipetting. Most of the cumulus-free oocytes were then mounted on glass slides. A coverslip supported by four droplets of a mixture of vaseline and paraffin oil (9:1, v/v) was placed onto the oocytes, and gentle pressure was applied to the coverslip to hold the oocytes. Oocytes were then fixed in fixation solution (acetic acid:ethanol, 1:3, v/v) for 24–48 h and stained with 1% (w/v) orcein in 40% (v/v) acetic acid. Some of oocytes were stained with 10 μ g/ml of bisbenzimidazole (Hoechst 33342, Sigma B-2261) for 2–5 min immediately after cumulus cells were removed. Oocytes displaying a disassembled nuclear membrane, chromatin condensation, and a first polar body were considered to be mature.

Parthenogenetic Activation of Oocytes

It has been reported that parthenogenetic activation of oocytes has been induced in several species by chemical reagents, including calcium ionophore [14], strontium (Sr²⁺) [15], ethanol [16], cycloheximide [16], and 6-dimethylaminopurine (6-DMAP) [17], and by electrical stimuli [18]. To our knowledge, however, the effects of chemical agents and/or electrical stimuli on parthenogenetic activation of ferret oocytes have not been previously reported. In the present study, different electrical and chemical stimuli were evaluated either individually or in combination for their effects on the initiation of cleavage and *in vitro* developmental potential of ferret oocytes. The oocytes matured in medium 2 for 20–48 h were removed from the culture medium and placed in Ca²⁺- and Mg²⁺-free Dulbecco PBS (DPBS) containing 0.1% (w/v) BSA and 0.2% (w/v) hyaluronidase for 1–3 min. On removing cumulus cells by pipetting, only oocytes with normal morphology and uniform cytoplasm were selected for parthenogenetic activation. After stimulation, the oocytes were cultured in

medium 1 under an atmosphere of 5% CO₂/95% air at 38.5°C for 1–6 days [8]. Cultured *in vitro* for 6 days, five normal blastocysts collected from the oviducts of mated ferrets at the 1-cell stage were stained with Hoechst 33342 and propidium iodide (PI; Sigma P-4170) and then examined by fluorescence microscopy as a control.

Electrical activation. Cumulus-free ferret oocytes matured in medium 2 for 20–48 h were transferred to activation medium (0.3 M mannitol, 0.1 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM Hepes, and 0.01% [w/v] BSA) and placed between the parallel electrodes (spacing, 1 mm) in the chamber of a BTX Electro-cell Manipulator 2001 (BTX, San Diego, CA). Electrical stimulation consisted of an alternating-current pulse of 3 V for 5 sec followed by one direct-current pulse of 180 V/mm for 30 μ sec. These conditions were determined for ferret oocytes in preliminary experiments (data not shown). The control oocytes were incubated in activation medium for 2 min before being transferred to the culture medium.

Chemical activation. Cumulus-free ferret oocytes matured in medium 2 for 24 or 28 h were subjected by incubation in TCM-199 containing either 5 μ g/ml of cycloheximide (Sigma C-7689) for 5 min or 2 mM/ml of 6-DMAP (Sigma D-2629) for 4 h. Effects of these chemicals on oocyte activation were then compared to the controls that were incubated in TCM-199 for 5 min or 4 h without chemical supplement.

Combined electrical and chemical activation. Cumulus-free ferret oocytes matured in medium 2 for 24 or 28 h were subjected to a combination of electrical and chemical activation stimuli in an effort to maximize the number of oocytes induced to undergo parthenogenetic embryogenesis. The experimental paradigm consisted of electrical stimulation followed by a sequential treatment with cycloheximide and 6-DMAP as described above. Effects of these electrical and chemical activations on oocytes were then compared to the controls as described above.

Assessment of Parthenogenetic Embryonic Development

Oocytes were collected from the each of the electrical and/or chemical activation groups at various postactivation time points for analysis of the relative success of the activation paradigms in inducing cleavage and embryonic development. The developmental stages of embryos (ranging from 2-cell to blastocyst) were assessed by staining with 10 μ g/ml of Hoechst 33342 for 2–5 min at 38.5°C, followed by fluorescent microscopy. The nuclei of cleaved oocytes showed blue fluorescence using a DAPI filter. Selected blastocysts were differentially stained with 10 μ g/ml each of Hoechst 33342 and PI [19]. Briefly, the blastocysts were treated with 0.5% (w/v) pronase (Sigma P-8811) for 30–60 sec at room temperature to dissolve the zona pellucida, then incubated for 1–2 h in RD medium (1:1 mixture of RPMI-1640 and Dulbecco modified Eagle medium, high glucose; Gibco). The blastocysts were then washed and incubated in 10 mM trinitrobenzenesulfonic acid (Sigma P-2297) for 10 min on ice before washing and incubation in rabbit antiniditrophenyl antiserum (Sigma D-9656) diluted 3:5 (v/v) for 30 min at 39°C under 5% CO₂/95% air. After washing and incubation in ferret serum diluted 1:2 (v/v) (as a source of complement), they were then fixed in 2.3% (w/v) sodium citrate in ethanol (3:1, v/v) and stained with 10 μ g/ml each of Hoechst 33342 and PI in sodium citrate:ethanol for 2.5 min at room temperature. Reagents were prepared in RD medium + 0.1% (w/v) polyvinyl alcohol, and the same medium was used for washing the blastocysts. Blastocysts were transferred into a 2- to 3- μ l drop of PBS:glycerol (1:1, v/v) on a glass slide, covered with a glass coverslip, and examined by fluorescent microscopy. Both nuclei of the inner cell mass (ICM) and nuclei of the trophectoderm (TE) fluoresced blue using a DAPI filter. However, nuclei of TE fluoresced red using a N2.1 filter. When the two fluorescent channels were merged (two photos into one), nuclei of ICM fluoresced blue; however, nuclei of TE fluoresced pink.

Parthenogenetic development of stimulated oocytes was assessed by microscopic observation at 24-h intervals, and samples were collected at various time points for staining to determine their developmental stages. Oocytes that cleaved with two or more nuclei after receiving artificial stimulation were considered to be activated, whereas all sham-stimulated (control) oocytes were arrested at the 1-cell stage.

Statistical Analysis

Before statistical analysis, raw data sets were transformed to percentages, and the mean percentages (\pm SEM) were calculated for each group. The normality assumption of the percentages for each data set was checked by the Kolmogorov-Smirnov test using statistical Minitab 13 software (Minitab Inc., State College, PA). In addition, the Bartlett test (also using Minitab 13 software) was performed to justify the equal variance assumption of the percentages for each data set. When either of these tests sug-

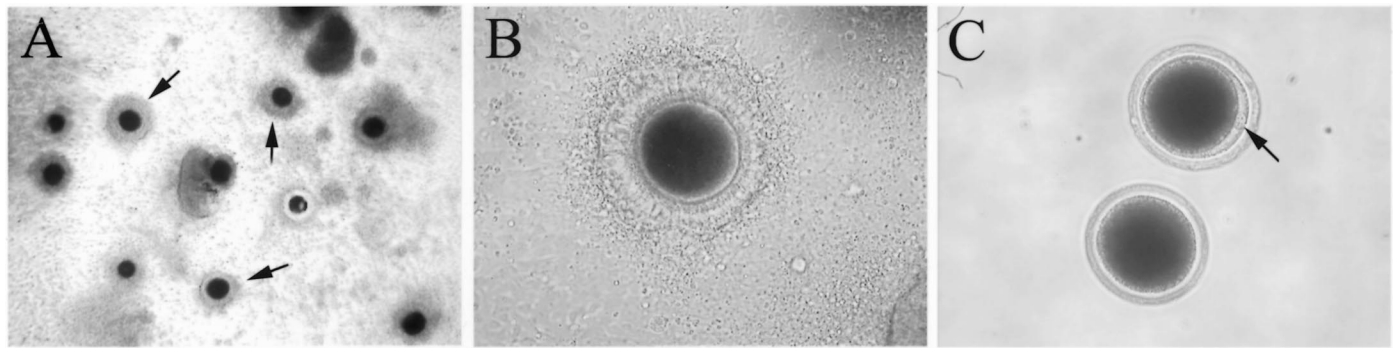


FIG. 1. Ferret oocytes cultured in vitro. **A**) Ferret COCs (arrows) harvested from ovarian follicles were cultured in medium 2 for 24 h. **B**) A single COC showing dispersion of cumulus cells from an oocyte (enlarged from **A**). **C**) Oocytes with or without the first polar body (PB1; arrow) after the cumulus cells were removed. Magnification $\times 40$ (**A**) and $\times 100$ (**B** and **C**).

gested that one of the two assumptions was not valid, arcsine transformation of percentages was performed, and both assumptions on the transformed variables were confirmed by re-evaluating the data by the Kolmogorov-Smirnov and Bartlett tests before statistical analysis. One-way ANOVA was used for statistical analysis of data sets with one independent variable (i.e., evaluating effects of media on IVM in Tables 1 and 2, and evaluating effects of time on development in Table 3). Two-way ANOVA was used for statistical analysis of data sets with two independent variables (i.e., evaluating effects of time and chemicals on development in Tables 4 and 5). When ANOVA demonstrated a significant difference, the follow-up Tukey multiple comparison test was performed to determine P values for all possible two-group comparisons within the data set. The Student t -test was applied to data sets with only two groups (i.e., Table 6). For all statistical analyses including ANOVA, Tukey, and Student t -tests, a difference was considered to be significant at $P < 0.05$. All statistical analyses were performed using the SAS 8.0 statistical software (SAS Institute Inc., Cary, NC).

RESULTS

Effects of Hormones on IVM of Ferret Oocytes

The initial experiment was designed to examine the effects of hormones in the culture media on IVM of ferret oocytes. The COCs (Fig. 1A) harvested from small vesicular follicles were cultured in TCM-199 + 10% FBS containing varied amounts of eCG, hCG, and/or 17β -estradiol for 24 h (Table 1) or 48 h (Table 2) as described in *Materials and Methods*. On completion of in vitro culture, COCs were removed from the culture medium, and cumulus cells were dislodged from the oocytes (Fig. 1, B and C). The cumulus-free oocytes were then fixed for 24 h and stained with 1% orcein (Fig. 2, A–C) or Hoechst 33342 (Fig. 2D) to determine the state of maturation.

Results from this analysis indicated that the percentage of mature oocytes cultured in medium 2 (70.1%, $n = 79$), which contained eCG and hCG, was significantly higher ($P < 0.01$) than the percentage of those cultured in either medium 1 (27.9%, $n = 73$) without added hormones or in

medium 3 (36.6%, $n = 67$), which contained $2 \mu\text{g/ml}$ of 17β -estradiol in addition to eCG and hCG, after 24 h of IVM (Table 1). In contrast to the results seen after 24 h of in vitro culture, no statistical differences were observed in all three kinds of maturation media (Table 2) after 48 h of IVM (56.2%–69.9%, $n = 76$ –87). These efficiencies of 48-h IVM did not differ significantly ($P > 0.05$) from that obtained at 24 h for medium 2. Doubling the incubation time caused a significant increase in maturation rates for both medium 1 (from 27.9% to 56.2%, $P < 0.01$, $n = 73$ –87) and medium 3 (from 36.6% to 69.9%, $P < 0.01$, $n = 67$ –76).

Electrical Activation of Ferret Oocytes

The next series of experiments was designed to examine the response of ferret oocytes matured in vitro to electrical and chemical artificial activation stimuli. Based on the findings described above, oocytes for the artificial activation experiments were matured in medium 2 for 20–48 h, because the maturation rate was significantly faster in medium 2 than in the other two media. The results showed that cleavage rates following electrical stimulation among in vitro-matured oocytes demonstrated a general increase as the IVM time was prolonged, from 21.3% ($n = 39$) at 20 h to 68.1% ($n = 41$) at 48 h (Table 3). Similar trends were observed for the percentage of electrically pulsed oocytes that developed to either morulae or blastocysts as a function of IVM time. In general, the best activation efficiency by electrical stimulation was achieved with oocytes matured in vitro for 48 h, with 68.1%, 32.7%, and 10.2% of oocytes ($n = 41$) developing to cleavage initiation, morulae, and blastocysts, respectively. However, given that blastocyst development was not significantly different between 28- and 48-h IVM, these results suggest that electrical activation is

TABLE 1. In vitro maturation of ferret oocytes in three different media for 24 h.

Culture media	No. of replicates	No. of oocytes examined	No. of mature oocytes	% Of mature oocytes (mean \pm SEM)*
Medium 1: TCM-199 + 10% FBS	6	73	20	27.9 \pm 2.2 ^a
Medium 2: TCM-199 + 10% FBS + 10 IU/ml of eCG + 5 IU/ml of hCG	6	79	56	70.1 \pm 3.7 ^b
Medium 3: TCM-199 + 10% FBS + 10 IU/ml of eCG + 5 IU/ml of hCG + 2 μg of 17β -estradiol	6	67	25	36.6 \pm 3.4 ^a

* One-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. Differences among percentages containing the different superscripted letters are significant ($P < 0.01$).

TABLE 2. In vitro maturation of ferret oocytes in three different media for 48 h.

Culture media	No. of replicates	No. of oocytes examined	No. of matured oocytes	% Of matured oocytes (mean \pm SEM)*
Medium 1: TCM-199 + 10% FBS	7	87	48	56.2 \pm 3.2 ^a
Medium 2: TCM-199 + 10% FBS + 10 IU/ml of eCG + 5 IU/ml of hCG	7	85	56	65.7 \pm 4.8 ^a
Medium 3: TCM-199 + 10% FBS + 10 IU/ml of eCG + 5 IU/ml of hCG + 2 μ g/ml of 17 β -estradiol	7	76	52	69.9 \pm 6.1 ^a

* One-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. Differences among percentages containing the same superscripted letter are not significant ($P > 0.05$).

most effective when performed after at least 28 h of maturation in medium 2.

Chemical Activation of Ferret Oocytes

Because results from electrical stimulation alone appeared to be suboptimal for somatic cell nuclear cloning, with only 6%–10% maximal development to the blastocyst stage, we next evaluated alternative approaches for activation using chemical treatment regimes. Strategies using cycloheximide and/or 6-DMAP have been successful in supporting or augmenting parthenogenetic activation in other species, such as mouse, cow, and *Xenopus* [20–22]. To this end, oocytes matured in vitro for 24 and 28 h were treated with cycloheximide (5 μ g/ml, 5 min) or 6-DMAP (2 mM/ml, 4 h), and parthenogenetic activation was assessed.

The rates of induced oocyte cleavage by either cycloheximide or 6-DMAP ranged from 34.8% ($n = 58$) to 53.8% ($n = 59$) and were not significantly different ($P > 0.05$) when either 24- or 28-h in vitro-matured oocytes were used (Table 4). Neither cycloheximide nor 6-DMAP treatment alone induced efficient activation, as demonstrated by the low percentages of oocytes that reached the morula or blastocyst stage under all conditions tested. Treatment with cycloheximide at 24 h post-IVM resulted in 5.7% and 3.1%

of the oocytes ($n = 58$) developing to morulae and blastocysts, respectively (Table 4). Of oocytes ($n = 59$) matured in vitro for 28 h, 7.1% developed to morulae and 5.9% to blastocysts after 4 h of treatment with 6-DMAP. Interestingly, the efficiency of development to the morula and blastocyst stages was significantly ($P < 0.05$) influenced by the time of IVM and chemical used for activation, with no oocyte development to these stages in the 24-h (6-DMAP) or 28-h (cycloheximide) treatment groups, respectively (Table 4).

Activation of Ferret Oocytes by a Combination of Electrical and Chemical Stimuli

Although the results presented above illustrate that electrical or chemical stimuli were each successful in inducing ferret oocyte activation, the efficiency of each type of stimulation alone was low. In an effort to increase the efficiency of activation, the effect of treating ferret oocytes with both cycloheximide and 6-DMAP after the electrical stimulus was evaluated. For these experiments, ferret oocytes matured for 24 or 28 h in medium 2 were electrically stimulated according to the parameters described above and then incubated sequentially in cycloheximide and 6-DMAP. As shown in Table 5, stimulation with an electrical pulse fol-

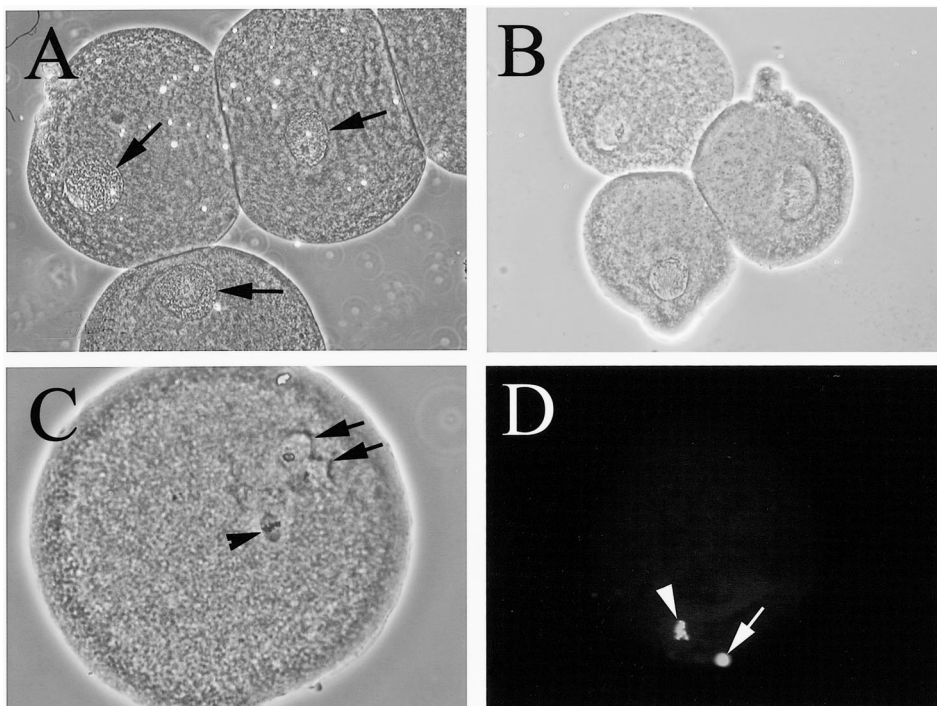


FIG. 2. Assessment of ferret oocyte maturity following IVM. Morphologic assessment of oocyte maturity following 24- to 48-h culture in medium 2 was performed by staining with 1% orcein (A–C) or 10 μ g/ml of Hoechst 33342 (D). A) Immature oocytes showing germinal vesicles (GV; arrows). B) Oocytes showing various stages of maturation from GV to germinal vesicle breakdown (GVBD). C) A mature oocyte showing condensed chromosomes (arrowhead) and polar bodies (PB; arrows). D) A mature oocyte stained with Hoechst 33342 showing condensed chromosomes (arrowhead) and PB1 (arrow). Magnification $\times 150$ (A), $\times 120$ (B), $\times 250$ (C), and $\times 130$ (D).

TABLE 3. Electrical activation of in vitro-matured ferret oocytes.

IVM (h)	No. of replicates	No. of oocytes examined	No. (mean % \pm SEM) of oocytes developed to*		
			Cleavage	Morula	Blastocyst [†]
20	4	39	8(21.3 \pm 2.6) ^a	6(14.4 \pm 2.8) ^a	0(0) ^a
24	4	37	13(35.8 \pm 1.6) ^{ab}	7(21.2 \pm 4.3) ^a	1(2.3 \pm 2.3) ^{ab}
28	4	41	18(42.6 \pm 3.5) ^b	9(23.5 \pm 4.1) ^a	3(6.4 \pm 2.2) ^{bc}
40	4	45	20(46.2 \pm 6.9) ^b	10(24.8 \pm 5.1) ^a	3(8.6 \pm 4.2) ^{bc}
44	4	47	23(50.2 \pm 4.0) ^b	12(27.3 \pm 5.0) ^a	4(9.9 \pm 4.2) ^{bc}
48	4	41	28(68.1 \pm 3.5) ^c	13(32.7 \pm 4.9) ^a	4(10.2 \pm 4.1) ^{bc}

* One-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. In the same column, differences among percentages containing the different superscripted letters are significant ($P < 0.05$).

[†] Blastocyst data points were transformed to arcsine values before statistical analysis.

lowed by treatment with cycloheximide and 6-DMAP markedly increased the percentage of oocytes developing to the cleavage, morula, and blastocyst stages in comparison to treatment with the electrical pulse alone. Although all increases at each stage were statistically significant ($P < 0.01$), the most notable enhancement resulting from combined electrical stimulation followed by chemical treatment occurred in oocytes that developed to the blastocyst stage (14- and 6-fold increases over electrical stimulation alone at 24 and 28 h of IVM, respectively). In addition, the increased activation seen following combined electrical and chemical stimulation was not dependent on the duration of IVM, because statistically equivalent numbers of oocytes ($n = 58$ – 65) developed to morulae (53.2% and 60.6%) or blastocysts (36.7% and 43.8%) following either 24 h or 28 h of IVM, respectively.

Normal Blastocyst Cytoarchitecture Is Retained by In Vitro-Matured, Artificially Activated Ferret Oocytes

To evaluate the cytoarchitecture of artificially activated oocytes, we performed differential staining with Hoechst 33342 and PI. This differential staining allows for the de-

termination of ICM and TE nuclei, which contribute to the total cellular mass of the blastocyst. Using this differential staining, all nuclei in the blastocyst stain positive with Hoechst and fluoresce blue, whereas only TE cells stain with PI and fluoresce red. When the two fluorescent channels are merged, nuclei in the ICM fluoresce blue, whereas the nuclei from TE cells fluoresce pink. Following development in vitro for 6 days, 11 blastocysts derived from activated in vitro-matured oocytes and five blastocysts from 1-cell stage embryos collected from the oviducts of mated ferrets were stained with Hoechst 33342 and PI. Stained blastocysts were examined by fluorescence microscopy (Fig. 3, B-D), and the ICM and TE cell counts were determined. The results (Table 6) demonstrated no statistical difference in the total, ICM, or TE nuclear counts.

DISCUSSION

Many reports have appeared stating that FSH/eCG, LH/hCG, and 17 β -estradiol in culture media facilitate oocyte maturation in vitro [9–13]. To our knowledge, however, the effects of these hormones on IVM of ferret oocytes have not been previously reported. The present study was carried

TABLE 4. Activation of in vitro-matured ferret oocytes with cycloheximide or 6-DMAP.

IVM (h)	No. of replicates	No. of oocytes subjected to*		No. (mean % \pm SEM) of oocytes developed to [†]		
		Cycloheximide	6-DMAP	Cleavage	Morula [‡]	Blastocyst [‡]
24	4	58	—	20(34.8 \pm 5.3) ^a	3(5.7 \pm 2.3) ^b	2(3.1 \pm 1.8) ^{ab}
	4	—	54	22(41.9 \pm 4.8) ^a	0(0) ^a	0(0) ^a
28	4	55	—	23(42.1 \pm 5.6) ^a	0(0) ^a	0(0) ^a
	4	—	59	31(53.8 \pm 5.5) ^a	4(7.1 \pm 0.9) ^b	3(5.9 \pm 2.0) ^b

* Cycloheximide at 5 μ g/ml for 5 min and 6-DMAP at 2 mM/ml for 4 h.

[†] Two-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. In the same column, differences among percentages containing the different superscripted letters are significant ($P < 0.05$).

[‡] Morula and blastocyst data were transformed to arcsine before statistical analysis.

TABLE 5. Activation of in vitro-matured oocytes using electrical pulses with or without chemical reagents.

IVM (h)	No. of replicates	No. of oocytes subjected to electrical pulses*		No. (mean % \pm SEM) of oocytes developed to [†]		
		Without cycloheximide & 6-DMAP	With cycloheximide & 6-DMAP	Cleavage	Morula	Blastocyst
24	4	37	—	13(35.8 \pm 1.6) ^a	7(21.2 \pm 4.3) ^a	1(2.3 \pm 2.3) ^a
	5	—	65	55(83.7 \pm 3.9) ^b	36(53.2 \pm 4.6) ^b	24(36.7 \pm 4.2) ^b
28	4	41	—	18(42.6 \pm 3.5) ^a	9(23.5 \pm 4.1) ^a	3(6.4 \pm 2.2) ^a
	5	—	58	50(85.8 \pm 4.7) ^b	35(60.6 \pm 5.5) ^b	25(43.8 \pm 5.4) ^b

* Electrical pulse: one AC pulse of 3 V for 5 sec followed by one DC pulse of 180 V/mm for 30 μ sec. Chemicals: cycloheximide at 5 μ g/ml for 5 min and 6-DMAP at 2 mM/ml for 4 h.

[†] Two-way ANOVA and the follow-up Tukey multiple comparison test were used to assess significant difference among groups. In the same column, differences among percentages containing the different superscripted letters are significant ($P < 0.01$).

out to examine the effects of eCG, hCG, and 17 β -estradiol in culture media on ferret oocyte maturation. Our results indicate that the maximum oocyte maturation rate during the first 24 h of culture in vitro was seen with TCM-199 medium supplemented with 10% FBS, 10 IU/ml of eCG, and 5 IU/ml of hCG. Exclusion of added hormones or a further addition of 17 β -estradiol to the culture medium significantly decreased the maturation rate over the same period of time. When assessed at 48 h, however, all media tested resulted in maturation rates comparable to the maximum rate achieved at 24 h. These results suggest that eCG and hCG have a significant effect on accelerating maturation of ferret oocytes during the first 24 h of culture in vitro. However, 17 β -estradiol appears to inhibit maturation of ferret oocytes during the first 24 h of culture, which seems to be different from its effects in cattle [9, 10], goats [11], and pigs [12, 13]. This difference may be due to species variation in oocyte biology. Because FSH/eCG and LH/hCG receptors are present on cumulus cells [23–26], eCG and hCG added to the culture medium may elicit their effects via cumulus-mediated interactions.

Both nuclear maturation and cytoplasmic maturation of oocytes are necessary for sperm penetration (i.e., fertilization) and oocyte activation. The nuclear maturation of oocytes can be characterized by morphologic changes. However, cytoplasmic maturation is not easily evaluated using this criterion. Because parthenogenetic activation provides a useful assessment regarding the quality of oocytes matured in vitro [27], further experiments were carried out to evaluate the response of the in vitro-matured ferret oocytes to artificial activation. In addition, artificial activation of mammalian oocytes is required for producing embryos by nuclear transplantation to generate cloned animal models. It is reported that parthenogenetic activation of oocytes has been induced in several species by chemical reagents, including calcium ionophore [14], strontium (Sr²⁺) [15], ethanol [16], cycloheximide [16], and 6-DMAP [17], and by electrical stimuli [18]. Despite these advances, oocyte activation remains one of the least efficient steps in the nuclear transplantation procedure, and to our knowledge, activation conditions for ferret oocytes have not been reported. In this study, ferret oocytes cultured in vitro for 20–48 h were initially tested for activation following electrical or chemical treatment. In a third series of activation experiments, electrical pulses were combined with cycloheximide and 6-DMAP treatments. Following electrical stimulation, the cleavage rates of ferret oocytes exhibited an age-dependent increase in the occurrence of cleavage and development to the morula and blastocyst stages. It has been reported that the activation responses of oocytes to various stimuli vary with maturational age. Bovine oocytes activate in response to ionophore A23187 treatment, electrical pulse, or ethanol treatment in an age-dependent manner [14, 28]. Electrically stimulated porcine oocytes show the same trend [29]. Our results support these findings.

Under the culture and stimulation conditions utilized in our experiments, neither an electrical nor a chemical stim-

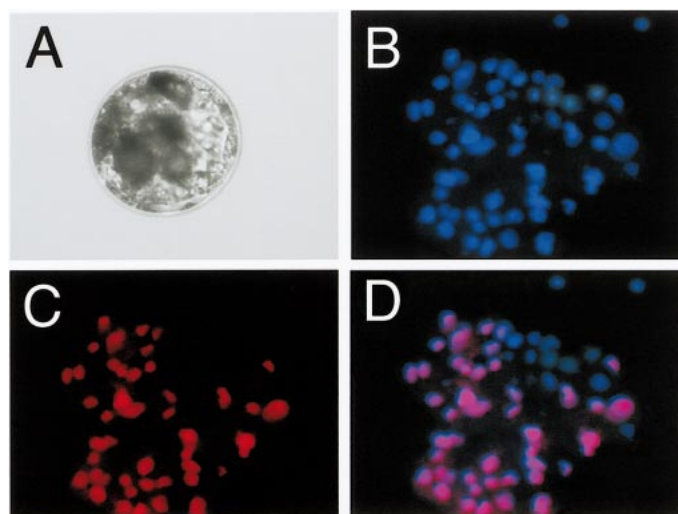


FIG. 3. Differential staining of ferret blastocysts with nuclear dyes. A blastocyst developed from an activated oocyte before (A) and after (B–D) Hoechst 33342 and PI differential staining is shown in phase-contrast image (A) and fluorescent (B–D) photomicrographs. A) The blastocyst before staining. B) Total cells of the blastocyst appear blue using a DAPI filter. C) TE cells appear red using a N2.1 filter. D) Merged DAPI and N2.1 images. The ICM cells appear blue, whereas the TE cells appear pink. Magnification $\times 110$ (A) and $\times 200$ (B–D).

ulus, acting by itself, activated in vitro-matured ferret oocytes at a high efficiency. However, exposing oocytes to cycloheximide and 6-DMAP in a sequential manner following electrical stimulation significantly ($P < 0.01$) increased the efficiency of oocyte activation and development to the blastocyst stage from 6.4% ($n = 41$, no chemicals) to 43.8% ($n = 58$, with chemicals) at 28 h of IVM. These results suggest that both electrical and chemical stimuli are needed for high activation efficiency of in vitro-matured ferret oocytes. It has been reported that electric pulses permeabilize the cell membrane, thereby inducing an influx of extracellular calcium into the cytoplasm [30] and a rise in calcium oscillations in oocytes [31]. Cycloheximide inhibits protein synthesis without causing any intracellular calcium changes [20], and 6-DMAP inactivates maturation-promoting factor (i.e., H1 histone kinase) but has no effect on overall protein synthesis or intracellular calcium changes [21]. Our results using both electrical and chemical stimuli for activation of IVM ferret oocytes suggest that these in vitro stimuli seem to act synergistically, because the overall activation efficiency was considerably higher than the sum of the rates of activation following individual stimuli. The differential staining of blastocysts ($n = 11$) derived from activated oocytes indicated that counts of total cells (71.1 ± 3.4), ICM cells (10.3 ± 1.1), and TE cells (60.8 ± 2.9) were similar to counts for blastocysts produced in vivo. This is indicative of the good quality of ferret embryos derived from IVM and activation.

In conclusion, the data presented here have begun to

TABLE 6. Cellular counts of ICM and TE in normal and artificially activated blastocysts.

Group	No. of blastocysts examined	Total cells (mean \pm SEM)	ICM cells (mean \pm SEM)	TE cells (mean \pm SEM)
Artificially activated	11	71.1 \pm 3.4 ^a	10.3 \pm 1.1 ^a	60.8 \pm 2.9 ^a
Normal	5	78.2 \pm 3.9 ^a	15.6 \pm 1.7 ^a	62.6 \pm 4.9 ^a

^a In the same column, differences among these groups are not significant ($P > 0.05$) as determined using the Student *t*-test.

develop IVM and artificial activation conditions for use in the development of ferret embryos by nuclear transplantation. For establishing these conditions, it was deemed to be advantageous to minimize the time in culture for maturation. The results indicate 28 h of IVM of ferret oocytes in medium 2, followed by artificial activation (combining electrical stimulation with chemical stimulation using cycloheximide and 6-DMAP), provides efficient conditions for embryonic development of immature ferret oocytes to a stage compatible with nuclear transfer and subsequent transplantation of embryos to a surrogate mother. These experiments, combined with our previous successful production of live young from transplanted ferret embryos, support the feasibility of somatic cell nuclear cloning of ferrets.

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