—Research Note—

Effect of Cycloheximide on *In Vitro* Development of Electrically Activated Feline Oocytes

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Abstract. This study was conducted to improve parthenogenetic development *in vitro* of feline oocytes following a combined activation treatment of electrical stimulation and cycloheximide. *In vitro* matured (IVM) oocytes were stimulated electrically by a DC electrical pulse of 2 kV/cm for 50 μ s. The stimulated oocytes were then incubated in MK-1 medium with or without cycloheximide and subsequently cultured *in vitro* for 6 days. No significant differences were observed between the two groups with respect to the proportions of cleavage, development to the morula stage, and the cell number of blastocysts. However, exposure of electrically stimulated oocytes to cycloheximide significantly increased the rate of development of the stimulated oocytes into the blastocyst stage compared with oocytes stimulated by electrical stimulation alone (31.0% vs 6.7%). The results from the present study suggested that a single electrical stimulation was insufficient to activate the IVM cat oocytes at 24 h of maturation and that exposure to cycloheximide following electrical stimulation improved the efficacy of the parthenogenetic development of domestic cat oocytes.

Key words: Cat, Electric field strength, Nuclear transfer, Parthenogenetic activation

(J. Reprod. Dev. 51: 783–786, 2005)

Establishing an effective artificial stimulation to activate oocytes and elicit their development is essential for the success of animal cloning by nuclear transfer (NT). Induction of proper activation in the nuclear transferred oocyte is considered to be essential for complete reprogramming of the donor nucleus because the activating signal from the sperm is absent. During

fertilization, sperm-induced oocyte activation is triggered by intracellular calcium oscillations [1]. This stimulus triggers the inactivation of maturation promoting factor (MPF), composed of cyclin B and p34^{cdc2}, which is present at high levels in metaphase II oocytes [2]. Therefore, artificial oocyte activation commonly includes the combination of an agent responsible for the increase of intracellular calcium and an inhibitor of MPF activity [3–5]. Oocyte activation procedures have been used successfully in several species that

combine a transient inhibition of MPF, induced by an increase of Ca^{2+} [6, 7] with a persistent inhibition of MPF, induced by the addition of protein synthesis inhibitors (cycloheximide) [8, 9] or nonspecific kinase inhibitor [3, 5]. However, since suitable conditions for activation *in vitro* vary with animal species, understanding of the events involved in oocyte activation for each family or genera of animals would offer a meaningful impact on the efficacy of somatic cell nuclear transfer procedures. At present, to our knowledge, little information is available concerning the parthenogenetic activation conditions for feline oocytes. Therefore, in an effort to develop a reliable and efficient method to activate recipient oocytes for nuclear transfer in the domestic cat, the present study was conducted to investigate the effects of electrical stimulation alone and when combined with cycloheximide on the *in vitro* developmental ability of feline oocytes after treatment.

Materials and Methods

Recovery and in vitro maturation of oocytes

Feline oocytes were matured according to procedures previously described by Karja *et al.* [10]. In brief, ovaries were obtained from local veterinary clinics following routine ovariohysterectomy. Ovaries were kept in physiological saline at 35 °C before oocyte recovery. Each ovary was sliced repeatedly with a scalpel blade to release cumulus-oocyte complexes (COCs) in a 90-mm culture dish containing modified-PBS (mPBS; Embryotech, Nippon Zenyaku Kogyo, Japan). Only COCs exhibiting uniform, darkly-pigmented ooplasm and an intact cumulus cell investment were used for further culture. The COCs were cultured in a 100- μl drop of maturation medium, consisting of tissue culture medium (TCM) 199 with Earle's salts (Gibco, Grand Island, NY, USA), supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), 0.1 IU/ml human menopausal gonadotropin (Teikokuzoki, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Teikokuzoki), 1 $\mu\text{g}/\text{ml}$ 17 β -estradiol (Sigma), and 50 $\mu\text{g}/\text{ml}$ gentamicin (Sigma). All cultures were performed at 38 °C in a humidified incubator containing 5% CO_2 in air.

Oocyte activation

Electrical stimulation to induce oocyte activation was delivered by a BTX 2001 (BTX, San Diego, CA) and monitored using a BTX Optimizor 500-Graphic Pulse Analyzer in a chamber with two parallel platinum wire electrodes spaced 1 mm apart covered with Zimmerman medium [11]. After 24 h of *in vitro* maturation, cumulus cells were removed from the oocytes by repeated pipetting. Only oocytes with the first polar body were used for the experiment. The oocytes were then transferred to Zimmerman medium and placed between the parallel electrodes of the chamber. A single DC pulse of 2 kV/cm for 50 μs was used for electrical stimulation. After a 5-min recovery, stimulated oocytes were then divided and allocated equally into two groups, either incubated in 10 $\mu\text{g}/\text{ml}$ cycloheximide (Sigma) in MK-1 [12] medium for 5 h or in MK-1 medium only.

In vitro culture of stimulated oocytes

Stimulated oocytes were cultured in MK-1 medium supplemented with 0.4% (w/v) BSA (Day 0). At 72 h of culture, all cleaved embryos were transferred to fresh MK-1 medium supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco) for an additional 3 days to evaluate their ability to develop to the morula and blastocyst stages.

Assessment of total cell number

Blastocysts derived from Day 6 of culture were fixed with 3.7% paraformaldehyde in PBS containing 1% (v/v) triton X-100 (Sigma) at room temperature for 15 min, and then placed in PBS supplemented with 0.3% (w/v) polyvinylpyrrolidone (PVP; Sigma). Embryos were then placed in a drop of mounting medium on a slide. The mounting medium consisted of 90% (v/v) glycerol containing 1.9 μM Hoechst 33342 (Sigma). A coverslip was placed on top of the embryos and the staining of the nuclei was visualized with a fluorescence microscope.

Statistical analysis

Each experiment was repeated five times. The results were pooled and then tested by Chi-square analysis with a Yates' correction. When some expected values were ≤ 5 , Fisher's exact probability test was used. The mean cell number of blastocysts was analyzed by Student's *t*-test. Differences at a probability of $P < 0.05$ were considered significant.

Table1. *In vitro* development of feline oocytes activated either by electrical stimulation only (None) or combined with cycloheximide (CYX)

Treatment	No. of oocytesx examined	No. (%) of cleaved oocytes	No. (%) of embryos developed to		Mean cell (No. $\pm$ SEM) of blastocysts
			Morula	Blastocyst	
None	45	34 (75.6)	22 (48.9)	3 (6.7) ^a	51.7 $\pm$ 4.1
CYX	42	36 (85.7)	22 (52.4)	13 (31.0) ^b	74.6 $\pm$ 6.3

^{a,b}Values with different superscript letters are significantly different ($P < 0.05$).

Fig.1. Developed cat embryos at the morula stage on Day 5 (a) and at the blastocyst stage on Day 6 (b) of *in vitro* culture (magnification 200 $\times$).

Results

No significant differences were observed between the two groups with respect to the proportions of cleavage, development to the morula stage, and the number of cells in blastocysts. However, exposure of electrically activated oocytes to cycloheximide significantly improved their development into the blastocyst stage compared with oocytes activated by electrical stimulation alone (31.0% vs 6.7%, $P < 0.01$) (Table 1). Representative developed cat embryos at the morula stage (Day 5) and blastocyst stage (Day 6) are shown in Fig.1.

Discussion

In the present study, we demonstrated that the combination of electrical stimulation and cycloheximide treatment increases the developmental ability of feline oocytes into the blastocyst stage. The positive effect of a protein synthesis inhibitor was also observed in pig oocytes

after their activation using an electrical pulse [4]. These results, including those of the present study, indicate that a single calcium stimulation induced by an electrical pulse can not adequately activate mature oocytes. It is known that electrical stimulation induces an increase of the intracellular free calcium concentration, which is triggered by an influx of extracellular Ca^{2+} immediately after electrostimulation [13]. However, the elevation of Ca^{2+} induces a temporal inactivation of MPF and is followed by an immediate reactivation of MPF to preactivation levels with the consequent cell cycle arrest [7, 14, 15]. Therefore, a specific stimulation in addition to the electrical stimulation treatment was required for full oocyte activation and to support their subsequent development. Moreover, in *Xenopus* oocytes, it is known that the presence of cytosolic factor (CSF) contributes to the inhibition of cyclin protease [4]. The inhibition of CSF or cyclin B following parthenogenetic activation will increase the incidence of oocyte activation. It has been suggested that the inhibition of protein synthesis prevents synthesis of CSF, which seems to be linked with MPF [16]. These phenomena also

support our results and explain our high activation rate following the combined electric stimulation and cycloheximide treatment. A single Ca^{2+} peak induced by the electric pulse would destroy the existing CSF [7], whereas the following cycloheximide treatment would prevent the renewal (new synthesis) of CSF in the oocyte. These reduced levels of CSF may result in degradation of cyclin B, a part of the MPF [17]. A high level of MPF is known to be essential for meiotic arrest of mammalian oocytes, and thus its degradation would induce resumption of meiosis or activation of oocytes.

However, several factors are believed to influence an oocyte's response to the electrical activation, including age [18], the applied voltage field strength, the pulse number [19], and the duration [7]. Therefore, further experiments are needed to elucidate the interactions of these factors.

In conclusion, the results from the present study suggest that culture in cycloheximide following the electrostimulation induces parthenogenetic activation in domestic cat oocytes at a high rate, and this activation method may be useful in a NT program.

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