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FULL PAPER

Effects of cycloheximide treatment on *in-vitro* development of porcine parthenotes and somatic cell nuclear transfer embryos

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Abstract

This study aimed to verify the beneficial effect of cycloheximide (CHX) treatment on the development of porcine somatic cell nuclear transfer (NT) embryos, which were constructed with enucleated oocytes and cumulus cells by using a single direct current (DC) pulse. In the first experiment, a single DC pulse applied to the induction of fusion and activation of NT embryos gave a high fusion rate. However, cleavage and subsequent development of fused couplets (NT embryos) to the blastocyst stage were poor. Experiment 2 was conducted to determine whether CHX treatment could enhance metaphase II (M II) oocyte activation and improve the subsequent parthenogenetic development. After giving the DC pulse and incubation with or without CHX, M II oocytes incubated with CHX showed higher cleavage and development to blastocysts compared with those incubated without CHX ($P<0.05$). Experiment 3 was carried out to verify the beneficial effect of CHX on the development of NT embryos. The NT embryos treated with the DC pulse and CHX treatment showed higher cleavage and subsequent development compared with those treated with the DC pulse alone ($P<0.05$). The present study demonstrates that CHX treatment enhances the electrical stimulus-induced activation of oocytes and NT embryos, and improves the subsequent development of parthenotes and NT embryos. The results indicate that protein synthesis inhibition treatment required for the induction of oocyte activation promotes the development of NT embryos.

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Introduction

Since the birth of the famous cloned sheep "Dolly" was reported by Wilmut *et al*. (1997)[37], cloned offspring has been produced by using somatic cell nuclear transfer (NT) in cattle[13], goats[2], pigs[3,22,27], rabbits[7] and cats[30]. Somatic cell NT has also been successfully applied to the production of transgenic sheep[28], cattle[8], goats[14] and pigs[18,23,24]. In the pig, transgenic technology is a valuable tool for the generation of animal models for human diseases and production of organs for xenotransplantation. In most successful porcine somatic cell NT protocols used for the production of cloned piglets, NT embryos at the 1- to 8-cell stages were transferred to the foster mothers (recipient animals)[4,22,24,27]. The transfer of transgenic NT embryos at the earlier developmental stages limits the monitoring of the expression of the foreign genes, which were transfected into the nuclear donor cells, compared with that at the morula and blastocyst stages. However, the *in-vitro* development of porcine somatic cell NT embryos to the blastocyst stage is very low[4,16,17,33,34]; therefore, there is a necessity to improve the *in-vitro* development of porcine somatic cell NT embryos to the blastocyst stage.

Many factors may affect the development of NT embryos such as genotype/cell line of nuclear donor cells, cell-cycle stages of nuclear donor cells and recipient oocytes, and methods of nuclear transfer including fusion/activation protocols[10]. Cycloheximide (CHX), a protein synthesis inhibitor, was reported to be effective on the induction of activation of porcine metaphase II (M II) oocytes when combined with electric stimulation[21], calcium ionophore A23187 or ethanol[5]. Our previous study[12] confirmed the effectiveness of the CHX treatment combined with an electrical stimulation for the induction of activation (pronuclear formation) of porcine young M II oocytes. Porcine NT embryos treated with an electrical stimulus followed by a CHX treatment showed a higher development compared with those treated with an electrical stimulus and calcium ionophore A23187[6]. However, a beneficial effect of CHX treatment in combination with an electrical stimulation on the development of porcine parthenotes and NT embryos to the blastocyst stage has not been confirmed.

In the present study, therefore, we treated porcine M II oocytes and NT embryos with an electrical stimulus combined with and without CHX to verify the beneficial effect of CHX treatment on the induction of activation of oocytes and NT embryos and on the subsequent development to the blastocyst stage.

Materials and Methods

Oocyte preparation

Abattoir-derived ovaries from prepubertal gilts were transported to the laboratory in a thermal flask with sterile physiological saline at 37℃ within 2 hr of slaughter. Oocyte-cumulus-granulosa cell complexes (OCGCs) were collected from 4- to 6-mm antral follicles by a dissection method[6]. Briefly, dissected non-atretic follicles with notable blood irrigation were kept in 25 mM Hepes-buffered TCM-199 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 0.1% polyvinyl alcohol (Sigma) at 30-35℃. Follicles were opened under a stereomicroscope, and OCGCs were released by scraping the inner surface of follicle wall with a pair of forceps. OCGCs were cultured in a 100-μl drop (5 OCGCs in each drop) of bovine serum albumin (BSA)-free

North Carolina State University Medium 23 (NCSU-23)[26] supplemented with 0.57 mM cysteine (Sigma), 10% porcine follicular fluid, 10 IU/ml equine chorionic gonadotropin (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Teikoku Hormone) and 50 μg/ml gentamicin sulfate (Sigma) covered with paraffin oil for 20 hr under a humidified atmosphere of 5 % CO_2 in air at 39℃. This was followed by the culture in the same medium without hormones for additional 22-24 hr. After the maturation culture, oocytes were freed of granulosa/cumulus cells by vortexing the OCGCs for 4 min in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (DPBS) containing 0.1% hyaluronidase (Sigma), and the oocytes with the first polar bodies (M II oocytes) were selected.

Oocyte enucleation

Matured-cumulus-free M II oocytes were mechanically enucleated as described previously[1]. In brief, the first polar body and around 20% of the adjacent cytoplasm were removed using a pipette under an inverted microscope (Eclipse TE 300, Nikon, Tokyo, Japan) in Hepes-buffered TCM-199 supplemented with 5 μg/ml cytochalasin B (Sigma) and 10% fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY, USA). After the enucleation, cytoplasts were incubated in Ca^{2+}- and Mg^{2+}-free DPBS supplemented with 5 μg/ml Hoechst 33342 and 3 mg/ml BSA for 20 min at 39℃. Enucleation was confirmed by exposing the oocytes to ultraviolet light for a few seconds under an inverted microscope equipped with an epifluorescence (TE-FM, Nikon) and UV-1A filter block (365 nm excitation and 400 nm emission).

Preparation of nuclear donor cells

Primary cultures of cumulus cells were established using OCGCs obtained after the maturation culture as described previously[1]. Briefly, cumulus cells were washed several times with Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12, Gibco). They were then cultured for 3-4 days in DMEM/F12 supplemented with 10% FCS and 50 μg/ml gentamicin sulfate at a concentration of 1 x 10^5 cells/ml in 4-well (16 mm diameter) tissue culture plate (Nalge Nunc International, Roskilde, Denmark). After establishing the primary cultures, the cells were further cultured in DMEM/F12 supplemented with 0.5% FCS and 50 μg/ml gentamicin sulfate for 4-5 days. A single-cell suspension was prepared by standard trypsinization, and middle-sized (20-24 μm) cumulus cells were used as nuclear donor cells for NT.

Production of nuclear transfer embryos

Nuclear donor cell was injected into the perivitelline space of an enucleated oocyte through the same slit in the zona pellucida as was made during enucleation. Couplets of enucleated oocytes and nuclear donor cells were placed and manually aligned between two wire electrodes, 1.0-mm apart, overlaid with 0.3M mannitol solution containing 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.05 mg/ml BSA. Cell fusion and activation were induced simultaneously by applying a single DC pulse of 150 V/mm for 100 μsec using an Electro Cell Fusion instrument (LF 100, Life Tec Co., Tokyo, Japan). Fusion of the couplets was determined 30 min after the electrical stimulus.

Production of parthenotes

The oocytes at the M II stage received a single electrical stimulation which was the same DC pulse as that was used for the induction of fusion/activation of NT embryos. They were then incubated for 6 hr in NCSU-23 containing 5 μg/ml cytochalasin B and 50 μg/ml gentamicin sulfate.

In-vitro culture of NT embryos and parthenotes

NT embryos and parthenotes were cultured in 30-μl drops of NCSU-23 containing 50 μg/ml gentamicin sulfate covered with paraffin oil for 6 days under humidified air with 5% CO_2 at 39℃. The cleavage and subsequent development to the blastocyst stage were determined 48 and 144 hr after the electrical stimulus, respectively. At the end of the culture, all of NT embryos and parthenotes, which reached the blastocyst stage, were assigned for the cell count by using an air-dry method as described elsewhere[32].

Experimental studies

In experiment 1, NT embryos were produced by using an electrical stimulus of a single DC pulse alone, and the rates of fusion, cleavage and development of NT embryos to the blastocyst stage were examined.

Experiment 2 was conducted to determine whether CHX treatment could enhance electrostimulation-induced oocyte activation, and could improve the subsequent parthenogenetic development to the blastocyst stage. In this experiment, *in vitro* mature M II oocytes were treated with a single DC pulse, and they were then incubated for 6 hr in NCSU-23 containing 5 μg/ml cytochalasin B in the presence or absence of 10 μg/ml CHX.

Experiment 3 was carried out to verify the beneficial effect of CHX on the development of NT embryos. Fused couplets of enucleated oocytes and cumulus cells (NT embryos) produced by using a single DC pulse were incubated for 5 hr in NCSU-23 with or without 10 μg/ml of CHX before assessing the *in vitro* development to the blastocyst stage.

Statistical analysis

Statistical differences between 2 means were analyzed by Student's t-test using StatView software (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Experiment 1

After applying the electrical stimulation, 87.6 ± 10.1% (mean ± SD of 7 replicates, n=107) of couplets were fused. The proportions of fused couplets which cleaved and developed to the blastocyst stage were 28.9 ± 7.3 and 5.5 ± 6.1% (means ± SD of 7 replicates, n=94), respectively.

Experiment 2

As shown in Table 1, the oocytes treated with CHX showed higher cleavage and blastocyst formation rates than those cultured without CHX ($P<0.05$).

Experiment 3

Although there was no difference in the fusion rate between the two groups, cleavage and blastocyst rates of NT embryos were im-

Table 1. Effects of cycloheximide (CHX) treatment on parthenogenetic development of porcine oocytes

Treatment	No. of oocytes examined	% of oocytes developed to		Cell no. in blastocysts (n)
		≥ 2-cell	blastocyst	
No CHX	114	31.4±9.8[a]	16.4±7.1[a]	24.3±5.8 (19)
With CHX	116	63.0±6.9[b]	46.8±8.3[b]	22.7±4.4 (54)

[a,b]Values (means±SD of 4 replicates) with different superscripts in the same column differ significantly ($P<0.05$).

Table 2. Effects of cycloheximide (CHX) treatment on the *in vitro* development of porcine NT embryos

Treatment	No. of couplets	% of fused couplets	% of couplets developed to[a]		Cell no.in blastocysts(n)
			≥ 2 -cell	blastocyst	
No CHX	65	98.7±2.3	43.3±16.3[b]	5.4±4.8[b]	27.0±7.9 (3)
With CHX	66	95.8±7.2	70.0±10.5[c]	16.9±3.5[c]	17.9±10.3 (11)

[a] % based on the number of fused couplets.
[b,c] Values (means±SD of 3 replicates) with different superscripts in the same column differ significantly ($P<0.05$).

proved when they were treated with CHX ($P<0.05$) as shown in Table 2.

Discussion

Excessive electrical stimuli for the induction of activation of porcine NT embryos was reported to reduce the development to the blastocyst stage[16]. To induce the fusion and activation of NT embryos, we used a single DC pulse of 150 V/mm for 100 μsec, which was the lowest voltage capable of inducing a high activation (pronuclear formation) rate of porcine M II oocytes in our preliminary experiment (64.3 ± 6.0%, mean of 3 replicates, n =40). In experiment 1, the single DC pulses efficiently induced the fusion between enucleated oocytes and nuclear donor cells; however, the cleavage and development of fused couplets (NT embryos) were poor. These results indicate that the single DC pulse used in the present study is insufficient to induce definitive activation of NT embryos.

Induction of activation of porcine young oocytes is more difficult than that of aging/aged oocytes[12,15]. Although CHX alone could not induce the activation of porcine oocytes[21], an electrical stimulus combined with CHX treatment was reported to enhance the activation (pronuclear formation) of porcine young M II oocytes[12]. In experiments 2 and 3, therefore, CHX treatment was applied to the induction of activation of M II oocytes and NT embryos after giving the same electrical stimulus as used in experiment 1. The higher rates of cleavage and development to blastocyts of parthenotes and NT embryos after the treatment with CHX clearly indicate that CHX treatment enhances the activation of M II oocytes and NT embryos, and leads the improvement in the subsequent development of parthenotes and NT embryos.

At the time of fertilization, penetration of sperm into the oocytes induces the oocyte activation and enables to exit from M II[11]. To exit from the M II stage and to enter the first cleavage, the inactivation of both cytostatic factor (CSF: c-mos/mitogen-activated protein kinase) and M-phase promoting factor (MPF: $p34^{cdc2}$/cyclin B) are required[11,20]. As mentioned above, more than half (approximately 65%) of porcine M II oocytes, which received the single DC pulse, formed pronuclei in our preliminary experiment. Therefore, the single DC pulse may induce reduction of CSF and MPF activities; however, the CSF activity may increase again before definitive inactivation[9]. CHX could inhibit resynthesis of components of CSF and MPF[19,31]. The present results indicate that protein synthesis inhibition treatment is required for the definitive inactivation of CSF and/or MPF, and to lead the initiation of cleavage and subsequent development of NT embryos when fusion and activation of NT embryos were induced simultaneously by using a single DC pulse.

Some groups have reported that the in-

terval between fusion and activation stimuli affected the development of somatic cell NT embryos in the pig[16], cattle[29,36] and mouse[35] : fusion several hours before activation was superior to the simultaneous fusion and activation treatment. In the present study, we induced fusion and activation of porcine NT embryos simultaneously by a single DC pulse before subjecting the NT embryos to the CHX treatment. Therefore, further studies are needed to clarify the role of CHX treatment in the development of somatic cell NT embryos with various intervals between fusion and activation stimuli.

Development of NT embryos to the blastocyst stage was poor compared with that of parthenotes even if they were treated with CHX. These results were in agreement with previous findings[16,25] ; however, it was not clear why the NT embryos showed a poor development compared with the parthenotes. Several factors may affect the *in vitro* development of somatic cell NT embryos, and one of most important factors is the nuclear reprogramming[10]. It remains to clarify the mechanisms involved and the effects of CHX on the inactivation of CSF/ MPF and reprogramming of somatic cell nuclei after transfer to enucleated oocytes.

In conclusion, the present study demonstrates that CHX treatment enhances the electrical stimulus-induced activation of M II oocytes and NT embryos, and leads the improvement in the cleavage and subsequent development of parthenotes and NT embryos to the blastocyst stage. The results also indicate that protein synthesis inhibition treatment required for the induction of M II oocyte activation promotes the development of NT embryos when fusion and activation of NT embryos were induced by a single DC pulse.

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