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Chromosome Aberrations in Porcine Embryo Produced by Nuclear Transfer with Somatic Cell

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ABSTRACT

This study was constructed the correlations of the embryonic developmental rates and the frequency of chromosome aberration using ear-skin-fibroblast cell in nuclear transfer (NT) derived embryos. Karyoplast-oocyte complexes were fused and activated simultaneously, then cultured for seven days to assess development. The developmental rates of NT and *In vitro* fertilization (IVF) embryos were 55.4% vs 63.5%, 31.7% vs 33% and 13.4% vs 16.8% in 2 cell, 8 cell and blastocyst, respectively. Firstly, the frequency of chromosome aberrations were evaluated using fluorescent *in situ* hybridization (FISH) technique with porcine chromosome 1 submetacentric specific probe. Chromosome aberration was detected at day 3 on the embryo culture, the percentages of chromosomal aneuploidy in NT and IVF embryos at 4-cell stage were 40%, 31.3%, respectively. Secondly, embryonic fragmentation was evaluated at 4-cell stage embryo. Frequency of embryonic fragmentations was in 51.3% of NT, 61.3% of IVF, 28.9% of parthenogenetic activation at 4-cell stage. The proportion of fragmentation in NT embryos was higher than activation embryos. This result indicates that chromosomal abnormalities and embryonic fragments are associated with low developmental rate in porcine NT embryo. It is also suggest that abnormal porcine embryos produced by NT related with lower implantation rate, increased abortion rate and production of abnormal fetuses.

(Key words : Porcine, Low development, Chromosome abnormality, Fragmentation)

Ⅰ. INTRODUCTION

The birth of live animal derived from NT embryos has significance not only for multiplication of valuable domestic animals but also for explanation of genomic totipotency of donor nuclei. Since the production of sheep from a differentiated cell line (Campbell et al., 1996), several species have been cloned from cultured somatic cells including lambs (Wilmut et al., 1997), cattle (Cibelli et al., 1998), mice (Wakayama et al., 1998), goats (Baguisi et al., 2000), and more recently pigs (Onishi et al., 2000; Polejaeva et al., 2000; Betthauser et al., 2000). However, efficiency of cloned porcine embryo is still low, and many embryos do not develop to full term. In addition, blastocysts produced *in vitro* have a small number of cells, which can be a cause for

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embryo loss after transfer. Cloned porcine embryos also appear to be more developmentally compromised compared with clones of other domesticated species, which may be related to early onset of maternal-zygotic transition (Kuhholzer and Prather, 2000). Activation of the embryonic genome occurs at the 4-cell stage in the pig, while which occurs during the 8- to 16-cell stage in sheep and cattle (Kopscny, 1989). Reconstructed embryos developing to blastocyst stage *in vitro* with low rates were reported in several species at less than 10% in porcine (Tao et al., 1999; Betthauser et al., 2000; Verma et al., 2000) compared with 20~50% for other species, including sheep (Wells et al., 1997), cattle (Wells et al., 1998), mice (Wakayama et al., 1998).

In molecular cytogenetics provide new approaches for investigating chromosomal disorders in gametes and embryos. Fluorescence *in situ* hybridization (FISH) has been used to study the incidence of aneuploidies in spermatozoa of boars (Kawarasaki et al., 1996) and bulls (Hassanane et al., 1999). The use of FISH with chromosome-specific probes has provided an opportunity to obtain more accurate estimates of the frequency of aneuploid cells. And also makes it possible to assess physical differences such as growth rate and abnormalities in early-stage male and female porcine embryos (Kawarasaki et al., 2000). With recent advances in technology to produce embryos *in vitro*, the study of early specific stage and preimplantation embryos has become more suitable, and it is possible to directly observe the impact of chromosomal abnormalities on embryo development (Viuff et al., 2000).

Chromosomal abnormalities are a major cause of embryonic loss or fetal loss in animal studies (Magnuson et al., 1985; Burgoyne et al., 1991), which include that sex chromosomes, autosomal trisomies or structural rearrangement of balanced structural rearrangements. Most of these abnormalities cause reproductive and health-associated problems of varying degrees (King, 1990). Incidence of chromosomally abnormal embryos in the domestic animals has been estimated to be at 7~10% (King, 1990). And also declines as the age of the embryo increases, further indicating a progressive loss of abnormal embryos at specific stages in early development (King, 1990; Iwasaki et al., 1992).

It can be suggest that chromosome analysis is important to develop markers of embryo quality for evaluating the viability of a given *in vitro* production (IVP) system. Therefore, the objective of this study is to estimate chromosome abnormalities detectable at 4-cell stage of NT embryos compared to parthenogenetic, IVF embryos and to examine the relationship between these abnormalities and embryonic development.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation of Porcine Oocytes

Prepubertal porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory in saline at 37℃. Cumulus-Oocyte complexes (COCs) were aspirated from the follicles (2~6 mm in diameter) using a 10ml syringe fitted with an 18 -gauge needle. The COCs were washed three times with TL-HEPES (Parrish et al., 1988). Then Groups of 50 COCs were matured into a 500 μl of Tissue Culture Medium 199 (TCM-199) (with Earle's salts: Gibco, Grand Island, NY) supplemented with 25 mM $NaHCO_3$, 10%(v:v) porcine follicular fluid, 0.57 mM cysteine, 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamycin sulfate (Sigma, St. Louis, MO), 0.5 μg/ml p-FSH (Follitropin V; Vetrepharm, Canada) and 1 μg/ml estradiol-17β (Sigma), 10 ng/ml epidermal growth factor. Culture was carried out at 39℃, 5% CO_2 in air for 42~44 hrs.

2. Preparation of Porcine Ear-skin Fibroblast

Porcine fibroblasts were harvested from an ear skin biopsy obtained from an adult pig. The tissue was finely minced and digested in 0.05% trypsin EDTA solution (Gibco BRL) for 2 h at 37℃. After digestion, the mixture was filtered through four layers of sterile gauze and cells were pelleted by centrifugation. Cells were cultured on 60 mm tissue culture plates (Falcon, Lincoln Park, NJ) in Dulbecco's Modified Eagles's medium (DMEM; Gibco BRL) supplemented with 10% FBS under 37℃, 5% CO_2 in air. After reaching confluence, cells were passaged. Passage 2 fibroblasts were trypsinized, suspended in the culture medium with 20%(v:v) dimethyl sulfoxide (Sigma) and stored as frozen aliquots. Donor cells were used for nuclear transfer between passages 3 and 8 of culture.

3. Nuclear Transfer of Porcine Oocytes

The matured oocytes were stripped of cumulus cells (CCs) in TL-HEPES supplemented with 0.1% hyaluronidase and washed three times in TL-HEPES containing 0.5% BSA. The oocytes were enucleated by the aspirating the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (30 μm in diameter) in NCSU23 (Petters and Wells, 1993) containing 0.3% BSA containing 7.5 μg/ml cytochalasin B (CB; Sigma). Enucleation was confirmed by staining aspirated cytoplasm with 5 μg/ml Hoechst 33342 (Sigma), then the enucleated oocytes were incubated in NCSU23 containing 0.3% BSA until injection of donor cells. The isolated single porcine ear-skin fibroblast was inserted into perivitelline space of each enucleated oocyte by the injection pipette (20 μm in diameter) in NCSU23 containing 0.3% BSA. Nuclear transferred oocytes were washed three times in fusion solution. The fusion solution composed of 0.3 M mannitol, 1.0 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Fusion was performed at room temperature, in a chamber with two platium electrodes at 1 mm apart overlaid with fusion solution. Membrane fusion was induced with single D.C. pulse of 2.1 kV/cm for 30 μsec delivered by a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA). This pulse was also utilized to simultaneously induce oocyte activation.

4. *In Vitro* Culture of Reconstructed Oocytes

Reconstructed oocytes were transferred into a 50 μl of NCSU23 supplemented with 0.4% fatty-acid -free BSA. Following culture for 48 hr, all embryos were cultured in 50 μl of NCSU 23 containing 10% FBS for 4 days.

5. Oocyte Activation

Non-manipulated (for controls) oocytes were places between 0.2 mm diameter platinum electrodes 1 mm apart in activation medium. Activation was induced with a D.C. pulse of 1.36 kV/cm for 80 μs on BTX Electro Cell Manipulater 200 (BTX, San Diego, CA).

6. *In Vitro* Fertilization of Porcine Oocytes

The oocytes were treated with 0.1% hyaluronidase solution to remove cumulus cells and washed three times with modified tris buffered medium (mTBM) containing 1 mM caffeine and 1 mg/ml BSA. After washing, 20~25 denuded oocytes were placed into 50 μl drops of the fertilization medium. The oocytes were kept in the incubator for 30 min until spermatozoa were added for fertilization. Semen was collected from cauda epididymis. Semen pellet was diluted into 5 ml Sp-TALP (Rosenkrans et al., 1993), and the extender was removed by washing two times. At the end of the washing procedure, the sperm pellet was resuspended in the mTBM containing 1 mM caffeine and 1 mg/ml BSA. After appropriate dilution, 50 μl of the sperm suspension was added to a 50 μl drop of the fertilization medium containing oocytes to give a

final sperm concentration of 1×10^5 cells/ml. Sperm and oocyte were coincubated in a 100 μl drops of mTBM for 6 hrs at 39, 5% CO_2 in air.

7. Preparation of DNA Probe

Porcine chromosome-specific DNA probes were produced by PCR as described by Kawarasaki et al (2000). Porcine chromosome 1-specific DNA was amplified and F-11 labeled with random prime system by PCR using primers designed according to the *Sus scrofa domestica* Mc1 satellite DNA sequence data (Jantsch et al., 1990).

8. Fluorescent *In Situ* Hybridization

The F-11 labeled chromosome-1 specific DNA probe was diluted with a denatured hybridization buffer. Briefly, 5 μl of the hybridization probe were dropped on slides and covered with a coverslip, after which the preparation were immediately denatured at 80℃ for 2 min on the heated plate and hybridized for overnight at 37℃. After hybridization, the slides were washed in a solution series of 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) containing 50% deionized formamide for 1 min, 2 ×SSC for 1 min by strong regular shaking in a water bath at 42℃. All the buffers and the moist chambers for washing and incubation had been warmed at treatment temperature before use.

The slides were then counterstained with 10 μl of 4',6-diamidino-2-phenylindole (DAPI; Vysis Inc., Downers Grove, IL 60515, USA), were sealed with nail polish and were viewed under a epifluorescence microscope (Nikon Co., Tokyo, Japan) using DAPI/FITC double bands filter (Nikon).

Approximately 20 min were required for the preparation of embryo nuclei and about 90 min to detect signals by *in situ* hybridization.

9. Statistical Analysis

At least three or four replicate were conducted for each experiment. The significant difference among treatment groups was determined by the ANOVA analysis (Abacus, Berkeley, CA). A value of $p<0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. *In Vitro* Development of Reconstructed Oocytes by Nuclear Transfer

As shown in Table 1, the cleavage rates of oocytes derived from activation, IVF and NT were 67.9%, 63.5% and 55.4%, respectively. The overall developmental rates to blastocyst stage were 26.4% in activation embryos, 13.4% in NT embryos and 16.8% in IVF embryos.

The developmental rate to the blastocyst derived from activation was higher than that of IVF and NT embryos. And there was significant difference ($p< 0.05$) between activation and IVF, NT group.

2. Detection of Chromosome Aberration Derived NT Embryos

Table 1. Comparison of developmental competence on porcine embryos by activation, IVF, NT

Group	No. of oocytes	No(%). of embryos				
		Fused	2-cell	8-16 cell	Morula	Blastocyst
Activation	212	-	144 (67.9)	102 (48.1)	64 (30.2)	56 (26.4)[a]
IVF	197	-	125 (63.5)	65 (33.0)	37 (18.8)	33 (16.8)[b]
NT	283	224 (79)	124 (55.4)	71 (31.7)	39 (17.4)	30 (13.4)[b]

[a,b] Different superscripts within a column differ significantly ($P<0.05$).

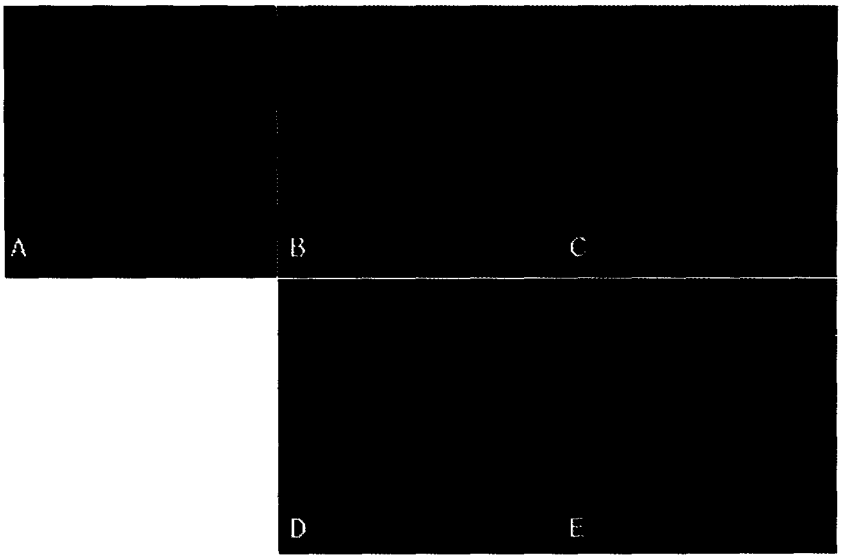

Fig. 1. FISH with F-11 labeled porcine chromosome-1 submetacentric (pMC1) DNA probe on extracted interphase nuclei from porcine embryos. A) haploid, B) diploid, C) triploid, D) tetraploid. (× 1000).

The porcine 4-cell embryos were analyzed for the detection of DNA fragmentation using FISH analysis and morphological features of chromosome aberration (Fig. 1). Control slides with metaphase chromosomes in porcine lymphocyte cells exhibited signals at the appropriate regions of the chromosome-1.

The incidence of chromosome abnormality was compared within porcine 4-cell embryos by activation, IVF and NT (Table 2). The rates of total amount of chromosome aberration were 31.3% and abnormal pattern in NT embryo (40.0%) were higher than other groups in IVF (31.3%), parthenogeneticactivation (21.9%) with significant difference ($p < 0.05$).

3. Comparison of Frequencies of Chromosome Abnormality by Activation, IVF, NT

As a shown Table 3, frequency of chromosome abnormality was compared in each experimental group. More haploid and mixoploid were present in entire porcine embryos. In addition, IVF and NT embryos were high incidence of haploid (50% vs 44.7%) and parthenogenetic activation embryos were

Table 2. Frequency of chromosome abnormality in porcine 4-cell embryos by activation, IVF, NT

Group	No. of 4-cell embryos	No. of analyzed blastomeres	No(%). of analyzed nucleus	No(%). of embryos	
				Normal	Abnormal
Activation	91	364	240 (65.9)	20 (21.9)[a]	71 (78.0)[a]
IVF	102	408	238 (58.3)	32 (31.3)[b]	70 (68.6)[b]
NT	95	380	214 (56.3)	38 (40.0)[c]	57 (60.0)[c]

[a,b,c] Different superscripts within a column differ significantly ($P < 0.05$).

Table 3. Different type of chromosome abnormality by activation, IVF and NT

Group	No(%). of abnormal embryos					
	Total	Haploid	Triploid	Tetraploid	Polyploid	Mixoploid
Activation	20 (21.9)	4 (20.0)	1 (5.0)	3 (15.0)	1 (5.0)	11 (55.0)
IVF	32 (31.3)	16 (50.0)	1 (3.1)	1 (3.1)	2 (6.2)	12 (37.5)
NT	38 (40.0)	17 (44.7)	4 (10.5)	4 (10.5)	2 (5.2)	11 (28.9)

Table 4. Frequency of fragmentation compare with activation, IVF and NT

Group	No. of 4-cell embryos	No(%). of analyzed nucleus	No(%). of embryos	
			Fragment	Normal
Activation	97	350 (90.2)	28 (28.9)[a]	69 (71.1)[a]
IVF	80	211 (65.9)	49 (61.3)[b]	31 (38.7)[b]
NT	78	226 (72.2)	40 (51.3)[c]	38 (48.7)[c]

[a,b,c] Different superscripts within a column differ significantly($P<0.05$).

mixoploid (55%).

4. Analysis of Fragment Incidence in NT and IVF Embryos

As shown in Table 4, type of embryonic fragmentations was in 51.3% of NT, 61.3% of IVF, 28.9% of activation at 4-cell stage. The proportion of fragmentation was higher in NT embryos than activation embryos. In these result, it was found that embryonic fragmentation was associate with lower development of NT embryos. And there was significant difference ($p < 0.05$) in activation and IVF, NT group.

Ⅳ. DISCUSSION

NT using somatic cells has been very difficult with less than 1% of reconstructed embryos developing to full term in pigs. However, little information has been gained about the developmental competence of NT porcine embryos. Thus, it is needed that try to find the reason for low development and production.

Then, analysis of porcine embryos in the third cell cycle revealed that caused reason for numerical chromosome aberrations. In this study, the incidence of chromosomal abnormalities in embryos derived by NT is generally higher than each experimental group. It is suggest that embryonic development associate with chromosome aberration and this may imply that NT embryos with a lower proportion of the diploid cell line have a low developmental potential. Thus, this result support the theory that a loss of abnormal embryos takes place at specific stages of development and also support the assumption that chromosome aberrations affecting the whole chromosome set are incompatible with development to term.

The timing of the transcriptional burst in mammalian embryos is species-specific (Kopecny et al., 1989; Telford et al., 1990). In porcine, the burst in activity occurs at the 4-cell stage, which is also the point when development usually stops *in vitro* under suboptimal conditions (Kopecny et al., 1989).

The other reports had shown that the sex of the embryo has an effect on the developmental rate of mouse (Tsunoda et al., 1985), pig (Cassar et al., 1994), and human embryos(Pergament et al., 1994).

It is suggest that a phase for chromosome aberration of porcine NT embryos associate with lower blastocyst formation, implantation rate. Thus, these results suggest that the developmental rate of the embryo is also governed by its ploidy. This abnormality in mammalian embryos was observed either as pure haploid or as mosaicism.

The total amount of aberrations was haploid 41.0 %, mixoploid 37.8%. Also, an aspect of chromosome aberration in NT group were haploid 44.7%, mixoploid 28.9%, triploid 10.5% and polyploid 5.2%. Thus, the incidence of haploid and mixoploid higher than other abnormal groups. Lack of an entire set of chromosomes may cause a reduction in developmental rate due to the lower levels of gene products and the suboptimal nucleocytoplasmic ratio in the haploid embryos (McGrath and Solter, 1986). The basis of mixoploidy is most likely established either in abnormal fertilization or abnormalities imposed during *in vitro* culture of the embryos. It has recently been reported that abnormal conditions can result in chromosomal mosaicism during subsequent cleavage divisions (Handyside and Delhanty, 1997).

In addition, abnormalities during *in vitro* culture of the embryo may cause abnormal chromosome segregation leading to mosaicism from the first cleavage division. These include mitotic nondisjunction, ploidy mosaicism, and chaotic chromosome complement in majority of nuclei. The high frequency of postzygotic chromosome abnormalities might be induced by lack of cell cycle checkpoints during cycles prior to the major activation of the embryonic genome (Dasso and Newport, 1990; Sullivan et al., 1993).

Porcine embryos produced *in vitro* have a small number of cells and low viability. Especially, NT embryos have a low cell number than other *in vitro* development embryos and embryonic fragment related low development. These results observed that NT embryos were high frequency of fragmentation comparison of activation embryos. It was found that embryonic fragmentation associated with lower development of NT embryos.

Fragmentation is a serious problem for pig embryos produced *in vitro* (Wang et al., 1999). It can be regarded as cytokinesis without karyokinesis, and it is a critical event in the development of NT embryo (Kawahara et al., 2002). One of reason for the fragmentation in embryos produced *in vitro* is that the embryos have an abnormal microfilament distribution (Wang et al., 1999). Also, these abnormalities in the NT embryos depend on the age of the recipient cytoplasm (Cheong et al., 2000). Accordingly, to avoid disruption of recipient oocytes and to improve the development competence of the NT embryo and an understanding of its process and involved factors are important.

In conclusion, results of this study have shown that chromosomal abnormalities are associated with low development in porcine NT embryos. It is possible that this phenomenon originates at an early stage in embryonic development. Chromosomal abnormalities in porcine NT embryos affect growth *in vitro* delaying cleavage, which leads to lower than expected cell numbers at specific times in development. This finding indicates that chromosomal abnormalities and embryo fragmentation were related to low developmental rate in porcine NT embryo. It is also suggest that abnormal porcine embryos produced by NT associate with lower implantation rate, increased abortion rates and production of abnormal fetuses. Therefore, advance of preselection technique for normal and healty porcine embryo contribute to the improvement of clone and transgenic animal production.

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