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The Use of Nuclear Transfer Procedure for Evaluation of Abattoir Derived Bovine Oocyte

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Abstract: Using nuclear transfer procedure, this study was conducted to investigate the effect of oocyte quality on the *in vitro* development of bovine embryos. Immature oocytes from slaughterhouse-collected ovaries were classified into good and poor quality based on their morphological appearance. After maturation culture, the good quality group showed a higher percentage of oocytes with first polar body than the poor one. The oocytes with first polar body from both groups were enucleated, activated, and fused with disaggregated donor blastomeres of 32-cell-stage embryos. Enucleation rates of the oocytes with the first polar body were the same for both groups. After nuclear transfer, no significant difference was detected in fusion and cleavage rates between the 2 groups. However, the embryos reconstituted from good quality oocytes showed significantly higher developmental rate to the blastocyst stage and blastocyst cell number than those from the poor ones. These results indicate the necessity of strict selection of oocytes with good morphology as recipient cytoplast for nuclear transfer.

Key words: Nuclear, procedure, abattoir derived, Oocyte

Introduction

Slaughterhouse oocytes, as byproducts, presents an abundant source for *in vitro* embryo production (Stice and First, 1993) however, different qualities are commonly encountered due to the variation between cows and/or within cows, which in turn is affected by the stage of the estrous cycle, stage of lactation, age, and stress at the time of collection (Hazeleger, *et al.*, 1995). It is well documented that the quality of abattoir-derived oocyte markedly affects the preimplantation developmental potential of bovine embryos produced by in vitro fertilization (IVF) (Blondin and Sirard, 1995, Hazeleger, *et al.*, 1995). The IVF system in assessing the oocyte quality has the disadvantages that, all oocytes after in vitro maturation are used without eliminating the oocytes failed to reach maturation, furthermore, disregard those went through fertilization failure. However, in nuclear transfer experiments only mature oocytes are used, and are dealt with individually, in terms of, enucleation, doner cell insertion, and fusion, therefore circumvent the IVF system failures.

In this study we investigated the effect of oocyte quality on the preimplantation developmental competence of bovine embryos using the nuclear transfer procedure.

Materials and Methods

Oocyte Collection, Classification and Maturation: Bovine oocytes were collected from slaughterhouse ovaries as described previously (Takahashi *et al.*, 1996). Briefly, cumulus-oocyte complexes (COCs) aspirated from small antral follicles were washed three times with HEPES-buffered modified Tyrode's medium (TALP-HEPES) (Bavister, *et al.*, 1983) supplemented with 3 mg/ml BSA (Fraction V, Sigma Co., St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma) and 50 μg/ml gentamycin sulfate (Sigma). As shown in Table 1 oocytes were then classified into good and poor quality based on the morphology of cumulus cells and ooplasm. This classification was modified from that of (Hazeleger *et al.*, 1995).

In vitro oocyte maturation for each of the designated oocyte group was separately conducted as described elsewhere (Takahashi, *et al.*, 1996) in HEPES-buffered TCM 199 (Gibco laboratories, Grand Island, NY, USA) supplemented with 10% FCS (Gibco), 0.02 units/ml FSH (from porcine pituitary, Sigma), 1 μg/ml estradiol-17ß (Sigma), 0.2 mM sodium pyruvate, and 50 μg/ml gentamycin sulfate (Sigma) under a humidified atmosphere of 5% CO_2 in air at 39°C for 20 hrs.

After maturation culture, cumulus cells were removed by vortexing the COCs in 0.1% hyaluronidase (Type 1-S, Sigma) in Ca^{2+}- and Mg^{2+}-free TALP-HEPES. Denuded oocytes were examined under an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan) to verify the extrusion of the first polar body.

Oocyte Enucleation and Activation: Denuded oocytes with first polar body (matured) were enucleated by removing the first polar body and the adjacent cytoplasm in a micromanipulation drop of TALP-HEPES supplemented with 10% FCS and 5μg/ml cytochalasin B (Sigma) (Mohamed Nour and Takahashi, 1999). After enucleation, cytoplasts

were incubated in TALP-HEPES containing 5μg/ml Hoechst 33342 (Sigma) for 15 min at 39°C. Enucleation was confirmed by exposing oocytes to UV light for a few seconds (Westhusin, *et al.*, 1992) under an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan) equipped with an epifluorescence and UV-2A filter block (330-380 nm excitation and 420 nm emission). Enucleated oocytes were activated by incubation with 10μM calcium ionophore A23187 (Sigma) in the embryo culture medium for 5 min. The oocytes were then incubated in the embryo culture medium supplemented with 10 μg/ml cycloheximide (Sigma) under a humidified atmosphere of 5% CO_2 in air at 39°C for 5 h (Mohamed Nour and Takahashi, 1999).

Donor Blastomere Preparation: Blastomeres of the 32-cell-stage embryos obtained at 5 days after *in vitro* fertilization were used as source of nuclear donors. *In vitro* fertilization and culture of fertilized embryos were performed as described previously (Takahashi *et al.*, 1996). Briefly, frozen semen from a single ejaculate of a Holstein bull was thawed at 37°C for 45 sec. Motile spermatozoa were separated using 45 and 90% Percoll gradient solution. The cumulus oocyte complexes were co-incubated with spermatozoa ($5x10^6$ cells /ml) in a 100 μl fertilization drop containing 3 mg/ml fatty acid-free BSA (Sigma) and 2.5 mM theophylline (Sigma) for 18 h at 39°C under a humidified air with 5% CO_2. Presumptive zygotes were cultured in modified synthetic oviduct fluid medium (Takahashi and First, 1992) supplemented with 1 mM L-glutamine (Sigma), essential amino acids for basal medium Eagle (Sigma), nonessential amino acids for minimum essential medium (Sigma), 1 mM glucose and 3 mg/ml fatty acid-free BSA. The zona pellucida of 32-cell-stage embryos was removed by incubation in TALP-HEPES containing 0.5% pronase (Actinase E, Kaken Pharmaceutical Co. Inc., Tokyo, Japan). Zona-free embryos were disaggregated with 0.25% trypsin (Sigma) and 0.2% EDTA (Kanto Chemical Co. Inc., Tokyo, Japan) in Ca^{2+}- and Mg^{2+}-free TALP-HEPES.

Nuclear Tansfer and Culture: Individual blastomeres were inserted in the perivitelline space of the activated cytoplasts. Fusion of the blastomere with the cytoplast was electrically induced. Manipulated couplets were placed in 0.3 M mannitol solution containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ between 2 electrodes (1 mm apart), and were then manually aligned. Two direct current pulses of 0.8 kv/cm for 30 μsec, 1 sec apart were delivered to the chamber using a somatic hybridizer (SSH-2, Shimadzu Co., Kyoto, Japan). Successfully fused couplets were cultured as mentioned above for *in vitro* fertilized embryos. Fusion and cleavage rates of the reconstituted embryos were investigated at 1 and 33 h after fusion, respectively. Seven days after fusion, development of the reconstituted embryos to the blastocyst stage and cell number of the blastocysts were assessed as described previously (Takahashi and First, 1992).

Statistical Analysis: Data were analyzed by Student's t-test with a computer software (StatView, Abacus Concepts Inc., Berkeley, CA, USA). A probability of less than 0.05 was considered significant.

Results

As shown in Table 2, good quality oocytes showed a higher first polar body extrusion rate than the poor quality ones ($P< 0.01$). No difference was detected in the enucleation rate when the oocytes with first polar body were used in both groups. Results of nuclear transfer experiments are shown in Table 3. No difference was detected in the fusion and cleavage rates between the oocytes of the 2 groups. The good quality oocytes showed a

Table 1: Classification of cumulus oocyte complexes

Oocyte quality	Classification criteria		
	No. of Cumulus cell layers	Cumulus cell condition	Cytoplasm
Good	≥3	Compact	Homogenous with fine granulation
	≥3	Compact	Homogenous with slightly coarse granulation
	≥3	Slight expansion in the outer layer	Dark clumps throughout
	≥3	Full expansion with dark clumps	Dark clumps throughout
Poor	1	Exposed corona cells	Variable
	0	Exposed zona pellucida	Variable

Table 2: First polar body extrusion and enucleation rates of *in vitro* matured oocytes

Parameters	Oocyte quality	
	Good	Poor
No. of oocytes examined	303	193
% of oocytes with first polar body	69.6 ± 7.5[a]	52.1 ± 8.8[b]
% of oocytes enucleated*	53.6 ± 9.5	46.4 ± 11.1

[a,b] % value (means ± SD of 5 replicates) with different superscripts differ significantly ($P<0.01$)
*Based on the number of oocytes showed the first polar body

Table 3: Development of bovine nuclear transfer embryos derived from good and poor quality oocytes

Parameters	Oocyte quality	
	Good	Poor
No. of replicates	5	5
No. of attempted fusion	136	107
% of fused	86.1 ± 4.6	79.7 ± 5.1
% of cleaved*	87.6 ± 6.4	79.6 ± 10.9
% of blastocysts*	49.0 ± 7.4[a]	19.8 ± 7.7b
Blastocyst cell No.	94.5 ± 28.0[a]	74.2 ± 24.1b
(No.)	(41)	(14)

Values are means ± SD.
[a,b]Values within rows with different superscripts differ significantly ($P<0.05$).
*Based on the number of oocytes fused

significantly higher developmental rate to the blastocyst stage than the poor quality oocytes ($P< 0.05$). Although the blastocysts derived from both oocyte groups were morphologically normal, the blastocysts derived from good quality oocytes had a higher cell numbers than those from poor quality oocytes ($P< 0.05$).

Discussion

The low oocyte maturation rate in the poor quality group was confirmed by the lower polar body extrusion rate. The abnormality or partial loss of the cumulus investment of the poor quality oocytes could be one of the contributing factors for their low maturation rate. The connection between cumulus cells and oocytes was reported to be necessary for oocytes to achieve normal maturation and acquire developmental competence (Buccione, *et al.*, 1990, Fukui and Sakuma, 1980, Leibfried and First, 1979).

There was no difference in the enucleation rate between the 2 groups. Enucleation rate is affected mainly by the location of the metaphase chromatin in relation to the polar body (Mohamed Nour and Takahashi, 1999). Present result indicates that the metaphase chromatin location in the ooplasm is the same for the oocytes of 2 groups if they had the first polar body.

In the previous *in vitro* fertilization report (Hazeleger, *et al.*, 1995), the oocytes with poor morphology resulted in a lower cleavage compared to those with good morphological features due to low fertilization rates. The similar cleavage rate for good and poor oocytes obtained in the present study could be attributed to the use of matured oocytes confirmed by possessing the first polar body. Despite the similar cleavage rate, poor quality oocytes failed to support the development of the reconstituted embryos to the blastocyst stage at the same rate as the good quality ones. The difference in the developmental potential between the oocytes with different morphological features is clearly attributed to the difference in the cytoplasmic developmental potential; however the exact reasons remained to be elucidated.

The present study indicated that the strict selection of abattoir-derived oocyte as recipient cytoplast is an essential factor to have better development of nuclear transfer embryos. The developmental potential of each morphological feature within the good and poor quality oocytes needs further investigation.

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