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Factors Affecting Enucleation Rates of Bovine and Porcine Oocytes After Removal of Cumulus Cells by Vortexing

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Abstract. *In vitro* matured bovine and porcine oocytes were enucleated by aspiration of the first polar body and adjacent cytoplasm after removing the cumulus cells by vortexing and the factors affecting enucleation rate were investigated. The results indicate that vortexing cumulus-oocyte complexes for long periods in hyperosmotic media moves the first polar body and reduces the enucleation rate. Removal of 20% cytoplasmic volume increased enucleation rate compared with that of 10% and showed no deleterious effect on subsequent developmental capacity of reconstructed bovine embryos. Aging of oocytes could also reduce enucleation efficiency by inducing the migration of the chromatin material to the center of the oocyte. Therefore, we recommend to enucleate newly matured bovine and porcine oocytes by removing the first polar body and adjacent 20% of ooplasm after the removal of cumulus cells by vortexing for a short period using a hypo-osmotic media to achieve a high enucleation rate.

Key words: Cattle, Enucleation, Nuclear transfer, Oocyte, Pig

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Transplantation of nuclei into enucleated oocytes is currently a major focus of interest in the production of cloned animals. The technique involves the preparation of karyoplast and cytoplast. Production of a suitable cytoplast in nuclear transfer (NT) procedures is considered a critical step. Enucleated MII oocytes are commonly used as recipient cytoplasts. The efficient removal of the cytoplasmic chromatin material before NT is of crucial importance in the production of reconstructed embryos with normal ploidy. However, enucleation of MII oocytes remains a major obstacle with 50–80% enucleation efficiency [1–4] in cattle. In the pig, enucleation efficiency varies from 60 to 90% [5–11]. Solving this limitation

would enable the sufficient preparation of recipient cytoplasts for the reconstruction of NT embryos.

The removal of the nuclear material of MII oocytes is typically performed blindly by aspirating a portion of the cytoplasm underlying the first polar body [1, 2, 4–15]. To achieve a high enucleation rate with this procedure, the chromatin material needs to be located near or adjacent to the first polar body. Removal of the cumulus cells surrounding the oocyte is required prior to enucleation. In general, the cumulus cells are removed by vortexing. This method, however, could possibly affect the relationship between the position of the first polar body and the chromatin material. Speculation exists that the possible movement of the first polar body away from the original site of extrusion [2], frequent displacement

of the chromatin material [16], and the volume of removed cytoplasm during aspiration/bisection might be major causes of low enucleation rates.

The present study was undertaken to investigate the factors affecting the enucleation efficiency of bovine and porcine oocytes after the removal of cumulus cells by vortexing and aspiration of the first polar body and 10 or 20% of the cytoplasm. We also determined the developmental capacity of bovine NT embryos constructed with cumulus cells and enucleated oocytes with different cytoplasmic volumes.

Materials and Methods

Oocyte preparation

In vitro matured, slaughterhouse-derived bovine oocytes were prepared as described elsewhere [17]. Briefly, cumulus-oocyte complexes (COCs) aspirated from small antral follicles (2–7 mm in diameter) were washed 3 times with HEPES-buffered modified Tyrodes's medium (TALP-HEPES) [18] supplemented with 3 mg/ml bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma) and 50 µg/ml gentamycin sulfate (Sigma). The COCs were then cultured in HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 0.02 units/ml FSH (from porcine pituitary, Sigma), 1 µg/ml estradiol-17β (Sigma), 0.2 mM sodium pyruvate and 50 µg/ml gentamycin sulfate. Ten to twelve oocytes were cultured for 18–20 h in a 50-µl droplet of maturation medium overlaid with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) under a humidified atmosphere of 5% CO₂ in air at 39 C.

Porcine oocyte-cumulus-granulosa-complexes (OCGCs) collected from abattoir-derived ovaries were cultured to induce maturation as described previously [10]. Briefly, healthy follicles with 4–6 mm of diameter were dissected in HEPES-buffered TCM 199 supplemented with 0.1% PVA (Sigma). OCGCs were collected in the same medium by scraping the inner surface of the follicles with a pair of forceps. Five OCGCs were cultured in a 100-µl drop of BSA-free North Carolina State University Medium 23 (NCSU-23) [19] supplemented with 0.57 mM cysteine (Sigma), 10% (v/v) porcine follicular fluid, 10 IU/ml eCG (Teikoku Hormone

Mfg. Co. Ltd., Tokyo, Japan) and 10 IU/ml hCG (Teikoku Hormone) under a humidified atmosphere of 5% CO₂ in air at 39 C for 20 h. The oocytes were further cultured in the same medium without hormones for another 22–24 h.

The bovine COCs and porcine OCGCs were vortexed (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for 0.5 or 3 min in a 1.5-ml test-tube (Dorika Co. Ltd., Sapporo, Japan) containing 200 µl of TALP-HEPES with 0.1% hyaluronidase (Type 1-S, Sigma). The cumulus cells of some bovine and porcine oocytes were removed by repeated pipetting.

Oocyte enucleation

After the removal of cumulus cells, denuded bovine and porcine oocytes were mechanically enucleated by removing the first polar body and approximately 10 (7.1 ± 3.2 , $n=42$) or 20% (19.8 ± 1.4 , $n=38$) of the adjacent cytoplasm with a 30-µm (outer diameter) glass pipette. To regulate the volume of aspirated cytoplasm, the reference point of removed ooplasm inside the injection pipette during enucleation was determined in a preliminary study by measuring the oocyte diameter before and after enucleation and calculation of cytoplasmic volume as described previously [20]. Enucleation was carried out using an inverted microscope (Diaphot, Nikon, Tokyo, Japan) with Narashige (Narashige Co. Ltd., Tokyo, Japan) micromanipulators in a 40-µl drop of TALP-HEPES supplemented with 10% FCS and 5 µg/ml cytochalasin B (Sigma). Enucleated oocytes were incubated in Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS) containing 3 mg/ml BSA and 5 µg/ml Hoechst 33342 (Sigma) for 15 min at 39 C. Oocytes were examined individually under an inverted microscope (Eclipse TE 300, Nikon) equipped with an epifluorescence (TE-FM, Nikon) and UV-1A filter block (365 nm excitation and 400 nm emission) to confirm enucleation.

Nuclear transfer, fusion, activation and culture

Primary cultured bovine cumulus cells were prepared as described previously [21]. Briefly, collected cumulus cells from *in vitro* matured bovine oocytes in TALP-HEPES with 0.1% hyaluronidase were washed three times with DMEM/F12 (Gibco) by centrifugation. The concentration and viability of the separated cells

were determined and then cultured in DMEM/F12 + 10% FCS at a concentration of $8-9 \times 10^4$ cells/ml in 60×10 mm dishes (Falcon 3802, Becton Dickinson Labware, Franklin Lakes, NJ, USA) under a humidified atmosphere of 5% CO₂ in air at 37 C. Cultured cells were allowed to multiply for 3–4 days, and thereafter were deprived of serum source (0.5% FCS) for 3 days. After serum-starved culture, the cells were disaggregated with Ca²⁺- and Mg²⁺-free DPBS containing 0.1% (w/v) trypsin (Sigma) and 0.1% (w/v) EDTA (Kanto Chemical Co. Inc., Tokyo, Japan) and used as karyoplasts. In a separate experiment, DNA content analysis using flow cytometry of serum-starved primary cumulus cells revealed that around 95% (n=3) of medium-sized cells were at the G0/G1-stage, and that the percentage of live cells was 95% (n=3).

Trypsinized cumulus cells were inserted individually into the perivitelline space of the enucleated oocytes. The couplets were placed and manually aligned between two wire electrodes (1.0 mm apart) overlaid with 0.3 M mannitol (Kanto) solution containing 0.1 mM CaCl₂ (Kanto) and 0.1 mM MgCl₂ (Kanto). Cell fusion and activation were simultaneously induced by two direct current pulses of 100 V/mm for 40 μ sec, 1 sec apart using an Electro Cell Fusion (LF-100, Life Tec Co., Tokyo, Japan). After 1 h, successfully fused couplets were transferred to a modified synthetic oviductal fluid [17] supplemented with 20 amino acids (Sigma), 10 μ g/ml insulin (Sigma), 5 mM glycine (Sigma), 5 mM taurine (Sigma), 1 mM glucose (Kanto), 1 mM L-glutamine (Sigma), 3 mg/ml fatty acid-free BSA (Sigma) and 10 μ g/ml cycloheximide (CHX, Sigma) for a period of 5 h at 39 C in 5% CO₂, 5% O₂ and 90% N₂. After CHX treatment, the reconstituted embryos were thoroughly washed and then placed in the culture medium excluding CHX at 39 C in 5% CO₂, 5% O₂ and 90% N₂. The fusion and cleavage rates were determined at 1 and 33 h post fusion (hpf), respectively. Development to blastocyst and the cell numbers [22] were determined 174 hpf.

Experimental design

In experiment 1, the cytoplasmic chromatin distribution of MII bovine and porcine oocytes were determined. *In vitro* matured bovine COCs were divided randomly into three treatment groups wherein the cumulus cells were removed by: 1) repeated pipetting in TALP-HEPES with 0.1% hyaluronidase, 2) pipetting and vortexing for

0.5 min, and 3) vortexing for 3 min. *In vitro* matured porcine oocytes were denuded following the procedure of 1) or 3) described above. Oocytes with the first polar body were incubated in Hoechst 33342 stain. The location of chromatin material was determined using the first polar body as a reference point (Fig. 1). In experiment 2, the enucleation efficiency with the removal of 10 or 20% of the cytoplasmic volume was determined. The oocytes were denuded and grouped as in experiment 1 and enucleated blindly. In experiment 3, the developmental capacity of reconstituted bovine embryos constructed with enucleated oocytes with approximately 80 or 90% of its original cytoplasmic volume and serum-starved primary cumulus cells was investigated to determine the effect of reduced ooplasmic volume in somatic NT. In experiment 4, we examined whether the first polar body of bovine and porcine oocytes could be moved by vortexing. After initial staining with Hoechst 33342, all oocytes with chromatin location at Position 1 (see Fig. 1) were further vortexed for 5 min and restained with Hoechst 33342. The cytoplasmic chromatin location was examined thereafter.

Statistical analysis

Data in experiments 1 and 2 were subjected to one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test using StatView software (Abacus Concepts Inc., Berkeley, CA, USA). Differences between two means in experiments 2 and 3 were analyzed using student's *t*-test.

Results

Experiment 1

After 3 min of vortexing, bovine oocytes, but not porcine oocytes, showed the lowest proportions that exhibited cytoplasmic chromatin material located at Positions 1 and 2 ($P < 0.05$) as shown in Table 1.

Experiment 2

Three minutes vortexing in bovine oocytes gave a significantly lower enucleation rate than the other treatments ($P < 0.01$) both in 10 and 20% removal of cytoplasmic volume. However, removal of 20% cytoplasmic volume revealed a significantly higher enucleation rate compared with that of 10%

Fig. 1. Schematic presentation of metaphase chromatin relative to the first polar body in *in vitro* matured bovine and porcine oocytes. 1) Position 1: the metaphase chromatin is located immediately adjacent to the polar body. 2) Position 2: at the cytoplasmic third near the polar body but not in close association. 3) Position 3: at the mid-cytoplasmic third. 4) Position 4: at the cytoplasmic third far from the polar body.

($P < 0.05$) both in bovine and porcine oocytes as shown in Table 2.

Experiment 3

As shown in Table 3, no significant differences were observed in fusion, cleavage, development to the blastocyst stage and cell number of blastocysts between the two experimental groups.

Experiment 4

As shown in Table 4, 40% (14/35) of porcine oocytes and 60% (12/20) of bovine oocytes showed displacement of the chromatin material from

Position 1 after vortexing for 5 min. The chromatin materials were located peripherally in all of the oocytes that were observed at Positions 2–4.

Discussion

The results of this study clearly indicate that prolonged vortexing changes the location of the first polar body relative to the chromatin material, and supports the previous finding that the metaphase spindle of 60% of bovine oocytes was displaced from the previously assumed location subjacent to the first polar body during cytoplasm preparation [16]. Under the same condition of vortexing (3 min), porcine oocytes kept the first polar body near the site of extrusion probably due to the smaller perivitelline space and the higher cytoplasmic osmolarity compared with bovine oocytes. The higher proportion of bovine oocytes which showed first polar body movement from the site of extrusion during cumulus removal might be due to the large perivitelline space. The use of a hyperosmotic media induces shrinkage of the ooplasm and polar body leading to an enlargement of the perivitelline space. Thus, a hypo-osmotic media should be used during the vortexing process.

This study demonstrates that minimizing the time of vortexing to remove the cumulus cells of MII oocytes supports a high enucleation rate by maintaining the first polar body close to the site of extrusion. After 10% removal of cytoplasmic volume during enucleation, only MII oocytes with chromatin material at Position 1 could be

Table 1. Cytoplasmic chromatin distribution after cumulus cell removal by repeated pipetting or vortexing of *in vitro* matured bovine and porcine oocytes

Treatment for cumulus removal	No.of oocytes (replicates)	% of oocytes with chromatin at position ^a				
		1	2	3	4	1 + 2
<i>Bovine</i>						
Pipetting	43 (3)	76.8 ± 3.3 ^b	11.5 ± 3.3 ^b	4.8 ± 4.2 ^b	7.0 ± 0.6 ^b	88.3 ± 4.2 ^b
Pipetting+ 0.5 min vortexing	65 (3)	76.5 ± 11.4 ^b	11.7 ± 1.4 ^b	5.8 ± 4.6 ^b	5.9 ± 6.8 ^b	88.2 ± 9.5 ^b
3 min vortexing	63 (3)	32.0 ± 5.5 ^c	32.0 ± 5.1 ^c	17.2 ± 3.8 ^c	18.7 ± 3.7 ^c	64.0 ± 4.8 ^c
<i>Porcine</i>						
Pipeting	75 (3)	77.6 ± 3.7 ^b	18.7 ± 1.2 ^b	3.7 ± 3.6 ^b	0 ^b	96.3 ± 3.6 ^b
3 min vortexing	79 (3)	76.5 ± 7.4 ^b	22.2 ± 6.8 ^b	1.3 ± 2.2 ^b	0 ^b	98.7 ± 2.2 ^b

^a See Fig. 1.

^{b,c} Values (means ± SD) with different superscripts within a column differ significantly ($P < 0.05$).

Table 2. Enucleation rate after the removal of 10 or 20% cytoplasmic volume of *in vitro* matured bovine and porcine oocytes after removal of cumulus cells by repeated pipetting or vortexing

Treatment for cumulus removal	10 % removal		20% removal	
	No. of oocytes (replicates)	% of enucleated	No. of oocytes (replicates)	% of enucleated
<i>Bovine</i>				
Pipetting	49 (3)	67.2 ± 4.1 ^a	43 (3)	88.2 ± 4.6 ^{a*}
Pipetting + 0.5 min vortexing	81 (3)	65.2 ± 0.8 ^a	77 (4)	89.2 ± 2.7 ^{a*}
3 min vortexing	89 (3)	26.4 ± 0.5 ^b	76 (4)	60.4 ± 1.2 ^{b*}
<i>Porcine</i>				
Pipetting	69 (3)	63.9 ± 0.8 ^a	75 (3)	84.2 ± 2.1 ^{a*}
3 min vortexing	86 (3)	63.3 ± 5.1 ^a	92 (4)	82.5 ± 8.3 ^{a*}

^{a,b} Values (means ± SD) with different superscripts within a column differ significantly (P<0.01).

* Values (means ± SD) with asterisks differ significantly from those of 10% removal (P<0.05).

Table 3. Developmental competence of bovine nuclear transfer embryos constructed with cumulus cells and enucleated oocytes derived from two different cytoplasmic volumes

% of removed cytoplasmic volume	No. of couplets (replicates)	% of fused couplets	% of embryos developed to ^a			Blastocyst cell no. (n)
			≥ 2-cell	≥ 8-cell	blastocysts	
10	86 (3)	68.8 ± 1.1	74.0 ± 2.8	59.6 ± 8.6	37.3 ± 8.8	177.7 ± 7.7 (22)
20	109 (6)	68.5 ± 1.8	76.5 ± 3.4	57.0 ± 1.6	35.4 ± 4.5	176.3 ± 9.5 (25)

^a Values (means ± SD) are based on the numbers of fused couplets.

Development of NT embryos at ≥ 2-cell, ≥ 8-cell and blastocyst stages were determined at 33, 72 and 174 h after fusion, respectively.

Table 4. Cytoplasmic chromatin distribution of bovine and porcine oocytes with initial chromatin location at Position 1 after vortexing for 5 min

Animal species	No. of oocytes used	% of oocytes with chromatin at position ^a			
		1	2	3	4
Bovine	20	40.0	40.0	15.0	5.0
Porcine	35	60.0	28.6	11.4	0

^a See Fig. 1.

Data were pooled from 3 replicates.

successfully enucleated both in cattle and pigs. Mohamed and Takahashi [4] indicated that only bovine MII oocytes with chromatin material at Positions 1 and 2 can be successfully enucleated after removing the first polar body and approximately 20% of adjacent cytoplasm. The present results support their findings. The results in the porcine oocytes showed that only a few oocytes at Position 2 were successfully enucleated. This is probably due to the use of the same size of pipette during enucleation; the pipette could not be

inserted deeply into the ooplasm due to the smaller size of porcine oocytes compared with bovine oocytes.

Enucleation of a bovine oocyte with a volume of cytoplasm similar to that of the nuclear donor cell was found to be more favorable for the development of bovine NT embryos than enucleation with minimum or large volumes of cytoplasm [20]. With embryonic blastomeres as donors, a 50% reduction of the recipient oocyte volume decreased blastocyst development after

nuclear transfer [20, 23] and lowered blastocyst cell number [24, 25] in cattle. However, replacing the removed cytoplasm during enucleation with an equal amount or increasing it to twice the original volume with cytoplasm obtained from another enucleated oocyte increased blastocyst development [23] and cell numbers [24] in cattle. Similar development rates of bovine NT embryos to the blastocyst stage and blastocyst cell numbers in both tested groups (10 and 20% reduced cytoplasm) indicate the absence of a deleterious effect on the subsequent developmental capacity of somatic NT embryos reconstructed with enucleated oocytes containing 80% of the original cytoplasmic volume.

Duration of *in vitro* maturation culture influences the enucleation efficiency of cattle [26], sheep [15] and pig [7] oocytes. Enucleation rates decrease in aged oocytes matured *in vitro*. Postovulatory aging in mouse oocytes induces the migration of the second meiotic spindle towards the center of the oocytes due to the disappearance of the microfilament-rich domain [27]. This microfilament-rich domain is responsible for the maintenance of the meiotic spindle and chromosomes in the peripheral position of rat [27], mouse [28], and pig [29] MII oocytes. In the present study, some bovine and porcine oocytes could have reached the MII stage earlier than the time allowed for the oocytes to mature; 18–20 h in cattle [30] and 32–36 h in pig [7], respectively. This might be the reason why not all of the oocytes had the

metaphase plate adjacent to the first polar body even after pipetting.

In summary, the present study indicated that prolonged vortexing of COCs using hyperosmotic media moved the first polar body from the original site of extrusion and reduced the enucleation rate both in bovine and porcine oocytes. A lesser volume (10%) of ooplasm removal decreased the enucleation rate without any advantages on the subsequent developmental capacity compared with 20% removal in bovine oocytes. Aging of oocytes may also reduce the enucleation rate by inducing the migration of chromatin material. Therefore, we recommend to enucleate newly matured bovine and porcine oocytes by removing the first polar body and adjacent 20% of ooplasm after the removal of cumulus cells by vortexing for a short period using a hypo-osmotic media.

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