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Somatic Cell Nuclear Transfer of Oocytes Aspirated from Postovulatory Ovarian Follicles of Superovulated Rabbits

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ABSTRACT : The aim of this study was to evaluate if oocytes, aspirated from postovulatory ovarian follicles of superovulated rabbits 14 h post-hCG administration, could be efficiently used as ooplasm recipients for somatic cell nuclear transfer (SCNT). Within a common SCNT protocol, a comparison between oocytes recovered by direct aspiration (aspirated) from available ovarian follicles and oocytes flushed out from oviducts (flushed) was carried out. The results showed that maturation and enucleation rates of aspirated oocytes were 70.7% and 69.2%, significantly lower than 95.3% ($p<0.01$) and 83.6% ($p<0.05$), respectively, from flushed oocytes. However, following enucleation of matured oocytes as ooplasm recipients for SCNT, no difference was recorded in fusion and cleavage rates, as well as blastocyst development from cleaved embryos or hatching of blastocysts between aspirated and flushed groups. Additionally, some matured aspirated and flushed oocytes were also used for immediate parthenogenetic activation and the resulting embryo development was not significantly different. Results from this study show the following: i) the majority of oocytes aspirated from postovulatory ovarian follicles of superovulated rabbits 14 h post-hCG administration are matured and can be used directly as ooplasm recipients for SCNT; ii) the reconstructed embryos derived from these oocytes have similar *in vitro* developmental ability to those flushed from the oviducts. (**Key Words :** Nuclear Transfer, Oocyte, Rabbit, Follicle, Somatic Cell)

INTRODUCTION

The success of somatic cell nuclear transfer (SCNT) has been confirmed by the birth of cloned animals in several species (reviewed by Campbell et al., 2005). SCNT has the potential to improve the productivity of highly valuable livestock and to produce transgenic animals that synthesize large quantities of human proteins for biomedicine (Brophy et al., 2003). Also, SCNT has also become one of the pivotal tools for producing cloning-derived embryonic stem cells for therapeutic purpose (Wakayama et al., 2001) and for interspecies cloning to preserve wild animals (Lanza et al., 2000). Rabbits are much easier to handle during oocyte micromanipulation compared to mice and rats. They also require lower costs of maintenance compared to cattle and sheep. Therefore such species provides an excellent model system for basic research elucidating mechanisms of nuclear reprogramming (Mitalipov et al., 1999; Dinnyes et al., 2001; Yang et al., 2003). Rabbit oocytes have also been used as ooplasm recipients in SCNT to obtain embryonic stem cells (ESCs) derived from human somatic nuclei (Chen et al., 2003) and blastocysts by interspecies cloning between giant panda, macaca or ibex and rabbit (Chen et al., 2002; Yang et al., 2003; Jiang et al., 2005). Thus, recovering more rabbit oocytes with lower cost for SCNT is valuable and practical.

Most rabbit nuclear transfer studies are dependent on the use of *in vivo* matured and ovulated oocytes, which are routinely collected by flushing of the oviducts removed aseptically together with ovaries following anaesthesia or killing of donors does. Usually, donor animals are used only once and their ovaries are not utilized at all. Currently, there are only a few studies reporting the use of *in vitro* matured oocytes (Yin et al., 2001), and no information are available on the direct use of oocytes aspirated from superovulated rabbit ovarian follicles in rabbit SCNT. Oocytes aspirated from ovarian follicles are immature and have to be matured *in vitro* prior to fertilization or nuclear transplantation in many mammalian species (Hong et al., 2005). However,

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rabbits are induced ovulators and mature preovulatory follicles are present constantly on the surface of their ovaries. Ovulation normally occurs between 10 to 14 h (Harper, 1963; Varian et al., 1967) with some unruptured larger follicles remain until 26 to 30 h (Varian et al., 1967) following hormonal administration. Fresh rabbit ova recovered from the ovarian surface 10 to12 h after hCG injection are capable of undergoing fertilization *in vitro* and their developmental competence has been confirmed by the birth of a living offspring (Brackett et al., 1972). Thus, it can be hypothesized that oocytes aspirated from postovulatory ovarian follicles of superovulated rabbits (14 h post hormone injection), together with the use of those flushed out from the oviducts, could be used as ooplasm recipients to increase total number of mature oocytes for SCNT.

Accordingly, the goals of this study were to compare the micromanipulation efficiency and the *in vitro* developmental potential of NT and parthenogenetic embryos produced from aspirated and flushed oocytes, and to evaluate whether aspirated from postovulatory ovarian follicles of superovulated rabbits (14 h post hCG injection) could be used directly as ooplasm recipients for SCNT.

MATERIALS AND METHODS

Chemicals

Chemicals and media were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Life Technologies (Grand Island, NY, USA), respectively unless otherwise stated.

Superovulation of rabbits and collection of oocytes

Mature female New Zealand White rabbits weighting 2.0 to 3.0 kg were purchased from Guangdong Center of Laboratory Animal, China. Hormonal treatment of ovum donors was similar to what reported previously (Yang et al., 2003) unless otherwise described. Briefly, does were treated with 150 IU of PMSG (Tianjin Center of Laboratory Animal, China) to induce multiple ovulation, followed 72 h later by 150 IU of hCG (Ningbo Hormone Product Co LTD, China). To evaluate possible differences in development potential between oocytes aspirated from ovarian follicles from animals receiving or not hCG, in some experiments three does were administered the same volume (1.5 ml) of physiological saline instead of hCG to serve as control. At 14 h following hCG or saline injection, animals were laparotomized and ovaries and oviducts were removed aseptically.

Recovery of flushed oocytes : Cumulus masses were collected by flushing oviducts with Dulbecco's modified PBS (D-PBS) supplemented with 10% (v/v) of fetal bovine serum (FBS) and washed twice in TCM-199 with Earle's salts, 25 mM Hepes supplemented with 10% FBS (mM-199).

Recovery of aspirated oocyte : Ovarian surface follicles of 1 to 3 mm in diameter were aspirated with needle in 10 ml disposal syringe containing 2 ml mM-199. Cumulus-oocyte complexes (COCs) were selected under stereomicroscopy and washed twice in the same medium.

Cumulus cells were removed by short exposure to 2 mg/ml of hyaluronidase in TCM-199 and subsequent gentle pipetting with a small-bore (125 to 130 μm i.d.) Pasteurpipette. Cumulus-free M II oocytes with a first polar body (PB1) were then subject to nuclear transfer or parthenogenetic activation experiments as discussed later.

Enucleation of the oocytes

Cumulus-free M II oocytes with a PB1 were enucleated by micromanipulation. Briefly, oocytes were incubated for 10 min in 10 μg/ml Hoechst 33342 and then transferred into a small drop of mM-199 containing 7.5 μg/ml of cytochalasin B (CB). The first polar body and the metaphase plate of an oocyte were drawn into an enucleation pipette (20 to 22 μm i.d.). The aspirated karyoplasts were exposed to ultraviolet-light to confirm the presence of nuclei. Only oocytes with removed chromosomes were used for nuclear transfer.

Donor cells preparation

Rabbit cumulus cells from primary cultures (within six passages) were used as nuclear donors. Cells removed from oocytes were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. Before being used as nuclear donors, cells were washed twice with Ca^{2+} and Mg^{2+}-free PBS and then trypsinized. Cells were then washed by centrifugation and resuspended in mM-199, incubated at 38°C in drops and used within 1 h. Small, smooth membrane-surfaced cells were selected for nuclear transfer.

Nuclear transfer and activation

Single donor cells were inserted into the perivitelline space in close contact with the plasma membrane of enucleated oocytes. For electrofusion and activation, the oocyte-cell complexes were manually oriented in a coaxial electrodes (FE-C25/400) of a PA4000/PA-101S Cell Fusion System (Cyto Pulse Science, USA) in Cytofusion Medium (CPS-LCM-C, Cyto Pulse Science), then induced by double DC pulses of 2.0 kV/cm for 20 μsec spaced 3 sec apart. The reconstructed embryos were activated by incubating for 4 min with 5.0 μM ionomycin in dark and culturing for 3 hours with 2.0 mM 6-dimethylaminopurine (6-DMAP) in mM-199. At the end of 6-DMAP treatment, the reconstructed embryos were assessed for fusion.

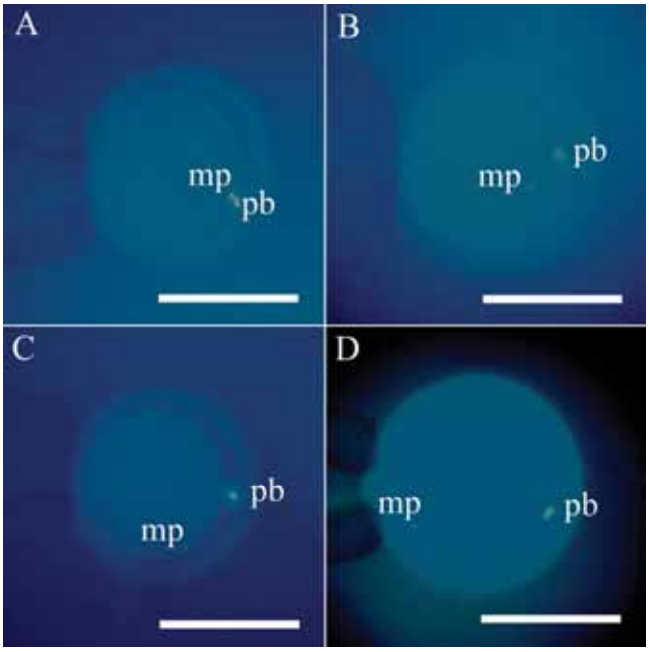

Figure 1. Representative micrographs of metaphase II oocytes showing different angles of deviation of the metaphase plate (mp) with regard to the location of the first polar body (pb) under UV lights microscope. (A): 0-5°; (B): 6-45°; (C): 46-90°; (D): >90°. 20×; Bars = 100 μm.

For parthenogenetic activation, oocytes (16 to 18 h after hCG) were treated with ionomycin and 6-DMAP with the same parameters and procedures for activation of the reconstructed embryos.

Embryo culture *in vitro*

All fused complexes and parthenotes retrieved after 6-DMAP treatment were washed 3 times and were cultured further for 144 h in 50 μl droplets of mM-199 under mineral oil in a humidified atmosphere of 5% CO_2 in air at 38°C. Half volume of fresh medium was replaced every 48 h. Cleavage and blastocysts development derived from reconstructed embryos and parthenotes were recorded. Total cell numbers of blastocysts fixed at day 5.5 (5.5 d) derived from parthenogenesis were counted following Hoechst 33342 epifluorescein staining.

Measurement of deviation angle between the metaphase plate and the PB1

Oocyte was exposed to UV lights for localization of metaphase plate and PB1 just before enucleation. The measurement was conducted as previously described by Rienzi et al. (2003). For this purpose, the oocyte was immobilized at the holding pipette with PB1 at the 3 o'clock position, and then rotated with the use of the enucleation pipette until the metaphase plate was clearly in focus in the oocyte equatorial plane under the UV lights. The angle was measured between the line connecting the oocyte with the metaphase plate and that connecting the oocyte centre with the PB1. The angle of deviation was divided into four groups of 0-5°, 6-45°, 46-90° and >90° for groups A to D, respectively according to the angle of metaphase plate deviation from the PB1 position (Figure 1).

Immunofluorescence localization of chromatin and microtubules

Oocytes were fixed and immunostained for chromatin and microtubules using a modification of the method described by Sun et al. (2001) to determine whether the oocytes with a PB1 obtained from aspiration were matured and M II arrested and to evaluate possible different localization of chromatin and microtubules in oocytes between aspirated and flushed groups. Briefly, rabbit oocytes were fixed with 4% paraformaldehyde in PBS for at least 2 h. They were first extracted in PBS containing 1% Triton X-100 overnight at room temperature and then blocked in PBS containing 0.3% BSA and 1% Triton X-100 for 30 min. After washing for 15 min, the oocytes were stained with FITC-conjugated anti-α-tubulin monoclonal antibody diluted 1:300 in PBS containing 0.3% BSA and 1% Triton for at least 2 h. After two washes in PBS, the oocytes were then stained with 1μg/ml propidium iodide (PI) for 5 min. Finally, the oocytes were mounted on glass slides and examined by using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Germany).

Statistical analysis

Values in each parameter were subjected to ANOVA in a generalized linear model (PROC-GLM) of SPSS 10.0 (SPSS Inc, Chicago, IL, USA). When model effect was significant for each parameter, each value after the treatment was compared by the least square method. $p<0.05$ was considered significant.

Table 1. Maturation and efficiency of micromanipulation in recovered aspirated and flushed oocytes from rabbits treated with PMSG and with or without hCG

Group	PMSG+hCG-		PMSG+hCG+				
	Oocyte	Mature	Oocyte	Mature (%)	Oocyte used (r)	Enucleated (%)	Nuclear injected (%)
Aspirated	62	0	246	174 (70.7)^{A}	107 (7)	74 (69.2)^{a}	56 (75.7)
Flushed	0	0	277	264 (95.3)^{B}	122 (7)	102 (83.6)^{b}	76 (74.5)

Values within the same column with different superscripts are significantly different ($^{A, B}$ $p<0.01$; $^{a, b}$ $p<0.05$). r: Replicates.

Table 2. Recorded angle between metaphase plate and PB1 in recovered aspirated and flushed oocytes from rabbits treated with PMSG and hCG

Group	Oocytes	A (0-5°, %)	B (6-45°, %)	C (46-90°, %)	D (>90°, %)
Aspirated	92	44 (47.8)	25 (27.7)	22 (23.9) [a]	1 (1.1)
Flushed	95	54 (58.9)	22 (23.2)	14 (14.7) [b]	3 (3.2)

Values within the same column with different superscripts are significantly different ([a, b] $p<0.05$). Seven replicates.

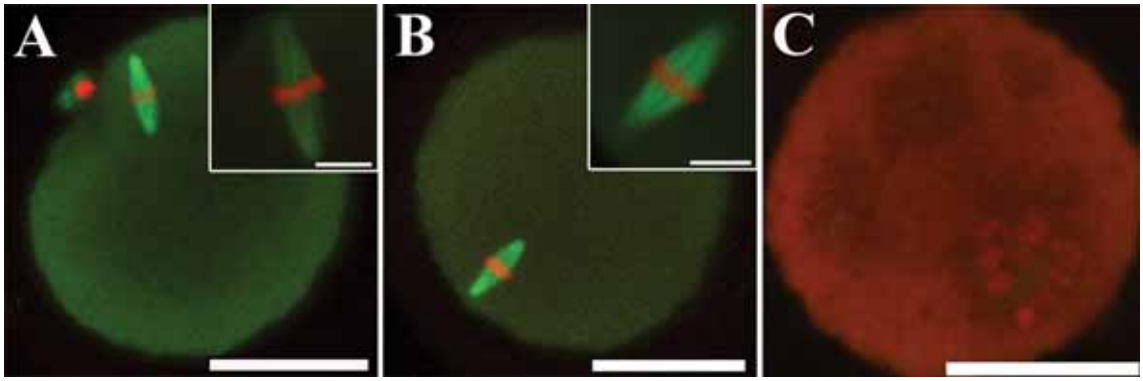

Figure 2. Immunofluorescence localization of microtubules (green) and chromosomes (red) under a laser scanning confocal microscope. Intact oocytes recovered 15 to 16 h following hCG administration displayed chromosomes with or without a spindle. Metaphase chromosomes, with (A) or without (B) adjacent to PB1, arranged on a compact plate at the equator with a typical barrel-shaped meiotic spindle (insert) close to the plasma membrane; absent spindle and dispersed chromosomes (C). Spindle labeled with FITC; nuclei in cytoplasm and PB1 labeled with PI. 20×; Bars = 50 μm (insert: 63×; Bars = 10 μm).

RESULTS

Maturation rates and efficiency of micromanipulation

The proportion of mature M II oocytes (indicated by the extrusion of the PB1) was assessed after the removal of cumulus cells. The survival of ooplasms to next manipulation step was considered a successful enucleation or nuclear injection. As shown in Table 1, neither oocytes flushed from oviducts nor oocytes aspirated from ovarian follicles had a PB1 from rabbits (n = 3) treated with PMSG and saline (PMSG+hCG-). In rabbits (n = 25) treated with PMSG and hCG (PMSG+hCG+), rate of PB1 extrusion as well as enucleation rate of oocytes with PB1 from aspirated group were 70.7% and 69.2%, significantly lower than 95.3% ($p<0.01$) and 83.6% ($p<0.05$) from the flushed group, respectively, and the nuclear injection rate was not statistically different between the two groups ($p>0.05$).

Deviation angle between the metaphase plate and the PB1

The deviation angle was divided into 0-5°, 6-45°, 46-90° and >90° for groups A to D and assessed 15 to 17 h after hCG administration. As shown in Table 2, overall distribution of oocytes with different groups of deviation angle was not significantly different between aspirated and flushed groups ($p>0.05$), however, the proportion of oocytes with angle 46-90° (group C) between the metaphase plate and the PB1 in aspirated group was significantly higher than control group (23.9% vs. 14.7%, $p<0.05$), indicating that more oocytes from the aspirated group had a longer distance between metaphase plate and PB1.

Immunofluorescence localization of chromatin and microtubules

A total of 24 aspirated and 30 flushed oocytes with a PB1 and 20 aspirated oocytes without a PB1 were examined. As spindle formation patterns and chromosome allocation were similar in oocytes with a PB1 in both aspirated and flushed groups, the data have been pooled. All oocytes with a PB1 recovered 15 to 16 h after hCG administration had metaphase chromosomes arranged on a compact plate at the equator with a typical barrel-shaped meiotic spindle close to the plasma membrane. Overall, 14/24 (58.3%) and 19/30 (63.3%) aspirated and flushed oocytes, respectively, had the metaphase plate adjacent to the PB1 (Figure 2a), whereas the remaining oocytes did not (Fig 2b). On the contrary, absent spindle and dispersed chromosomes were observed in all oocytes without a PB1 (Figure 2c).

Developmental ability of reconstructed complexes and parthenotes

Following enucleation, oocytes were used as nuclear recipients for SCNT together with donor cumulus cells. As shown in Table 3, no significant differences were obsevered in fusion and developmental rates at all stages examined between aspirated and flushed groups ($p>0.05$). Similar results were observed in parthenogenetically activated oocytes (Table 4). Furthermore, total cell number of

Table 3. Development of reconstructed complexes by using cumulus cells as nuclear donors in recovered aspirated and flushed oocytes from rabbits treated with PMSG and hCG

Group	No. of complexes	Fusion (%)	Cleavage (%)	No. (%) of blastocysts	No. (%) of hatching BL
Aspirated	56	41 (73.2)	31 (75.6)	9 (29.0)	5 (55.6)
Flushed	76	59 (77.6)	45 (76.3)	14 (31.1)	9 (64.3)

% of hatching BL: percentage of hatching blastocysts developed from blastocysts. Seven replicates.

Table 4. Development of parthenotes derived from mature oocytes recovered by aspiration and flush from rabbits treated with PMSG and hCG

Group	No. of oocytes	No. (%) cleaved	No. (%) of blastocysts	TCN (n) (Mean±SD)	No. (%) of Hatching BL
Aspirated	42	33 (78.6)	18 (54.5)	135.7±17.2(7)	9/11 (81.8)
Flushed	49	40 (81.6)	22 (55.0)	143.0±20.9(9)	10/13 (77.3)

TCN: Total cell number of counted blastocysts (fixed at D5.5). n: Number of blastocysts counted.
% of Hatching BL: Percentage of hatching blastocysts developed from blastocysts. Four replicates.

blastocysts that were derived from matured oocytes and fixed at 5.5 d showed no difference between the two groups (Table 4).

DISCUSSION

To our knowledge, this is the first report on SCNT of oocytes recovered from postovulatory ovarian follicles of superovulated rabbit without *in vitro* maturation. The results showed that maturation rate of oocytes aspirated was significantly lower ($p<0.01$) than those of flushed, which is consistent with the result showing that maturity and fertility of ova recovered from ovarian follicles are not as good as ova flushed from oviducts (Brackett et al., 1972). Although the proportion of oocytes with PB1 by aspiration is lower than that of flush, it still reaches similar rate of follicular ova matured *in vitro* for *in vitro* fertilization (Lorenzo et al., 1996) or nuclear transfer (Yin et al., 2001).

It is well known that hCG is used to mimic the spontaneous LH surge for induction of follicle maturation, rupture and ovulation in mammals. In rabbits, lower doses of hCG trigger nuclear maturation, whereas luteinization requires higher doses and it is only with the highest doses of hCG that follicle rupture is induced (Bomsel-Helmreich et al., 1989). In the present study, none of oocytes was flushed out from oviducts and none of oocytes aspirated from ovarian follicles had a PB1 from rabbits treated with PMSG but without hCG (Table 1). Such results are in agreement with studies performed on *in vitro* perfused rabbit ovaries (Kobayashi et al., 1981). In addition, Varian et al. (1967) reported a high number of unruptured follicles without ovulation in rabbit receiving FSH and a low dose of LH (0.10 mg/kg body weight).

Recent observations have shown that PB1 position does not predict accurately the location of the meiotic spindle in metaphase II mammalian oocytes (Hewitson et al., 1999; Silva et al., 1999). It has been reported that the metaphase plate was adjacent to the polar body in 55% of the rabbit oocytes (Dinnyes et al., 2001). Even Mitalipov et al. (1999) reported that, in more than 50% of oocytes evaluated, the metaphase plate was located far from the PB1. Our observations of the metaphase plate adjacent to the PB1 within aspirated and flushed oocytes (47.8% and 58.9%, respectively) support these findings.

In the present study, the efficiency of micromanipulation in terms of successful enucleation in oocytes aspirated from ovarian follicles is significantly lower compared to oocytes flushed from oviducts ($p<0.05$). The reason for this deviation is not clear but it might partly be due to the larger proportion of oocytes with the angle 46-90° between the metaphase plate and the PB1 found in the aspirated group rather than in the flushed group (23.9% vs. 14.7% respectively; $p<0.05$), indicating a higher number of aspirated oocytes displaying a longer distance between metaphase plate and PB1 (Table 2). The relative position of M II spindle and PB1 is still object of discussion. It has been reported that the method of cumulus cell removal could be affecting the relationship between the first polar body and the MII spindle in both hamster (Silva et al., 1999) and human (Rienzi et al., 2003). However, Hardarson et al. (2000) suggested that the distance between the PB1 and the MII spindle cannot be explained solely as being a result of lateral displacement during denudation of oocyte. Furthermore, Moon et al. (2005) reported that the spindle displacement is a time-dependent process affected by unknown factors instead of the result of displacements caused by the cumulus removal process, and the different spindle positions observed in *in vivo* and *in vitro* matured mouse oocytes may reflect differences in their cytoplasmic maturation processes. Results from this study, in both similar *in vitro* developmental potential of SCNT and parthenogenetic embryos as well as the spindle formation patterns and chromosome allocation, suggest that aspirated and flushed oocytes share similar characteristics. A previous report from Oh et al. (1975) has also shown that the ultrastructural details of rabbit ova recovered from ovarian follicles revealed no distinguishable characteristics when compared to flushed ova. During the preparation of cumulus-free oocytes, we found that the time of oocyte denudation for COCs aspirated from follicles was much

longer than cumulus masses flushed out from oviducts, and even the cumulus of aspirated oocytes could not be striped off thoroughly. In addition, we observed that a reduction in deviation angle in some oocytes could be achieved by pushing the PB1 to move towards the metaphase plate with the injection pipette during enucleation (Data not shown). Thus, we speculate that the increased distance between M II nucleus and PB1 may have been caused mainly by the cumulus removal process.

Results obtained in this study give indications that the developmental potential of reconstructed embryos from aspirated and flushed oocytes is similar. Similarly, Oh et al. (1975) reported in rabbits a similar fertilization and developmental rate when using ova recovered from ovarian follicles and fertilized *in vitro*, and ovulated ova undergoing *in vivo* fertilization. Hayashi et al. (1987) also reported in superovulated mice similar fertilization rate between ovulated and unovulated matured ova following inhibition of ovulation by indomethacin.

In conclusion, for the first time this study has shown that i) the majority of oocytes aspirated from postovulatory ovarian follicles of superovulated rabbits are matured and can be used directly as ooplasm recipients for SCNT and ii) the reconstructed embryos from these oocytes have similar *in vitro* developmental potential to those flushed from oviducts.

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