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—Original Article—

Improvement of the developmental competence of porcine oocytes collected from early antral follicles by cytoplast fusion

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Abstract. In the present study, we propose an alternative technique called cytoplast fusion to improve the maturation rate and developmental competence of growing oocytes collected from early antral follicles in pigs. We examined whether the fusion of a growing oocyte with the cytoplast from a fully-grown oocyte (CFR group) could better promote maturation and developmental competence of the growing oocyte compared to germinal vesicle (GV) transfer (GVTR group). After 44 h of *in vitro* maturation (IVM), most growing oocytes (GR group) were still arrested at the GV stage (64.0 ± 5.1%); this number was significantly higher ($P < 0.01$) than that of the other groups. No matured oocyte was observed in the GR group. The maturation rate of GVTR oocytes was significantly improved (18.8 ± 3.5%) compared with that of growing oocytes. The proportion of oocytes that reached the metaphase-II (M-II) stage in the CFR group (37.8 ± 2.0%) was significantly higher ($P < 0.05$) than that in the GVTR group, although still lower than that in the control group (75.2 ± 4.4%). No blastocyst was derived from growing oocytes. Among *in vitro* fertilized GVTR oocytes, 3.0 ± 1.9% developed into blastocysts; however, this percentage showed an insignificant increase compared with the GR group. On the other hand, the percentage of CFR embryos that developed into blastocysts (12.0 ± 4.3%) was significantly higher than that of GR embryos (0.0%), although still lower than that of control embryos (27.0 ± 5.5%). Total cell number in blastocysts in the GVTR group (23.3 ± 6.9) was significantly lower ($P < 0.05$) than that in the control group (50.4 ± 5.0). Meanwhile, the total cell number in blastocysts derived from CFR oocytes (36.3 ± 4.8) was comparable to that of the control group. In summary, cytoplast fusion significantly improves maturation rate and developmental competence of growing oocytes compared with GV transfer.

Key words: Cytoplast fusion, Early antral follicle, Germinal vesicle transfer, Growing oocyte

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In domestic animals, ovaries contain hundreds of follicles at different developmental stages and with different sizes. Each follicle contains one oocyte, and the size of the oocyte correlates with the size of the follicle [1]. Normally, only oocytes from fully-grown follicles are collected and used for further *in vitro* experiments, whereas growing oocytes are generally discarded. Improvement of maturation and developmental competence of early antral oocytes is important for both infertility treatment and animal conservation, since it helps increasing the number of oocytes and embryos that can be used for embryo transfer and, thus, increase the chance of success.

With recent improvements in the *in vitro* culture (IVC) techniques, it is possible to develop oocytes of domestic animals from pre- or early antral follicles to fully-grown follicles [2, 3]. However, the developmental competence is still low. Recently, addition of resveratrol during *in vitro* growth was shown to support developmental competence of growing oocytes after *in vitro* follicular culture [4]. The germinal vesicle (GV) from growing oocytes could also be transferred to the cytoplast of fully-grown oocytes to improve the maturation and developmental competence of growing oocytes [5, 6]. GV transfer has been used to exchange nuclear material between oocytes with low and high quality such as aged and young oocytes [7, 8], growing and fully-grown oocytes [5, 6], or vitrified and normal oocytes [5, 9, 10]. Although GV transfer helps improving the maturation rate in aged, growing, and vitrified oocytes to a certain extent, no reports indicate that their developmental competence is significantly improved.

We propose another technique to improve the maturation rate and developmental competence of low quality oocytes. The technique involves fusion of a low quality oocyte with the cytoplast (enucleated GV oocytes) of a fully-grown oocyte, which is known to be normal. In this study, growing oocytes collected from early antral follicles are rescued by fusing them with the cytoplast of fully-grown oocytes to generate big fusion oocytes (CFR group). The maturation and development to blastocysts of the CFR oocytes were then compared

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with those of oocytes collected from fully-grown follicles (control group), growing oocytes collected from early antral follicles (GR group), and growing oocytes rescued by GV transfer (GVTR group).

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were collected from pre-pubertal cross-bred gilts (Landrace × Large White × Duroc) at a local abattoir and transported to the laboratory in phosphate buffered saline (PBS; Takara Bio, Otsu, Shiga, Japan) at 35°C within 1 h. Growing oocytes were collected from < 1-mm diameter follicles, whereas fully-grown oocytes were collected from 2–6-mm follicles. After collection from follicles, cumulus-oocyte complexes were cultured in 500 µl of modified North Carolina State University (NCSU)-37 medium [11] according to Kikuchi *et al.* [12] in 4-well dishes (Thermo Scientific, Roskilde, Denmark) for 22 h. The IVM medium was modified by adding 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 50 mM β-mercaptoethanol (Sigma), 1 mM dibutyryl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan), and 10 IU/ml hCG (Puberogen, Novartis Animal Health, Tokyo, Japan). The oocytes were then transferred to IVM medium without dbcAMP and hormones and cultured for another 22 h. IVM was performed in 5% CO_2, 5% O_2, and 90% N_2 at 39°C.

Measurement of oocyte diameter

A number of fully-grown oocytes and growing oocytes were immediately denuded by mechanical pipetting, transferred to a drop of TCM199 (with Hanks salts; Sigma) supplemented with 5% (v/v) fetal bovine serum (FBS; Gibco, Waltham, MA, USA). The oocytes were placed under an inverted microscope (Olympus IX70; Olympus Optical, Tokyo, Japan) equipped with an eyepiece micrometer with × 400 magnification for measurement of diameter. Fully-grown oocytes are oocytes collected from fully-grown follicles (diameter 2–6 mm). Growing oocytes are oocytes collected from early antral follicles (diameter < 1 mm). A total of 100 fully-grown oocytes and 100 growing oocytes were examined in three replications.

GV transfer and GV-cytoplast fusion

Transfer of GV from a growing oocyte to an enucleated cytoplast from a fully-grown oocyte was performed using an Olympus IX70 inverted microscope equipped with Narishige hydraulic micromanipulator (Figure 1A'–F'). Briefly, after 22 h of IVM, fully-grown and growing oocytes were freed of the cumulus by gentle pipetting in the presence of 150 IU/ml hyaluronidase (Sigma). Denuded oocytes were then centrifuged in TCM 199 supplemented with 5% FBS, 1 mM dbcAMP, and 5 µg/ml cytochalasin D (Sigma) at 10,000 × *g* for 10 min at 38°C for visualization of the GV. Isolation of GV was performed in TCM 199 supplemented with 5% FBS, 1 mM dbcAMP, and 5 µg/ml cytochalasin D. A slit was made on the zona pellucida by using a sharp and thin needle. The GV and the surrounding cytoplasm were separated from the remaining cytoplast by squeezing the needle against the oocyte. The GV from a growing oocyte was then transferred to the perivitelline space of an enucleated cytoplast of a fully-grown oocyte in TCM 199 medium supplemented with 5% FBS, 1 mM dbcAMP, and 300 µg/ml phytohemagglutinin (PHA, Sigma). The GV-cytoplast complex was sandwiched with a pair of electrodes in fusion medium consisting of 0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.5 mM HEPES, and a single direct current pulse of 2.0 kV/cm for 20 µsec was applied for GV-cytoplast fusion using Nepagene ECFG21 electroporator (Nepa Gene, Ichikawa, Chiba, Japan). GV-cytoplast complexes were then kept in IVM medium supplemented with 1 mM dbcAMP. The fusion rate was checked after 1 h, and only fused GV-cytoplast complexes were cultured in IVM medium without hormones and dbcAMP for 22 h.

Fusion of a growing oocyte with an enucleated cytoplast from a fully-grown oocyte was performed by using an Olympus IX70 inverted microscope equipped with Narishige hydraulic micromanipulator (Fig. 1A–E). Briefly, after 22 h of IVM, fully-grown and growing oocytes were freed of the cumulus by gentle pipetting in the presence of 150 IU/ml hyaluronidase. Fully-grown oocytes were then centrifuged in TCM 199 supplemented with 5% FBS, 1 mM dbcAMP, and cytochalasin D at 10,000 × *g* for 10 min at 38°C for visualization of GV. Isolation of GV from fully-grown oocytes was performed in TCM 199 supplemented with 5% FBS, 1 mM dbcAMP, and 5 µg/ml cytochalasin D. A slit on zona pellucida was made by using a sharp and thin needle. The GV and the surrounding cytoplasm were isolated from the remaining cytoplast by squeezing the needle against the oocyte to obtain enucleated cytoplast from fully-grown oocytes. Growing oocytes and enucleated cytoplast from fully-grown oocytes were then treated with 0.5% pronase (Sigma) to remove the zona pellucida. Then, one growing oocyte was paired with one enucleated cytoplast and incubated in 300 µg/ml phytohemagglutinin for 15 min. Oocyte-cytoplast complexes were fused with a DC pulse of 2.0 kV/cm for 20 µsec using a Nepagene ECFG21 electroporator in a fusion chamber. Oocyte-cytoplast complexes were then kept in IVM medium supplemented with 1 mM dbcAMP. The fusion rate was checked after 1 h, and fused oocyte-GV complexes were cultured in IVM medium without hormones and dbcAMP for another 22 h.

In vitro fertilization

The medium for *in vitro* fertilization (IVF) was a modified Pig-FM [13], which consisted of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 10 mM HEPES, 8 mM $CaCl_2$, 2 mM sodium pyruvate, 2 mM caffeine, and 5 mg/ml bovine serum albumin (BSA; Fraction V, Sigma). IVF was conducted as previously described by Kikuchi *et al.* [12]. Briefly, after 44 h of maturation, fully-grown, growing, and reconstructed oocytes were transferred into 100-µl droplets of fertilization medium covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). About 20 oocytes in each droplet were fertilized by epididymal spermatozoa from a Landrace boar that had been frozen-thawed [14] and pre-incubated for 15 min [15]. The final concentration of spermatozoa was 1×10^5/ml. Oocytes and spermatozoa were co-incubated for 3 h at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2. The day of IVF was defined as Day 0.

In vitro culture

After co-incubation of the gametes, cumulus cells and attached spermatozoa were removed from the oocytes by gentle pipetting through a fine glass pipette. They were then transferred into IVC

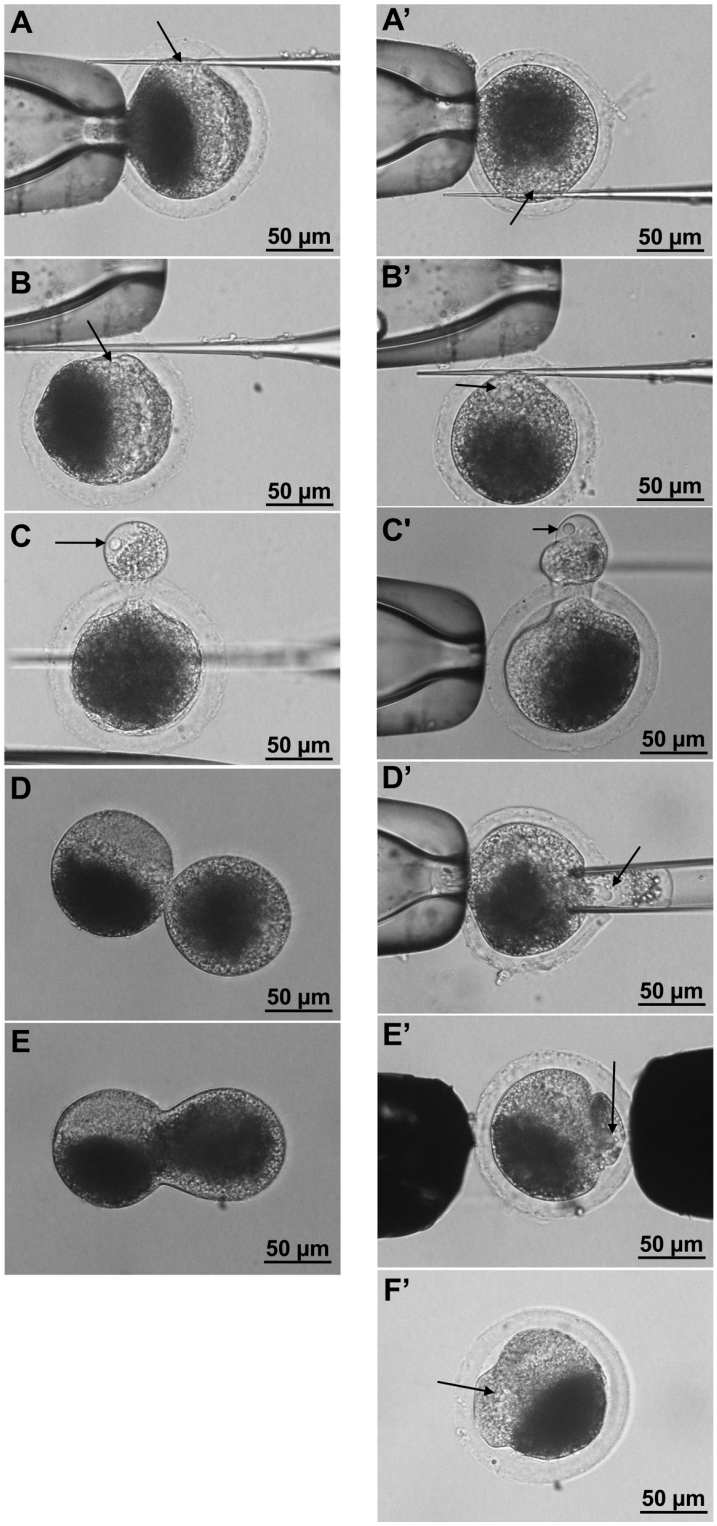

Fig. 1. Cytoplast fusion (CF) (A–E) and germinal vesicle transfer (GVT) (A'–F'). A sharp and thin needle was used to penetrate the zona pellucida nearby the GV (A), and the needle was then rubbed against the holding pipette (B) to make a slit on the zona pellucida. The GV and the surrounding cytoplasm were separated from the remaining cytoplast by squeezing the needle against the oocyte (C). After removal of the zona pellucida, one growing oocyte was paired with one enucleated cytoplast (D). An oocyte-cytoplast complex was fused after electrical fusion (E). Similar to CF, isolation of GV for GVT were performed by using a sharp and thin pipette (A'–C'). Then, a GV from a growing oocyte was transferred to the perivitelline space of an enucleated cytoplast of a fully-grown oocyte (D'). The GV-cytoplast complex was sandwiched with a pair of electrodes for induction of electrical fusion (E'). Oocyte-cytoplast complexes were fused after electrical fusion (F'). Arrows indicate the location of GV.

medium. Two types of IVC medium were prepared [12]. The basic IVC medium was NCSU-37 modified by the addition of 0.4% (w/v) BSA and 50 μM β-mercaptoethanol. Embryos were cultured at 38.5°C in IVC-PyrLac (basic IVC medium with the addition of 0.17 mM sodium pyruvate and 2.73 mM sodium lactate) from Day 0 (the day of IVF was defined as Day 0) to Day 2 and in IVC-Glu (basic medium supplemented with 5.55 mM glucose) until Day 6. Zona-free embryos were cultured in microwells (Dai Nippon Printing, Tokyo, Japan) (Fig. 2). Zona-intact embryos were cultured in a group of 20 to 30 in 500 μl IVC medium in 4-well dishes. IVC was carried out in 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C.

Orcein and Hoechst 33342 staining

Some oocytes in each experimental group were collected after 44 h of IVM to examine the maturation, whereas some zygotes in the group were collected 10 h after fertilization for examination of the fertilization status. Oocytes/zygotes were fixed with acetic acid: ethanol 1:3 for at least 3 days, then stained with 1% (w/v) orcein in acetic acid, mounted in glycerol:acetic acid:water (1:1:3), and examined under a phase-contrast microscope with × 100 to × 400 magnification (Olympus BX51, Olympus).

To evaluate the total cell number in blastocysts, Day 6 blastocysts were washed in PBS, fixed in 4% paraformaldehyde for 15 min, and then stained with 50 μg/ml Hoechst 33342. The blastocysts were then mounted on a glass slide and covered with a coverslip. The blastocysts were flattened and the cells appeared on a plane. Total cell numbers were counted under an epifluorescence microscope with × 100 to × 400 magnification (Olympus BX51). Blastocysts were defined as embryos with clear blastocoels and had over 10 cells.

Experimental groups and statistical analysis

There are four experimental groups in this study. The control group includes oocytes collected from fully-grown follicles (2–6-mm in diameter). The growing oocyte (GR) group includes growing oocytes collected from early antral follicles (smaller than 1 mm in diameter). The oocytes rescued by germinal vesicle transfer (GVTR) group includes growing oocytes rescued by transferring GV into enucleated cytoplasts of fully-grown oocytes. The oocyte rescued by cytoplast fusion (CFR) group includes growing oocytes rescued by fusion with enucleated cytoplasts of fully-grown oocytes.

The data of oocyte diameter, maturation status, fertilization status, blastocyst rate, and total cell numbers in blastocysts were analyzed by one-way ANOVA followed by Tukey's test by using Statview 5 software package (SAS Institute, Cary, NC, USA). Percentage data were arcsine transformed before analysis. Data are expressed as mean ± SEM.

Results

Diameter of oocytes collected from fully-grown follicles and early antral follicles

The average diameter of oocytes collected from fully-grown follicles (112.1 ± 1.8 μm) was significantly larger than that of oocytes collected from early antral follicles (88.1 ± 1.2 μm; $P < 0.0001$).

In vitro maturation of fully-grown oocytes, growing oocytes, and rescued oocytes

The *in vitro* maturation of fully-grown, growing, and reconstructed oocytes is shown in Fig. 3. After 44 h of IVM, most GR oocytes were still arrested at the GV stage (64.0 ± 5.1%); this number was significantly higher ($P < 0.01$) than that of the other groups. No matured oocyte was observed in the GR group. GVTR oocytes presented a significantly improved maturation rate (18.8 ± 3.5%) compared with growing oocytes. The proportion of oocytes that reached the metaphase-II (M-II) stage in the CFR group (37.8 ± 2.0%) was significantly higher ($P < 0.05$) than that in the GVTR group, although still lower than that in the control group (75.2 ± 4.4%). The incidence of degenerated oocytes significantly increased in the GR (8.1 ± 0.6%) and GVTR (8.3 ± 0.5%) groups in comparison with the control (0.8 ± 1.0%) and CFR (1.6 ± 1.1%) groups. A significant increase in the incidence of abnormal oocytes with two metaphase plates and oocytes with two polar bodies was also detected in the GVTR (10.5 ± 3.6% and 9.0 ± 2.3%, respectively) and CFR (11.0 ± 3.5% and 9.4 ± 2.6%, respectively) groups compared with those of the control (0.0% and 0.0%, respectively) and GR (2.2 ± 0.7% and 0.0%, respectively) groups.

In vitro fertilization after in vitro maturation of fully-grown oocytes, growing oocytes, and rescued oocytes

Figure 4 shows the proportion of fertilization rate, male pronuclear formation, and monospermy of fully-grown, growing, and rescued oocytes. The percentage of fertilized oocytes and MPN formation in the GVTR (68.2 ± 17.5%, and 68.0 ± 7.2%, respectively) and CFR (63.6 ± 5.2% and 72.0 ± 9.0%, respectively) groups were similar to those of the control group (75.8 ± 7.0% and 86.8 ± 7.5%, respectively), and significantly higher ($P < 0.05$) than those in the GR group (36.1 ± 5.8% and 9.1 ± 1.8%, respectively). The proportions of monospermic-fertilized oocytes were similar in all four groups. Fertilized oocytes are oocytes that contain a female pronucleus or a metaphase plate and one to several sperm heads and/or MPNs. The percentage of fertilized oocytes was calculated based on the total number of M-I and M-II oocytes. The percentage of MPN was calculated based on the number of fertilized oocytes.

Developmental competence of in vitro matured fully-grown oocytes, growing oocytes, and rescued oocytes

Table 1 shows the developmental competence of embryos derived from fully-grown, growing, and rescued oocytes in terms of blastocyst rate and cell number in blastocysts. No blastocyst was derived from growing oocytes. Among *in vitro* fertilized oocytes in the GVTR group, 3.0 ± 1.9% were able to develop into blastocysts. However, this is an insignificant increase compared with the GR group. On the other hand, the percentage of embryos in the CFR group that developed to blastocysts (12.0 ± 4.3%) was significantly higher than that in the GR group (0.0%), although still lower than that in the control group (27.0 ± 5.5%). The total cell number in blastocysts in the GVTR group was significantly lower than that in the control group. Meanwhile, the total cell number in blastocysts derived from the oocytes in the CFR group was comparable to that of the control group.

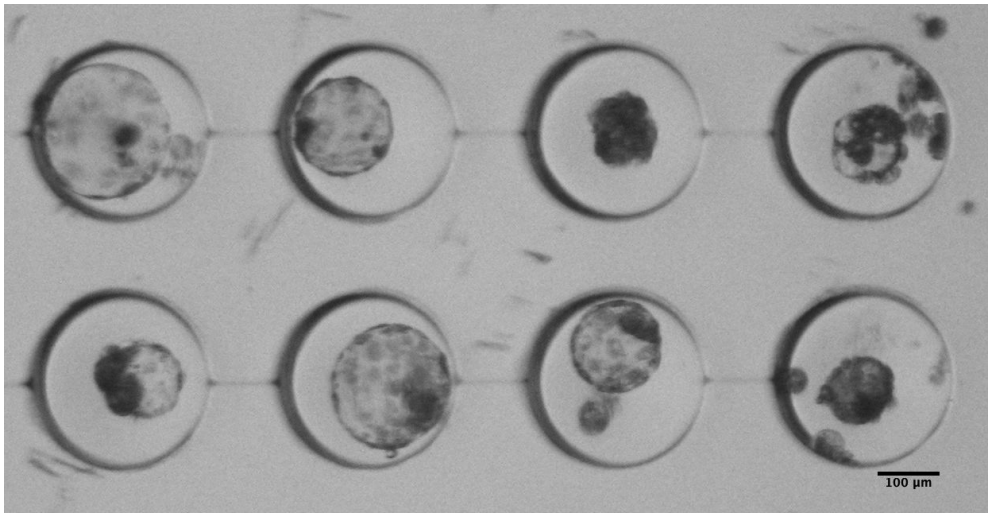

Fig. 2. Culture of zona-free embryos in microwells at Day 6 of IVC. Zona-free embryos derived from oocytes rescued by cytoplast fusion were cultured in microwells during 6 days.

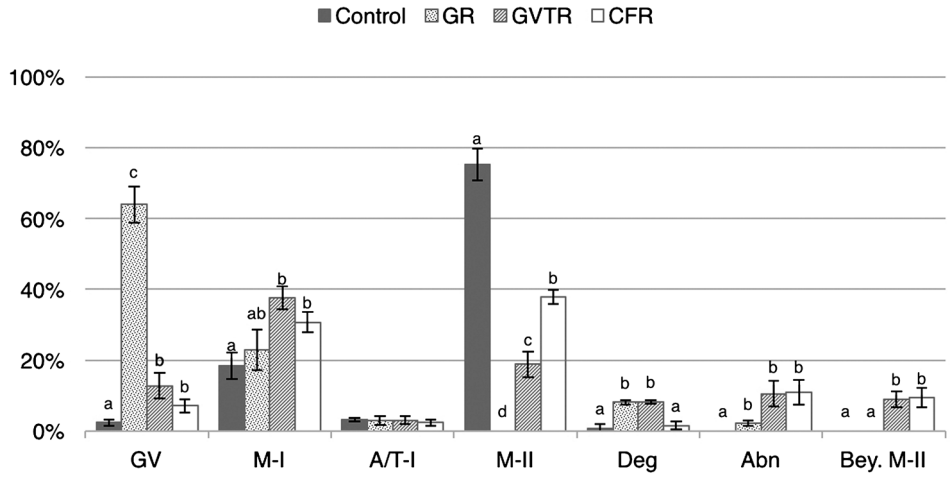

Fig. 3. *In vitro* maturation of fully-grown, growing, and rescued oocytes. Control: fully-grown oocytes after 44 h of IVM (N = 125). GR: growing oocytes after 44 h of IVM (N = 136). GVTR: growing oocytes, rescued by transferring germinal vesicles (GV) into enucleated cytoplasts of fully-grown oocytes, after 44 h of IVM (N = 133). CFR: growing oocytes, rescued by fusion with enucleated cytoplasts of fully-grown oocytes, after 44 h of IVM (N = 127). M-I: Metaphase-I; A/T-I: Anaphase/Telophase-I; M-II: metaphase-II; Abnormal: oocytes with two metaphase plates; Bey. M-II: oocytes beyond M-II such as Anaphase/Telophase-II or spontaneously activated oocytes (oocytes with two polar bodies and one set of chromosome with no sperm penetration). Four trials were performed. Different letters denote significant differences in the same categories ($P < 0.05$).

Discussion

In the present study, we assessed whether fusion with the cytoplast from fully-grown oocytes could improve the maturation and developmental competence of oocytes collected from early antral follicles compared with GV transfer. First, we assessed the diameter of fully-grown and growing oocytes. Fully-grown oocytes are the oocytes collected from fully-grown follicles with a diameter of 2–6 mm, whereas growing oocytes are the oocytes collected from early antral follicles with a diameter of < 1 mm. Fully-grown oocytes presented a significantly larger diameter compared with growing oocytes. This result is in agreement with previous reports [1–3].

Previous studies revealed that oocytes with diameter smaller than 100 µm collected from pre- and early antral follicles cannot mature *in vitro* [2,3]. Our result on the maturation (Fig. 3) of growing and fully-grown oocytes is consistent with these reports. No matured oocyte was detected in the GR group, whereas over 75% of fully-grown oocytes matured after 44 h of IVM. Meanwhile, almost 40% of growing oocytes reconstructed by fusion with the cytoplast reached the M-II stage after IVM. This is a significant improvement

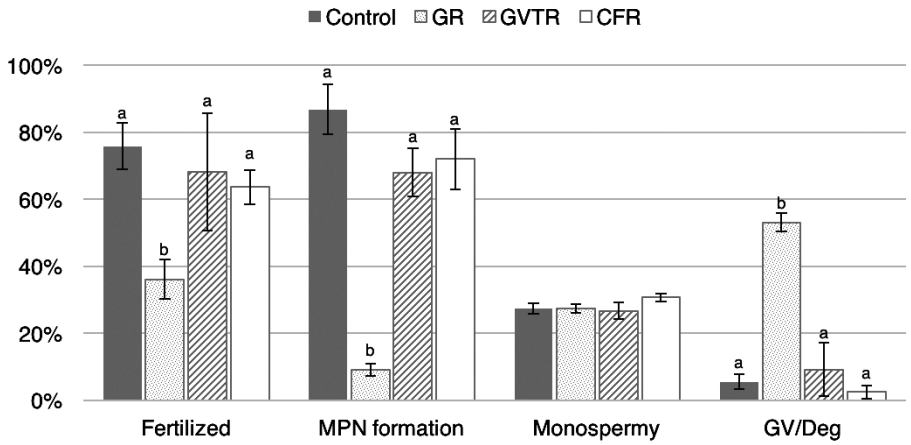

Fig. 4. *In vitro* fertilization of *in vitro* matured fully-grown, growing, and rescued oocytes. Control: zygotes 10 h after IVF, derived from *in vitro* matured fully-grown oocytes (N = 127). GR: zygotes 10 h after IVF, derived from growing oocytes (N = 130). GVTR: zygotes 10 h after IVF, derived from growing oocytes reconstructed by transferring germinal vesicles (GV) into enucleated cytoplasts of fully-grown oocytes (N = 121). CFR: zygotes 10 h after IVF, derived from growing oocytes rescued by fusion with enucleated cytoplasts of fully-grown oocytes (N = 121). MPN: male pronucleus. GV/Deg: the total number of GV and degenerated oocytes. The percentage of fertilized oocytes was calculated based on the total number of M-I and M-II oocytes. The percentage of MPN was calculated based on the number of fertilized oocytes. Four trials were performed. Different letters denote significant differences in the same categories ($P < 0.05$).

Table 1. Development of embryos derived from fully-grown, growing, and rescued oocytes

Group	No. of embryos cultured	Blastocysts (%)	Total cell number in blastocysts
Control	100	27 (27.0 ± 5.5) ^{a}	50.4 ± 5.0 ^{a}
GR	100	0 ^{c}	--
GVTR	100	3 (3.0 ± 1.9) ^{bc}	23.3 ± 6.9 ^{b}
CFR	100	12 (12.0 ± 4.3) ^{b}	36.3 ± 4.8 ^{ab}

Control: oocytes collected from fully-grown follicles (2–6 mm in diameter) were fertilized and then cultured *in vitro*. GR: growing oocytes collected from early antral follicles (smaller than 1 mm in diameter) were fertilized and then cultured *in vitro*. GVTR: growing oocytes, rescued by transferring germinal vesicles (GV) into enucleated cytoplasts of fully-grown oocytes, were fertilized and then cultured *in vitro*. CFR: growing oocytes, rescued by fusion with enucleated cytoplasts of fully-grown oocytes, were fertilized and then cultured *in vitro*. Four trials were performed. Different letters denote significant differences in the same column ($P < 0.05$).

compared with not only the GR, but also the GVTR group. Although the maturation rate of growing oocytes fused with the cytoplast was still lower than that of the control oocytes, it should be noted that, after cytoplast fusion, the oocytes were cultured without cumulus and zona pellucida for 22 h of IVM. Culturing oocytes without cumulus during IVM significantly reduces the maturation rate [16]. Our data are in agreement with a previous study showing that a lower percentage of cumulus-free and zona-free oocytes reached the M-II stage when compared with the control oocytes (Supplementary Fig. 1: online only). Therefore, an improvement of 40% in the maturation rate can be considered remarkable. The incidence of chromosomal abnormalities, including oocytes with two M-II plates and those with two polar bodies and one set of chromosomes, in the GVTR and CFR groups was significantly higher than that in the control and GR groups. These data suggest that oocyte manipulation and/or electrical fusion procedures may have some negative effects on normal oocyte maturation. Nevertheless, this incidence is not considered high, because they were limited to less than 10% of the examined oocytes.

We examined whether cytoplast fusion could promote fertilization. There has been a concern that using the same sperm concentration than that used regularly for cumulus-oocyte complexes might change the fertilization and monospermy rates for the oocytes without cumulus cells and/or zona pellucida [17]. However, in the present study, the percentages of fertilization, MPN formation, and monospermy did not differ among zona-free, cumulus-free, and control oocytes (Supplementary Fig. 2: online only). We, therefore, used the same sperm concentration (1×10^5 sperm/ml) and IVF conditions for all treatments. IVF data showed that both GV transfer and cytoplast fusion techniques significantly improved fertilization and MPN formation rates compared with those of the growing oocytes (Fig. 4) with no significant difference between the two groups as well as between each of these two groups and the control group.

We then, followed the embryo developmental ability to the blastocyst stage (Table 1). As expected, no blastocyst was derived from growing oocytes after 6 days of IVC. Although 3% of GVTR embryos managed to develop to blastocysts, this is an insignificant increase compared to that in the GR group. Moreover, the total cell number in blastocysts in the GVTR group was significantly lower compared with that of the control group. On the other hand, the percentage of embryos that developed to the blastocyst stage in the CFR group was significantly higher than that in the GR oocytes, and the total cell number in blastocysts in the CFR group was comparable to that of the control group. These data suggest that cytoplast fusion

can promote developmental competence of growing oocytes better than GV transfer can. Further study is necessary to examine whether cytoplast fusion could support development to term.

It should be noted that, in order to keep the embryos intact and support embryonic development, embryos derived from CFR oocytes were cultured in microwells with the size of 280 μm in diameter and 160 μm in depth (Fig. 2). In a preliminary experiment, zona-free embryos cultured in these microwells had similar blastocyst rates and total cell numbers in blastocysts compared with control embryos suggesting that these microwells provided suitable conditions for zone-free embryos to grow to blastocysts (Supplementary Table 1: online only).

The present study indicates that cytoplast fusion could improve maturation rate and developmental competence of growing oocytes when compared with GVT. However, the possible reasons for these observations, which remain difficult to explain, are not known. Nevertheless, it is likely not due to the amount of cytoplasm because previous studies showed that the amount of cytoplasm in an oocyte *per se* does not affect *in vitro* preimplantation development in mice [18–20].

One of the major concerns about cytoplast fusion is that the CFR group would contain two different sources of mitochondria, and this state of mitochondria heteroplasmy might affect the development of the oocytes in the CFR group. However, a recent study proved that hetero-mitochondria have no negative influence on the development to blastocysts [21]. Research on how mitochondrial heteroplasmy affects the development to term should be carried out in the future.

In summary, cytoplast fusion significantly improves maturation rate and developmental competence of growing oocytes compared with GV transfer. This technique could be very useful for basic studies on oocyte maturation, fertilization, and embryonic development as well as for applications in the conservation of endangered animals and infertility treatment.

Acknowledgements

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