Effects of In Vitro Aging of Mouse Oocytes on Metaphase II Spindle Morphology, In Vitro Fertilization and Subsequent Embryonic Development

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Abstract: *The aim of the present study was to reveal the effects of in vitro aging of mouse oocytes without cumulus cells on morphological alteration of their metaphase II (MII) spindles, ability to be fertilized in vitro and the developmental competence of the resulting embryos. To observe the MII spindles by immunofluorescence staining, oocytes without cumulus cells were divided into four groups: a non-aged control and three others aged for 10, 15 or 25 h. Although the incidence of morphologically normal spindles was significantly lower in the 25 h group, no significant increase of chromosome misalignment was observed in any of the aged groups ($P < 0.05$). For the in vitro fertilization (IVF) experiments, denuded oocytes were aged for 15, 16.5 or 18 h and the resulting normal zygotes with a second polar body were cultured in vitro for 120 h to assess their embryonic development. The fertilization rate was significantly lower only in the 18-h aged group. The rates of blastocyst formation were significantly lower in all aged oocyte groups, compared with non-aged controls, and blastocysts derived from aged oocytes had lower total cell numbers. Therefore, a significant decline of developmental competence appears in cumulus cell-removed oocytes aged for more than 15 h, even if they retain fertilizability.*

Key words: *Mouse oocyte, In vitro aging, MII spindle, In vitro fertilization, Embryonic development*

Received: October 10, 2009
Accepted: November 25, 2009
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Introduction

With the recent progress and widespread use of assisted reproductive technology (ART), *in vitro* aging of oocytes under culture raises as concern for the success of *in vitro* fertilization (IVF). In human IVF, sometimes there are instances of failure of IVF despite the use of normal spermatozoa. To salvage these human oocytes after IVF failure, reinsemination of the unfertilized and aged oocytes has been tried using intracytoplasmic sperm injection (ICSI) [1–10]. In these reports, the possibility of fertilization and cleavage after reinsemination has been demonstrated, but the clinical results of the subsequent pregnancies were not promising. Ethical issues concerning the experimental use of human oocytes prevent their use in the elucidation of *in vitro* oocyte aging. In order to improve clinical results in such cases, research into *in vitro* oocyte aging using animal oocytes is necessary.

It has been demonstrated that ovulated mouse oocytes retain competence for fertilization for 4–6 h *in vivo* [11]. The *in vivo* aged mouse oocytes fertilized rapidly compared with non-aged oocytes [12], and the development of embryos derived from aged oocytes was accelerated [13–16]. Postovulatory aging of mouse oocytes *in vivo* caused aneuploidy due to premature centrosome separation [17], and it resulted in a reduction of newborns [18, 19]. Significant disruption of microtubules in MII spindles in the *in vitro* cultured mouse oocytes occurred at 16 h and incidence of

chromosomal misalignment increased significantly 29 h post-ovulation [20]. Bai *et al.* demonstrated that the developmental competence of mouse oocytes aged for 42 h post injection of human chorionic gonadotrophin (hCG) could be rescued by transferring karyoplast of aged oocytes into cytoplast of fresh oocytes, and suggested that cytoplasm is more crucial than nucleus for the aging process [21]. Miao *et al.* have demonstrated that the existence of cumulus cells in *in vitro* culture for aging increased the rate of oocyte activation and decreased the activity of maturation promoting factor, i.e. cumulus cells accelerated aging of oocytes [22]. However, in these reports, the developmental competence of oocytes aged for different lengths of time in culture was not compared. Therefore, in the present study we examined the morphological alteration of metaphase II (MII) spindles in mouse oocytes without cumulus cells during aging *in vitro*, compared the developmental competence of embryos derived from IVF using oocytes aged for different periods, and estimated the quality of the resulting IVF blastocysts.

Material and Methods

Animals

Mouse oocytes used in all experiments were obtained from 4–5 months old (BALB/c × C57BL/6J) F1 females. The F1 females were bred from BALB/c females (CLEA, Tokyo, Japan) and C57BL/6J males (CLEA). Spermatozoa used for insemination in the IVF experiments were obtained from 5–6 months old ICR male mice (CLEA). We maintained all mice at 24°C with a photoperiod of 14 h light and 10 h darkness, and all mice had free access to food and water. The animals were used according to the Guide for the Care and Use of Laboratory Animals of Utsunomiya University.

Observation of changes in shape of aged oocytes and their MII spindles

A series of procedures from superovulation to IVF was performed as described previously [23]. Female mice were superovulated by an intraperitoneal injection of 5 IU of equine chorionic gonadotrophin (eCG, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) followed by 5 IU of human chorionic gonadotrophin (hCG, ASKA Pharmaceutical Co., Ltd.) 48 h later. Fourteen hours after hCG injection, the mice were euthanized by cervical dislocation, and cumulus oocyte complexes were collected from their oviductal ampullae. Collected cumulus oocyte complexes were washed in phosphate-buffered saline (PBS, Nissui, Tokyo, Japan), and cumulus cells around the oocytes were removed by pipetting in PBS containing 0.1% hyaluronidase (Sigma, St. Louis, MO, USA) [24]. After washing with PBS without hyaluronidase, oocytes showing abnormal shape (i.e. shrunk, fragmented or cleaved oocytes) were removed. The oocytes were divided into 4 groups, a control (non-aged) group and 3 aged groups according to the planned aging times of 10, 15, and 25 h in human tubal fluid (HTF) medium [25] supplemented with 0.5% bovine serum albumin (BSA, Sigma) at 37°C in 5% CO_2 and 95% air. The oocytes of the control group were immediately fixed in PBS containing 3.7% formaldehyde (Wako, Osaka, Japan) for 30 min at room temperature. After culture, oocytes of the *in vitro* aged groups were fixed by the same procedure. All the fixed oocytes were stored in PBS containing 0.1% BSA and 0.1% NaN_3 (Wako) at 4°C until immunofluorescence staining.

After a permeabilization treatment with 0.25% Tween-20 (Wako) in PBS for 5 min at room temperature, the oocytes were washed 3 times with PBS containing 0.1% BSA. Staining of microtubules was performed using mouse anti-β-tubulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (MP Biomedicals, Morgan Irvine, CA, USA) as the secondary antibody. After incubation with the primary antibody for 45 min at room temperature, the oocytes were washed 3 times with PBS containing 0.5% Triton X-100 (Wako) and 0.5% BSA and then incubated with the secondary antibody for 1 h at room temperature. After washing 3 times with PBS containing 0.5% Triton X-100 and 0.5% BSA, oocyte chromosomes were stained with 1 μg/ml Hoechst 33342 (Sigma) solution for 30 min at room temperature. Following washing, the oocytes were mounted on a glass bottom dish (Asahi Glass Co., Tokyo, Japan), and observed using a fluorescence microscope, Axiovert 200MOT-LSM (Carl Zeiss Inc., Oberkochen, Germany).

In vitro fertilization of aged oocytes

To examine the time limits of *in vitro* aging of mouse oocytes, we first performed a pre-experiment of IVF in 10-h and 20-h aged groups. As the rates of 2nd PB extrusion, cleavage, and blastocyst formation after IVF in the 10-h aged group were comparable to the rates in the non-aged group, the cell numbers of the blastocysts derived from 10-h oocytes had not decreased significantly compared with the non-aged oocytes. However, in the 20-h aged group, most oocytes had

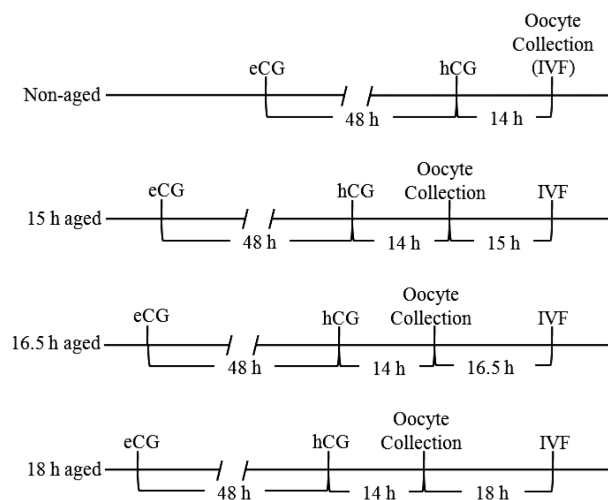

Fig. 1. Time schedule of superovulation for *in vitro* aging of mouse oocytes.

degenerated or fragmented at 5 h after IVF and very few oocytes extruded the 2nd PB. There were few cleaved oocytes at 29 h after IVF, and no embryos developed to the blastocyst stage. Therefore, we decided to compare the developmental competence of the embryos derived from IVF of oocytes aged *in vitro* for 0, 15, 16.5, and 18 h.

Oocytes from the control and aged groups were inseminated using spermatozoa obtained from the same male at the same time. Induction of superovulation, oocyte collection and removal of cumulus cells were performed as described above. For *in vitro* aging of oocytes prior to IVF, eCG was injected into mice at 15, 16.5, and 18 h earlier than the injection time for the control group mice (Fig. 1) followed by hCG administration 48 h later. Oocytes for *in vitro* aging were stored in HTF medium supplemented with 0.5% BSA and 1 mg/ml fetuin (Sigma) at 37°C in 5% CO_2 and 95% air for 15, 16.5, and 18 h until insemination. Fetuin was added to the medium [26] to inhibit zona pellucida hardening which occurs in mouse oocytes aged *in vitro* in serum-free medium [27].

Male mice were euthanized by cervical dislocation 2 h before insemination, and their spermatozoa were retrieved from the cauda epididymes by squeezing. Retrieved spermatozoa were suspended in HTF medium supplemented with 0.5% BSA and incubated at 37°C in 5% CO_2 and 95% air for 2 h, then transferred to drops of the IVF medium (a final concentration of 200 sperm/μl). The oocytes of the control and aged groups were introduced to 400-μl drops of the insemination medium separately and cultured for 5 h at 37°C in 5% CO_2 and 95% air. After 5 h incubation, they were washed with fresh media and checked for extrusion of the second polar body (2nd PB), degeneration or fragmentation using an inverted microscope. The zygotes with 2nd PB were transferred to KSOM medium, which was used for developmental culture, and the embryonic development was observed every 24 h. Rates of 2nd PB extrusion, cleavage at 29 h after insemination, and blastocyst formation were compared between the control and aged groups.

Differential staining of ICM and TE cells of resulting blastocysts

Blastocysts obtained from the IVF experiments were fixed with PBS containing 3.7% formaldehyde for 30 min at room temperature to count the cells. The fixed blastocysts were permeabilized with 0.25% Tween-20 in PBS for 5 min at room temperature, and then washed 3 times with PBS containing 0.1% BSA. To stain the inner cell mass (ICM) cells, goat anti-Oct4 polyclonal antibody (Santa Cruz Biotechnology Inc.) was used as the primary antibody and Alexa 594-conjugated donkey anti-goat antibody (Zymed Laboratories, San Francisco, CA, USA) was used as the secondary antibody [28]. To stain the trophectoderm (TE) cells, mouse anti-Cdx2 monoclonal antibody (Biogenex, San Ramon, CA, USA) was used as the primary antibody and FITC-conjugated goat anti-mouse antibody (MP Biomedicals) was used as the secondary antibody. Before incubation with antibody, blastocysts were immersed with 2.5% Tween-20 in PBS for 5 min at room temperature, and then treated with anti-Oct4 antibody overnight at 4°C and with other antibodies for 1 h at room temperature. Each time after incubation with antibodies, blastocysts were washed 3 times in PBS containing 0.5% Triton X-100 and 0.5% BSA, and then immersed in 1 μg/ml Hoechst 33342 solution for 30 min at room temperature to stain the nuclei. After washing, blastocysts were mounted on glass slides and covered with glass slips, and observed using the fluorescence microscope.

Statistical analysis

Statistical analysis was performed using the chi-square test to compare the normality of MII spindle morphology between the control and aged groups. Rates of 2nd PB extrusion, cleavage at 29 h after insemination, and blastocyst formation were compared using Student's *t*-test or one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered to be significant.

Table 1. Results of observation of shape and spindle morphology in aged mouse oocytes

Time of *in vitro* aging (h)	No. of oocytes cultured	No. of normal shape oocytes (%)	No. of spindles observed	No. of normal spindles (%)	Normal alignment of chromosomes (%)
0	36	–	17	14 (82.4)[a]	14 (82.4)[a]
10	32	32 (100)[a]	15	15 (100)[a]	15 (100)[a]
15	37	36 (97.3)[a]	24	19 (79.2)[a]	23 (95.8)[a]
25	29	27 (93.1)[a]	19	1 (5.3)[b]	14 (73.7)[a]

The values with different superscripts (a, b) in the same column are significantly different ($P < 0.05$: chi-square test).

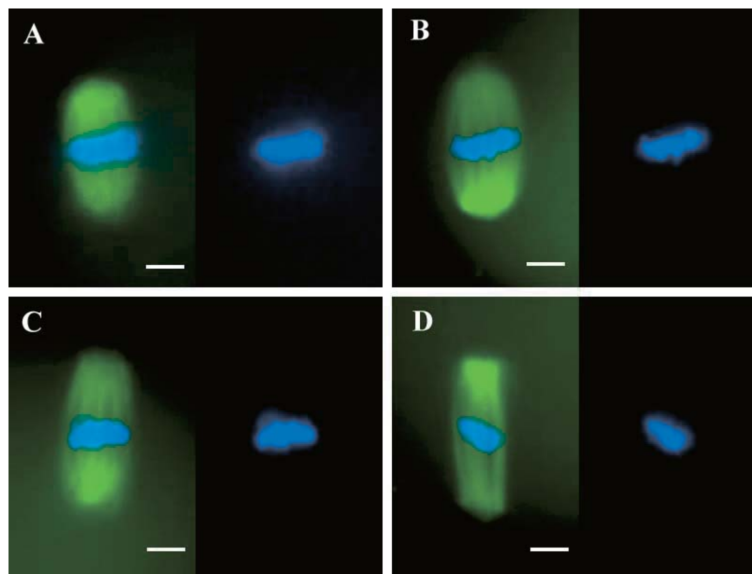

Fig. 2. Morphology of MII spindles in mouse oocytes after *in vitro* culture for aging. Fig. A shows the spindle of an MII oocyte fixed immediately after retrieval. Figs. B, C, and D show spindles cultured *in vitro* for 10, 15, and 25 h, respectively. Morphology of spindles in B and C differ slightly from that in A, but marked disruption of microtubules and chromosomal misalignment are not seen and their barrel shapes are similar to that in A. Morphology of microtubules of the spindle in D is markedly different from those in A, B, and C, but chromosomal misalignment is not seen. Scale bar = 5 μm.

Results

Changes in shape of oocytes and their MII spindles

The results observed for shape of oocytes and morphology of MII spindles after *in vitro* culture for aging are shown in Table 1. The percentages of oocytes which maintained normal shape in the 10-, 15-, and 25-h aged groups were 100%, 97.3% and 93.1%, respectively, and the remainder were shrunk, fragmented or cleaved oocytes. A significant increase in the number of abnormally shaped oocytes was not observed, not even in the 25-h aged group. The morphologies of the spindles in the non-aged oocytes and aged oocytes are shown in Fig. 2. In a non-aged oocyte, the spindle kept the barrel shape which is typical of the mouse MII oocyte, and the chromosomes which aligned in the equatorial plane were considered to be normal (Fig. 2A). We defined the MII oocyte which has the typical barrel shape MII spindle and such chromosome alignment as normal. In some oocytes, the normally shaped spindle and chromosome alignment was gradually lost with aging. The

Table 2. Developmental competence of mouse embryos derived from IVF of oocytes aged *in vitro* for 0, 15, 16.5, and 18 h

Time of *in vitro* aging (h)	No. of Exp.	Total number of oocytes	Oocytes with 2nd PB (%)	Embryos cleaved at 29 h (%)	Blastocysts formed at 96 h (%)	Blastocysts formed at 120 h (%)
0	3	209	61.4 ± 6.8^{a}	60.4 ± 8.8^{a}	90.1 ± 3.4^{a}	94.5 ± 1.5^{a}
15	3	164	67.3 ± 6.7^{a}	68.5 ± 10.9^{a}	32.8 ± 10.9^{b}	56.3 ± 14.3^{ab}
16.5	3	123	53.4 ± 18.0^{a}	44.3 ± 12.1^{a}	22.4 ± 10.9^{b}	48.4 ± 12.8^{b}
18	3	118	32.5 ± 12.6^{b}	44.9 ± 7.8^{a}	6.7 ± 3.7^{b}	42.4 ± 4.7^{b}

The values with different superscripts in the same column are significantly different ($P < 0.05$: ANOVA).

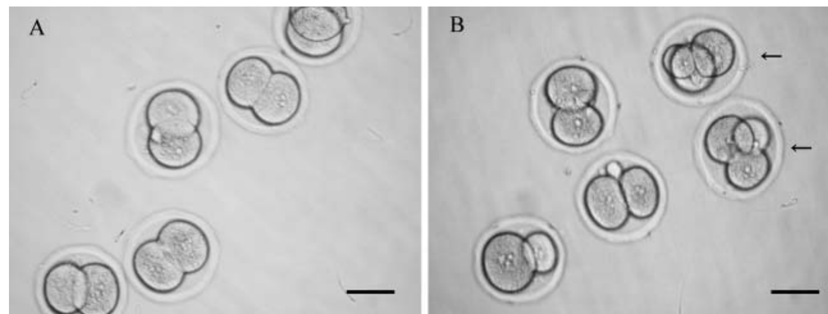

Fig. 3. Mouse embryos after first cleavage division at 29 h after *in vitro* fertilization (IVF) of non-aged and aged oocytes. Figs. A and B show the 2-cell stage embryos derived from IVF of non-aged oocytes and the 2- and 3-cell (arrows) stage embryos derived from *in vitro* culture of 15 h aged oocytes, respectively. Scale bar = 50 μm.

percentages of spindles which maintained normal morphologies in 0-, 10-, 15-, and 25-h aged groups were 82.4%, 100%, 79.2%, and 5.3%, respectively. The percentage of morphologically normal spindles decreased significantly in the 25-h aged group, but a significant increase in chromosome misalignment was not observed in this group.

Developmental competence of embryos derived from IVF of in vitro aged oocytes

Table 2 shows the results of the comparison among the control, 15-, 16.5-, and 18-h aged groups for the rates of 2nd PB extrusion, cleavage at 29 h after IVF, and blastocyst formation in 96- and 120-h culture after IVF. The rate of 2nd PB extrusion in the 18-h aged group was significantly lower than those in the other groups. There was no significant difference among the groups for the rate of cleavage at 29 h after IVF, although both the 16.5- and 18-h groups had lower cleavage rates than those of the other groups. The rates of blastocyst formation at 96 h after IVF in all aged groups were significantly lower than that of the control group. However, the number of blastocysts in each aged group increased at 120 h after IVF. Furthermore, there was no significant difference between the control and 15-h aged groups at this time.

It is interesting to note that embryos which directly cleaved into more than 3 blastomeres were observed in the 15-, 16.5-, and18-h aged groups (Fig. 3) after the first cleavage division at 29 h after IVF. The percentage of such embryos gradually increased with the period of oocyte aging *in vitro* (Table 3).

Fig. 4 shows the proportion of different developmental stages in the cleaved embryos at 48 h *in vitro* culture (IVC) after IVF in the control and each aged group. At 48 h IVC, many embryos of the control group developed to the 8-cell stage, but few embryos derived from the aged oocytes developed beyond the 4-cell stage.

The number of cells in blastocysts derived from IVF of aged oocytes

The number of cells in blastocysts derived from IVF of the control and each aged oocyte are shown in Table 4. Fig. 5A shows Oct4 expression in the ICM (red) cells

Table 3. The number of cells in mouse embryos cleaved at 29 h after IVF of non-aged and aged oocytes

Time of *in vitro* aging (h)	No. of Exp.	No. of cleaved embryos	Percent of cell number			
			2 cells	3 cells	4 cells	5 cells
0	3	127	100.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]
15	3	109	81.9 ± 2.2[ab]	14.7 ± 3.1[ab]	3.4 ± 1.9[a]	0.0 ± 0.0[a]
16.5	3	58	65.7 ± 8.3[bc]	27.6 ± 3.3[bc]	6.7 ± 5.1[a]	0.0 ± 0.0[a]
18	3	55	44.7 ± 4.5[c]	48.6 ± 10.0[c]	3.3 ± 3.3[a]	3.3 ± 3.3[a]

The values with different superscripts in the same column are significantly different ($P < 0.05$: ANOVA).

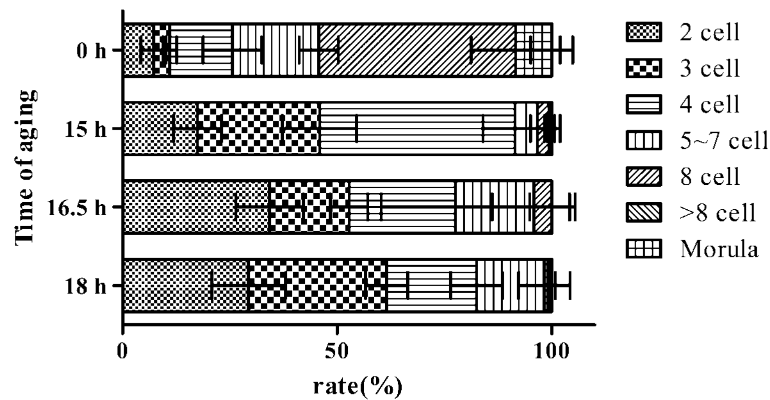

Fig. 4. Proportion of mouse embryos at different developmental stages at 48 h of *in vitro* culture after IVF of non-aged and aged oocytes.

Table 4. Comparison of the number of inner cell mass (ICM) and trophectoderm (TE) cells in mouse blastocysts derived from IVF of aged oocytes

Time of *in vitro* aging (h)	No. of blastocysts observed	Oct4 positive (ICM cells)	Cdx2 positive (TE cells)	Oct4/Cdx2 (%) (ICM/TE)
0	20	11.4 ± 1.0[a]	94.6 ± 5.0[a]	10.8 ± 0.8[a]
15	18	7.2 ± 1.0[b]	67.7 ± 6.1[b]	9.1 ± 1.0[a]
16.5	15	5.2 ± 0.8[b]	56.3 ± 5.5[bc]	8.6 ± 1.4[a]
18	7	4.6 ± 1.3[b]	40.1 ± 6.9[c]	10.7 ± 2.5[a]

The values with different superscripts in the same column are significantly different ($P < 0.05$: ANOVA).

and Cdx2 expression in TE (green) cells in the blastocysts derived from IVF of non-aged and aged oocytes. The numbers of Oct4 and Cdx2 positive cells were significantly fewer in the embryos of each aged group than those of the control group; however, there was no significant difference in the ratio of Oct4/Cdx2. This result indicates that the total number of cells in the blastocysts derived from aged oocytes was poor, and especially, the blastocysts in the 18-h aged group were poorly developed (Fig. 5B).

Discussion

In the present study, the shapes of zona pellucida and cytoplasm of aged oocytes were stable during *in vitro* culture, i.e., after 25 h *in vitro* aging, and most oocytes maintained normal cytoplasm as MII oocytes. Wakayama *et al.* [29] demonstrated that 60–80% of oocytes maintained normal morphology at 24 h after *in vitro* aging at 37°C. Their results were slightly lower than the results of the present study. In the present study, we selected normally shaped oocytes for *in vitro* aging after removing cumulus cells, whereas

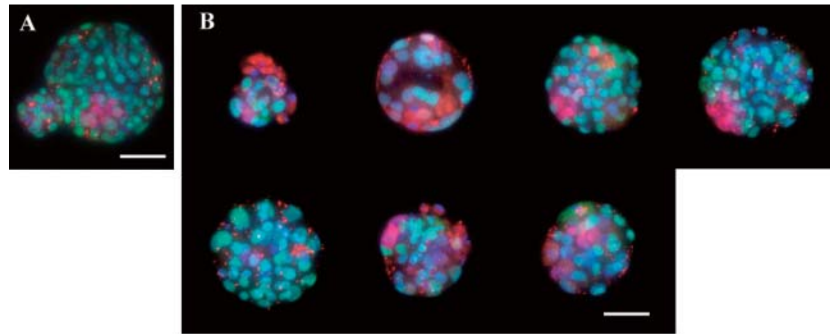

Fig. 5. Immunofluorescence staining of mouse blastocysts using anti-Oct4 and anti-Cdx2 antibodies. Figs. A and B show the blastocyst derived from IVF of a non-aged oocyte and the blastocysts derived from IVF of oocytes aged for 18 h *in vitro*, respectively. Fluorescence of red and green indicates nuclei of ICM and of TE cells, respectively. Scale bar = 50 μm.

Wakayama *et al.* selected oocytes with cumulus cells. As described in their study, the shrunk or fragmented abnormal oocytes were probably already present at the time of oocyte collection from oviducts, and the shrinkage or fragmentation may not have been associated with oocyte aging. In addition, they speculated that the existence of cumulus cells during *in vitro* culture for aging affected the rate of normal MII oocytes. They suggested that 6–20% oocytes, which cleaved after *in vitro* aging were spontaneously activated. It has also been reported that existence of cumulus cells during culture for aging increases the sensitivity of oocytes to activation [22]. In the present study, spontaneous activation of oocytes cultured without cumulus cells was inhibited and it resulted in a well maintained shape of mouse MII oocytes in a day old *in vitro* culture.

Because the MII spindle abnormalities during oocyte aging might disrupt the equal segregation of sister chromatids in the oocyte and the 2nd PB at fertilization, there is a possibility that chromosomal abnormality is caused in the embryos derived from IVF of aged oocytes. In fact, it has been reported that the incidence of chromosomal abnormality increases in embryos produced by ICSI of one-day-old oocytes in humans [30]. In the present study, a significant increase of morphologically abnormal spindles was not observed at 15 h of *in vitro* aging, which was consistent with the results of George *et al.* [20] who stated that abnormal spindles in mouse oocytes increased significantly 16 h post ovulation. Furthermore, they demonstrated that a significantly high incidence of chromosomal abnormalities was evident at 29 h *in vitro* aging. Wakayama *et al.* [29] reported that spindle abnormalities are observed even in oocytes with normally aligned chromosomes. In the present study also, similarly abnormal spindles were observed. Therefore, it seems that microtubule disruption occurs before chromosomal misalignment in the alteration of the spindle in oocyte culture for aging.

In the present study, although the number of oocytes with morphologically normal spindles did not decrease significantly, the rate of blastocyst formation in the embryos derived from IVF of aged oocytes and the cell number of resulting blastocysts were significantly reduced after 15 h. It has been suggested that the cytoplasm is more crucial than the nucleus in oocyte aging [21], indicating the possibility that cytoplasmic aging influences the developmental competence of the embryos derived from IVF of aged oocytes.

The percentage of blastocysts formed in aged oocytes on day 5 was approximately 10% in 12-h aged oocytes [31], 0% in 42-h aged oocytes post hCG [21], and approximately 20–25% in 6-h aged oocytes [32]. The percentages of blastocysts formed in aged oocytes in studies conducted by Tarin *et al.* [31] and Rausell *et al.* [32] were lower than those of the present study, although the aging times of the oocytes used in their experiments were shorter than those in used in the present study. There was no cytogenetically harmful effect seen with doses of 5 IU each of PMSG (eCG) and hCG administered to induce superperovulation in murine *in vivo* fertilization [33] and IVF [34]. However, it is possible that a larger dose of gonadotropin for superovulation, strains (C57/6 x DBA/2 [21], C57BL/6JIco x CBA/JIco [31, 32]) of mice used as oocyte

donors, the media used for *in vitro* aging or IVC and addition of fetuin into medium to inhibit zona pellucida hardening [26] might be responsible for the success of IVF and subsequent blastocyst formation.

In the present study, at 24 h of IVC after IVF, embryos that cleaved directly into more than 3 blastomeres appeared in the aged oocyte group. It has been reported that mouse zygotes derived from oocytes aged postovulation *in vivo* and fertilized *in vivo* showed shorter stages of the first cell cycle [13]. Furthermore, it has also been demonstrated that mouse zygotes derived from IVF of oocytes aged in oviducts developed more rapidly to the pronuclear stage than non-aged ones [12, 14]. Ishikawa *et al.* have reported that the mean cell number of embryos at 77 h postcoitus in delayed mating groups was greater than that of the normal mating group [15], and they suggested that the ovulation clock should be used as the start point of the time scale of growth and differentiation of embryos rather than the fertilization clock [16]. However, in contrast, after 48 h of IVC in the present study, many embryos derived from IVF of non-aged oocytes developed to the 8-cell stage, but few embryos derived from IVF of oocytes aged for more than 15 h developed beyond the 4-cell stage. Furthermore, the rates of blastocyst formation in the aged groups at both 96 and 120 h of IVC were lower than those in the non-aged group. However, the number of blastocysts in the aged groups increased in IVC up to 120 h, though it was not on a par with that in the non-aged group. Therefore, we propose that fertilization and first cleavage of oocytes aged *in vitro* are accelerated. In addition, previous reports [12, 13] have demonstrated the developmental acceleration of embryos derived from oocytes aged *in vivo*, but not their subsequent development. The negative effect of *in vitro* aging appears at 48 h of IVC as developmental delay; however, the intracellular alteration which causes this phenomenon remains to be elucidated. It has been suggested that the developmental competence after IVF of an oocyte without cumulus cells is retained for a considerable period of time, considering the effect of cumulus cells on aging of oocytes in culture [22, 35]. In human IVF in ART, human oocytes are usually cultured with cumulus cells under normal conditions. Although it is necessary to consider the effects of other extrinsic factors (i.e. spermatozoa), it may be possible that human oocytes retain the potential for embryonic development due to the removal of cumulus cells in IVF. In the present study, we did not compare the length of time over which mouse oocytes and human oocytes retain their potential for embryonic development. It may be possible to retain the potential of human oocytes which fail to fertilize, and are kept for one day, by removing cumulus cells, although the results of the present study indicate that a significant decline of the developmental competence appears in oocytes aged for more than 15 h even if they retain fertilizability. This is a problem which require further research.

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