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Poor Embryo Development in Mouse Oocytes Aged In Vitro Is Associated with Impaired Calcium Homeostasis[1]

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ABSTRACT

We examined whether impairment of intracellular Ca^{2+} homeostasis is related to poor embryo development in in vitro-aged oocytes. We found that in vitro aging of mouse oocytes affected the patterns of Ca^{2+} oscillations at fertilization: these Ca^{2+} oscillations were lower in amplitude and higher in frequency compared with oocytes without in vitro aging. We also observed that the intracellular Ca^{2+} store was decreased in in vitro-aged oocytes. A decrease in the Ca^{2+} store induced by thapsigargin, a specific endoplasmic reticulum (ER) membrane Ca^{2+}-ATPase inhibitor, resulted in a lower fertilization rate and in poorer embryo development. The frequency of Ca^{2+} oscillations was significantly increased at fertilization, whereas their amplitude was decreased in thapsigargin-treated oocytes. These results suggest that impairment of intracellular Ca^{2+} homeostasis (such as a decrease in the ER Ca^{2+} store) caused an alteration in Ca^{2+} oscillations and the poor embryo development in in vitro-aged oocytes. Because embryo fragmentation is closely related to apoptosis, we examined expression of BAX (a proapototic protein) and BCL2 (an antiapoptotic protein) in in vitro-aged oocytes. Although BCL2 was strongly expressed in oocytes without in vitro aging, expression of BCL2 was significantly reduced in oocytes of other culture conditions and treatments such as those in in vitro aging and those that were pretreated with H_2O_2 or thapsigargin. Acting together, alteration in Ca^{2+} oscillations and decrease in BCL2 expression in in vitro-aged oocytes may lead to poor embryo development.

calcium, calcium oscillation, calcium store, cumulus cells, embryo, in vitro aging, in vitro culture, in vitro fertilization, ovum, oxidative stress

INTRODUCTION

In mammals, ovulated oocytes are arrested at the metaphase stage of the second meiotic division until fertilization. The optimal period for oocyte fertilization lasts less than 10 h [1–3]. Fertilization within this narrow window of developmental opportunity results in normal embryo development. If fertilization does not occur during this optimal period, unfertilized oocytes that remain in the oviduct or in vitro culture go through a time-dependent aging process [4–7]. Numerous investigations have shown that, if fertilized, aged oocytes in vivo and in vitro frequently exhibit abnormal embryo development [8]. However, the mechanism underlying this abnormal embryo development in aged oocytes is still unclear.

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are widely used as treatments for assisted reproduction or in animal experiments. For human IVF or ICSI treatment, cumulus-oocyte complexes (COCs) are collected from preovulatory follicles that are subjected to oocyte maturation by human chorionic gonadotropin (hCG) [9]. Then, the collected COCs are cultured for several hours until insemination or injection with spermatozoa. This in vitro culturing of COCs could compromise their subsequent competence for embryo development. In fact, there are several reports that the preincubation time of COCs until insemination or injection of spermatozoa for subsequent embryo development is a limiting factor for successful IVF or ICSI [10–13]. Although a preincubation time of 9 h or less does not influence the number of good-quality embryos, a preincubation time longer than 9 h results in poor embryo development in human ICSI treatment cycles [10]. In addition, in vitro culture for about 3 h resulted in a low blastocyst formation rate during mouse subzonal insemination treatment [14]. Thus, the prolonged in vitro culture of oocytes (i.e., in vitro-aged oocytes) before micromanipulation resulted in poor embryo development. Therefore, control of oocyte aging in vitro could have advantages for reproductive technologies.

In mammalian oocytes at fertilization, sperm induces drastic changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which consist of a single long-lasting rise in $[Ca^{2+}]_i$, followed by short repetitive changes in $[Ca^{2+}]_i$ lasting for several hours. These temporal changes in $[Ca^{2+}]_i$ are termed "Ca^{2+} oscillations" [15]. The increase in intracellular Ca^{2+} has an important role in fertilization and is involved in cortical granule exocytosis, resumption of meiosis, and pronucleus formation. In addition, investigations have shown that the oscillatory pattern of $[Ca^{2+}]_i$ is a prerequisite for normal embryo development [16].

In view of the importance of Ca^{2+} oscillations for embryo development, we have been studying the molecular mechanisms of poor embryo development in in vivo-aged mouse oocytes. We have previously reported that in vivo postovulatory aging (insemination 20 h after hCG injection) significantly alters the pattern of Ca^{2+} oscillations in fertilized mouse oocytes [17]. Compared with freshly ovulated oocytes (14 h after hCG injection), in vivo-aged oocytes showed altered Ca^{2+} oscillations that were high in frequency and low in amplitude [17, 18]. Regarding the molecular mechanism of these alterations, we showed that there is an impairment of Ca^{2+} uptake by Ca^{2+}-ATPases in the endoplasmic reticulum (ER) in aged oocytes [17]. Moreover, we showed that there is a reduction in Ca^{2+} release from the 1,4,5-triphosphate ($InsP_3$)-sensitive Ca^{2+} stores in in vivo-aged oocytes [19]. Thus,

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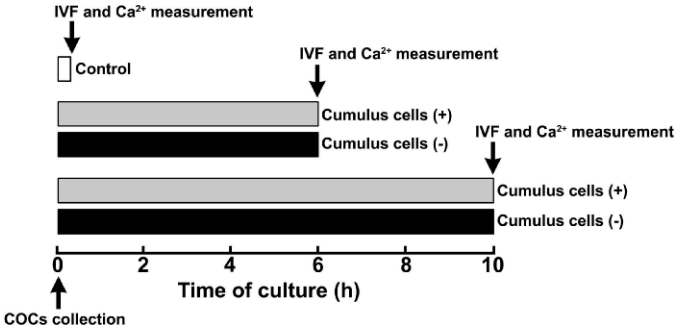

FIG. 1. Schematic diagram of the experiments. Individual experimental protocols are described in detail in the text.

impaired Ca^{2+} handling of intracellular Ca^{2+} stores affects Ca^{2+} oscillations in in vivo-aged oocytes.

Concerning in vitro aging of oocytes, Gordo et al. [20] reported that the disrupted Ca^{2+} signaling in in vitro-aged mouse oocytes leads to apoptosis of oocytes. They demonstrated that injection of Ca^{2+} oscillators (such as sperm cytosolic factor, adenophostin A [a potent agonist of the $InsP_3$ receptor], or sperm) into in vitro-aged oocytes causes fragmentation but not activation of oocytes. They also reported that injection of Ca^{2+} oscillators into in vitro-aged oocytes induced abnormal Ca^{2+} oscillations with low amplitude and abrupt cessation. Although the authors clarify the effects of in vitro aging of oocytes on early events of oocyte activation and Ca^{2+} signaling induced by artificial stimuli, they did not examine the effects of in vitro aging of oocytes on subsequent embryo development and Ca^{2+} oscillations induced by IVF. In this study, we examined mechanisms of poor embryo development in in vitro-aged mouse oocytes. We hypothesized that poor embryo development in in vitro-aged oocytes might be related to impairment of Ca^{2+} homeostasis, and we observed that in vitro aging of mouse oocytes affected the patterns of Ca^{2+} oscillations at fertilization and decreased the ER Ca^{2+} store.

MATERIALS AND METHODS

Reagents

Human tubal fluid (HTF) medium was used in all the experiments. Hepes-HTF medium was used for handling oocytes in air. The compositions of these media were described previously [19]. The media contained 0.5% bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO) unless otherwise noted. The HTF medium was used after equilibration with 5% CO_2 in air at 37°C (pH 7.4). Antibody for cleaved caspase 3 was purchased from Promega (Madison, WI). Antibodies for BCL2 and BAX were purchased from GeneTex (San Antonio, TX). Fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Hydrogen peroxide (H_2O_2) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were obtained from Sigma unless otherwise indicated.

Oocyte Preparation and In Vitro Aging

This study was performed with permission from the Committee of Animal Experimentation, Yamagata University School of Medicine. The methods for oocyte recovery and preparation were described previously [19]. Briefly, B6C3F1 hybrid female mice that were aged 4–6 wk were superovulated by i.p. injection of 10 IU of equine chorionic gonadotropin (Teikokuzouki, Tokyo, Japan), followed after 48 h by i.p. injection of 10 IU of hCG (Mochida, Tokyo, Japan). As shown in the schema of the experiments in Figure 1, COCs were removed from the oviduct 14 h after hCG was injected using a fine needle and were placed into droplets of BSA-free Hepes-HTF medium. The collected COCs were cultured with cumulus cells for 6 h or without cumulus cells for 10 h in 5% CO_2 in air at 37°C. These oocytes were termed "in vitro-aged oocytes". To remove the surrounding cumulus cells, the COCs were treated with 300 IU/ml of hyaluronidase. For the IVF experiment, the zona pellucida was removed by 25 IU of α-chymotrypsin.

In Vitro Fertilization

The IVF procedure and the assessment of embryo development were described previously [18]. Briefly, denuded oocytes in 300 μl of HTF medium were placed in a 35-mm plastic dish covered with mineral oil and cultured in 5% CO_2 in air at 37°C until sperm insemination. The sperm were collected from the cauda epididymidis of mature ICR male mice and were preincubated for 90 min in 400 μl of HTF medium to allow capacitation. After capacitation, the sperm were introduced into 300-μl droplets containing denuded oocytes (final concentration, $1–2 \times 10^5$ spermatozoa/ml). After 4 h of incubation, oocytes were recovered from the droplets, washed three times in Hepes-HTF medium to remove the remaining spermatozoa, and then cultured again in HTF medium for 5 days at 37°C in 5% CO_2 in air. The fertilized oocytes were assessed as two-cell embryos 24 h after insemination. Blastocyst formation and the number of embryos with cellular fragmentation were assessed visually 5 days after insemination.

Measurement of Intracellular Ca^{2+}

The measurement of intracellular Ca^{2+} in mouse oocytes was described previously [19]. Briefly, intracellular Ca^{2+} was determined by fluorometry using fura-PE3 acetoxymethyl ester (fura-PE3/AM; Wako Pure Chemical Industries). Fura-PE3/AM is the same as Fura-2 in its spectral properties but is retained within the cell for a longer period, thus being suitable for the present study. Zona pellucida-free oocytes were incubated for 30–40 min at 37°C in HTF medium containing 3 μM fura-PE3/AM. Then, the dye-loaded oocytes were washed five times in Hepes-HTF medium. After washing, oocytes were placed on a Cell-Tak-coated (Becton Dickinson Labware, Bedford, MA) nylon mesh in a measuring cuvette to prevent movement artifacts during perfusion [21]. The cuvette was placed on the stage of an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan). The cuvette temperature was maintained at a mean ± SEM of 37.0 ± 0.5°C using circulating warm water. Fura-PE3/AM fluorescence (excitation at 340 or 380 nm and emission at 420 nm) was measured, followed by ratio metric determination of $[Ca^{2+}]_i$. The method for calculating the absolute $[Ca^{2+}]_i$ value was described previously [19].

Assessment of Ca^{2+} Stores

The assessment of Ca^{2+} stores in oocytes using thapsigargin or ionomycin has been reported previously [19, 22, 23]. Briefly, after the baseline $[Ca^{2+}]_i$ was measured for 5 min during perfusion with HTF medium, the perfusate was switched to Ca^{2+}-nominally free HTF medium containing 5 μM thapsigargin or 10 μM ionomycin for 15 min. Thapsigargin inhibits the Ca^{2+}-ATPase of the ER, and this causes an increase in $[Ca^{2+}]_i$ [22, 24, 25]. Then, we assessed the extent of the ER Ca^{2+} stores from the increase in $[Ca^{2+}]_i$ during the application of 5 μM thapsigargin. The Ca^{2+} ionophore, ionomycin, is also widely used in the assessment of total Ca^{2+} stores in oocytes and other types of cells [22, 23, 26]. Jones et al. [22] reported that the ionomycin-sensitive Ca^{2+} store is larger than the thapsigargin-sensitive Ca^{2+} store in oocytes. Then, we assessed the total Ca^{2+} stores in oocytes by using ionomycin.

Assessment of Oxidative Stress

Assessment of oxidative stress in mouse oocytes was described previously [18]. Briefly, the level of reactive oxygen species (ROS) was assessed with 5-(and 6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCF diacetate; Molecular Probes, Eugene, OR), a fluorescent dye. Carboxy-H_2DCF diacetate is a carboxylated analogue of H_2DCF diacetate, which passes through the oolemma during loading and stays in the intracellular space for a prolonged period because of its negative charges. In the intracellular space, oxidation of the compound produces fluorescent carboxydichlorofluorescein (carboxy-DCF). Thus, carboxy-H_2DCF diacetate is an indicator of ROS. Denuded oocytes were incubated for 25 min at 37°C in HTF medium containing 10 μM carboxy-H_2DCF diacetate. After oocytes were washed five times in Hepes-HTF medium, carboxy-DCF fluorescence was measured (excitation at 480 nm and emission at 520 nm), and the images were captured using ImageJ software (version 1.3; National Institutes of Health, Bethesda, MD) [27, 28] under an inverted fluorescence microscope.

Immunofluorescence

To examine expression of cleaved caspase 3, BCL2, and BAX in mouse oocytes in different culture conditions and treatments, immunofluorescence

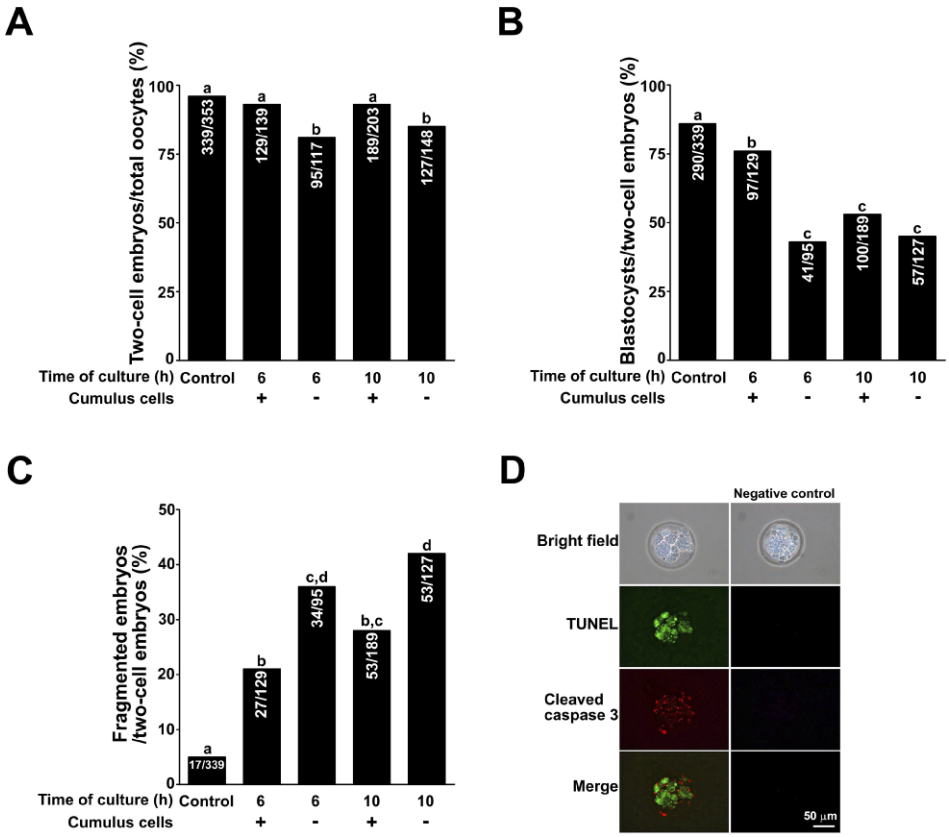

FIG. 2. Effects of in vitro aging on embryo development. Superovulated COCs were collected from the fallopian tubes of female mice 14 h after hCG injection. Collected COCs were cultured with or without cumulus cells for 6 or 10 h. After in vitro culture, these oocytes were subjected to IVF. Oocytes without additional culture were used as a control. **A**) Fertilized oocytes were assessed as two-cell embryos 24 h after insemination with epididymal spermatozoa. The numbers inside the bars indicate the number of two-cell embryos/total number of oocytes. **B**) The number of blastocysts formed from fertilized embryos was counted after 5 days of culture. The numbers inside the bars indicate the numbers of blastocysts/two-cell embryos. **C**) The number of fragmented embryos was counted after 5 days of culture. The numbers inside the bars indicate the numbers of fragmented embryos/two-cell embryos. **D**) Fragmented embryos were subjected to TUNEL analysis, and expression of cleaved caspase 3 is shown in the left panel. The negative control is shown in the right panel. Bars with different letters represent a significant difference ($P < 0.05$). Data shown are representative of at least three independent experiments.

staining was performed as described previously [29]. Briefly, denuded oocytes were fixed in 4% buffered formalin at room temperature for 30 min. After washing them three times with 0.1% BSA containing PBS, the denuded oocytes were incubated overnight in specific primary antibodies, including cleaved caspase 3 (1:100), BCL2 (1:50), and BAX (1:50) in 0.1% BSA containing PBS at 4°C. Oocytes were washed three times with 0.5% Triton X-100 in 0.5% BSA containing PBS and then incubated with FITC- or TRITC-conjugated secondary antibodies diluted (1:100) in 0.1% BSA containing PBS for 1 h at room temperature. Then, they were washed three times with 0.5% Triton X-100 in 0.5% BSA containing PBS to eliminate excess antibodies. Thereafter, the samples were mounted on a slide and observed under an inverted fluorescence microscope. The captured images were processed using ImageJ software (version 1.3) [27, 28]. Negative control studies were performed in which PBS was used instead of primary antibodies.

Immunoblotting

Fifty denuded oocytes were lysed in Laemni sample buffer, and protein extracts were stored at −80°C until use. After denaturing by boiling for 5 min, 20 μl of each protein sample was separated by SDS-PAGE and then transferred to polyvinylidene fluoride membrane. The membrane was blocked for 1 h at room temperature in 5% nonfat dry milk in 0.1% Tween 20/PBS (TBS-T). Primary antibody of BCL2 (1:500) or α-tubulin (1:1000) was added in 5% nonfat milk in TBS-T and incubated overnight at 4°C. Immunoreacted bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit IgG secondary antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) by using the enhanced chemiluminescence Western blotting system.

Apoptosis Detection

DNA fragmentation during apoptosis was detected by the TUNEL technique using a DeadEnd Fluorometric TUNEL System (catalog No. G3250; Promega). A TUNEL assay was performed according to the manufacturer's instructions. Briefly, the embryos were washed twice in PBS and then were fixed in 4% buffered formalin (pH 7.4) at room temperature for 30 min. After washing them three times in PBS, the embryos were permeabilized with 0.25% Tween 20 in PBS for 5 min. After washing them another three times in PBS, the embryos were incubated with equilibration buffer at room temperature for 5 min. The COCs were incubated with TUNEL reaction mixture containing fluorescein-12-deoxyuridine triphosphate and terminal deoxynucleotidyl transferase (TdT) at 37°C for 60 min in a humidified chamber. The embryos were washed twice with standard saline citrate for 15 min to stop the reaction. For the negative control, TdT was omitted from the reaction mixture. After thorough washing in 0.1% BSA containing PBS, the samples were mounted on a slide and observed under an inverted fluorescence microscope. The captured images were processed using the ImageJ software (version 1.3) [27, 28].

Statistical Analysis

All experiments consisted of at least three independent experimental runs. Differences between means were calculated by one-way ANOVA, followed by Fisher least significant difference post hoc test using StatView software (Abacus Concepts, Berkeley, CA). Values are given as mean ± SEM. Data expressed as percentages were analyzed using Chi-square test. Significant differences are defined as $P < 0.05$.

RESULTS

Effects of In Vitro Aging on Embryo Development

We tested whether the conditions of in vitro aging affect embryo development. In vitro fertilization was performed, and the embryo was allowed to grow in culture for 5 days during which its development was assessed. Although the fertilization rate in the group in which oocytes were cultured with cumulus cells for 6 or 10 h was not impaired, it was significantly ($P < 0.05$, Chi-square test) reduced in oocytes cultured without cumulus cells (Fig. 2A). The blastocyst formation rate was significantly ($P < 0.05$, Chi-square test) decreased in the in vitro-aged groups compared with the control (Fig. 2B). The blastocyst formation rate in the group in which oocytes were cultured with cumulus cells for 6 h was about 70%, while it was significantly ($P < 0.05$, Chi-square test) lower in the other in vitro-aged groups. Embryos with cellular fragmentation were significantly ($P < 0.05$, Chi-square test) increased in the

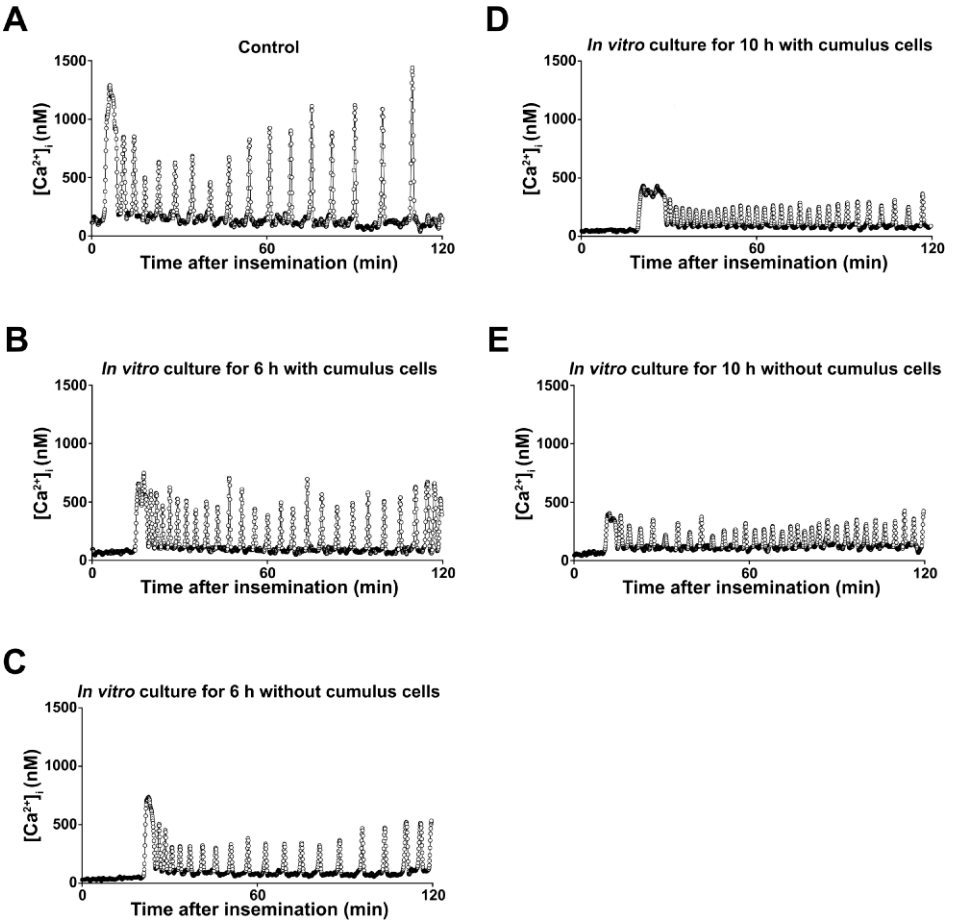

FIG. 3. The effects of in vitro aging on Ca^{2+} oscillations following IVF. Collected COCs were cultured with or without cumulus cells for 6 or 10 h. After in vitro culture, oocytes were subjected to IVF and Ca^{2+} measurement. The Ca^{2+} oscillations after IVF are shown in oocytes under the following various culture conditions: no additional culture as a control (**A**), in vitro culture for 6 h with cumulus cells (**B**), in vitro culture for 6 h without cumulus cells (**C**), in vitro culture for 10 h with cumulus cells (**D**), and in vitro culture for 10 h without cumulus cells (**E**). Insemination occurred at time 0. Data shown are representative of at least three independent experiments.

in vitro-aged groups compared with the control. The fragmented embryos were increased in oocytes cultured without cumulus cells compared with oocytes cultured with cumulus cells for 6 or 10 h (Fig. 2C). The apoptosis pathway has a role in the formation of fragmented embryos [30]. As expected, TUNEL positivity and cleaved caspase 3, the active form of caspase 3, were observed in many of the fragmented embryos (about 90%, data not shown) (Fig. 2D).

Effects of In Vitro Aging on Ca^{2+} Oscillations Following IVF

Ozil et al. [31, 32] and Bos-Mikich et al. [33] reported that the pattern of Ca^{2+} oscillations following fertilization in rabbit or mouse oocytes was closely associated with embryo development. Therefore, we examined the effects of in vitro aging on Ca^{2+} oscillations following fertilization in mouse oocytes. Figure 3 shows that Ca^{2+} oscillations follow IVF in various culture conditions. Table 1 summarizes the analyzed data of individual Ca^{2+} changes among the groups. The basal $[Ca^{2+}]_i$ in oocytes cultured without cumulus cells for 10 h was significantly higher than that in the other groups. The amplitudes of Ca^{2+} oscillations in both the control and the 6-h culture with cumulus cells were significantly higher than those in the other in vitro-aged groups. In contrast, the frequency of Ca^{2+} oscillations in the in vitro-aged groups was significantly higher than that in the control. Moreover, rates of rise and fall of $[Ca^{2+}]_i$ in the in vitro-aged groups were significantly slower than those in the control.

Effects of In Vitro Aging on Intracellular Ca^{2+} Stores

Changes in Ca^{2+} oscillations at fertilization in in vitro-aged oocytes were consistent with those in in vivo-aged oocytes [17, 18]. We previously reported that intracellular Ca^{2+} stores, presumably of the ER, were decreased in aged mouse oocytes in vivo [19]. To reveal the mechanism underlying these changes in Ca^{2+} oscillations at fertilization in in vitro-aged oocytes, we examined whether in vitro aging affected

TABLE 1. The effects of in vitro aging on the pattern of Ca^{2+} oscillation at fertilization in mouse oocytes.[a]

Treatment (n)	Basal $[Ca^{2+}]_i$ (nM)	Amplitude (nM)	Frequency (oscillations/h)	Rate of rise of $[Ca^{2+}]_i$ (nM/sec)	Rate of decline of $[Ca^{2+}]_i$ (nM/sec)
Control (10)	118 ± 4[b]	841 ± 85[b]	5 ± 0.3[b]	30 ± 4[b]	28 ± 3[b]
6 h culture with cumulus cells (11)	120 ± 5[b]	830 ± 63[b]	13 ± 1[c]	9 ± 1[c]	8 ± 1[c]
6 h culture without cumulus cells (6)	118 ± 4[b]	629 ± 39[c]	13 ± 2[c]	10 ± 2[c]	10 ± 2[c]
10 h culture with cumulus cells (12)	123 ± 5[b]	501 ± 25[c]	18 ± 2[d]	6 ± 0.4[c]	7 ± 1[c]
10 h culture without cumulus cells (14)	136 ± 5[c]	502 ± 27[c]	19 ± 2[d]	6 ± 1[c]	7 ± 1[c]

[a] Data are the mean ± SEM.
[b–d] Values with different superscript letters within the same column are significantly different; $P < 0.05$.

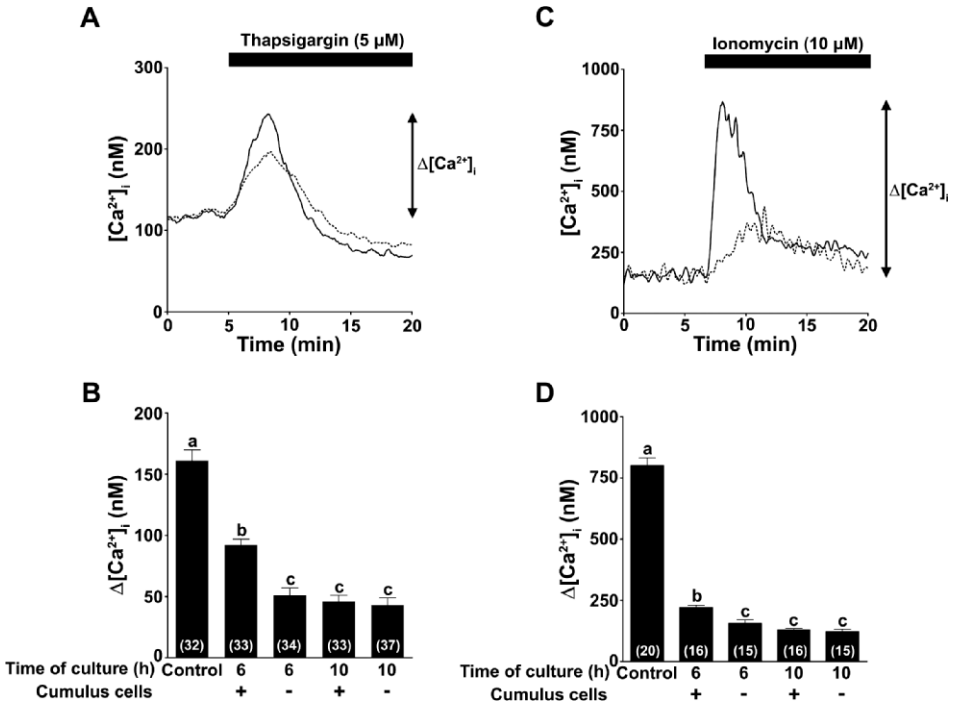

FIG. 4. The effects of in vitro aging on intracellular Ca^{2+} stores. Collected COCs were cultured with or without cumulus cells for 6 or 10 h. After in vitro culture, oocytes were subjected to an assessment of Ca^{2+} stores by thapsigargin or ionomycin. **A**) Representative data demonstrating the release of Ca^{2+} from thapsigargin-sensitive Ca^{2+} stores in endoplasmic reticulum in oocytes without in vitro culture (control, solid line) and in oocytes cultured with cumulus cells for 10 h (dotted line). **B**) Peak Ca^{2+} measured from baseline ($\Delta[Ca^{2+}]_i$) following administration of 5 μM thapsigargin in oocytes under various culture conditions. **C**) Representative data demonstrating the release of Ca^{2+} from ionomycin-sensitive Ca^{2+} stores in oocytes without in vitro culture (control, solid line) and in oocytes cultured with cumulus cells for 10 h (dotted line). **D**) Peak Ca^{2+} measured from baseline ($\Delta[Ca^{2+}]_i$) following administration of 10 μM ionomycin in oocytes under various culture conditions. Values are shown as mean ± SEM. Numbers inside the bars indicate the number of tested oocytes. Bars with different letters represent a significant difference ($P < 0.05$). Data shown are representative of at least three independent experiments.

intracellular Ca^{2+} stores. Intracellular Ca^{2+} stores were assessed using thapsigargin (a specific inhibitor of the Ca^{2+}-ATPase of the ER) or the Ca^{2+} ionophore ionomycin [19, 22]. Thapsigargin or ionomycin treatment in oocytes induced a Ca^{2+} change in the Ca^{2+}-nominally free HTF medium (Fig. 4, A and C). The peak $[Ca^{2+}]_i$ induced by thapsigargin or ionomycin in in vitro-aged oocytes was decreased compared with that in the oocytes without in vitro culture. However, the $\Delta[Ca^{2+}]_i$ in oocytes cultured with cumulus cells for 6 h was higher than that in the other in vitro-aged groups (Fig. 4, B and D). From these results, it was suggested that in vitro aging decreased intracellular Ca^{2+} stores in oocytes. Next, we examined whether thapsigargin treatment affects embryo development and Ca^{2+} oscillations at fertilization in oocytes without in vitro aging (collected 14 h after hCG injection).

Effects of Thapsigargin Treatment on Embryo Development and Ca^{2+} Oscillations Following IVF

The fertilization rates of thapsigargin-treated oocytes were significantly and dose dependently decreased (Fig. 5A). The blastocyst formation rates of oocytes treated with 1 or 5 μM thapsigargin were significantly decreased compared with the control after 5 days of culture (Fig. 5B). Rates of fragmented embryos from thapsigargin-treated oocytes were dose dependently increased (Fig. 5C). Next, we examined whether thapsigargin treatment affects the pattern of Ca^{2+} oscillations at fertilization. The Ca^{2+} oscillations following fertilization were recorded in oocytes pretreated with 0.02% dimethyl sulfoxide (DMSO) (control) or 1 μM thapsigargin for 1 h (Fig. 6). Table 2 summarizes the patterns of Ca^{2+} oscillations. Although the basal $[Ca^{2+}]_i$ levels were not changed in either group, the amplitude of Ca^{2+} oscillations in the thapsigargin-pretreated group was significantly lower than that in the control. In contrast, the frequency of Ca^{2+} oscillations in the thapsigargin-pretreated group was significantly higher than that in the control group. Moreover, rates of rise and fall of $[Ca^{2+}]_i$ in the thapsigargin-pretreated group were significantly slower than those in the control. A similar observation that preincubation of oocytes with thapsigargin results in lower amplitude and slower rise of Ca^{2+} oscillations has previously been reported [22, 24].

Effects of In Vitro Aging on Oxidative Stress in Mouse Oocytes

We previously reported that in vivo-aged oocytes are subjected to lipid peroxidation [18]. Results suggested that in vivo-aged oocytes are exposed to oxidative stress. In this study, we examined whether in vitro-aged oocytes are also subjected to oxidative stress. Oxidative stress in oocytes was assessed by measurement of carboxy-DCF fluorescence, a nonspecific indicator of ROS (Fig. 7A). H_2O_2 was used as a positive control for oxidative stress. We previously reported that carboxy-DCF fluorescence following treatment of oocytes with H_2O_2 increased in a dose-dependent manner [18]. Carboxy-DCF fluorescence was significantly increased in in vitro-aged oocytes compared with the control. The increase in carboxy-DCF fluorescence in oocytes cultured with cumulus cells was much lower than that in oocytes cultured without

TABLE 2. The pattern of Ca^{2+} oscillations at fertilization in thapsigargin pretreated-oocytes.[a]

Treatment (n = 11)	Basal $[Ca^{2+}]_i$ (nM)	Amplitude (nM)	Frequency (oscillations/h)	Rate of rise of $[Ca^{2+}]_i$ (nM/sec)	Rate of decline of $[Ca^{2+}]_i$ (nM/sec)
0.02% DMSO pretreated-oocytes (control)	124 ± 7	830 ± 77	6 ± 0.4	29 ± 4	25 ± 3
1 μM thapsigargin-pretreated oocytes	122 ± 5	271 ± 14[b]	24 ± 2[b]	1 ± 0.2[b]	1 ± 0.2[b]

[a] Data are the mean ± SEM.
[b] Significantly different from the control group; $P < 0.01$.

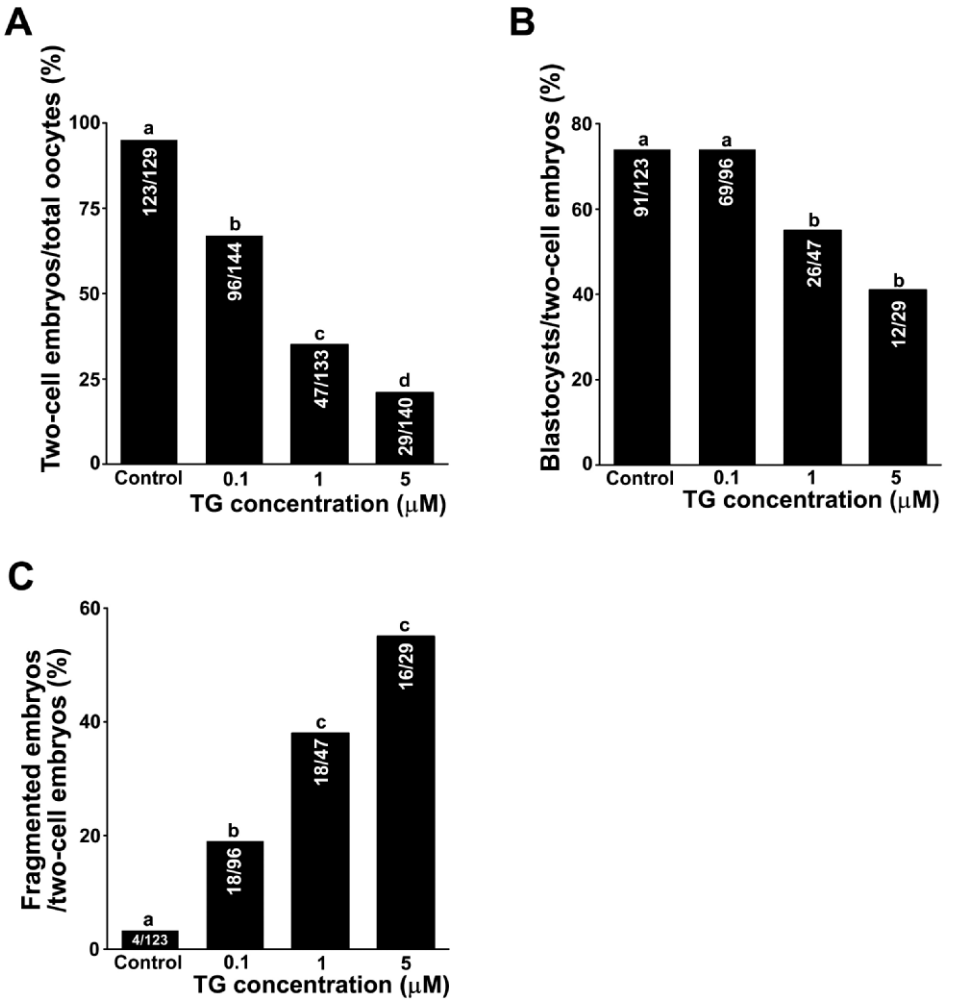

FIG. 5. The effects of thapsigargin treatment on IVF rate and embryo development. The COCs were treated with hyaluronidase to remove cumulus cells, and then the denuded oocytes were incubated in media containing various concentrations of thapsigargin (TG) for 30 min and were then subjected to IVF. Treatment of oocytes with 0.02% DMSO (vehicle) was used as a control. **A**) Fertilized oocytes were assessed as two-cell embryos 24 h after insemination with epididymal spermatozoa. Numbers inside the bars indicate the number of two-cell embryos/total number of oocytes. **B**) The number of blastocysts formed from fertilized embryos was counted after 5 days of culture. Numbers inside the bars indicate the numbers of blastocysts/two-cell embryos. **C**) The number of fragmented embryos was counted after 5 days of culture. Numbers inside the bars indicate the numbers of fragmented embryos/two-cell embryos. Bars with different letters represent a significant difference ($P < 0.05$). Data shown are representative of at least three independent experiments.

cumulus cells for 6 or 10 h (Fig. 7B). When all in vitro-aged groups of oocytes were exposed to 200 μM H_2O_2, the levels of carboxy-DCF fluorescence were similarly high as in the positive control (data not shown).

Expression of BCL2 in Mouse Oocytes

Fragmented embryos were found to be TUNEL positive and expressed the active form of caspase 3 (Fig. 2D). Because fragmentation of embryos is known to be involved in apoptosis [30], we examined the effects of in vitro aging on expression of BCL2 (an antiapoptotic protein) and BAX (a proapoptotic protein) by immunofluorescence. Although BCL2 fluorescence was strong in the control, BCL2 fluorescence was significantly lower in oocytes of the other treatment groups, including those after in vitro culture for 10 h without cumulus cells, those after pretreatment with 200 μM H_2O_2 for 30 min, and those after pretreatment with 5 μM thapsigargin for 1 h (Fig. 8, A and B). We confirmed decreased BCL2 expression in oocytes with in vitro culture for 10 h without cumulus cells by immunoblotting (Fig. 8C). In contrast, there were no differences in BAX fluorescence among the four groups (data not shown).

DISCUSSION

In the present study, we examined the mechanisms of poor embryo development in in vitro-aged oocytes, with particular regard to Ca^{2+} regulation. We have shown that poor embryo development in in vitro-aged oocytes is related to alteration of Ca^{2+} oscillations at fertilization, which is caused by a decrease in Ca^{2+} stores in the ER.

We have previously shown that changes in Ca^{2+} oscillations at fertilization (such as lower amplitude or higher frequency compared with fresh oocytes) occur in in vivo-aged oocytes [17, 18]. These changes in Ca^{2+} oscillations in in vivo-aged oocytes are caused by a decrease in intracellular ER Ca^{2+} stores [19]. Moreover, we have shown that impairment of Ca^{2+} uptake by Ca^{2+}-ATPase and Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores in the ER is observed in in vivo-aged oocytes [17, 18]. In this study, we observed consistent results between in vivo-aged and in vitro-aged oocytes with regard to Ca^{2+} oscillations and Ca^{2+} stores (Figs. 2 and 3). In addition, we found that experimentally decreased Ca^{2+} stores resulted in changes similar to those seen in in vitro-aged oocytes with regard to Ca^{2+} oscillations at fertilization (Fig. 6) and poor embryo development (Fig. 5). Collectively, these results suggest that an altered Ca^{2+} homeostasis occurs in in vitro-aged oocytes.

The molecular mechanism for this defective Ca^{2+} homeostasis in in vitro-aged oocytes has not been elucidated but may involve a decrease in ATP production. Impaired availability of ATP may inhibit function of the ER Ca^{2+}-ATPase, which is responsible for refilling the ER Ca^{2+} stores. In fact, Chi et al. [34] reported that in vitro aging decreases ATP content in mouse oocytes compared with freshly ovulated oocytes. Alternatively, the production of ATP generated from mito-

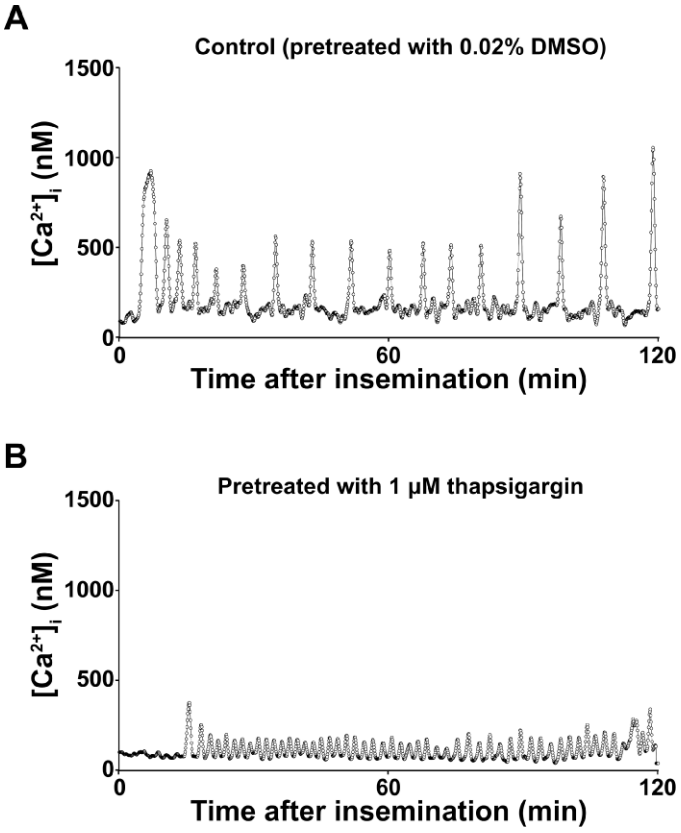

FIG. 6. The effects of thapsigargin treatment on Ca^{2+} oscillations following IVF. The COCs were treated with hyaluronidase to remove cumulus cells, and then the denuded oocytes were incubated in media containing 1 μM thapsigargin for 1 h and were subjected to IVF and Ca^{2+} measurement. Treatment of oocytes with 0.02% DMSO (vehicle) was used as a control. **A**) Typical Ca^{2+} oscillations following IVF in an oocyte in the control. **B**) Ca^{2+} oscillations following IVF in an oocyte treated with 1 μM thapsigargin. Insemination occurred at time 0. Data shown are representative of at least three independent experiments.

chondrial oxidative phosphorylation is closely related to Ca^{2+} homeostasis in various cells [35]. Recently, we and another group reported that Ca^{2+} oscillations stimulate an ATP increase at fertilization in mouse oocytes [36–38]. We have shown that this increase in ATP content at fertilization was inhibited in in vivo-aged oocytes [36]. Because the pattern of Ca^{2+} oscillations has a significant effect on embryo development [31, 32], impaired ATP regulation in in vitro-aged oocytes may be responsible, through Ca^{2+} oscillations, for this poor development. Taken together, the changes in Ca^{2+} oscillations at fertilization in in vitro-aged oocytes might be related to poor embryo development.

We showed that poor embryo development (such as decreased blastocyst formation and increased fragmented embryos) was observed in thapsigargin-treated oocytes compared with oocytes treated with vehicle (Fig. 5). We manipulated Ca^{2+} stores of the ER in oocytes by use of thapsigargin, which is a specific inhibitor of ER Ca^{2+}-ATPase. We previously demonstrated that there is about 80% inhibition of Ca^{2+} reuptake from cytosol to ER in mouse oocytes treated with 5 μM thapsigargin [17]. Therefore, we used the concentration of 5 μM thapsigargin to measure ER Ca^{2+} stores (Fig. 4, A and B). In this study, we used the concentration of 1 μM thapsigargin to partially deplete ER Ca^{2+} stores. We examined the effects of thapsigargin on the Ca^{2+} store in oocytes. The Ca^{2+} increase in thapsigargin-pretreated oocytes exposed to ionomycin was 64% decreased compared with that in vehicle-treated oocytes 3 h after treatment (Supplemental Figure 1 available online at www.biolreprod.org). Moreover, we tested the long-term effects of thapsigargin on depletion of Ca^{2+} stores. We demonstrated that the Ca^{2+} increase in thapsigargin-pretreated oocytes exposed to ionomycin was still decreased compared with that in vehicle-treated oocytes 24 h after treatment (Supplemental Figure 1). These results suggest that thapsigargin remains bound to the ER Ca^{2+}-ATPase 1 day after treatment. Therefore, this long-term effect of thapsigargin might be related to the poor embryo development in thapsigargin-pretreated oocytes after fertilization. On the other hand, thapsigargin is well known as a potent inducer of ER stress [39]. In this study, we did not examine the effects of thapsigargin on ER stress in oocytes. Because ER stress induces apoptosis in many cells [40], poor embryo development (especially an increase in fragmented embryos) in thapsigargin-treated oocytes may be related to ER stress-mediated cell death.

Oxidative stress is involved in the etiology of defective embryo development [41]. It was reported that exposure of mouse oocytes to H_2O_2 (200 μM) completely inhibited cleavage to two-cell stage embryos [42]. Moreover, Yang et al. [43] reported that there is a link between apoptosis and endogenous H_2O_2 levels in human embryos. We also reported that exposure of freshly ovulated oocytes to 100 μM H_2O_2 significantly reduces their fertilization rate and results in poor embryo development such as decreased blastocyst formation and increased fragmented embryos [18]. Tarin [44] proposed a mechanism based on "the oxygen radical mitochondrial injury hypothesis of aging" to explain the effects of aging on oocytes. In the present study, we found that carboxy-DCF fluorescence intensity, which indicates ROS, was increased in in vitro-aged oocytes (Fig. 7). Based on the hypothesis by Tarin, the increase in ROS in oocytes cultured in vitro may be related to poor embryo development. On the other hand, ROS affect Ca^{2+} regulation in various types of cells through Ca^{2+} regulatory proteins (such as calmodulin) and Ca^{2+}-ATPase and the $InsP_3$ receptor in the ER [45]. We previously reported that H_2O_2-treated fresh oocytes showed changes in their Ca^{2+} oscillations (lower amplitude and higher frequency) at fertilization, as well as poor development [18]. The changes in Ca^{2+} oscillations in oxidative-stressed oocytes are similar to those in aged oocytes both in vivo [18] and in vitro (Fig. 3). Taken together, these results suggest that the high level of ROS in in vitro-aged oocytes may be the cause of the alteration in Ca^{2+} oscillations and the poor embryo development.

Cumulus cells have multiple roles in mammalian reproduction such as oocyte maturation, ovulation, fertilization, and embryo development [46]. In the present study, we found that cumulus cells partly prevented poor embryo development during in vitro aging (Fig. 2, B and C). In addition, we found that cumulus cells prevented decreases in amplitude of Ca^{2+} oscillations (Fig. 3 and Table 1) and in Ca^{2+} stores of the ER (Fig. 4) at least until 6 h of in vitro culture. Moreover, we found that cumulus cells prevented the increase of ROS during in vitro aging (Fig. 7). Because follicular and oviduct fluid contains scavengers of ROS [47, 48], the COCs released from the oviduct are sensitive to oxidative stress. In fact, scavengers of ROS such as glutathione (GSH) are decreased in in vivo-aged mouse oocytes [49]. Cumulus cells also produce a large amount of GSH in hamster [50] and pig [51] COCs; therefore, cumulus cells may maintain Ca^{2+} homeostasis in ovulated oocytes by preventing ROS generation. On the other hand, Miao et al. [52] reported that rates of oocytes activation and

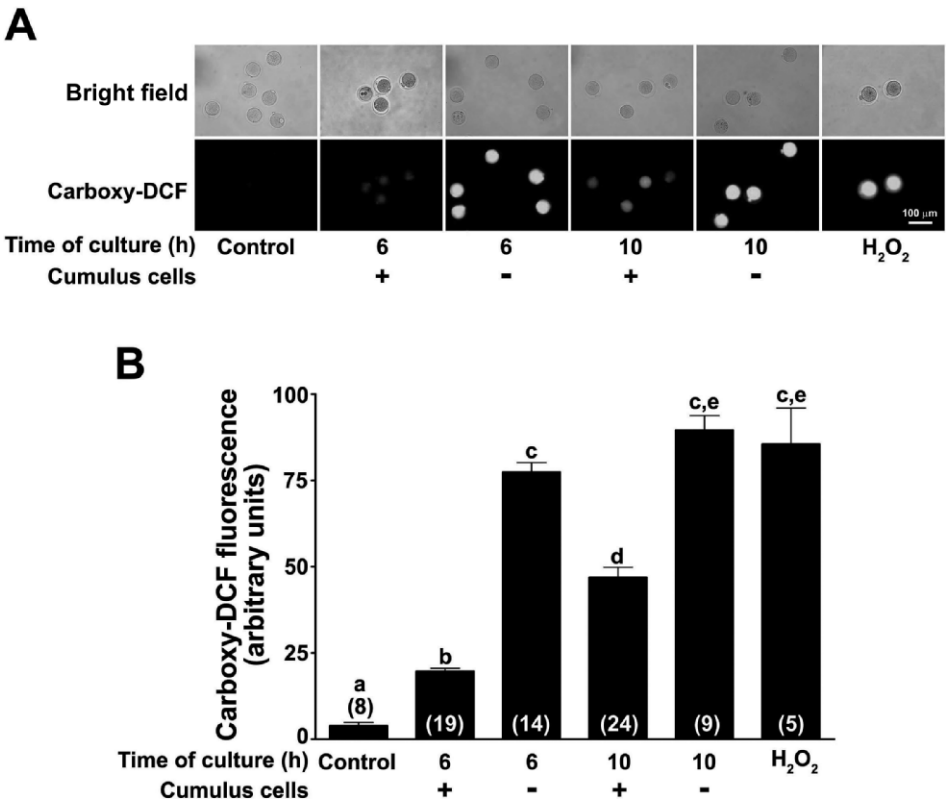

FIG. 7. The effects of in vitro aging on oxidative stress in mouse oocytes. Collected COCs were cultured with or without cumulus cells for 6 or 10 h. **A**) After in vitro culture, oocytes were subjected to analysis of oxidative stress by using a fluorescent probe, carboxy-DCF. Oocytes without additional culture were used as a control. For a positive control, oocytes treated with 200 μM H_2O_2 were used. Bar = 100 μm. **B**) Carboxy-DCF fluorescence was measured in these groups. Values are shown as mean ± SEM. Numbers above or inside the bars indicate the number of tested oocytes. Bars with different letters represent a significant difference ($P < 0.01$). Data shown are representative of at least three independent experiments.

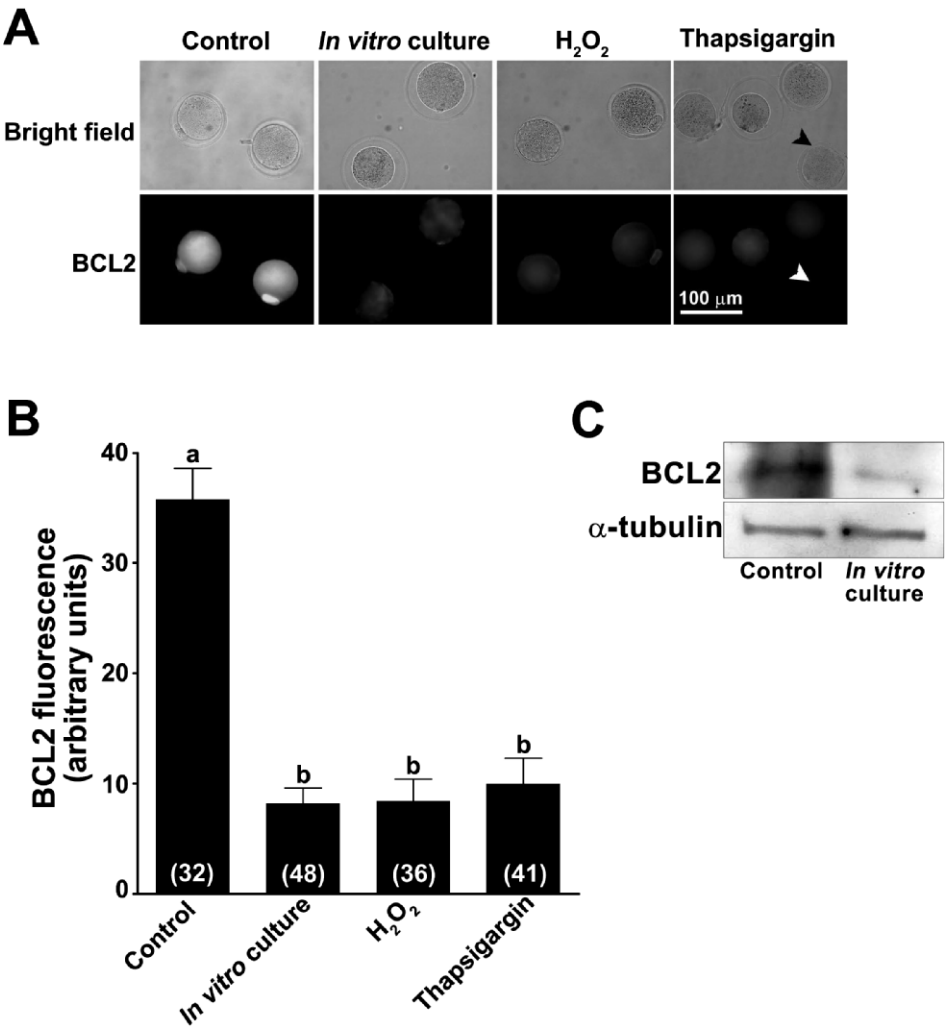

FIG. 8. Expression of BCL2 in mouse oocytes. The COCs were treated by hyaluronidase to remove cumulus cells, and then the denuded oocytes were cultured for 10 h or treated with 200 μM H_2O_2 or 5 μM thapsigargin. After treatments, oocytes were subjected to BCL2 immunofluorescence or immunoblot analysis. Oocytes without additional in vitro culture were used as a control. **A**) Representative data demonstrating expression of BCL2 under various treatments by immunofluorescence. Arrowheads indicate loss of expression of BCL2 in the thapsigargin-treated oocytes. Bar = 100 μm. **B**) BCL2 fluorescence was measured. Values are shown as mean ± SEM. Numbers inside the bars indicate the number of tested oocytes. Bars with different letters represent a significant difference ($P < 0.01$). **C**) Immunoblot analysis of BCL2 expression in oocytes without in vitro culture (control) and in oocytes with in vitro culture without cumulus cells for 10 h. Lysates equivalent to 50 oocytes were loaded into each lane. Expression of α-tubulin was used as an internal control. Data shown are representative of at least three independent experiments. The molecular weight of BCL2 is 30 kDa.

cortical granule release were increased in mouse oocytes aged in vivo and in vitro in the presence of cumulus cells. They concluded that cumulus cells accelerate aging of oocytes. However, they did not examine subsequent embryo development after activation of oocytes by artificial stimuli in oocytes aged in vivo and in vitro. We showed that cumulus cells prevent poor embryo development (Fig. 2) and oxidative stress (Fig. 7) in in vitro-aged oocytes.

BCL2 protein, originally described in lymphoma cells [53], is widely distributed in a variety of cells and is a potent inhibitor of apoptosis [54]. BCL2 is located in biological membranes, including mitochondria [55], and acts to inhibit the mitochondrially controlled steps that lead to cell death through preventing Ca^{2+}-induced cytochrome C release [56]. In the present study, we found that expression of BCL2 (an antiapoptotic protein) was decreased and expression of BAX (a proapoptotic protein) was unchanged in in vitro-aged oocytes (Fig. 8). In addition, we found that fragmented embryos, which were TUNEL or cleaved caspase 3 positive, were increased in in vitro-cultured oocytes (Fig. 2D). In mouse and pig oocytes, expression of BCL2 protein was decreased, and TUNEL-positive unfertilized oocytes were increased in in vitro-aged oocytes [57, 58]. In addition, Gordo et al. [20] reported that expression of BCL2 protein was decreased and expression of BAX protein was unchanged in oocytes cultured in vitro for about 24 h. Moreover, Gordo et al. reported that the high frequency of Ca^{2+} oscillations in in vitro-cultured oocytes induced by sperm factors or adenophostin A (a potent $InsP_3$ analogue) resulted in fragmented and TUNEL-positive embryos. These findings are consistent with our results and suggest a possible role for apoptosis in poor embryo development in in vitro-aged oocytes.

Recent evidence has suggested that the mechanism by which BCL2 exerts its antiapoptotic effect is by modulating intracellular Ca^{2+} fluxes and, in particular, by dampening mobilization of Ca^{2+} from the ER [59]. Kuo et al. [60] suggested that BCL2 may upregulate expression of Ca^{2+}-ATPase mRNA in the ER and may interact directly with the Ca^{2+} pump, possibly modulating its function. There is a possibility that decreased BCL2 expression in in vitro-aged oocytes may affect Ca^{2+} oscillations at fertilization [3]. However, the mechanism underlying the decreased expression of BCL2 during in vitro aging is unclear. We found that expression of BCL2 protein was significantly decreased in oocytes treated with H_2O_2 or thapsigargin (Fig. 8). Reactive oxygen species are known to regulate cell death, including apoptosis, in a variety of cell types [61]. It is known that overexpression of BCL2 can protect cells from apoptosis mediated by ROS [62]. Alternatively, ROS control expression of BCL2 through expression of Bim, a proapoptotic protein, in T cells [63]. It was also reported that BCL2 overexpression maintains ER Ca^{2+} homeostasis [64]. In addition, Kuo et al. [60] reported that BCL2 preserves the ER Ca^{2+} store by upregulating ER Ca^{2+}-ATPase. These results suggest that ROS and depletion of the ER Ca^{2+} store may be involved in the decreased BCL2 expression seen in oocytes.

In summary, we showed that the pattern of Ca^{2+} oscillations was altered in in vitro-aged oocytes, which was caused by a decrease in the ER Ca^{2+} stores. We also showed that expression of BCL2 was decreased in in vitro-aged oocytes, which may be related to oxidative stress. Both the abnormal Ca^{2+} oscillations and the decreased BCL2 expression in in vitro-aged oocytes might affect subsequent embryo development. We are investigating whether the addition of antioxidant agents to the culture media restores the embryo development of in vitro-aged oocytes.

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