Effect of Oocyte Aging on Parthenogenetic Activation with Cycloheximide and Alteration of the Activity of Maturation Promoting Factor during Aging of Bovine Oocytes

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Abstract: *This study was carried out to examine the underlying mechanism for the high susceptibility of bovine aged oocytes to activation stimulus. The oocytes were matured for 21, 27, 33, and 39 h and were then parthenogenetically activated with different concentrations of cycloheximide (CHX); their activation rates were then assessed. Additionally, the p34^{cdc2} kinase activities in oocytes matured for different time periods were determined. The activation rates of oocytes treated with CHX increased with oocyte aging. In oocytes cultured for as long as 39 h in maturation medium, the treatment led to maximal activation rate of oocytes even in those with the lowest concentration of CHX. The p34^{cdc2} kinase activity in oocytes decreased progressively with prolonged incubation time and the activity of the oocytes cultured for 39 h was approximately 40% of that of oocytes cultured for 21 h. In conclusion, the sensitivity of bovine oocytes to activation stimulus increases during in vitro aging; also, the time dependent decline in the capacity of oocytes to synthesize the regulatory protein required for maintaining MII stage may contribute to a high susceptibility of aged oocytes to protein synthesis inhibitor (activation stimulus).*
Key words: *Bovine oocyte, Aged oocyte, Cycloheximide, MPF, Activation*

In a study to explore the influence of interactions between aged gametes on fertilization and early development, Smith and Lodge [1] found that pronuclear development of long-term aged ova was higher than that of short-term aged ova when fertilized by all groups of aged sperm. This showed that the aging of matured oocytes primed their intrinsic ability to undergo pronuclear formation. In nuclear transferred embryos reconstructed with aged oocytes as recipient cytoplasm, it has been reported that not only pronuclear formation and 2-cell progression but also blastocyst formation were improved [2–4]. Additionally, higher fertilization and pronuclear formation rates were noted in aged oocytes which were used for intracytoplasmic sperm injection [5, 6]. These results suggest the possibility that aging of oocytes may result in increasing sensitivity to various activation stimulus treatments.

Meiotic arrest of mammalian oocytes is known to be maintained by persistently high activity of maturation promoting factor (MPF). The sustained high MPF activity is maintained by cytostatic factor (CSF) activity [7]. The key component of CSF, Mos, may regulate MAPK which is activated by the MAP kinase cascade [8, 9]. Inactivation of these kinases (MPF, MAPKs, and Mos) is caused by fertilization or other parthenogenetic stimuli through the oscillations of intracellular Ca^{2+}, leading to release from MII arrest and pronuclear formation [10, 11]. Accordingly, it is thought that much readier occurrences of activation in aged oocytes are dependent on the decline in the activities of enzymes, including MPF, MAPK, and Mos. However, the relationship between the activation of aged oocytes and the changes in their enzyme activities remains to be fully elucidated.

To account for the high susceptibility to activation of aged oocytes, matured bovine oocytes were cultured for several hours in maturation medium and their activation rates were evaluated after parthenogenetic activation using protein synthesis inhibitor. The alteration of the activity of p34^{cdc2} kinase during the aging of oocytes was also examined.

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Materials and Methods

Collection of oocytes and in vitro maturation

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% saline at 30–35°C. Using a 21 gauge needle with a 10 ml syringe, cumulus oocyte complexes (COCs) were aspirated from small antral follicles (2–8 mm) of the ovaries. Only oocytes with intact cytoplasm and surrounded by compact cumulus cells were used. These COCs were washed with Dulbecco's phosphate buffer saline containing 0.05% polyvinylpyrrolidone (Sigma, St. Louis, MO, USA) and then washed twice in TCM-199 with Earle's salt (Gibco BRL, Grand Island NY) supplemented with 15% bovine follicular fluid, 0.6 μg/ml p-FSH (Sigma), 1.3 μg/ml e-LH (Sigma), 0.2 mM Na-pyruvate, and 50 μg/ml gentamicin (Sigma). Then, 15–20 COCs were cultured in a 100 μl droplet of the medium overlaid with mineral oil (Sigma) for various times at 39°C, 5% CO_2, and 95% air with humidity.

Histone H1 kinase assay

The histone H1 kinase activity in oocytes was determined as described previously [12]. Briefly, the cultured oocytes were kept on ice for 30 min and then denuded from their cumulus cells by vortexing in PBS for 2 min. Denuded oocytes were put into plastic tubes containing 20 μl of assay buffer. After the tube contents were frozen and thawed, the oocyte suspensions were centrifuged at 15,000 × g for 25 min at 4°C. Five microliters of oocyte extract were mixed with 20 μl of a kinase assay buffer containing 50 μM histone H1 (type III-S, Sigma), and the kinase reactions commenced upon the addition of [γ-^{32}P]ATP and continued for 60 min at 36°C. Assays were terminated by the addition of 0.4 ml 20% trichloroacetic acid solution. Radioactivity was measured using a liquid scintillation counter (LCS-1000, Aloka, Tokyo, Japan). The value of blank tubes containing all materials except for the oocyte extract was subtracted from each value to obtain the histone H1 kinase activity.

p34^{cdc2} kinase assay

p34^{cdc2} kinase assay was carried out by measuring the activity of its catalytic subunit, p34^{cdc2} kinase, with a MESACUP cdc2 Kinase Assay Kit (MBL, Nagoya, Japan). This kit uses an enzyme linked immunosorbent assay that utilizes a synthetic peptide substance (SLYSSSPGGAYC). At designated time intervals in the preparation of the cultures, COCs were collected and their cumulus cells were removed, as in the histone H1 kinase assay. Twenty denuded oocytes were washed twice with the p34^{cdc2} kinase sample buffer containing 50 mM Tris HCl, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA (Katayama Kagaku, Osaka, Japan), 0.01% Brij 35, 1 mM phenylmethylsulfonyl fluoride, 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate, and 1 mM Na-orthovanadate (Sigma). The oocytes were then transferred to a microtube containing 5 μl of the buffer and stored at –80°C.

At the time of assay, the oocytes were lysed by successive freezing and thawing with liquid nitrogen and water (10°C). The cdc2 kinase activity in the oocyte lysate was measured according to the manufacturer's protocol.

Parthenogenetic activation

At 21, 27, 33, and 39 h after the onset of maturation, COCs were freed from cumulus cells by vortexing with 0.1% hyaluronidase. A portion of the denuded oocytes was collected as mentioned above for the cdc2 kinase assay. Another portion of the oocytes was washed twice and 15–20 of them were transferred into 30 μl droplets of activation medium. The activation medium was Hepes-buffered TALP containing MEM amino acids with glutamine, MEM non-essential amino acids (Gibco), and 5% newborn calf serum (Gibco), and it was further supplemented with different concentrations of cycloheximide (CHX, Sigma). The oocytes were incubated for 6 h in each activation medium then cultured for an additional 17 h without CHX to encourage the progress to the pronuclear formation stage. The oocytes were examined for pronuclear formation.

Evaluation of nuclear status

At the end of developmental culture, the oocytes were mounted on slides with coverslips and fixed with ethanol: acetic acid (3:1) for more than 48 h at room temperature. The oocytes were stained with 1% lacmoid in 45% acetic acid and sealed with acetoglycerol. These samples were examined by phase-contrast microscopy (× 125, × 600). Oocytes with single or multiple pronuclei were classified as activated.

Statistical analysis

The linear correlation coefficient between values for cdc2 kinase and histone H1 kinase activities in Exp. 1 were statistically analyzed by Pearson's correlation coefficient test. All data of Exp. 2 and 3 were statistically analyzed by one-way ANOVA. When significance was found in the ANOVA analysis, the treatments were compared by the Tukey-Kramer test. A P value<0.05 was considered significant. All statistical analyse were car-

ried out with Stat View 5.0 (SAS Institute Inc.).

Experimental design

Experiment 1: p34^{cdc2} kinase activity in 10, 20, 30, and 40 denuded oocytes from COCs, cultured for 21 h in maturation medium, was measured by the MESACUP cdc2 Kinase Assay Kit. Histone H1 kinase activity was measured in the same number of oocytes under the same conditions using a radioactive method. The two activities were compared.

Experiment 2: This experiment was conducted to examine the effect of aging on parthenogenetic activation of bovine oocytes. Oocytes cultured for 21, 27, 33, and 39 h in maturation medium were parthenogenetically activated by 6 h cultivation in 0, 2, 10, and 20 μg/ml cycloheximide. Pronuclear formation was evaluated after 17 h incubation in a drug-free medium.

Experiment 3: To investigate the relative ease of activation of aged oocytes, COCs were matured for 21, 27, 33, and 39 h and then were denuded. Their cdc2 kinase activities were measured with the MESACUP cdc2 Kinase Assay Kit as described above.

Results

Experiment 1: Correlation between histone H1 kinase activity and p34^{cdc2} kinase activity

To determine whether the procedure for measuring p34^{cdc2} kinase activity used in this study might be useful, a comparison of activities obtained by p34^{cdc2} kinase assay and those by histone H1 kinase assay, which is one of the major procedures for measuring the MPF kinase activity, was conducted by multiple regression analysis. When histone H1 kinase activity in 10, 20, 30 and 40 oocytes was measured by the radioactive method, increased activity was found to be proportional to the increases in oocyte numbers (Fig. 1). A similar tendency in the relationship between the oocyte numbers and p34^{cdc2} kinase activity was noted when using the MESACUP cdc2 Kinase Assay Kit. Correlation coefficients for them were as high as 0.9961. This indicated that the method used in this study for p34^{cdc2} kinase assay might be useful for measuring MPF activity in bovine oocytes.

Experiment 2: Effects of oocyte aging on parthenogenetic activation of bovine oocytes

When oocytes cultured for 21 h were treated with 0, 2, 10, and 20 μg/ml CHX, the activation rates of oocytes in the treatment groups were significantly higher than that of the control (27–34% vs 2.2%, respectively), but no significant differences were detected within the treatment groups. In oocytes cultured for 27 h, the activation rates of all treatment groups were significantly higher than those in oocytes cultured for 21 h. The activation rate in 27 h cultured oocytes which were treated with 2 μg/ml CHX was significantly higher than that of the control groups, but lower than those for the oocytes treated

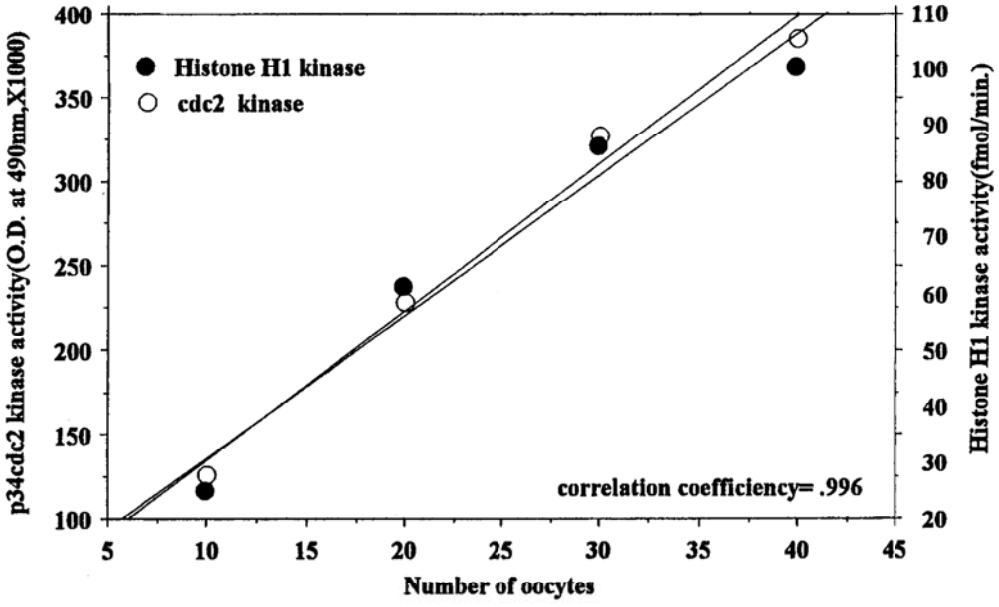

Fig. 1. Correlation between p34^{cdc2} kinase activity and histone H1 kinase activity.

with higher concentrations of CHX (10 and 20 μg/ml), and these rates with higher concentrations of CHX were reached in about 50% of the oocytes. In oocytes cultured for 33 h and treated with or without CHX, each activation rate was significantly increased compared with the rates for oocytes cultured for 21 and 27 h. The treatment with CHX drove the increase in activation rates of 33 h cultured oocytes in a dose dependent manner. Nearly all of the 33 h cultured oocytes which were treated with 20 μg/ml CHX were activated. In oocytes cultured for as long as 39 h in maturation medium, no significant differences in activation rates were detected within treatment groups. Even with the lowest concentration of CHX (2 μg/ml), the treatment could effectively lead to activation of 39 h cultured oocytes (88.7%). The activation rate of 39 h cultured oocytes treated with the lowest concentration of CHX (2 μg/ml) was significantly increased over that in 33 h cultured oocytes which were treated with the same concentration of CHX.

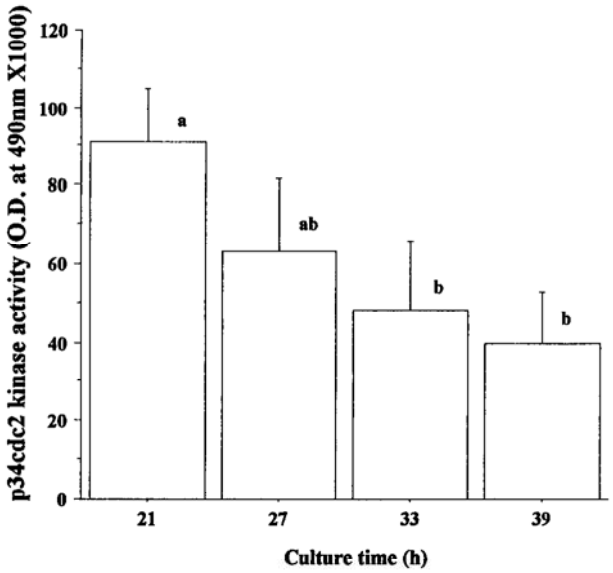

Fig. 2. p34^{cdc2} kinase activity during oocyte aging. Twenty oocytes were harvested at each time point and subjected to MESACUP cdc2 kinase assay. Means bearing different letters are significantly different, P<0.05.

Experiment 3: Changes of p34^{cdc2} kinase activity during oocyte aging

To compare the difference in p34^{cdc2} kinase activities in newly matured and aged oocytes, the activity of 10 oocytes in each group was determined. The fluctuations during oocyte aging are summarized in Fig. 2. The p34^{cdc2} kinase activity in oocytes cultured for 27 h in maturation medium was considerably decreased compared with that of 21 h cultured oocytes, though the difference between them was not significant. In the oocytes cultured for 33 h, the activity further decreased and the mean activity was significantly lower than that in 21 h cultured oocytes. The p34^{cdc2} kinase activity in oocytes cultured for 39 h was about 40% of that in 21 h cultured oocytes.

Discussion

Results from the present study demonstrated that when bovine oocytes, matured for various lengths of time, were parthenogenetically activated with different concentrations of cycloheximide (CHX), a high activation rate in oocytes cultured for 39 h was obtained, even after treatment with a low concentration of CHX (Table 1). Similar results were reported by Nagai [13] who used another activation agent (ethanol) for parthenogenetic activation. The promotion of activation in oocytes by aging has also been shown with other activation agents including Ca^{2+} ionophore A23187 and electrical stimulation in cattle [14–16], and with a variety of mammals including mice [17, 18], rabbits [19], and pigs [20]. Taken together, these results suggest that

Table 1. Effects of oocyte aging on parthenogenetic activation of bovine oocytes treated with various concentrations of CHX*

	Culture time (h)			
	21	27	33	39
0 μg/ml	2/91 (2.2 ± 0.58) ^{aA}	4/98 (4.2 ± 0.82) ^{aA}	20/137 (14.6 ± 0.82) ^{bA}	14/77 (18.2 ± 0.58) ^{bA}
2 μg/ml	36/133 (27.1 ± 0.82) ^{aB}	56/144 (38.9 ± 1.63) ^{bB}	69/101 (68.3 ± 2.99) ^{cB}	110/125 (88.0 ± 4.44) ^{dB}
10 μg/ml	36/107 (33.7 ± 0.82) ^{aC}	76/156 (48.7 ± 1.83) ^{bC}	86/104 (82.7 ± 2.08) ^{cC}	103/118 (87.3 ± 2.63) ^{cB}
20 μg/ml	32/106 (30.2 ± 2.16) ^{aC}	80/151 (53.0 ± 2.16) ^{bC}	95/100 (95.0 ± 1.71) ^{cD}	91/105 (86.7 ± 3.86) ^{cB}

*Experiments were repeated 4 times. The date within parentheses are expressed as mean ± SD. $^{A, B, C, D}$ Values with different superscripts in the same column are significantly different, P<0.05. $^{a, b, c, d}$ Values with different superscripts in the same line are significantly different, P<0.05.

the agedoocytes are more sensitive to various activation stimuli than newly matured oocytes in all mammalian species.

It is well known that when matured oocytes are treated with activation stimuli such as ethanol [21–23], Ca^{2+} ionophore A23187 [24, 25], IP_3 [26, 27], thimerosal [26, 28], strontium [24, 29], and electric pulses [30, 31], transient intracellular calcium $[Ca^{2+}]_i$ increases are triggered. Additionally, it is postulated that the sensitivity of the oocyte's activation mechanism to calcium increases with culture time following maturation. For example, Fissore and Robl [32] observed no differences between recently ovulated and aged oocytes in their Ca^{2+} response to electric pulse even though activation rates were significantly higher in aged oocytes. They concluded that the higher activation rates of aged oocytes might be due to increases of Ca^{2+} sensitivity in the oocytes.

CHX has been shown to halt protein synthesis without causing or affecting $[Ca^{2+}]_i$ release in oocytes [29, 33–36]. Apparently, the oocyte activation via CHX in this experiment was provoked independently of calcium oscillations. The present results suggested that the greater the reduction in the activity of the regulatory proteins, including MPF, due to the blocking of new protein synthesis with low concentrations of CHX leads to higher activation rates of the aged oocytes.

In porcine oocytes, it has been reported that the inactivation of MPF during aging might not depend on cyclin B degeneration, but might be due to inhibitory phosphorylation of $p34^{cdc2}$ [37]. However, Moos *et al.* [38] used the Western blotting technique to determine the amount of cyclin B, the regulatory subunit of MPF, in matured mouse oocytes after CHX treatment. They found that the amount of cyclin B gradually decreases from 1 h after CHX treatment, and the product is undetectable 7 h after the treatment. Further, de novo synthesis of the protein is essential for induction and maintenance of MPF activation of Xenopus oocytes [39]. It has been reported that the decrease in MPF activity is involved in the initiation of oocyte activation, i.e. in exit from MII and pronuclear formation [40]. Consequently, it is thought that the reason for the higher activation rate in aged oocytes treated with low concentrations of CHX may be related to the rapid decrease below the limited level of MPF activity sufficient to maintain meiotic arrest, depending on the depression of the capacity of their oocytes to synthesize new cyclin B.

In experiment 3, $p34^{cdc2}$ kinase activities of bovine oocytes were investigated at given times after the initiation of culture. The results showed that $p34^{cdc2}$ kinase activity decreased progressively with prolonged maturation time and the activity in the oocytes cultured for 39 h reached approximately 40% of that in the oocytes cultured for 21 h. These results were in close agreement with the observation of Wu *et al.* [41] who determined the MPF kinase activity using endogenous histone H1 as a substrate. They showed that histone H1 kinase activity in bovine oocytes cultured for more than 26 h in maturation medium gradually decreased and reached a basal level at 48 h. These time dependent decreases in MPF activity were also noted in porcine and mouse oocytes [42–44]. These results confirm the finding that the activity of MPF in bovine oocytes gradually decreases with the progression of oocyte aging. Therefore, slight protein synthesis inhibition with low concentrations of CHX may be sufficient to reduce MPF activity below the threshold level for oocyte activation, since MPF activity is already decreased in aged oocytes.

In conclusion, the time dependent decline in the capacity for oocytes to synthesize the regulatory protein(s) required for maintaining the MII stage contributes to the increased susceptibility of oocyte activation by the protein synthesis inhibitor (CHX).

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