

Effect of Cycloheximide on Bovine Oocyte Nuclear Progression and Sperm Head Transformation after Fertilization *In Vitro*

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ABSTRACT: Bovine oocytes with compact and complete cumulus cells were cultured in 6 groups for up to 24 h in TCM199 buffered with 25 mmol/l HEPES and supplemented with 10% FCS, 1 mg/ml 17 β -estradiol, 20 IU/ml hCG. Half of the oocytes at each group cultured in the presence of 25 μ g/ml cycloheximide at different times during maturation (0, 6, 12, 18, 20, 22 h) were fixed at 24 h of maturation to examine the nuclear progression. The rests of them were inseminated with frozen-thawed spermatozoa in medium BO with 10 mg/ml BSA and 10 mg/ml heparin and fixed after additional 18-20 h culture to evaluate the sperm head transformation. When a protein synthesis inhibitor was added at the onset of the maturation, the oocytes were prevented to proceed GVBD. A few of the oocytes (16%) were able to be penetrated and sperm head decondensation was inhibited

either. Addition of cycloheximide after 6-12 h of culture resulted in an increasing percentage of GVBCD (more than 80%), but the oocytes became arrested in M-I (69.2%). More than half of the oocytes was penetrated with a decondensing sperm head. Formation of male pronucleus was first obtained at 12 h of culture in the presence of cycloheximide. When cycloheximide was added from 18 h of culture onwards, nuclear progression to M-II was increasingly restored (80.4-85.5%). The proportion of male and female pronuclear formation increased from 17.9% to 46.2%. It is concluded that protein synthesis is necessary not only for GVBD and development from M-I to M-II, but also for sperm head decondensation and male pronuclear formation in bovine oocytes.

(Key Words: Protein Synthesis, Bovine Oocytes, Maturation, Sperm Nucleus)

INTRODUCTION

Fully grown oocytes of mammal species are arrested in prophase-I of meiosis until they are committed to ovulation or atresia. Resumption of meiosis can be induced by the transfer of oocytes from their ovarian follicles into a suitable culture medium. In the ensuing period of culture, oocytes undergo nuclear progression from diplotene to metaphase-II (M-II) and the change is characterized by dissolution of the nuclear membrane (Germinal Vesicle Breakdown, GVBD) and extrusion of the first polar body. Both the roles of protein synthesis and the pattern of proteins synthesized are involved in oocytes undergoing maturation. However, mature oocytes have not only undergone nuclear, but also cytoplasmic changes necessary for fertilization and subsequent embryonic development (Moor and Trounson, 1977). Structural rearrangements of organelles (Cran, 1985) and

major changes in protein synthesis patterns take place during cytoplasmic maturation and have been described for several mammalian species (pig and mouse, Fulka et al., 1986; cattle, Sirard et al., 1989 and Kastrop et al., 1990; horse, Alm and Henrichs, 1996). Our previous studies indicated that the period of 2-4 h after nuclear maturation is important for fertilization and subsequent embryo development (Qian et al., 1994).

So far, although it has been known that protein synthesis is necessary for oocyte nuclear maturation *in vitro*, it remains unclear about the relationship between protein synthesis during oocyte maturation and subsequent fertilization as well as sperm head transformation *in vitro*. Cycloheximide, which inhibits peptidyl transferase, suppresses oocyte maturation by blocking synthesis of stage-specific protein. In the present experiment, we have examined the effect of cycloheximide on bovine oocyte nuclear progression and sperm head transformation post inseminated *in vitro*.

MATERIALS AND METHODS

Cumulus-oocyte complexes collection and treatment

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Ovaries were removed from cows at a local slaughterhouse and returned to the laboratory in saline at 30-32°C within 3 h. Oocytes were aspirated through a 24-gauge needle into a disposable 10 ml syringe from follicles of 2-7 mm in diameter. The collected cumulus-oocyte complexes (COCs) were washed three times with maturation medium. Only oocytes surrounded by a complete coronal cell layer and 2-6 compact cumulus layers were used in this study (figure 1). Dark- and pale yellow-looking cumuli were also discarded from this study. Ten-15 COCs were cultured in 100 ml drops of maturation medium under paraffin oil at 39°C with 5% CO₂ in air and a high humidity atmosphere. The maturation medium was TCM199 medium (with Earle's Salts, GIBCOBRL) buffered with 25 mmol/l HEPES (Sigma) and supplemented with 10% fetal calf serum (FCS, GIBCO), 1 mg/ml 17β-estradiol (Sigma) and 20IU/ml hCG (Mochida Pharmaceutical CO. LTD.). The processing time from aspiration to culture was 1 h.

After culture for 0, 6, 12, 18, 20, 22, 24 h (control), cycloheximide (Sigma) was added into the maturation medium at 25 μg/ml final concentration for each group respectively. All groups were cultured for a total of 24 h before being processed for the next.

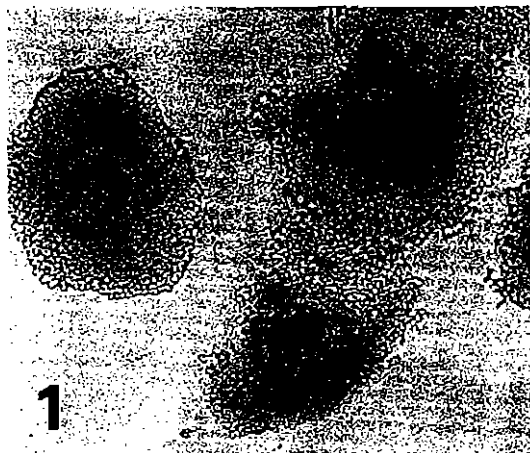


Figure 1. Cumulus-oocyte complexes × 80.

In vitro fertilization

One 0.5 ml straw of frozen semen obtained from a Japanese Black Bull was thawed in a water bath at 37°C. Spermatozoa were washed twice in medium BO supplemented with 3 mg/ml BSA (Sigma) by centrifugation at 600 g for a period of 10 min each. A 50 ml sample of final sperm suspension ($5-10 \times 10^6$ spermatozoa/ml) with capacitation medium BO containing 10 mg/ml heparin and 10 mg/ml BSA was introduced into 50 ml of the capacitation medium that include the oocytes. The

mixture gave a final concentration of $2.5-5 \times 10^6$ spermatozoa/ml. Eight h later, oocytes were washed and transferred into maturation medium without hCG for another 10-20h culture.

Half of the oocytes at each group was inseminated after 24 h culture and the rests were fixed for examination of oocyte nuclear progression.

Evaluation of nuclear status

Oocytes for 24 h maturation culture and at 18-20 h after insemination were denuded and mounted respectively between slides and coverslips supported by vaseline, fixed for 48-72 h at room temperature in 25% (v/v) acetic alcohol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined for evidence of nuclear status under a phase-contrast microscope at 400x magnification. Germinal vesicle (GV) stage defined by intact visible nuclear membrane, and GVBD by absence of visible nuclear membrane, chromosome condensation characterized by a cluster of DNA material without individual chromosomes.

Statistical analyses

Data were analyzed using the chi-squared test with $p < 0.05$ considered statistically significant (Steel and Torrie, 1980).

RESULTS

Effect of cycloheximide on bovine oocyte nuclear progression

Oocytes were fixed 24 h after the beginning of maturation and were therefore cultured in the presence of cycloheximide for 0-24 h. The total numbers of oocytes at each time point (117-165) are from four replicates. Distribution of oocytes at different developmental stages in the different treatment groups is presented in the table 1. When a protein synthesis inhibitor was added at the beginning of the maturation period, the proportion of germinal vesicle stage oocytes (figure 3) was significantly higher than that of any other treatment groups ($p < 0.01$), and the cumulus cells expansion was inhibited. None of the oocytes reached metaphase-I (M-I). When cultured for 6 h in inhibitor-free medium, more than 80% of the oocytes underwent GVBD and developed to chromosome condensation stage, but when cultured for 12 h under this condition, 69.2% were able to attain M-I. There was no significant change in the proportion of these oocytes developed to M-II between 18, 20, 22 and 24 h (control) preincubation treatments without cycloheximide ($p > 0.05$). No typical spindles were formed in M-I and M-II when cycloheximide was added. In control, 88.9% of the oocytes reached to M-II with normally developed spindles.

Cycloheximide was ineffective in suppressing cumulus cells expansion (figure 2) after 12 h culture in the medium without cycloheximide.

Table 1. Nuclear status when protein synthesis inhibitor cycloheximide was added at different times during maturation culture

Nuclear configuration	Time (h) of cycloheximide addition (25 μ g/ml)						
	0	6	12	18	20	22	24 (Control)
Geminal vesicle intact (%)	140 (94.0)	22 (18.0)	2 (1.5)	0	0	0	0
Geminal vesicle breakdown (%)	6 (4.0)	14 (11.5)	0	0	0	0	0
Chromosome condensation (%)	3 (2.0)	85 (69.7)	11 (8.5)	7 (5.1)	5 (4.0)	4 (2.4)	2 (1.7)
M-I (%)	0	0	90 (69.2)	12 (8.7)	4 (3.2)	6 (3.6)	5 (4.3)
Anaphase-I and telophase-I (%)	0	0	3 (2.3)	1 (0.7)	7 (5.6)	12 (7.3)	6 (5.1)
M-II (%)	0	0	21 (16.2) ^b	111 (80.4) ^a	103 (83.1) ^a	141 (85.5) ^a	104 (88.9) ^a
One pronucleus (%)	0	1 (0.8)	3 (2.3)	4 (2.9)	3 (2.4)	1 (0.6)	0
Two pronucleus (%)	0	0	0	3 (2.2)	2 (1.6)	1 (0.6)	0
Total	149	122	130	138	124	165	117

^{a, b}; $p < 0.01$.

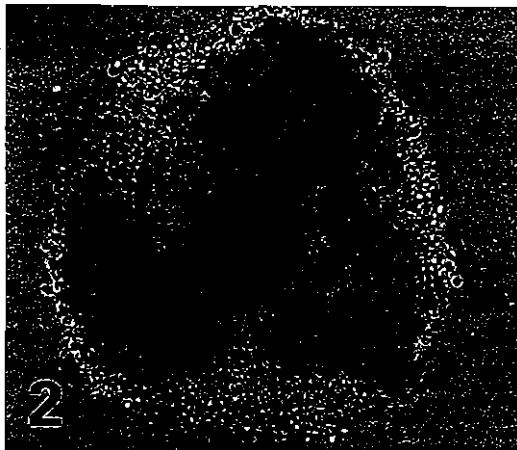


Figure 2. Oocyte with expanded cumulus cells $\times 80$.



Figure 3. Oocyte at GV stage after 6 h of maturation culture without cycloheximide $\times 400$.

Effect of cycloheximide on sperm head transportation

As shown in table 2, very low penetration rate (16.0%) was obtained in the oocytes that had been cultured in the presence of cycloheximide for 24 h. All of these oocytes were at the germinal vesicle stage and had undecondensed sperm heads (figure 4). When more than 6 h of incubation in the absence of cycloheximide, sperm heads were able to undergo decondensation (figure 5). Oocytes penetrated with male pronuclei were first obtained in group C. The proportion of the oocytes with male and female pronuclei (figure 6) was significantly higher in group F than in group D and group E ($p < 0.01$), but was significantly lower than in group (p < 0.01). Extremely



Figure 4. Fertilized ovum with undecondensed sperm head following 24 h of maturation culture with cycloheximide $\times 800$.

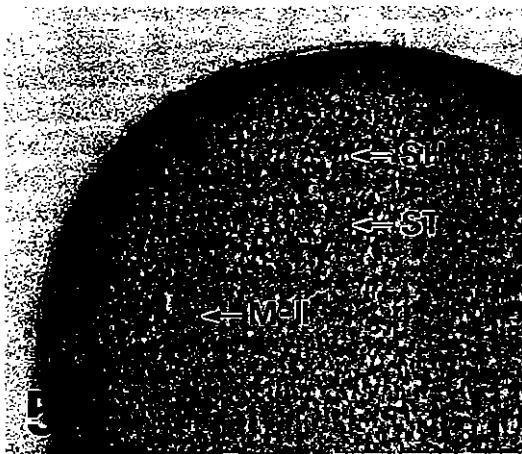


Figure 5. Fertilized ovum with decondensing sperm head after 18 h of maturation culture without cycloheximide \times 800.

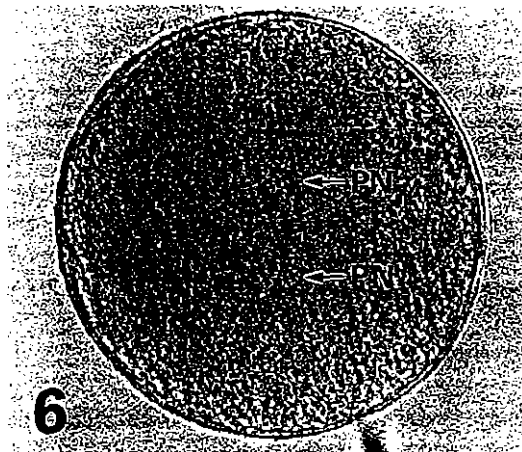


Figure 6. Fertilized ovum with male and female pronuclei following 22 h of maturation culture without cycloheximide \times 400.

high proportions of polyspermy were obtained in group A, B and C. In control, 2.2% of the oocytes exhibited polyspermy.

DISCUSSION

Successful *in vitro* maturation of mammalian oocytes requires continual and/or episodic protein synthesis by cumulus-oocyte complexes. Germinal vesicle breakdown is accompanied by qualitative and quantitative changes in the protein synthesis pattern (Fulka et al., 1977; Moor and Trounson, 1977; Kastrop et al., 1991a; 1991b; Alm, 1996; Wu et al., 1996). The results of this study demonstrate that treatment with cycloheximide from the onset of culture inhibits the occurrence of GVBD. Addition of cycloheximide after maturation for 6 h allows

chromosome condensation, and more than 80% of the oocytes undergoes GVBD. It appears that protein synthesis during the first 6 h of maturation is necessary for the GV to undergo breakdown. This was shown previously in several mammals (Hunter and Moor, 1987; Sirard, 1989; Kastrop et al., 1991b; Pawshe et al., 1994).

In present study, majorities of the oocytes were arrested in chromosome condensation stage at 6 h and in M-I at 12 h of maturation without the inhibitor. A few of the oocytes (16.2%) reached to M-II at 12 h treatment. It seems that oocyte development from GVBD to M-I depends on a short period of protein synthesis during 6-12 h of culture. Similarly, Kastrop et al. (1991a) reported that addition of cycloheximide after 6 or 8 h culture resulted in an increasing percentage of GVBD, but the oocytes became arrested in M-I. Only parts of the changes in protein synthesis after GVBD were observed. However, metabolic labeling with (S-35)-methionine demonstrated that oocytes exhibited a steady or slightly increasing rate of protein synthesis during the first 12 h of maturation (Wu et al., 1996). As soon as 18 h or more after removal from the follicles, more than 80% of the oocytes completed meiosis-I and reached to M-II. It appears that newly synthesized proteins are necessary for nuclear progression from M-I to M-II. It is well known that formation of M-I and M-II plates are associated with high activity of maturation/mitosis promoting factor (MPF). MPF activity decreases during the M-I to M-II transition in pigs (Naita, 1991). In this experiment, arrest of oocytes at M-I in the presence of cycloheximide suggests that proteins needed for nuclear progression from M-I may participate in deactivation of MPF.

Mitogen activated protein (MAP) kinases, known as extracellular signal regulated kinases in mammalian cells, are serine/threonine kinases that are activated by phosphorylation on both tyrosine and serine/threonine residues (Davis, 1993). In porcine oocytes, the MAP kinase activity abruptly increases at M-I and remains significantly higher than that at GV stage until M-II. MAP kinase activation requires protein synthesis (Inoue, et al., 1996). In the present study, the inhibition of formation and maintenance of typical metaphasic spindle seems involved in the suppression of MAP kinase activation.

In mammals, it is well known that sperm nuclear decondensing factor is synthesized in the egg cytoplasm, transported to and accumulated in the GV, and then released into the egg cytoplasm at the time of GVBD (Usui et al., 1976). However, 100% of the bovine oocytes at GV stage can be penetrated and have a decondensing sperm head (Niwa, 1991). It is possible that the activation of the factor occurs before the GVBD by these chemicals of heparin and caffeine used for fertilization. In the present study, the inhibition of oocyte protein synthesis

during first 6 h of maturation inhibited both sperm penetration and sperm head decondensation. The oocytes cultured for 6 h without protein synthesis inhibitor were able to induce sperm head decondensation, although some of them were at GV stage (table 2). The results indicate that the proteins synthesized during the first 6 h of maturation are necessary for synthesis and/or activation of the sperm nuclear decondensing factor.

Some kind of factor(s) for male pronucleus growth or sperm pronucleus development might be closely involved in the male pronuclear development. The activation of the factor may occur only partly just after GVBD and be

intensified gradually as the oocytes develop from GVBD to M-II in mammals (Niwa et al., 1991). In this experiment, development of male pronuclei was first observed in the oocytes cultured for 12 h of maturation in the absence of protein synthesis inhibitor, and the proportion of male pronuclei formation increased significantly ($p < 0.01$) at and after 22 h of maturation culture (table 2). It is speculated that some kinds of factor (s) for male pronucleus growth may be synthesized during the early stage of maturation culture and be fully activated at and after 22 h in the cattle.

Table 2. Effect of protein synthesis inhibitor cycloheximide on sperm head transformation post inseminated *in vitro*

Group	Time (h) of cycloheximide addition	No. of oocytes inseminated	No. of oocytes penetrated							No. of polyspermic oocytes
			Total (%)	With decondensing sperm head at the stage of				With male and female pronuclei		
				GV	Chromosome condensation	M-I T-1	M-II		Female pronucleus	
A	0	106	17 (16.0)	0	0	0	0	0	0	15 (88.2)
B	6	99	47 (47.5)	10	34	2	0	1	0	38 (80.9)
C	12	117	72 (61.5)	0	10	36	16	5	5 (6.9)	35 (48.6)
D	18	112	84 (75.0)	0	4	12	44	9	15 (17.9) ^c	13 (15.5)
E	20	112	98 (87.5)	0	2	6	70	4	16 (16.3) ^c	14 (14.3)
F	22	118	104 (88.1)	0	0	4	10	42	48 (46.2) ^b	3 (2.9)
G	24 (Control)	101	90 (89.1)	0	0	2	0	4	84 (93.3) ^a	2 (2.2)

(T-I: telophase-I) ^{b vs a, c vs a, c vs b}; $p < 0.01$.

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