Timing of Completion of the First Meiotic Division in Bovine Oocytes after Maintenance of Meiotic Arrest with Cycloheximide and Their Subsequent Development

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ABSTRACT. This study examined the timing of completion of meiosis I of bovine oocytes in which meiotic resumption had been inhibited by cycloheximide (CHX), and also determined the optimum interval of maturation in culture for subsequent fertilization and development. Most oocytes treated with CHX reached metaphase II at 16 hr in the maturation culture, while control oocytes did at 20 hr. CHX-treated oocytes cultured for 16 hr were normally fertilized but failed to develop into blastocysts. Maturation in culture for 20 hr resulted in comparable development for control oocytes. The results indicate that nuclear maturation of CHX-treated oocytes was completed 4 hr faster than for control oocytes, however the same interval of maturation as that of control oocytes is necessary for subsequent development to blastocysts. — **KEY WORDS:** cycloheximide, inhibition of bovine meiotic resumption, maturation.

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Mammalian extrafollicular oocytes resume meiosis spontaneously [15]. Active protein synthesis is required for the resumption in oocytes of pigs, sheep, goats and cattle [5, 6, 8, 9]. Bovine oocytes do not undergo germinal vesicle breakdown (GVBD) in media with protein synthesis inhibitors such as cycloheximide (CHX) [6, 19, 21] and puromycin [10]. The inhibition of GVBD is fully reversible, since after washing the inhibitors, oocytes undergo GVBD [6, 10, 21]. Moreover, oocytes maintained in meiotic arrest by CHX can mature *in vitro* [21] and develop to blastocysts following *in vitro* fertilization [17]. It has been reported that normal fertilizability and developmental capacity are kept only within several hours after nuclear maturation of bovine *in vitro* matured oocytes [2]. However, the remarkable fact with oocytes after reversal of meiotic arrest with protein synthesis inhibitors is that the time sequence of GVBD is twice as fast as the control oocytes [7, 8, 10, 21]. The timing of completion of the first meiotic division (meiosis I) in oocytes treated with CHX and subsequent fertilizability and developmental capacity of oocytes have not yet been reported.

Here, examinations were made to determine the time sequence of nuclear maturation of bovine oocytes which had been maintained in meiotic arrest with CHX for 24 hr and to determine the optimum interval of maturation in culture of oocytes for subsequent fertilization and development to the blastocyst stage.

Bovine cumulus-oocyte-complexes (COCs) were collected from the small antral follicles (2 to 5 mm in diameter) of slaughterhouse ovaries transported and stored in saline (0.9% w/v NaCl) for 7–9 hr at 18–22°C. COCs were washed in 25 mM HEPES-buffered TCM-199 with

Earle's salts (Cat. No. 12340, Gibco Laboratories, Grand Island, NY, U.S.A.), supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; M.W. 40,000, Sigma Chemical, St Louis, MO), 0.5 mM Na-pyruvate (Nacalai Tesque, Kyoto, Japan) and 1% (v/v) antibiotic antimycotic solution (Gibco, m-TCM199) three times. Maintenance of meiotic arrest was carried out as described by Saeki *et al.* [17]. Ten COCs were incubated in 50 μ l drops of m-TCM199 supplemented with 10% (v/v) fetal calf serum (Gibco, m-TCM-199+FCS) and 10 μ g/ml cycloheximide (CHX, Sigma), 0.02 AU/ml FSH (Antoine, Denka Seiyaku, Tokyo, Japan) and 1 μ g/ml estradiol-17 β (E₂, Sigma) in a plastic dish (Falcon 1007, Becton and Dickinson Labware, Lincoln Park, NJ, U.S.A.) covered with mineral oil (Squibb, Princeton, NJ, U.S.A.) for 24 hr at 39°C in 5% CO₂ in air at high humidity. Following incubation, some oocytes were fixed in acetic alcohol (1:3) and stained with aceto-orcein for examination of the nuclear stage [18].

The COCs were washed five times thoroughly and cultured in m-TCM199 supplemented with 0.02 AU/ml FSH and 1 μ g/ml E₂ (maturation medium) [16] in a plastic dish covered with mineral oil (10 COCs/50 μ l droplet) for 12–24 hr at 39°C in 5% CO₂ in air at high humidity.

In vitro fertilization was carried out as described by Nagao *et al.* [11]. Briefly, COCs cultured for 16, 20 and 24 hr in the maturation medium were inseminated with Percoll-washed spermatozoa [16] (1 $\times$ 10⁶ cells/ml) in defined medium [1] modified by excluding glucose and supplemented with 1 μ g/ml heparin [14] (10 COCs/100 μ l droplet). Oocytes and spermatozoa were co-incubated for 6 hr at 39°C in 5% CO₂ in air with high humidity. Following co-incubation, the oocytes were freed from the spermatozoa and cumulus cells and cultured in modified synthetic oviduct fluid medium (m-SOFM) [20] supplemented with 3 mg/ml bovine serum albumin (A-4378, Sigma) for 7 days (168 hr) at 39°C in 5% CO₂, 5% O₂ and 90% N₂ with high humidity (20–30 embryos/50 μ l droplet) [12]. Some embryos were fixed and stained 18 hr after insemination for assessment of fertilization [16]. Total fertilization included all penetrated

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Fig. 1. Time sequence of nuclear maturation of bovine oocytes after reversal of meiotic arrest by CHX. CHX-treated oocytes were incubated in a medium containing 10 $\mu\text{g}/\text{ml}$ CHX for 24 hr, then washed and cultured in maturation medium. Control oocytes were collected from ovaries 24 hr after the treated group and matured without inhibition. Points are the mean and bars indicate SEM of three replicates. Means with different letters are significantly different ($P < 0.05$) in the same treatments. The total number of oocytes within each treatment ranged from 52 to 58.

oocytes; normal fertilization represented only those oocytes with two pronuclei and one sperm tail. Polyspermic fertilization was also recorded as oocytes with more than two male pronuclei and/or decondensed sperm heads with their associated tails. The remaining embryos were examined with a stereomicroscope ($\times 60$) for cleavage and blastocyst development 7 days after insemination.

Each experiment was repeated three times. Percentage data obtained from each experiment were analyzed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test with Stat View software (Abacus Concepts, Stat View, version J-4.11, Abacus Concepts Inc., Berkeley CA, U.S.A.). The data were expressed as mean $\pm$ SEM.

In the first experiment, the time course of nuclear maturation of bovine oocytes treated with CHX for 24 hr was examined. CHX-treated oocytes were cultured for 12, 14, 16, 18, 20, 22 and 24 hr in the maturation medium. Oocytes were fixed and stained at each end point for

assessment of nuclear maturation. Oocytes collected from ovaries on the day that treated oocytes were moved to the maturation medium served as a control. The percentage of oocytes arrested at the germinal vesicle stage after 24 hr culture in medium with CHX was $98 \pm 4\%$. CHX effectively inhibited meiotic resumption in bovine oocytes. As shown in Fig. 1, at 12 hr following onset of maturation culture, very few oocytes in both groups completed nuclear maturation (2–5%). Maturation rates gradually increased as the culture interval increased, and reached a maximum rate at 16 hr in the CHX-treated group ($66 \pm 6\%$) and at 20 hr in the control group ($86 \pm 4\%$). The results indicate that the interval for nuclear maturation of bovine oocytes which were maintained in meiotic arrest with CHX is 4 hr shorter than that of oocytes just released from the follicles. The time sequence of GVBD in bovine, ovine, caprine and porcine oocytes after maintenance of meiotic arrest with protein synthesis inhibitors is two to three times as fast as in untreated oocytes [7–10, 21]. The interval required for nuclear maturation of CHX-inhibited oocytes may thus be shortened by the acceleration of the GVBD sequence.

In the second experiment, fertilizability and developmental capacity of CHX-treated oocytes matured for different intervals were examined. COCs were cultured for 16, 20 or 24 hr in the maturation medium. They were then fertilized *in vitro* and cultured to the blastocyst stage. Oocytes collected from ovaries on the day that treated oocytes were moved to the maturation medium served as a control. High penetration rates were obtained in nearly all oocytes in all groups ($P > 0.05$, Table 1). A higher rate of polyspermic fertilization was obtained with control oocytes, cultured for 16 hr in the maturation medium, which had not completed meiosis I at insemination, than for the other groups ($P < 0.05$). It was also noted by Niwa *et al.* [13] that bovine maturing oocytes could be fertilized *in vitro* but with high incidence of polyspermy. They suggested that there seemed to be no block to polyspermy in immature bovine oocytes [13]. On the other hand, CHX-treated oocytes were fertilized normally, even though they were cultured for only 16 hr (Table 1). Therefore, CHX-treated bovine oocytes which have completed meiosis I may become capable of

Table 1. *In vitro* maturation, fertilization and development of CHX-treated oocytes for 16 and 20 hr maturation culture after reversal^{a)}

Duration of Maturation (h)	Treatment	Maturation		Fertilization				Development		
		No. of oocytes	% ^{b)} matured	No. of oocytes	% ^{b)} fertilized			No. of embryos	% ^{b)} cleaved	% ^{b)} of blastocysts
					Total	Normal	Poly-spermic			
16	Control ^{c)}	38	19 $\pm$ 5 ^{e)}	42	87 $\pm$ 6	29 $\pm$ 5 ^{e)}	54 $\pm$ 14 ^{e)}	121	70 $\pm$ 6	16 $\pm$ 9 ^{e)}
16	CHX ^{d)}	36	73 $\pm$ 8 ^{f)}	35	86 $\pm$ 8	73 $\pm$ 4 ^{f)}	13 $\pm$ 4 ^{f)}	109	65 $\pm$ 1	11 $\pm$ 3 ^{e)}
20	Control ^{c)}	39	88 $\pm$ 5 ^{f)}	40	90 $\pm$ 6	69 $\pm$ 1 ^{f)}	21 $\pm$ 7 ^{f)}	117	79 $\pm$ 4	35 $\pm$ 6 ^{f)}
20	CHX ^{d)}	36	74 $\pm$ 4 ^{f)}	39	90 $\pm$ 6	64 $\pm$ 7 ^{f)}	26 $\pm$ 2 ^{f)}	119	73 $\pm$ 1	31 $\pm$ 8 ^{f)}

a) Data from three replicates.

b) Percentages are expressed as mean $\pm$ SEM.

c) Oocytes were collected from ovaries 24 hr after CHX treatment and matured without inhibition.

d) Oocytes were treated with CHX for 24 hr, then washed and matured.

e), f) Values differ significantly in the same columns ($P < 0.05$, ANOVA followed by Fisher's PLSD test).

Table 2. *In vitro* maturation, fertilization and development of CHX-treated oocytes for 20 and 24 hr maturation culture after reversal^{a)}

Duration of maturation (h)	Treatment	Maturation		Fertilization				Development		
		No. of oocytes	% ^{b)} matured	No. of oocytes	% ^{b)} fertilized			No. of embryos	% ^{b)} cleaved	% ^{b)} of blastocysts
					Total	Normal	Poly-spermic			
20	Control ^{c)}	40	77 ± 3	55	83 ± 5 ^{e)}	57 ± 9	32 ± 9	130	83 ± 2	32 ± 3
20	CHX ^{d)}	45	82 ± 2	54	98 ± 2 ^{f)}	73 ± 8	22 ± 10	160	78 ± 1	24 ± 9
24	Control ^{c)}	41	85 ± 3	55	98 ± 2 ^{f)}	68 ± 3	31 ± 3	134	85 ± 2	33 ± 10
24	CHX ^{d)}	40	82 ± 2	57	83 ± 4 ^{e)}	68 ± 2	15 ± 5	154	77 ± 5	26 ± 11

a) Data from three replicates.

b) Percentages are expressed as mean ± SEM.

c) Oocytes were collected from ovaries 24 hr after CHX treatment and matured without inhibition.

d) Oocytes were treated with CHX for 24 hr, then washed and matured.

e), f) Values differ significantly in the same columns (P<0.05, ANOVA followed by Fisher's PLSD test).

blocking polyspermy even in a short culture interval. Most embryos were cleaved similarly in all groups (P>0.05). In control groups, maturing oocytes cultured for 16 hr in the maturation medium, which were fertilized but with high incidence of polyspermy, consequently failed to develop into blastocysts (P<0.05). After reaching metaphase II, the control oocytes developed into blastocysts following *in vitro* fertilization. Although CHX-treated oocytes cultured for the shorter interval (16 hr) were successfully matured and fertilized, they failed to develop into blastocysts (P<0.05, Table 1). A longer interval (20 hr) in the maturation culture was necessary for the CHX-treated oocytes to obtain a similar rate of blastocyst development to that of the control oocytes. Since development of oocytes inseminated 4 hr after completion of meiosis I (20 hr) following maintenance of meiotic arrest with CHX was superior to those inseminated immediately after nuclear maturation (16 hr), an experiment was carried out to determine if 4 hr-extended culture of control oocytes in maturation medium after nuclear maturation enhances subsequent development. Table 2 indicates that the development into blastocysts of control oocytes cultured for 24 hr did not increase as compared to that for 20 hr (P>0.05). The present results confirm our previous observation [17] that CHX-treated oocytes were normally fertilized and developed to blastocysts, when they were cultured in maturation medium after reversal of meiotic arrest as long as control oocytes completed meiosis I (Tables 1 and 2). CHX-treated oocytes cultured for 16 hr were successfully fertilized but failed to develop into blastocysts, although the majority reached metaphase II when inseminated. Development of CHX-treated oocytes comparable to that of control oocytes was observed when inseminated 4 hr after nuclear maturation (20 hr, Table 1). Cytoplasmic maturation of mammalian oocytes originally described by Thibault *et al.* [22] involves the development of cytoplasmic signals essential for completing fertilization and directing early embryonic development. These signals appear to result from translation of maternal mRNA synthesized during oogenesis [3]. Eppig [4] recently hypothesized with mouse experiments that for

embryogenesis, immature oocytes must be competent to complete the 2-cell stage to blastocyst transition, and the factor (s) for this transition are synthesized in oocytes during maturation. If the hypothesis is acceptable for bovine oocytes, the interval (16 hr) for completion of nuclear maturation of CHX-treated oocytes may not be long enough for the synthesis of such transition factor (s) in oocytes. It is possible that the synthesis in CHX-treated oocytes may take at least a similar interval (20 hr) as that which allows control oocytes to complete nuclear maturation.

In conclusion, nuclear maturation of CHX-treated oocytes is accelerated and completed 4 hr faster than control oocytes, however the same interval in the maturation culture as control oocytes is necessary to complete meiosis I for subsequent development to blastocysts.

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