Effect of Meiotic Arrest by Cycloheximide on the In Vitro Maturation of Bovine Oocytes and Their Subsequent Development Following In Vitro Fertilization

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Abstract: *Cycloheximide (CHX) is a reversible inhibitor of bovine meiotic resumption. This study examined the timing of nuclear maturation in bovine oocytes treated with CHX and determined the optimum maturation interval in cultures for their subsequent development. CHX prevented the nuclear maturation of nearly all oocytes for 24 h. In an inhibitor-free medium, the majority of the CHX-treated oocytes underwent germinal vesicle breakdown (GVBD) after 6 h of culture, while the control oocytes did so at 10 h. In the maturation culture, the majority of the CHX-treated oocytes had reached metaphase II by 16 h whereas the control oocytes took 20 h. However, the CHX-treated oocytes that matured for 16 h developed into blastocysts at low rates while those that were matured for 20 h had a development rate similar to that of the control oocytes. These results indicate that the nuclear maturation of CHX-treated oocytes did accelerate, but these oocytes needed the same maturation time as required by the control oocytes for their subsequent development to blastocysts.*

Key words: *Meiotic arrest, Cycloheximide, Oocytes, Maturation, Bovine*

Introduction

Mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, also known as the germinal vesicle stage (GV). When oocytes are stimulated by the LH surge or are removed from Graafian follicles and cultured *in vitro* under suitable conditions, they spontaneously resume meiosis and progress to the metaphase II (MII) stage. Resumption of meiosis is regulated by a complex known as maturation promoting factor (MPF) which is formed by the association of cyclin-dependent kinases (Cdk1 or p34cdc2) and cyclin B [1]. For the resumption of meiosis, mouse oocytes do not require protein synthesis [2]. In contrast, porcine [3], sheep [4], goat [5], and bovine [6] oocytes require *de novo* protein synthesis for the resumption of meiosis.

Bovine oocytes do not undergo germinal vesicle breakdown (GVBD) in media with protein synthesis inhibitors such as cycloheximide (CHX) [7] and puromycin [8]. After the inhibition of GVBD by CHX, oocytes can undergo GVBD reversibly [7, 9, 10] and can mature and develop into blastocysts following *in vitro* fertilization [7, 9]. The time sequence of nuclear maturation of CHX-treated oocytes is faster than that of the control oocytes [3, 4, 7, 8]. However, few studies have reported the optimum maturation interval of bovine oocytes in culture with respect to the subsequent development after meiotic arrest by CHX.

In this study, we aimed to determine the time sequence of the nuclear maturation of bovine oocytes after meiotic arrest with CHX for 24 h and to determine the optimum maturation interval for their subsequent development to the blastocyst stage.

Materials and Methods

Oocyte collection

Bovine ovaries were collected from a local slaughterhouse and brought to the laboratory in 0.9% saline at 18–25° C. The ovaries were washed in 0.9%

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saline. Cumulus-oocyte complexes (COCs) were collected by aspiration from small follicles (2–6 mm in diameter) by using an 18-gauge needle connected to a 5-mL disposable syringe. The COCs were washed three times, each with Dulbecco's phosphate-buffered saline (D-PBS; Gibco Laboratories, Grand Island, NY, USA) supplemented with 3% heat-treated calf serum (CS; Gibco), 5 μg/mL of gentamicin sulfate (Sigma chemical, St Louis, MO, USA) and the subsequent culture medium. Only oocytes with an intact and compact cumulus cell were selected for the experiments.

Maintenance of meiotic arrest and in vitro maturation

Twenty COCs were transferred to a 100-μL droplet of TCM-199 (Gibco), supplemented with 5% CS, 10 μg/ml CHX (Sigma), and 5 μg/ml gentamicin sulfate, under paraffin oil in a 60-mm plastic dish (Falcon 1007; Becton Dickinson Labware, Lincoln Park, NJ, USA). These COCs were cultured for 24 h at 38.5° C in humidified air with 5% CO_2. After meiotic arrest, the COCs were washed three times with the maturation medium and cultured in TCM-199 supplemented with 5% CS and 0.02 mg/mL FSH (maturation medium; Antrin, Denka, Japan) in a plastic dish covered with paraffin oil (20 COC/100-μL droplet) for 0 to 20 h at 38.5° C in humidified air with 5% CO_2.

In vitro fertilization

In vitro fertilization was performed as described by Takahashi *et al*. [11]. Frozen semen from a single ejaculate of a Holstein bull was used (semen donated by National Livestock Breeding Center, Japan). The frozen sperms were thawed at 37° C for 30 sec and subsequently layered onto Percoll gradient (45% and 90%). A 90% Percoll stock solution was prepared by using a 9:1 mixture of Percoll (Pharmacia, Uppsala, Sweden) and 10-strength modified Brackett and Oliphant isotonic solution [12] without BSA but with 5 μg/ml gentamicin sulfate (mBO). The Percoll gradient was prepared in a 15-mL plastic centrifuge tube (Corning, N.Y., USA) containing 2 mL of 90% Percoll that was layered with 2 mL of 45% Percoll (1:1 mixture of 90% Percoll and single strength mBO). The gradient was centrifuged at 700 $\times$ g for 20 min. After removing the top layers, the sperm pellet was resuspended in mBO (approximately 6 mL) and washed again by centrifugation at 500 $\times$ g for 5 min. After maturation, the COCs were transferred to a 100-μL fertilization droplet (20 COCs per drop) that was prepared by adding a 95-μL aliquot of sperm suspension to 5 μL mBO containing 6 mg/mL fatty acid-free BSA (Sigma) to achieve a final concentration of 5×10^6 cells/mL. These cells were co-cultured for 18 h in the presence of sperms at 38.5° C in humidified air with 5% CO_2.

In vitro culture

Presumptive zygotes were stripped of the cumulus cells after co-incubation with sperms by vortexing in CR1aa medium [13] supplemented with 5% CS and 5 μg/mL gentamicin sulfate (mCR1). The zygotes were then washed three times with mCR1, and incubated in a 50-μL droplet (25 to 30 zygote per drop) covered with paraffin oil at 38.5° C in an atmosphere comprising 5% CO_2, 5% O_2, and 90% N_2.

Evaluation of nuclear status

At the end of the maturation period, the COCs were denuded mechanically, and all oocytes were mounted on slides, fixed with aceto-alcohol (1:3) solution for 48 h, stained with aceto-orcein, and observed under a phase-contrast microscope to determine their nuclear status.

Experimental design

In the first experiment, the CHX-treated and control COCs were cultured in the maturation medium for 0 to 20 h, and at the end of culture, they were fixed for light microscopic analysis of their nuclear status. In the second experiment, control and CHX-treated COCs that were cultured in the maturation medium for 16, 18, or 20 h were inseminated (Cont or CHX-16, -18, -20h). The cleavage and embryonic development to the blastocyst stage were examined under an inverted microscope at 3 days (72 h) and 9 days (216 h) after *in vitro* insemination.

Statistical analysis

Each experiment was repeated three times. All data were compared on the basis of Chi-square analysis or Fisher's exact probability test.

Results

Effect of pretreatment of CHX on time course of nuclear maturation

As shown in Fig. 1, all oocytes that were immediately removed from their follicles and the majority of oocytes treated with CHX for 24 h arrested at the GV stage. At 2 h after the onset of maturation culture, very few oocytes in both groups underwent complete GVBD. The GV rates gradually decreased as the culture interval

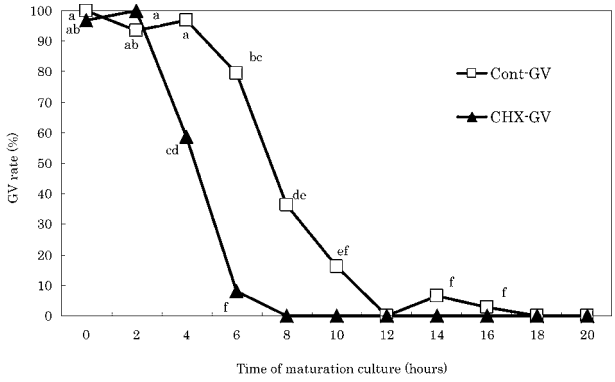

Fig. 1. Time sequence of the germinal vesicle (GV) rates of bovine oocytes after the reversal of meiotic arrest by CHX. CHX-treated oocytes were incubated for 24 h in a medium containing 10 μL/mL CHX and were then washed and cultured in the maturation medium. The control oocytes were collected and matured without inhibition. The points indicated here are the means of three experimental replicates. The means with different letters are significantly different (P<0.05).

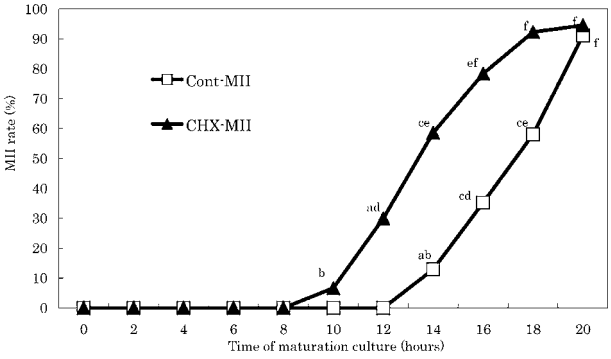

Fig. 2. Time sequence of the maturation (MII) rates of bovine oocytes after the reversal of meiotic arrest by CHX. CHX-treated oocytes were incubated for 24 h in a medium containing 10 μL/mL CHX and were then washed and cultured in the maturation medium. The control oocytes were collected and matured without inhibition. The points indicated here are the means of three experimental replicates. The means with different letters are significantly different (P<0.05).

Table 1. Effect of meiotic arrest by cycloheximide on the *in vitro* maturation of bovine oocytes and their subsequent development following *in vitro* fertilization[a]

Treatment	Duration of Maturation (h)	Maturation		Development		
		No. of oocytes	% matured	No. of embryos	% cleaved	% of blastocysts
Control[b]	16	34	12 (35.3)^{de}	93	34 (36.6)d	14 (15.1)^{de}
	18	31	18 (58.1)^{ef}	97	48 (49.5)^{de}	18 (18.6)^{df}
	20	34	31 (91.2)^{g}	104	60 (57.7)^{ef}	29 (27.9)^{f}
CHX[c]	16	37	29 (78.4)^{fg}	96	60 (62.5)^{ef}	8 (8.3)^{e}
	18	39	36 (92.3)^{g}	90	54 (60.0)^{ef}	13 (14.4)^{de}
	20	37	35 (94.6)^{g}	105	70 (66.7)^{f}	22 (21.0)^{df}

[a]Data from three replicates. [b]Oocytes were collected from ovaries matured without inhibition. [c]Oocytes were treated with CHX for 24 h, then washed and matured. [d, e, f, g]Different superscripts indicate significant differences in the same column (P<0.05).

increased and reached a minimum rate at 6 h in the CHX-treated (8.1%) and at 10 h in the control group (16.1%). On the other hand, the maturation (MII) rates gradually increased as the culture interval increased and reached a maximum rate at 16 h in the CHX-treated group (78.4%) and at 20 h in the control group (91.2%) (Fig. 2).

Effect of maturation time on embryonic development

Most embryos in the CHX-treated group cleaved in a similar manner, and the cleavage rates in the control groups gradually increased as the culture interval increased (Table 1). The cleavage rate in Cont-16h was significantly lower than those in Cont-20, CHX-16, -18 and -20h (P<0.05). The rate of blastocyst formation gradually increased as the culture interval increased in both the groups. Blastocyst formation in Cont-16, CHX-16 and -18h were significantly lower than that in Cont-20h (P<0.05), which was similar to that of CHX-20h.

Discussion

In this study, we examined the effect of meiotic arrest on the *in vitro* maturation of bovine oocytes using CHX and the subsequent development of these oocytes following *in vitro* fertilization.

In the first experiment, the time sequence of the nuclear maturation of bovine oocytes treated with CHX for 24 h was examined. The data of the present study shows that in the control oocytes, the GV rate

decreased at 6 h after the onset of maturation, but the MII rate increased at 14 h, reaching 91.2% at 20 h. In contrast, in the CHX-treated oocytes, the GV rate suddenly decreased at 4 h after the onset of maturation, but the MII rate increased at 10 h reaching 78.4% at 16 h. This rate was identical to the GV rate of control oocytes at 20 h ($P<0.05$). These results indicate that in bovine oocytes arrested by CHX, nuclear progression was shorter than in those just released from follicles. This acceleration in the nuclear progression after the removal of a protein synthesis inhibitor such as CHX or puromycin has been previously described in cattle [7, 8, 10], pigs [3], and goats [4]. Recently, an identical phenomenon was observed following the meiotic arrest caused by a cyclin-dependent kinase inhibitor such as butyrolactone-I (BL-I) [14, 15]. The reason for this acceleration in the nuclear maturation is not clear; however during the inhibition of protein synthesis or cyclin-dependent kinase, some change in the nucleus may progress into GVBD.

In this study, the cleavage rate of Cont-16h was lower than that observed in CHX-16h; this was different from the cleavage rate of embryos reported by Saeki *et al*. [7] who reported that following meiotic arrest by CHX for 24 h and maturation for 16 h the cleavage rate was high and similar to the control group. Niwa *et al*. [16] reported that bovine maturing oocytes could be fertilized *in vitro*; however, a high incidence of polyspermy was observed. In this study, the assessment of fertilization was not done. However, to the best of our knowledge, no studies have reported that polyspermy affects the cleavage of embryos. The reason for the low cleavage in Cont-16h in this study is unclear.

In this study, the MII rate in CHX-16h was similar to that of Cont-20h; however, the blastocyst rate in CHX-16h was lower than that in Cont-20h. This result was similar to that presented by Saeki *et al.* [7]. The reason for the low blastocyst rate in CHX-16h may be insufficient cytoplasmic maturation. Hashimoto *et al*. [14] have reported that the blastocyst rate in oocytes treated with BL-I and matured for 15.5 h was significantly higher than the rate in control oocytes that were matured for 21 h. Campos-Chillon *et al.* [17] have also reported that the blastocyst rate in oocytes treated with roscovitine (meiotic inhibitor) and matured for 16 h was similar to the rate of control oocytes that were matured for 22 h. In oocytes that were meiotically arrested using BL-I or ROS, both of which are specific cyclin-dependent kinase inhibitors [18–20], there is some possibility of accelerating both nuclear progression and cytoplasmic maturation. The relationship between cytoplasmic maturation and protein synthesis is not clear, however the results of the present study indicated that CHX may inhibit some factor dependent on the protein synthesis during cytoplasmic maturation. Furthermore, this factor is not accelerated after the removal of inhibition. Thus, the cytoplasm in CHX-16h had not completed maturation, and its blastocyst rate was reduced.

In conclusion, the nuclear maturation of CHX-treated oocytes is accelerated. However, CHX-treated oocytes require 20 h of maturation for subsequent development to the blastocyst; this is equivalent to the time required by control oocytes. Further studies are required to understand the relationship between cytoplasmic maturation and protein synthesis.

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