Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology

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The period of protein synthesis necessary for meiotic maturation in horse oocytes initially having compact or expanded cumulus cells was studied. Oocytes incubated in the presence of cycloheximide after 0, 4, 8, 12 or 16 h maturation *in vitro* (total incubation time 24 h) were matured for 24 h, or were incubated with cycloheximide for 24 h and then matured for 24 h. Incubation with cycloheximide from 0 h was effective in suppressing maturation (no significant increase in maturing oocytes compared with controls fixed directly after removal from the follicle) in both expanded and compact groups and was completely reversible, as there was no difference in the proportion of oocytes reaching metaphase II between controls and treatment groups of either cumulus type. Addition of cycloheximide after 4 h maturation resulted in no significant difference in distribution of oocytes compared with addition at 0 h in either cumulus group. A significant decrease in the proportion of germinal vesicle stage oocytes, and an increase in oocytes in metaphase I occurred in oocytes with expanded cumulus cells in the 8 h treatment and in oocytes with compact cumulus cells in the 12 h treatment, compared with oocytes treated after 0 h. A significant increase in the proportion of oocytes at metaphase II occurred in the 12 h treatment for expanded cumulus–oocyte complexes and in the 16 h treatment for compact cumulus–oocyte complexes. These data show that nuclear maturation of horse oocytes can be reversibly suppressed by incubation with cycloheximide from the onset of culture. Oocytes with different initial cumulus type differed in the time required for protein synthesis essential for maturation: expanded cumulus–oocyte complexes required less time to prepare for germinal vesicle breakdown, maturation to metaphase I, and maturation to metaphase II, than did compact cumulus–oocyte complexes.

Introduction

Fully grown oocytes of mammalian species are arrested in prophase I of meiosis until the preovulatory surge of LH, or release from the follicle *in vitro* (Pincus and Enzmann, 1935). Resumption of oocyte maturation involves both synthesis and phosphorylation of proteins. Cycloheximide, which inhibits peptidyl transferase, suppresses oocyte maturation by blocking synthesis of stage-specific proteins. In pigs, the pattern of proteins synthesized, as visualized by gel electrophoresis, changes significantly before germinal vesicle breakdown (GVBD; McGaughey and Van Blerkom, 1977). In cows, although no protein changes are seen in the protein synthesis pattern of oocytes before GVBD (Kastrop *et al.*, 1990), culture of oocytes in the presence of cycloheximide inhibits protein phosphorylation (Kastrop *et al.*, 1991). This led these authors to hypothesize that small amounts of enzymes involved in phosphorylation of existing proteins must be synthesized for GVBD to occur.

The period of protein synthesis essential for the resumption of the first meiotic division appears to be related to the period needed for GVBD and maturation. Mouse oocytes take 12–14 h to mature and GVBD occurs within 2 h (Donahue, 1968); treatment with cycloheximide from the onset of culture does not prevent GVBD. Bovine oocytes take 24 h to mature and GVBD occurs within 6–9 h (Sirard and First, 1988; Sirard *et al.*, 1989); treatment with cycloheximide up to 6 h after the onset of maturation prevents GVBD (Sirard, 1990; Kastrop *et al.* 1991; Tatemoto and Terada, 1995). Pig oocytes take 44 h to mature and GVBD occurs after approximately 16–20 h (Motlik and Fulka, 1976); GVBD occurred in 15, 46 and 75% of oocytes when cycloheximide was added after 6, 12 and 16 h of culture, respectively (Fulka *et al.*, 1986). Maturation to metaphase II requires synthesis of additional new proteins. In mice, while oocytes cultured in the presence of protein synthesis inhibitors undergo GVBD, they do not proceed beyond metaphase I (Stern *et al.*, 1972; Wassarman *et al.*, 1976; Schultz and Wassarman, 1977). In bovine oocytes, when cycloheximide is added after 9 h of maturation, essentially all oocytes undergo GVBD (Sirard *et al.*, 1989; Kastrop *et al.*,

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1991). However, maturation to metaphase II occurs only if cycloheximide is added after 12 h of culture. Little work has been done on meiotic arrest of horse oocytes. Hinrichs *et al.* (1995a) reported that follicular fluid alone did not suppress maturation, but follicle wall tissue (sheets of granulosa, follicle wall sections or intact follicle) did maintain oocytes in the germinal vesicle stage. Differences in viability (maturation to metaphase II) after suppression were found between oocytes originally having compact (CP) or expanded (EX) cumulus cells, with EX oocytes maturing in significantly higher proportions.

Previous studies have shown that although EX oocytes are recovered mainly from atretic follicles (Hinrichs, 1991), there is no difference in the proportion originally at the germinal vesicle stage when compared with CP oocytes (Hinrichs *et al.* 1993a), and there is no difference in the ability of EX and CP oocytes to mature to metaphase II (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993a). However, the time sequence of maturation is different for EX and CP oocytes. Significantly more EX than CP oocytes had undergone GVBD at 8 h and at 16 h of culture, and maximum maturation occurred at 24 h for EX oocytes but at 32 h for CP oocytes (Hinrichs *et al.*, 1993a).

This study was designed to investigate whether maturation of horse oocytes is suppressed by culture with cycloheximide, and the degree to which such suppression is reversible. In addition, we wished to define the period of protein synthesis necessary for meiotic maturation in horse oocytes, and to determine whether the timing of this critical period varies with different initial cumulus morphology.

Materials and Methods

Experimental design

Oocytes were assigned to one of eight groups: direct control (Dir), fixed directly upon removal from the follicle; maturation control (Mat), culture in maturation medium for 24 h; suppression groups, matured *in vitro* for 0, 4, 8, 12 or 16 h, and then transferred to cycloheximide-containing medium – total incubation time 24 h; or suppression followed by maturation (Cyc–Mat), culture in the presence of cycloheximide for 24 h and then transfer to maturation medium for a further 24 h. Each treatment was carried out for each cumulus type (CP and EX). Oocytes (12 of each cumulus type per replicate) were assigned to the eight treatments in randomized order. Each replicate of eight groups was randomized separately. If at the end of a collection day fewer than 12 oocytes were available for one treatment, the replicate was filled on the next collection day. The experiment was continued until there was a minimum of 30 analysable oocytes and three replicates in each treatment.

Methods

Horse ovaries were obtained from a local abattoir, placed in saline containing 0.1 mg ticarcillin ml⁻¹ (Smith Kline Beecham Pharmaceuticals, Philadelphia, PA), and transported (1 h) to the laboratory at room temperature. Oocytes were collected from all follicles of under 30 mm diameter. Oocytes were recovered by opening the follicles visible on the surface with a scalpel

blade and scraping the interior of follicles with a bone curette (Hinrichs *et al.*, 1993a). After all visible follicles were opened, the ovaries were cut into 5 mm sections to locate any follicles within the ovarian stroma. The scraped granulosa cells thus collected were washed into individual Petri dishes using modified M199 (M199 with Hank's salts and 25 mmol Hepes buffer l⁻¹ (Gibco Life Technologies, Inc., Grand Island, NY) and 0.1 mg ticarcillin ml⁻¹).

Cumulus–oocyte complexes were located using a dissecting microscope at a magnification of $\times 10$, and were classified as having compact (CP) or expanded (EX) cumulus cells (Fig. 1). The cumulus–oocyte complexes were dissected from attached mural granulosa and held at room temperature in modified M199 until incubation (maximum 2.5 h). Oocytes without cumulus or with only corona radiata were not used in the experiments.

For chromatin evaluation, cumulus cells were removed by pipetting in a 0.25% (v/v) solution of trypsin and 1 mmol l⁻¹ EDTA in Hank's salt solution without CaCl₂, MgCl₂ or MgSO₄ (Gibco Life Technologies, Inc.). The denuded oocytes were fixed for at least 24 h in buffered formol saline, and then placed on a slide and covered with 6 μ l mounting medium (3:1 glycerol:PBS) containing 2.5 μ g Hoechst 33258 ml⁻¹ (Hinrichs *et al.*, 1993b). The oocytes were examined using a fluorescence microscope with a G 365 nm exciter filter. The chromatin was classified as fluorescent nucleus (FN), diffuse fluorescence throughout the germinal vesicle; condensed chromatin (CC), the chromatin condensed in one mass (within the germinal vesicle, as shown by Hinrichs *et al.* (1993a)); crinkled fluorescent nucleus, the germinal vesicle was fluorescent but was misshapen, with a granular or crosshatched appearance; diakinesis (D), metaphase I, metaphase II or degenerating (chromatin spread throughout oocyte, abnormal chromatin or no chromatin visible) as described by Hinrichs *et al.* (1993b). Oocytes classified as FN or CC were considered to represent germinal vesicle stage oocytes (Hinrichs *et al.*, 1993a).

For culture, cumulus–oocyte complexes were incubated in 200 μ l drops of maturation medium (M199 with Earle's salts supplemented with 10% neonatal calf serum, 25 μ g gentamycin ml⁻¹, 1.25 μ g Fungizone ml⁻¹ (Gibco Life Technologies, Inc.) and 5 μ g FSH ml⁻¹ (Schering Corp. USA, Kenilworth, NJ) under paraffin oil. The culture was carried out at 38.2°C in a humidified atmosphere of 5% CO₂. Inhibition treatments consisted of incubation in 200 μ l drops of the maturation medium containing 10 μ g cycloheximide ml⁻¹ (Sigma Chemical Co., St Louis, MO).

Statistical analyses

Data were analysed using the chi-squared test with $P < 0.05$ considered statistically significant. Fisher's exact test was used in comparisons in which fewer than five values were expected in any cell.

Results

From the 140 ovaries that were processed, 651 oocytes were recovered from 915 follicles, a 71.1% oocyte recovery rate. Of the collected oocytes, 330 (50.7%) had a compact (CP)

Fig. 1. Horse oocytes at the time of recovery. (a) Compact cumulus; (b) expanded cumulus. Scale bar represents 200 μ m.

cumulus, 307 (47.2%) had an expanded (EX) cumulus, and 14 (2.2%) had no cumulus, corona radiata only, or had a visibly degenerated or damaged ooplasm. Only CP and EX oocytes ($n = 288$ for each cumulus type) were used in the study.

On evaluation of oocyte chromatin, we changed the classification system slightly from that used by Hinrichs *et al.* (1993a, b). Because the proportion of germinal vesicle stage oocytes that had the crinkled fluorescent nucleus configuration increased markedly over incubation, from 3/50 for (combined EX and CP) directly fixed oocytes to 4/5 for matured oocytes

and 4/4 for oocytes matured after suppression, we postulated that crinkled fluorescent nuclei are associated with abnormal or nonviable oocytes and placed this chromatin configuration in the degenerating category rather than with oocytes with fluorescent nuclei (germinal vesicle stage).

The distribution of oocytes among chromatin classifications in the different treatment groups is presented (Table 1). In direct controls, the proportion of oocytes in the germinal vesicle stage was not significantly different between EX and CP oocytes (66% versus 67%; $P > 0.1$). The proportion of germinal

Table 1. Percentage of horse oocytes in the different chromatin categories according to treatment and cumulus type

Treatment	n	Fluorescent nucleus	Condensed chromatin	Diakinesis	Metaphase I	Metaphase II	Crinkled fluorescent nucleus	Degenerating
CP Direct	36	25.0	41.7	2.8	0	0	2.8	27.7
EX Direct	35	5.7	60.0	2.8	2.8	0	5.7	22.9
CP 0 h	36	0	33.3	5.5	0	0	8.3	52.8
EX 0 h	34	0	58.8	2.9	11.8	2.9	2.9	20.6
CP 4 h	36	0	36.1	5.6	5.5	0	0	52.8
EX 4 h	36	0	41.7	2.8	25.0	0	8.3	22.2
CP 8 h	36	0	16.7	2.8	11.1	0	5.5	63.9
EX 8 h	35	0	17.1	5.7	65.7	5.7	2.9	2.9
CP 12 h	35	0	2.9	2.9	17.1	5.7	2.9	68.5
EX 12 h	32	0	12.5	3.1	21.9	25.0	12.5	25.0
CP 16 h	34	0	0	5.9	2.9	17.6	5.9	67.6
EX 16 h	33	0	6.1	0	12.1	51.5	9.1	21.2
CP Mat	34	0	2.9	0	5.9	20.6	2.9	64.7
EX Mat	32	0	0	3.1	15.6	62.5	6.3	12.5
CP Cyc-Mat	35	0	0	0	0	31.4	8.6	60.0
EX Cyc-Mat	35	0	0	0	14.3	62.8	2.9	20.0

CP: compact cumulus; EX: expanded cumulus. h: hours of maturation before treatment with cycloheximide (total culture time 24 h); Mat: maturation control (24 h culture); Cyc-Mat: treatment with cycloheximide for 24 h followed by maturation for 24 h; Direct: fixed directly upon removal from the follicle.

vesicle stage oocytes in the fluorescent nucleus configuration was significantly higher for CP than for EX oocytes (38% versus 9%; $P < 0.05$). There was no difference between EX and CP oocytes in the proportion of degenerating oocytes (29% versus 31%; $P > 0.1$).

Ability of cycloheximide to inhibit nuclear maturation reversibly

Cycloheximide was effective in suppressing maturation in both EX and CP oocytes, as there was no significant increase in maturing oocytes (diakinesis, MI and MII combined) between direct controls and 0 h suppression for either cumulus type ($P > 0.1$). There was no significant change in the proportion of germinal vesicle stage oocytes (combined fluorescent nucleus and condensed chromatin configurations) between direct controls and 0 h suppression for EX oocytes ($P > 0.1$). In the CP group, the proportion of oocytes in the fluorescent nucleus chromatin configuration decreased significantly between direct controls and 0 h suppression treatment (25% versus 0%; $P > 0.01$), whereas the proportion of oocytes in the condensed chromatin configuration did not (42% versus 33%; $P > 0.1$). Concomitant with the loss of fluorescent nucleus oocytes in the CP group after suppression, there was a significant increase in the proportion of degenerating oocytes ($P < 0.05$). The proportion of degenerating oocytes was significantly higher in the CP than in the EX group in the 0 h treatment ($P < 0.01$). In the EX group, there were no oocytes in the fluorescent nucleus configuration after 0 h suppression but this was not significantly different from the direct control (6% versus 0%; $P > 0.1$). The proportion of oocytes in the condensed chromatin stage was significantly higher in EX than in CP oocytes in the 0 h suppression group ($P < 0.05$).

The suppression of maturation by cycloheximide in both EX and CP oocytes was completely reversible, as there was no difference between the proportion of oocytes reaching metaphase II in maturation controls and in suppression–maturation treatments (CP, 21% versus 31%; EX, 63% versus 63% respectively; $P > 0.1$). In the maturation controls, a significantly greater proportion of EX than CP oocytes matured when only MII were compared ($P < 0.01$) or when MI and MII combined were compared ($P < 0.01$). In the suppression–maturation treatment, a significantly greater proportion of EX than CP oocytes matured when comparing only MII ($P < 0.01$) or when comparing MI and MII combined ($P = 0.01$). In EX oocytes, there tended to be more abnormal metaphase configurations (chromosomes displaced from the metaphase plate or appearing to decondense) in the suppression–maturation treatment than in the maturation controls (4/27 versus 0/25; $P = 0.07$). There were no abnormal configurations in metaphase CP oocytes in either the maturation or suppression–maturation treatments.

Effect of addition of cycloheximide after 0, 4, 8, 12 or 16 h maturation

For both CP and EX oocytes, there was no significant difference in the distribution of oocytes when cycloheximide was added after 4 h compared with at 0 h ($P > 0.1$). For the EX group, addition of cycloheximide at 8 h was associated with a significant decrease in the proportion of germinal vesicle stage oocytes ($P < 0.01$) and a significant increase in the proportion of oocytes in MI ($P < 0.01$) compared with at 0 h. At 12 h and 16 h, there was a significant increase in MII oocytes compared with at 0 h ($P < 0.05$ and $P < 0.01$, respectively) and the

proportion of oocytes at MII tended to increase between 12 h and 16 h ($P = 0.052$).

For CP oocytes, there was no significant change in the distribution until 12 h, when the proportion of oocytes in the germinal vesicle stage decreased ($P < 0.01$) and the proportion in MI increased ($P < 0.05$) compared with the distribution at 0 h. A significant increase in MII oocytes occurred at 16 h compared with at 0 h ($P < 0.05$).

Discussion

The results of this study demonstrate that treatment with cycloheximide from the onset of culture inhibits the maturation of horse oocytes *in vitro*, and that this inhibition is reversible. An interesting feature of this study was the differential effect of cycloheximide treatment on different chromatin configurations in oocytes at the germinal vesicle stage. Horse oocytes possessing a germinal vesicle can have either diffuse fluorescence throughout the nucleus (the fluorescent nucleus configuration) or have chromatin condensed in only one area of the nucleus (condensed chromatin configuration; Hinrichs *et al.*, 1993a). As seen in this study, the fluorescent nucleus configuration is more common in CP than in EX oocytes (38% versus 9% of direct GV oocytes, respectively); this has been shown previously (31% versus 12% of direct GV oocytes, respectively; Hinrichs *et al.*, 1993a). In the present study, the proportion of oocytes in the fluorescent nucleus configuration decreased from 25% in CP oocytes fixed directly upon removal from the follicle to 0% in CP oocytes after 24 h incubation with cycloheximide; however, the proportion of CP oocytes in the condensed chromatin configuration did not change. Despite the loss of the fluorescent nucleus oocytes after suppression, there was no difference in the proportion of oocytes reaching MII between CP maturation controls and those maturing after suppression. This suggests that treatment with cycloheximide is associated with degeneration of oocytes in the fluorescent nucleus configuration, but not of oocytes in the condensed chromatin configuration. These data also suggest that oocytes in the fluorescent nucleus configuration do not contribute to the population of CP oocytes that reaches MII during maturation *in vitro*.

The superior performance of EX horse oocytes during maturation *in vitro* and related manipulations has been shown previously; EX oocytes matured to MII in equal (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993a) or greater (Hinrichs *et al.*, 1995a, b) proportions maintained viability better after suppression of maturation with follicular components (Hinrichs *et al.*, 1995a) and were activated at higher rates after maturation *in vitro* (Hinrichs *et al.*, 1995b) than were CP oocytes. This is in spite of the fact that EX oocytes are recovered from atretic follicles (Hinrichs, 1991; K. Hinrichs, unpublished).

EX horse oocytes mature more rapidly than do CP oocytes, reaching maximum proportions at MII by 24 h (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993a), whereas the proportion of CP oocytes reaching metaphase II increases between 24 h and 30–32 h (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993a; Alm and Torner, 1994). Correspondingly, in the present study, we found that EX oocytes required a shorter period for protein synthesis before becoming competent to undergo GVBD than did CP

oocytes (maturation to MI: 8 h in EX versus 12 h in CP; and maturation to MII: 12 h in EX versus 16 h in CP).

The requirement for protein synthesis in relation to morphological stages of nuclear maturation for horse oocytes in this study appears to differ from that reported for bovine oocytes, in that addition of cycloheximide at 8 h to EX oocytes was associated with development of oocytes to MI; however, GVBD has not occurred at this time (Hinrichs *et al.*, 1993a). In cattle, development to MI does not result unless oocytes have matured in inhibitor-free media to, or past, the time GVBD would normally occur (6.5 h; Sirard *et al.*, 1989; Kastrop *et al.*, 1991; Tatemoto and Terada, 1995). Similarly, in goats, GVBD occurs between 2 h and 6 h of maturation, yet addition of cycloheximide at 6 h allows chromatin condensation only, without progression to MI (Pawshe *et al.*, 1994). Our results for horses more closely parallel those for sheep, in which GVBD occurs at about 8 h, and yet 50% of oocytes undergo GVBD and progression to MII when cycloheximide is added after 7 h of maturation (Moor and Crosby, 1986).

However, in horses as in other species, it appears that newly synthesized proteins are necessary for progression from MI to MII. These must include proteins necessary for the completion of meiosis I (extrusion of the polar body) as well as resumption of meiosis II. Formation of the MI plate is associated with high activity of MPF (histone H1 kinase), as reported in pigs (Naito and Toyoda, 1991). Histone H1 kinase activity decreases during the MI to MII transition in pigs. The proteins needed for progression from MI may therefore be related to deactivation of MPF (dephosphorylation of P34cdc2 or proteolysis of cyclin B), or of cytosolic factor (CSF). CSF, which is a gamete-specific protein probably similar to the product of the gene, *c-mos*, stabilizes the cyclin-B component of MPF (O'Keefe *et al.*, 1991). In mice, the *c-mos* product is required for resumption of meiosis II; however, oocytes injected with *c-mos* anti-sense oligonucleotides which block synthesis of the *c-mos* protein, do not remain in MI but extrude the polar body and form a pronucleus (O'Keefe *et al.*, 1989). Arrest of oocytes at MI in the presence of cycloheximide suggests that MPF and CSF concentrations remain high when protein synthesis is inhibited.

In cattle and goat oocytes, addition of cycloheximide after maturation for 12 h or more is associated with abnormal metaphase configurations and pronucleus-like structures (Sirard *et al.*, 1989; Pawshe *et al.*, 1994). In cattle, 46% and 60% of oocytes form pronuclear structures when cycloheximide is added after 12 h or 16 h of maturation, respectively (Sirard *et al.*, 1989). Similarly, in mice, addition of cycloheximide after MI leads to extrusion of a polar body and formation of a nucleus (Clarke and Masui, 1983). This led these authors to the conclusion that maintenance of MII requires protein synthesis. In our study, however, in the 12 h and 16 h cycloheximide treatments combined, only 1/69 CP oocytes and 3/65 EX oocytes formed pronuclear-type structures. This may relate to the refractoriness of matured horse oocytes to activation: ethanol, ethanol plus cycloheximide, or calcium ionophore alone did not significantly activate horse oocytes; however, treatment with calcium ionophore plus cycloheximide was effective in doing so (Hinrichs *et al.*, 1995b).

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