Activation of cumulus-free equine oocytes: effect of maturation medium, calcium ionophore concentration and duration of cycloheximide exposure

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Two different culture media (TCM-199 and follicular fluid), two activation treatments (10 and 50 μmol calcium ionophore I^{-1}) and three culture periods with cycloheximide were evaluated to find effective culture conditions for activation of cumulus-free equine oocytes. Oocytes were collected by scraping the follicle walls of ovaries obtained from an abattoir. Oocytes with expanded cumuli were matured at 38.2°C in a humidified atmosphere of 5% CO_2 in air, in either TCM-199 with 10% fetal bovine serum (FBS) and 5 μU FSH ml^{-1} , or in 100% follicular fluid derived from a preovulatory follicle 24 h after injection of hCG. After 40–42 h of *in vitro* maturation, oocytes were denuded by gentle pipetting in TCM-199 plus 10% FBS with hyaluronidase. Oocytes with intact cytoplasmic membranes ($n = 398$; 94% presumed metaphase II) were treated in protein-free PBS with 10 or 50 μmol calcium ionophore I^{-1} for 5 min. After washing, the oocytes were cultured in TCM-199 containing

10% FBS and 10 μg cycloheximide ml^{-1} for 6 h, in cycloheximide for 6 h and then in cycloheximide-free medium for 18 h, or in cycloheximide for 24 h. The oocytes were fixed and evaluated by fluorescence microscopy. Oocytes with pronucleus I–II (dense to decondensing chromatin), pronucleus III–IV (decondensed chromatin) or progressing towards the first cleavage division were considered activated. The activation rate for oocytes matured in TCM-199 was significantly ($P < 0.05$) higher than for oocytes matured in follicular fluid (49% (99/204) versus 35% (60/171), respectively; $P < 0.05$). Culture with cycloheximide for 24 h resulted in a significantly higher rate of activation (67%, 74/111) than did the 6 h (33%, 44/136) or 6 h plus 18 h (32%, 41/128) treatments. The highest rate of activation (82%) was observed in oocytes matured in TCM-199, treated with 50 μmol calcium ionophore I^{-1} and cultured with cycloheximide for 24 h.

Introduction

Offspring cloned from somatic cells have been reported in several species, including sheep (Wilmut *et al.*, 1997), mice (Wakayama *et al.*, 1998), cattle (Kato *et al.*, 1998), goats (Baguisi *et al.*, 1999) and pigs (Onishi *et al.*, 2000; Polejaeva *et al.*, 2000). However, little is known about methods for nuclear transfer in horses. In contrast to other species, there are no reports on equine nuclear transfer using embryonic blastomeres, due to the difficulty of recovering early embryos *in vivo* and the failure of IVF to be successful reproducibly in this species. Somatic cell nuclear transfer has been investigated in horses only recently (Hinrichs *et al.*, 2000; Li *et al.*, 2000a; Reggio *et al.*, 2000; Choi *et al.*, 2001). Hinrichs *et al.* (2000) found that the activation rate of equine oocytes was low (3/28, 11%,

estimated by cleavage rates) compared with that for bovine oocytes (49/93, 53%) after transfer of equine somatic cells. Cleavage rates after nuclear transfer in equine oocytes were also low in two other studies (0/24: Reggio *et al.*, 2000; and 1/7 (14%): Li *et al.*, 2000a).

Activation of host cytoplasts is a crucial step in the production of embryos by nuclear transfer (Mitalipov *et al.*, 1999; Polejaeva *et al.*, 2000). Two studies have compared methods for activation of equine oocytes (Hinrichs *et al.*, 1995a; Li *et al.*, 2000b). Hinrichs *et al.* (1995a) treated cumulus-enclosed oocytes with 5–7% (v/v) ethanol or 5–10 μmol calcium ionophore A23187 I^{-1} in combination with cycloheximide, and obtained an estimated 11–70% activation rate per metaphase II oocyte. Activation rates for oocytes that originally had expanded cumuli were significantly higher than the activation rates of oocytes that originally had compact cumuli. Li *et al.* (2000b) obtained 56–79% activation rates (resumption of meiosis or decondensation of the sperm head) after intracytoplasmic sperm injection (ICSI) and treatment of oocytes with

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10 μmol ionomycin l^{-1} , 7% (v/v) ethanol, 100 μmol thimerosal l^{-1} or injections of 250 μmol inositol 1,4,5-trisphosphate l^{-1} . The highest rate of female pronucleus formation/first mitosis in that study was 56%. Activation rates after ICSI, estimated by cleavage or pronuclear formation, were similar after treatment with 10 μmol calcium ionophore l^{-1} for 5 min (27–48%: Kato *et al.*, 1997; Schmid *et al.*, 2000) or with no activation treatment (54–63%: Dell'Aquila *et al.*, 1999). In pig oocytes, increases in concentration of calcium ionophore from 10 to 50 μmol l^{-1} were associated with increased rates of nuclear activation (Wang *et al.*, 1998); however, no work has been done with concentrations of calcium ionophore > 10 μmol l^{-1} in equine oocytes.

Mature oocytes are arrested in metaphase II in association with a high activity p34^{cdc2}–cyclin B complex (maturation-promoting factor; MPF). MPF activity is maintained during metaphase II arrest by the activity of cytotstatic factor, which prevents the degradation of the cyclin B component (O'Keefe *et al.*, 1991). Cytostatic factor activity is dependent on the activity of the c-mos protein, its substrate mitogen-activation protein (MAP) kinase kinase and its substrate, MAP kinase (O'Keefe *et al.*, 1991; Cross and Smythe, 1998). Oocyte activation, marked by completion of meiosis and formation of the female pronucleus, is induced by multiple oscillations of intracellular free calcium (Ca^{2+}) during fertilization. The increase in Ca^{2+} induces inactivation of MPF and subsequently of cytotstatic factor. Calcium ionophore is also able to induce an increase in cytoplasmic Ca^{2+} concentration and, thus, may serve to activate oocytes parthenogenetically. However, if the oocyte is still actively synthesizing protein, it appears to replenish the activity of cytotstatic factor, which causes a recrudescence of MPF activity, thus holding oocytes at 'metaphase III' (Liu and Yang, 1999). Treatment with cycloheximide, which blocks synthesis of proteins, can prevent MPF reactivation after an initial calcium influx (Presicce and Yang, 1994; Yang *et al.*, 1994; Moos *et al.*, 1996). When cycloheximide is used in combination with activating stimuli such as ethanol, calcium ionophore or an electric pulse, synergistic effects on activation have been reported in bovine (Shi *et al.*, 1993; Presicce and Yang, 1994; Yang *et al.*, 1994; Liu *et al.*, 1998), pig (Jilek *et al.*, 2000) and equine (Hinrichs *et al.*, 1995a) oocytes.

Follicular fluid has been added to maturation media to support cytoplasmic maturation, as assessed by pronucleus formation and embryonic development after IVF, in pigs (Naito *et al.*, 1988; Yoshida *et al.*, 1992; Vatzias and Hagen, 1999), sheep (Sun *et al.*, 1994) and cattle (Kim *et al.*, 1996; Romero-Arredondo and Seidel, 1996). In horses, Dell'Aquila *et al.* (1997) reported a positive effect of 20% (v/v) follicular fluid during maturation on rates of pronucleus formation after ICSI of oocytes. Culture of equine oocytes in 100% follicular fluid yielded higher rates of fertilization after IVF than did culture in medium with added serum or added follicular fluid (K. Hinrichs, D. D. Varner and C. C. Love, unpublished). The present study was

conducted to examine the effects of maturation medium, calcium ionophore concentration and duration of cycloheximide culture on activation of cumulus-free equine oocytes.

Materials and Methods

Preparation of follicular fluid

Mares in oestrus with a preovulatory follicle > 33 mm in diameter were treated with hCG (2000 iu, i.v.). After 24 h, follicular fluid was aspirated from the follicle by flank puncture (Vogelsang *et al.*, 1988). The granulosa cells recovered were examined under a dissection microscope and only fluid from follicles yielding expanded granulosa cells, indicative of response to hCG (Hinrichs *et al.*, 1998), was used for the experiment. The follicular fluid was centrifuged immediately at 2800 *g* for 10 min at room temperature, decanted, frozen in aliquots and kept at -20°C until used.

Oocyte collection

Ovaries were transported in PBS from local abattoirs to the laboratory (3–4 h transport time). The ovaries were trimmed with scissors and cleaned with sterilized gauze. All visible follicles were opened with a scalpel blade and the granulosa layer of each follicle was scraped using a 0.5 cm bone curette. The contents of the curette were washed into individual Petri dishes with Hepes-buffered TCM-199 with Hank's salts (Gibco Life Technologies Inc, Grand Island, NY) plus ticarcillin (0.1 mg ml^{-1} ; SmithKline Beecham Pharmaceuticals, Philadelphia, PA). The contents of the Petri dishes were examined using a dissection microscope at $\times 10$ –20 magnification. Cumulus–oocyte complexes were classified as compact, expanded or degenerating depending on the expansion of both mural granulosa and cumulus as described previously (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000). Oocytes with any sign of expansion of either the cumulus or the mural granulosa (from having individual cells visible protruding from the surface to having full expansion with copious matrix visible between cells) were classified as expanded. Oocytes with both compact cumulus and compact mural granulosa were classified as compact. Only expanded oocytes were used for activation treatments.

In vitro maturation

Selected oocytes were washed twice in either maturation medium (TCM-199 with Earle's salts (Gibco), 5 μU FSH ml^{-1} (Schering, Kenilworth, NJ), 10% fetal bovine serum (FBS) and 25 μg gentamycin ml^{-1}) or in 100% follicular fluid. Oocytes were cultured in droplets of the corresponding medium or fluid (10 μl per oocyte) under light white mineral oil (Sigma Chemical Co, St Louis, MO) at 38.2°C in 5% CO_2 in air for 40–42 h.

Activation treatment

After culture, oocytes were denuded of cumulus cells by pipetting in TCM-199 with Hank's salts and 25 mmol Hepes l^{-1} (Gibco) with 10% FBS, mixed 50:50 with 0.1% (w/v) hyaluronidase (Sigma) in Hank's salt solution (Gibco). Oocytes with an intact cytoplasmic membrane were selected and put into TCM-199 with Hank's salts and 25 mmol Hepes l^{-1} supplemented with 20% FBS. The oocytes were washed twice in modified PBS without protein and treated with 10 or 50 μmol calcium ionophore A 23187 l^{-1} (Sigma) in PBS at 37°C for 5 min. The stock solution of calcium ionophore was 10 mmol l^{-1} in dimethylsulphoxide and was kept at -20°C until used. After calcium ionophore treatment, the oocytes were washed in TCM-199 containing 20% FBS and assigned to one of three treatments: (i) culture in TCM-199 with Earle's salts, containing 10% FBS and 10 μg cycloheximide ml^{-1} (Sigma), for 6 h followed by fixation and staining; (ii) similar culture in cycloheximide-containing medium for 24 h; or (iii) culture in the cycloheximide-containing medium for 6 h followed by washing and culture in the same medium but without cycloheximide for 18 h (6 h plus 18 h).

Concurrent with the above experiment, equine oocytes were matured for 42 h in follicular fluid, and then cultured for an additional 24 h in the presence of supernatant from a sperm preparation to evaluate the rate of spontaneous activation.

Selection for membrane integrity

One hundred and sixty-three oocytes with compact cumuli were cultured in maturation medium for 24 h to determine the efficiency of selecting metaphase II oocytes on the basis of membrane integrity after maturation. After denuding the cumulus, oocytes were selected either for membrane integrity (possession of a clearly defined, smooth membrane around the ooplasm) or for the presence of an apparent polar body.

Evaluation of oocytes

For evaluation, oocytes were fixed briefly in buffered formal saline and placed on a glass slide with 6.5 μl mounting medium (3:1 glycerol:PBS containing 2.5 μg Hoechst 33258 ml^{-1}). The sample was examined using an epifluorescence microscope with a 365 nm excitation filter. Oocytes were classified as described in cows (Presicce and Yang, 1994) and horses (Li *et al.*, 2000b) as metaphase, pronucleus I-II (dense to decondensing chromatin), pronucleus III-IV (decondensed chromatin) or as progressing toward the first cleavage division as marked by bivalents condensed within the pronuclear area (Fig. 1). Other chromatin configurations (dispersed chromatin, abnormal chromatin or no chromatin) were considered as degenerated. Oocytes in metaphase were grouped together regardless of the number of polar bodies observed, as the ability to visualize the equine polar body in metaphase II oocytes

Fig. 1. Activation of equine oocytes with calcium ionophore and cycloheximide. (a) Pronucleus (pronuclear I; dense chromatin, arrow) with two polar bodies. (b) Pronucleus (pronuclear IV) with polar body. (c) Progression toward the first mitotic division (condensation of bivalents) with polar body. Scale bar represents 50 μm .

Table 1. Chromatin configuration of equine oocytes after activation treatment

Maturation medium	Follicular fluid						TCM-199					
	10			50			10			50		
	6 ^a	6 + 18 ^b	24 ^c	6 ^a	6 + 18 ^b	24 ^c	6 ^a	6 + 18 ^b	24 ^c	6 ^a	6 + 18 ^b	24 ^c
Treated (<i>n</i>)	34	35	27	31	31	26	40	35	32	39	34	34
Degenerated (<i>n</i>)	6	1	2	0	2	2	1	1	3	1	3	1
Basis (<i>n</i>)	28	34	25	31	29	24	39	34	29	38	31	33
Nuclear stage												
Pronuclear I–II (%)	6 (21)	0 (0)	3 (12)	3 (10)	1 (3)	3 (13)	1 (3)	0 (0)	0 (0)	5 (13)	0 (0)	2 (6)
Pronuclear III–IV (%)	2 (7)	14 (41)	5 (20)	2 (6)	4 (14)	7 (29)	3 (8)	3 (9)	9 (31)	16 (42)	10 (32)	17 (52)
First mitosis (%)	1 (4)	0 (0)	7 (28)	0 (0)	0 (0)	2 (8)	3 (8)	3 (9)	11 (38)	2 (5)	6 (19)	8 (24)
Total activated (%)	9 (32)	14 (41)	15 (60)	5 (16)	5 (17)	12 (50)	7 (18)	6 (18)	20 (69)	23 (61)	16 (52)	27 (82)
Metaphase (%)	19 (68)	20 (59)	10 (40)	26 (84)	24 (83)	12 (50)	32 (82)	28 (82)	9 (31)	15 (39)	15 (48)	6 (18)

Calcium ionophore: A23187; treated: number of oocytes treated; degenerated: oocytes with degenerating chromatin at the time of staining; basis: oocytes that were not degenerated at the time of staining (presumed metaphase II at the time of activation).
^aCulture in cycloheximide-containing medium for 6 h.
^bCulture with cycloheximide for 6 h then 18 h further culture with no cycloheximide.
^cCulture with cycloheximide for 24 h.
Pronuclear I–II: dense to decondensing chromatin; pronuclear III–IV: decondensed chromatin (pronucleus).

decreases as the duration of culture increases (Gable and Woods, 1999).

Statistical analysis

Each activation treatment was replicated three times. The two media and two calcium ionophore treatments were repeated on every collection day; however, only one culture treatment was performed on a given day. The proportions of oocytes with intact cytoplasmic membranes after maturation were compared using chi-squared analysis. Activation rates were determined on the basis of activated oocytes plus non-activated (metaphase) oocytes; oocytes with degenerating chromatin were disregarded.

A logistic regression model was used to analyse the main effects of media, calcium ionophore treatment and culture treatment, in addition to the three two-way interactions and the three-way interaction (Liang and Zeger, 1986). The model used generalized estimating equations to account for any within-day correlation that may have been attributable to factors not included in the model. Statistical testing was performed using the likelihood ratio chi-squared test. The level of significance was *P* < 0.05.

Results

Seventy-nine oocytes with compact cumuli were cultured, and 23 oocytes were selected as having an intact, smooth cytoplasmic membrane. Twenty-one (91%) of the selected oocytes were found to be in metaphase II upon staining and evaluation. Two of 56 rejected oocytes (4%) were found to be in metaphase II. An additional 84 oocytes with compact cumuli were cultured, and 26 oocytes were selected for the presence of a polar body. Twenty-three (89%) of these oocytes were found to be in metaphase II upon staining and

evaluation. Two of 58 oocytes in which no polar body was visible (4%) were found to be in metaphase II after staining.

For the activation study, 1858 follicles were scraped and 1065 oocytes were collected, for a recovery rate of 57%. Of the cumulus– oocyte complexes collected, 672 were expanded, 344 were compact and 49 were degenerating at the time of recovery. For this experiment, 621 expanded oocytes were used. The percentages of oocytes with an intact cytoplasmic membrane after maturation were 76% (241/317) in TCM-199 and 70% (213/304) in follicular fluid. This difference was not significant. After activation, one FSH replicate (27 oocytes) was discarded due to contamination and one follicular fluid replicate (20 oocytes) was discarded because of a break in protocol (use of the wrong batch of follicular fluid).

The percentages of oocytes in the different chromatin stages after activation treatment are shown (Table 1). The proportion of activation-treated oocytes categorized as degenerating after staining was not significantly different between maturation media (10/214 (5%) for TCM-199 and 13/184 (7%) for follicular fluid). Activation rates are expressed as a proportion of the oocytes presumed to be in metaphase II at the time of treatment (disregarding degenerating oocytes). The activation rate for oocytes matured in TCM-199 was significantly higher than that for oocytes matured in follicular fluid (99/204 (49%) versus 60/171 (35%), respectively; *P* < 0.05). Culture with cycloheximide for 24 h resulted in a significantly higher rate of activation (67%; 74/111) than did the 6 h (33%; 44/136) or 6 h plus 18 h (32%; 41/128) treatments. There was no overall difference in activation rates between 10 μmol l^{−1} (38%; 71/189) and 50 μmol l^{−1} (47%; 88/186) calcium ionophore treatments, but there was a significant interaction between maturation medium and calcium ionophore treatment (*P* < 0.01). TCM-199-matured oocytes exposed to

cycloheximide for 6 h (the 6 h and 6 h plus 18 h groups) had significantly higher activation rates when treated with 50 μmol calcium ionophore I^{-1} than when treated with 10 μmol calcium ionophore I^{-1} ($P < 0.001$). The highest activation rate (82%) was observed in oocytes matured in TCM-199 and activated with 50 μmol calcium ionophore I^{-1} followed by cycloheximide treatment for 24 h. This activation rate tended to be higher than that for TCM-199 maturation, 10 μmol calcium ionophore I^{-1} , and 24 h cycloheximide treatment ($P = 0.059$), and was significantly higher than that for all other treatments.

One of 71 oocytes cultured for 42 h in follicular fluid and then an additional 24 h after addition of sperm suspension supernatant (1%) had a pronucleus when evaluated after fixation and staining.

Discussion

The medium used during *in vitro* maturation is a key factor in preparation of oocytes for subsequent embryo development (Mermillod *et al.*, 1999; Kruip *et al.*, 2000). Follicular fluid has been used in two different ways to promote cytoplasmic maturation of oocytes: to suppress meiosis in medium without gonadotrophins, while cytoplasmic maturation proceeds (Sirard *et al.*, 1992), or to enhance cytoplasmic development during maturation through supplementation of gonadotrophin-containing media (Naito *et al.*, 1988). In equine oocytes, 100% follicular fluid, even that recovered from viable immature follicles, does not suppress meiosis (Hinrichs *et al.*, 1995b). In the present study, the hypothesis that follicular fluid from viable preovulatory follicles collected approximately 12 h before ovulation (24 h after hCG stimulation) would support both nuclear and cytoplasmic maturation of the oocytes was tested.

Equine oocytes that do not mature in culture appear to degenerate, as the proportion remaining in the germinal vesicle stage after culture is negligible (Alm and Hinrichs, 1995). Therefore, evaluation of oocytes for an intact cytoplasmic membrane was used in the present study to select for metaphase II oocytes before activation. Selection for membrane integrity has similar efficiency to selection for presence of an apparent polar body in equine oocytes. Selection of oocytes for the presence of an intact cytoplasmic membrane in the activation study was effective in selecting for metaphase oocytes, as overall only 23 of 398 (6%) oocytes selected in this way had degenerating chromatin upon staining; the remaining oocytes were in metaphase II or were activated. Neither the percentages of oocytes with an intact cytoplasmic membrane (70% for follicular fluid versus 76% for TCM-199) nor the proportions of degenerating oocytes after activation and staining (7% for follicular fluid and 5% for TCM-199) were different between media treatments. The overall maturation rate, (the proportion of metaphase or activated oocytes observed after staining), taken as a percentage of all oocytes put into maturation medium, was 65% for follicular fluid and 72% for TCM-199.

Although nuclear maturation rates were comparable in TCM-199 and follicular fluid, culture in TCM-199 yielded oocytes that were activated more easily by calcium ionophore treatments than the oocytes matured in follicular fluid. This finding is interesting in view of a recent report on the rate of fertilization and early embryo development of *in vitro*-matured oocytes after transfer to the oviduct of inseminated mares (Love *et al.*, 2000). In that study, oocytes matured in 100% follicular fluid had similar fertilization rates, but lower embryo development rates, than did oocytes matured in TCM-199. Taken together, these data indicate that cytoplasmic maturation may be somehow impaired after *in vitro* maturation in 100% follicular fluid.

Follicular fluid recovered 24 h after hCG administration would be expected to have all the hormonal signals present to support normal cytoplasmic maturation. Equine oocytes *in vivo* progress to metaphase I by 24 h after hCG administration (Bezard *et al.*, 1997). One major difference between the follicular fluid and TCM-199 treatments is that follicular fluid may have a low concentration of FSH, as the peripheral concentrations of FSH are at their nadir at the time that the preovulatory follicle reaches its maximum diameter immediately before ovulation (Snyder *et al.*, 1979). It is possible that some direct effect of the FSH added to the TCM-199 may be responsible for the responsiveness of these oocytes. Cumulus–oocyte complexes removed from follicles in an immature state may need FSH stimulation to trigger normal cytoplasmic maturation, either directly or through formation of receptors for factors present in the fluid.

Differences between the suppression of activation by follicular fluid observed in the present study and enhancement of normal fertilization observed in other experiments (Dell'Aquila *et al.*, 1997) may be due to the proportion of follicular fluid used (100% in the present study) or to the nature of the activation stimulus. In ICSI or conventional IVF, the spermatozoa induce serial calcium transients. However, parthenogenetic activation is induced by one calcium transient (for example, calcium ionophore or ionomycin) followed by suppression of MPF after activation by inhibitors of protein kinase activation, such as cycloheximide or 6-dimethyl amino purine (Susko-Parrish *et al.*, 1994; Hinrichs *et al.*, 1995a; Liu *et al.*, 1998). It is possible that the failure of oocytes matured in follicular fluid to respond to the activation stimulus is in fact a sign of vigour; that is, that the MPF concentrations are higher or replenished more rapidly in these oocytes. Further study on the comparison of MPF concentrations in oocytes matured in the different media would be of interest.

The oocytes used as controls in the present study showed a low rate of spontaneous activation (1/71) in agreement with previously reported findings (spontaneous activation rate for equine oocytes of 4–5%: Hinrichs *et al.*, 1995a; 0%: Dell'Aquila *et al.*, 1999; and 0%: Li *et al.*, 2000b).

In pig oocytes, increasing calcium ionophore concentration from 10 to 50 μmol I^{-1} resulted in higher nuclear activation rates (Wang *et al.*, 1998). The increase in acti-

vation was associated with a greater Ca^{2+} influx achieved with the higher concentration of ionophore. In the present study, activation rates of TCM-199-matured oocytes treated with 50 μmol calcium ionophore I^{-1} tended to be higher than those for 10 μmol calcium ionophore I^{-1} , and in those groups exposed to cycloheximide for only 6 h, activation rates were significantly higher in the 50 μmol I^{-1} group. This finding indicates that a higher initial calcium transient may induce more profound inhibition of MPF activity, thus overcoming the need for prolonged exposure to cycloheximide for an effective decrease in MPF concentrations in equine oocytes.

Li *et al.* (2000b) used thimerosal to activate equine oocytes after ICSI to mimic the calcium transients of fertilization. Thimerosal induces multiple intracellular calcium transients in unfertilized mammalian oocytes by promoting inositol 1,4,5-triphosphate-induced intracellular calcium release (Swann, 1991; Fissore and Robl, 1993). Li *et al.* (2000b) matured the equine oocytes in TCM-199 with added FSH, LH and oestradiol, and 20% FBS. A 79% activation rate (resumption of meiosis) was obtained using thimerosal; however, only 39% of oocytes formed a female pronucleus and an additional 7% were in the first mitotic division after treatment. The rates of these stages in the most effective treatment in the present study (maturation in TCM-199, activation with 50 μmol calcium ionophore I^{-1} and cycloheximide incubation for 24 h) were 52% (formation of female pronucleus) and 24% (first mitotic division). This finding indicates that inhibition of cytostatic factor activity after activation may be more effective in equine oocytes than induction of multiple calcium oscillations.

In cattle, ethanol-treated oocytes fixed after only 3 h exposure to cycloheximide had high rates of activation (96%, Presicce and Yang, 1994); however, low rates of activation were obtained in preliminary trials when equine oocytes were exposed to cycloheximide for only 6 h (K. Hinrichs, unpublished). As good activation rates have been obtained after culture of equine oocytes with cycloheximide for 24 h (Hinrichs *et al.*, 1995a), we wished to determine whether the important factor in activation was duration of exposure to cycloheximide, or simply increased duration of culture. The results of the present study indicate that 24 h incubation with cycloheximide after activation treatment of equine oocytes produces a higher activation rate than does 6 h exposure, even if the 6 h exposure is followed by additional culture time (as seen in the 6 h plus 18 h treatment). The requirement for relatively intense stimuli (high calcium ionophore concentrations and long duration of cycloheximide exposure) to achieve activation in equine oocytes, especially in light of low rates of spontaneous activation (Hinrichs *et al.*, 1995a; Dell'Aquila *et al.*, 1999; Li *et al.*, 2000b; present study) and low activation rates after nuclear transfer (Hinrichs *et al.*, 2000; Li *et al.*, 2000a; Reggio *et al.*, 2000) indicate that metaphase II equine oocytes have robust mechanisms for maintaining high MPF activity.

When germinal vesicle stage oocytes are cultured with cycloheximide, chromosomes form bivalents inside the

intact germinal vesicle membrane (Kubelka *et al.*, 1995). Long-term cycloheximide treatment appeared to have a similar effect on the activated oocytes in the present study. Thus, although long term culture with cycloheximide (24 h after activation treatment) had a positive effect on parthenogenetic activation, the effect on developmental capability of nuclear transferred or intracytoplasmic sperm injected oocytes should be investigated further.

In conclusion, equine oocytes matured in TCM-199 with serum and FSH for 40–42 h may be activated effectively with 50 μmol calcium ionophore I^{-1} followed by incubation with cycloheximide for 24 h. This may be a useful protocol for activation after cloning or ICSI.

This work was supported by the Link Equine Research Endowment Fund, Texas A&M University. The authors would like to thank L. B. Love for excellent technical assistance.

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Received 2 January 2001.

First decision 19 February 2001.

Accepted 27 March 2001.