REPRODUCTION
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Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection

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Abstract

This study was conducted to evaluate the effect of initial cumulus morphology (expanded or compact) and duration of *in vitro* maturation (24, 30 or 42 h) on the developmental competence of equine oocytes after intracytoplasmic sperm injection (ICSI). The effect of manipulation temperature (room temperature vs 37 °C) at the time of ICSI and concentration of glucose (0.55 vs 5.5 mM) during embryo culture was also investigated. The nuclear maturation rates of expanded (Ex) oocytes were significantly ($P < 0.001$) higher than those of compact (Cp) oocytes at all maturation times (61–72 vs 23–25% respectively). Forty-eight hours after ICSI of mature Ex oocytes, the rate of cleavage with normal nuclei was significantly ($P < 0.05$) higher for oocytes matured for 24 h than for those matured for 30 or 42 h (73 vs 57–59% respectively). For Cp oocytes, the morphologic cleavage rates for oocytes matured for 30 h were significantly higher ($P < 0.05$) than for those matured for 24 or 42 h (86 vs 55–61% respectively). The overall proportion of embryos having more than four normal nuclei at 48 h culture was significantly higher ($P < 0.05$) for Cp than for Ex oocytes. Manipulation temperature did not affect development of embryos from Ex or Cp oocytes at 96 h after ICSI. Culture in high-glucose medium significantly increased morphologic cleavage of Cp, but not Ex, oocytes ($P < 0.05$). Embryos from Cp oocytes had a significantly higher average nucleus number after 96-h culture than did embryos from Ex oocytes. These data indicate that developmental competence differs between Ex and Cp equine oocytes, and is differentially affected by the duration of maturation and by composition of embryo culture media.
Reproduction (2004) **127** 187–194

Introduction

Research on developmental competence of *in vitro* matured equine oocytes has been scant, largely due to low availability of oocytes and lack of efficient techniques for *in vitro* fertilization (IVF) (Squires 1996, Hinrichs 1998). Recently, techniques for intracytoplasmic sperm injection (ICSI) using the piezo drill have been developed in the horse, which have resulted in high fertilization and cleavage rates (Choi *et al.* 2002*a*, Galli *et al.* 2002*a*). Several laboratories have produced equine blastocysts by *in vitro* culture (IVC) of ICSI zygotes (Dell'Aquila *et al.* 1997*a*; Guignot *et al.* 1998, Li *et al.* 2001, Galli *et al.* 2002*a*, Choi *et al.* 2003*a*), but the proportion of embryos reaching the blastocyst stage *in vitro* has typically been less than 10%. The limited success of *in vitro* embryo production in the horse may be related to the low developmental competence of the oocytes used; however, few studies have been performed to evaluate factors affecting developmental competence of equine oocytes.

Methods for classification of recovered equine oocytes vary among laboratories. Many laboratories have selected oocytes with compact cumuli (Cp) for culture and discarded oocytes with expanded cumuli (Ex) (Fulka & Okolski 1981, Willis *et al.* 1991, Choi *et al.* 1993, Goudet *et al.* 1998, Li *et al.* 2000, Tremoleda *et al.* 2001), as is done in most other species. However, nuclear maturation rates of Ex equine oocytes have been shown to be similar to (Zhang *et al.* 1989, Hinrichs *et al.* 1993, Dell'Aquila *et al.* 2001), or higher than (Hinrichs *et al.* 1995, Alm & Hinrichs 1996, Hinrichs & Williams 1997, Hinrichs & Schmidt 2000, Choi *et al.* 2002*b*), those for Cp oocytes. Information on the developmental competence of these two types of oocytes is limited. Compact oocytes had a reduced ability to respond to activation stimuli (Hinrichs *et al.* 1995), and reduced ability to form a male pronucleus after ICSI (Dell'Aquila *et al.* 1997*b*) when compared to Ex oocytes. After ICSI, Cp oocytes have been reported to have lower (Hinrichs *et al.* 2002: duration of maturation

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.00087
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24 h) or equal (Franz *et al.* 2003: duration of maturation 30 h) rates of cleavage when compared to Ex oocytes.

The lower meiotic, and possibly developmental, competence of Cp oocytes may be related to their follicular origin. Compact oocytes are recovered from viable follicles, whereas Ex oocytes are recovered from atretic follicles (Hinrichs & Williams 1997). Viable follicles appear to keep the enclosed oocyte in a more juvenile state; in contrast, atretic follicles allow prematurational changes, such as chromatin condensation, to occur within the oocyte (Hinrichs & Williams 1997). Compact oocytes take longer to reach metaphase II than do Ex oocytes (Zhang *et al.* 1989, Hinrichs *et al.* 1993, Alm & Hinrichs 1996, Choi *et al.* 2002*b*). The duration of *in vitro* maturation (IVM) that affords maximum developmental competence for equine oocytes is unknown; it is possible that this may vary according to oocyte type. This possibility is supported by the finding that culture in the presence of the meiosis inhibitor, roscovitine, for 24 h before starting culture for IVM increased the developmental competence of Cp, but not of Ex, equine oocytes (Franz *et al.* 2003).

Assessment of developmental competence is dependent on having a culture system that supports embryo development; however, little work has been done on requirements for culture of early equine embryos. We previously found that development at 96 h was similar for embryos cultured in G1.2 or in a modified CZB medium (Choi *et al.* 2003*b*), establishing the possibility of using CZB as a base medium for culture of early equine embryos. One of the most influential components of embryo culture media is glucose, which can have an inhibitory effect on development of preimplantation embryos depending on stage, species and medium composition (review, Bavister 1995). There is only one previous report on the effect of glucose concentration on development of equine embryos: Azuma *et al.* (1995) investigated glucose concentrations of 0.5 and 5.5 mM for early equine embryo culture, and found that culture in the low concentration on days 1–4, followed by increasing glucose concentration, improved development to the morula stage at day 8.

Procedures performed during ICSI may also affect subsequent embryonic development. In the first report of use of a piezo drill for ICSI, performed in mice, it was suggested that oocytes should be manipulated at low temperatures and held briefly before culture to prevent membrane lysis (Kimura & Yanagimachi 1995). This group later performed ICSI at room temperature (23–25 °C) without a holding period and obtained similar survival rates (Wakayama & Yanagimachi 1998*a*,*b*). In the horse, some researchers use a heated stage for conventional ICSI (Dell'Aquila *et al.* 1997*a*,*b*, 2001, Cochran *et al.* 1998), but there is no information available on the effect of manipulation temperature on the survival or early development of equine embryos.

The aim of this study was to evaluate differences in developmental competence between Ex and Cp oocytes, and the effect of different durations of oocyte maturation on their developmental competence, by studying embryo development after ICSI. Because methods for culture of later-stage equine embryos have not yet been optimized, embryo development was assessed at 48 h after fertilization to minimize the effect of culture conditions on embryo performance. In addition, we evaluated the effects of manipulation temperature (room temperature vs 37 °C) and two glucose concentrations (0.55 vs 5.5 mM) during *in vitro* culture after ICSI of Ex and Cp oocytes on development at 96-h culture.

Materials and Methods

Experiment 1: competence of Ex and Cp oocytes after different periods of maturation

Oocyte collection

This study was conducted in the months of November to June. Ovaries were obtained from two abattoirs and transported to the laboratory at room temperature (26–34 °C, 3–4-h transport time). Adnexa were trimmed from the ovaries with scissors, and the ovaries were 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 TCM199 with Hanks' salts (Gibco Life Technologies, Inc., Grand Island, NY, USA) plus ticarcillin (0.1 mg/ml, SmithKline Beecham Pharmaceuticals, Philadelphia, PA, USA). The contents of the petri dishes were examined using a dissection microscope at ×10–20 magnification. Oocyte–cumulus complexes were classified as compact, expanded or degenerating depending on the expansion of both mural granulosa and cumulus, as described previously (Hinrichs & Williams 1997, Hinrichs & Schmidt 2000). Oocytes with any sign of expansion of either the cumulus or the mural granulosa (from having individual cells visibly protruding from the surface to having full expansion with copious matrix visible between cells) led to the classification of Ex. Oocytes having both compact cumulus and compact mural granulosa were classified as Cp. Oocytes showing signs of degeneration (shrunken, dense or fragmented cytoplasm) were discarded.

In vitro *maturation*

The oocytes were washed twice in maturation medium (TCM199 with Earle's salts (Gibco), 5 μU/ml follicle-stimulating hormone (FSH) (Sioux Biochemical, Inc., Sioux Center, IA, USA), 10% fetal bovine serum (FBS) (Gibco) and 25 μg/ml gentamycin (Gibco)), and then cultured in droplets of this medium at a ratio of 10 μl medium per oocyte, under light white mineral oil (Sigma Chemical Co., St Louis, MO, USA) at 38.2 °C in 5% CO_2 in air for 24, 30 or 42 h. After maturation, oocytes were denuded of cumulus by pipetting in a solution of 0.05% hyaluronidase

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in TCM199 with 5% FBS. Denuded oocytes were selected for presence of a polar body. Oocytes not exhibiting a polar body were fixed in buffered formol saline, mounted on a slide with 6.5 μl of 9:1 glycerol:PBS containing 2.5 μg/ml Hoechst 33258, and examined using fluorescence microscopy to determine the chromatin configuration.

Sperm preparation

Frozen equine semen was used for ICSI. Semen straws were thawed at 37 °C for 30 s, and 200 μl of the semen was placed on the bottom of a 5 ml tube containing 1 ml of modified Tyrode's albumin lactate pyruvate (Sp-TALP) (Parrish *et al.* 1988) for swim-up. After 20-min incubation at 38.2 °C in an atmosphere of 5% CO_2 in air, 0.6 ml of medium was collected from the top of the tube, and centrifuged at 327 ***g*** for 3 min in a 1.7 ml polypropylene tube. The sperm pellet was resuspended and washed once with the same medium. The supernatant was removed and the pellet was used to provide sperm for ICSI.

Intracytoplasmic sperm injection

The ICSI procedure was conducted as previously described (Choi *et al.* 2002*a*) using a piezo drill. Briefly, the outside diameter of the sperm injection pipette was 7–8 μm. A 120–140 μm (outside diameter) pipette was used to hold the oocytes. Immediately before injection, 1 μl of sperm suspension was placed in 3 μl of Sp-TALP containing 10% PVP (Sigma) under oil. Sperm injection was carried out in a separate 50 μl drop of Hepes-buffered TCM199 containing 10% FBS. Each spermatozoon was immobilized by applying a few pulses with the Piezo drill (Burleigh Instruments, Inc., Fishers, NY, USA) to the sperm tail immediately before injection. All manipulations were performed at room temperature. Injected oocytes were held for 20 min at room temperature in the same medium to heal the broken membrane slowly.

In vitro *culture*

After ICSI, fertilized oocytes were washed twice in G1.2 medium (G1.2/G2.2, IVF Science, Denver, CO, USA) and cultured in the same medium at 38.2 °C under 5% CO_2 in air. At 48 h post-ICSI, all oocytes/embryos were fixed and stained to examine the number and status of nuclei. Only nuclei that appeared to be normal were included in the embryo nucleus number; nuclei showing signs of degeneration (vacuolization, condensation or fragmentation) were disregarded.

Experiment 2: effects of manipulation temperature and glucose concentration

This study was conducted in the months of April to August. Media used for holding oocytes, swim-up for sperm, manipulation and culture were CZB-H, Sp-CZB, CZB-M and CZB-C respectively, which were modified from CZB (Chatot *et al.* 1989, Choi *et al.* 2003*b*). The osmolarity of these media was 298 ± 3 mOsm.

Oocytes were recovered and matured as described above; however, based on results of experiment 1, Ex and Cp oocytes were denuded after 24–25-h IVM and held in CZB-H until 30-h IVM. Sperm swim-up for ICSI was performed as described above, but using Sp-CZB for sperm preparation instead of Sp-TALP. Sperm were injected into oocytes in a microdroplet of CZB-M either at room temperature or at 37 °C. For the 37 °C treatment, a heating stage (Fryer A-50; Fryer Co., Carpentersville, IL, USA) was used. Oocytes manipulated at 37 °C were immediately transferred to CZB-H and incubated at 38.2 °C; oocytes injected at room temperature were held for 20 min at room temperature in a separate drop of manipulation medium before being placed in culture. The presumptive zygotes were cultured in CZB-C with either 0.55 mM glucose or 5.5 mM glucose at 38.2 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After 96-h culture, without a change of medium, embryos were fixed and stained as described above to examine the number and status of nuclei.

Statistical analysis

Differences in maturation rates, fertilization rates, morphologic and normal cleavage rates, and proportions of embryos developing to the different stages among groups were evaluated using chi-square analysis, with Fisher's exact test being used when the expected value for any parameter was less than 5. Differences in average nucleus number among maturation durations, glucose concentrations and manipulation temperatures, and between cumulus morphologies, were evaluated using analysis of variance.

Results

A total of 1198 ovaries were processed, and 6861 follicles were scraped, for an average of 5.7 follicles per ovary. Of recovered oocytes, 1139 were Cp, 2514 were Ex and 318 were degenerating. For this study, 1165 Ex and 1103 Cp oocytes were used, and the remainder were used on a separate project.

Experiment 1

Of the 500 Ex and 485 Cp oocytes cultured for IVM, 12 Ex and 6 Cp oocytes were broken during denuding of cumulus cells. After denuding, 319 Ex (65%) and 114 Cp (24%) oocytes were selected for having a first polar body. Of 178 Ex and 365 Cp oocytes without visible polar bodies, 10 (2% of total oocytes) Ex and 24 (5% of total oocytes) Cp were found to be in metaphase I (MI) on fixation and staining, and 6 (3%) Ex and 1 (0.3%) Cp were in metaphase II (MII). Maturation rates to MI and MII

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(visible polar body plus oocytes in MII on staining) in the different treatments are presented in Table 1. Maturation rates of Ex oocytes increased significantly from 24 to 30 h. The maturation rates for Ex oocytes were significantly ($P < 0.001$) higher than those for Cp oocytes at each time period. The proportion of Cp oocytes in MI at 24 h was significantly ($P < 0.05$) higher than that for 42 h.

Thirteen Ex oocytes having a polar body were sham injected with medium only; after fixation and staining at 48 h, all of these were found to be in metaphase.

A total of 402 oocytes with a polar body were manipulated for ICSI, and 394 (98%) were successfully injected. The rates of morphologic cleavage and cleavage with normal nuclei after ICSI in the different treatments are shown in Table 2. For Ex oocytes, the rate of normal cleavage (more than two nuclei) was significantly higher in the 24-h than in the 30- or 42-h treatments. For Cp oocytes, the morphologic cleavage rate was significantly higher ($P < 0.05$) in the 30-h than in the 24- or 42-h treatments. When Ex and Cp oocytes were compared within treatments, Ex oocytes had a higher rate of normal cleavage than did Cp oocytes when oocytes were matured for 24 h or 42 h, but there was no significant difference in normal cleavage between Ex and Cp oocytes when matured for 30 h.

There were no differences in further embryonic development among time periods within cumulus type; therefore, the data for time periods were combined. Overall, Ex oocytes produced significantly more embryos with delayed development (2–3 cells at 48 h) and significantly fewer embryos with advanced development (more than four cells at 48 h) than did Cp oocytes. The average nucleus number of embryos resulting from Ex oocytes matured for 30 or 42 h was significantly lower than that for Cp oocytes at the corresponding time periods ($P < 0.05$), and over all treatments, the average nucleus number for embryos resulting from Ex oocytes was significantly lower than that for Cp oocytes ($P < 0.001$). A six-cell embryo produced by ICSI of a Cp oocyte matured for 24 h is shown in Fig. 1.

Experiment 2

A total of 665 Ex and 618 Cp oocytes were used. Of these, 13 Ex oocytes were broken during removal of cumulus cells. Rates of polar body formation were 71% (460/652) in Ex and 22% (138/618) in Cp. Of 192 Ex and 480 Cp

Table 1 Percentages of oocytes with expanded (Ex) and compact (Cp) cumulus maturing after 24, 30 or 42 h culture *in vitro*.

Oocyte type	Duration of *in vitro* maturation (h)	Number of oocytes examined	Number of oocytes in metaphase I (%)	Number of oocytes in metaphase II (%)*
Ex	24	192	6 (3)	117 (61)[a]
	30	134	1 (1)	97 (72)[b]
	42	162	3 (2)	111 (69)
Totals		488	10 (2)	325 (67)
Cp	24	157	14 (9)[a]	38 (24)
	30	145	6 (4)	36 (25)
	42	177	4 (2)[b]	41 (23)
Totals		479	24 (5)	115 (24)

* Metaphase II is the total of oocytes with a visible polar body and those in metaphase II on fixation and staining.
[a,b] Values with a different superscript within columns are significantly different ($P < 0.05$).

Table 2 *In vitro* development of equine oocytes having expanded (Ex) and compact (Cp) cumulus matured for 24, 30 or 42 h and fertilized by intracytoplasmic sperm injection followed by 48 h culture.

Oocyte type	Duration of *in vitro* maturation (h)	Number of oocytes cultured	Number of oocytes fertilized (%)*	Number of oocytes cleaved (%)	Number of oocytes cleaved with normal nuclei (%)**	Number of embryos (% of embryos with normal nuclei) with			Average number of nuclei (mean ± S.E.M.)
						2–3 nuclei	4 nuclei	5–8 nuclei	
Ex	24	90	73 (81)	69 (77)[a,b]	66 (73)[a]	27 (41)	31 (47)	8 (12)	3.6 ± 0.1[a,b]
	30	94	74 (79)	71 (76)[a,b]	55 (59)[b]	24 (44)	27 (49)	4 (7)	3.5 ± 0.2[a,c]
	42	99	71 (72)	70 (71)[a,b,c]	56 (57)[b,c]	32 (57)	17 (30)	7 (13)	3.3 ± 0.2[a]
Totals		283	218 (77)	210 (74)	177 (63)	83 (47)[a]	75 (42)	19 (11)[a]	3.5 ± 0.1[a]
Cp	24	36	24 (67)	22 (61)[b,c]	14 (39)[c,d]	2 (14)	8 (57)	4 (29)	4.3 ± 0.3[b]
	30	35	30 (86)	30 (86)[a]	19 (54)[b,c,d]	4 (21)	8 (42)	7 (37)	4.2 ± 0.2[b]
	42	40	23 (58)	22 (55)[c]	14 (35)[d]	5 (36)	3 (21)	6 (43)	4.2 ± 0.5[b,c]
Totals		111	77 (69)	74 (67)	47 (42)	11 (23)[b]	19 (40)	17 (36)[b]	4.2 ± 0.2[b]

* The numbers of fertilized oocytes were calculated by adding oocytes with pronuclei and mitotic figures to cleaved embryos.
** Calculated as the number of oocytes that have cleaved and have ≥2 normal nuclei, as a percentage of injected oocytes.
[a–d] Within columns, values not having a common superscript are significantly different ($P < 0.05$).

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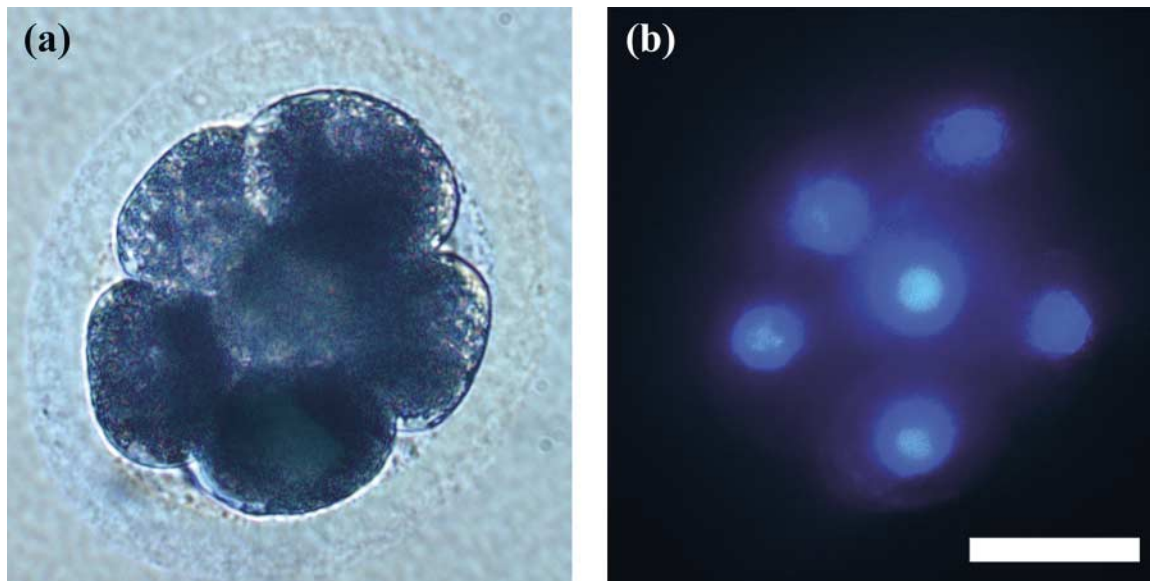

Figure 1 A fertilized six-cell equine embryo originating from an oocyte with compact (Cp) cumulus and cultured for 48 h in G1.2 medium after intracytoplasmic sperm injection as visualized by (a) bright field and (b) fluorescence and bright-field microscopy after Hoechst 33258 staining (six nuclei). Scale bar represents 50 μm.

oocytes without polar bodies, 14 (2% of total oocytes) of Ex and 32 (5% of total oocytes) of Cp oocytes were found to be in MI on fixation and staining, and 4 (0.6%) of Ex and 3 (0.5%) of Cp oocytes were in MII. Of the 232 Ex and 138 Cp oocytes manipulated for ICSI, 356 (96%) were successfully injected. The *in vitro* development at 96 h of Ex and Cp oocytes manipulated either at room temperature or at 37 °C, and then cultured in either 0.55 or 5.5 mM glucose, is shown in Table 3. For both Ex and Cp oocytes, there were no significant differences in morphologic cleavage, cleavage with normal nuclei or average number of nuclei between the two manipulation temperatures. For Ex oocytes, there was no effect of glucose concentration on embryo development. For Cp oocytes, when data for the two manipulation temperatures were combined, the higher glucose concentration significantly increased the morphologic cleavage rate of Cp oocytes compared to the lower glucose concentration (63/68, 93% vs 49/67, 73%; $P < 0.01$). This effect of glucose was also seen in room-temperature Cp oocytes when considered separately (Table 3). There were no significant differences in further embryo development among treatments within cumulus types. Overall, Cp oocytes had a significantly greater proportion of embryos reaching over 16 nuclei than did Ex oocytes (12/85, 14%, vs 7/134, 5% respectively; $P < 0.05$) and a significantly higher average nucleus number (10.9 vs 7.6 respectively; $P < 0.001$) than did Ex oocytes. The superior development of the Cp oocytes was not attributable only

Table 3 Effects of oocyte type, manipulation temperature and glucose concentration in culture media on the development of ICSI embryos cultured *in vitro* for 96 h.

Oocyte type	Manipulation temperature	Glucose (mM)	Number of oocytes cultured	Number of oocytes cleaved (%)	Number of oocytes cleaved with normal nuclei (%)*	Number of embryos (% of embryos with normal nuclei) with 2–7 nuclei	8–16 nuclei	>16 nuclei	Average number of nuclei (mean ± S.E.M.)
Ex	Room temp	0.55	55	41 (75)	29 (53)	18 (62)	11 (38)	0 (0)	6.6 ± 0.8
		5.5	57	47 (82)	37 (65)	20 (54)	14 (38)	3 (8)	8.6 ± 0.9
	37 °C	0.55	57	47 (82)	35 (61)	22 (63)	10 (29)	3 (9)	7.4 ± 0.8
		5.5	52	41 (79)	33 (63)	21 (64)	11 (33)	1 (3)	7.5 ± 0.9
Totals			221	176 (80)	134 (61)	81 (61)[a]	46 (34)[a]	7 (5)[a]	7.6 ± 0.4[a]
Cp	Room temp	0.55	32	22 (69)[a]	18 (56)	8 (44)	8 (44)	2 (11)	9.9 ± 1.6
		5.5	34	31 (91)[b]	22 (65)	6 (27)	12 (55)	4 (18)	12.0 ± 1.8
	37 °C	0.55	35	27 (77)[a,b]	19 (54)	7 (37)	10 (53)	2 (11)	9.6 ± 1.1
		5.5	34	32 (94)[b]	26 (72)	6 (23)	16 (62)	4 (15)	11.6 ± 1.4
Totals			135	112 (83)	85 (63)	27 (32)[b]	46 (54)[b]	12 (14)[b]	10.9 ± 0.8[b]

Ex: oocytes initially having expanded cumuli; Cp: oocytes initially having compact cumuli.
* Calculated as the number of oocytes that have cleaved and have ≥2 normal nuclei, as a percentage of injected oocytes.
[a,b] Within columns, values not having a common superscript are significantly different ($P < 0.05$).

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to their response to the higher glucose treatment: when the two manipulation temperatures were combined, the average nucleus number for Cp oocytes was higher than that for Ex oocytes for both low- (7.1 vs 9.7 for Ex and Cp respectively; $P < 0.05$) and high- (8.1 vs 11.8 for Ex and Cp respectively; $P < 0.01$) glucose treatments. Three embryos from Cp oocytes cultured for 96 h, fixed and stained with Hoechst 33258, are shown in Fig. 2.

Discussion

The increased meiotic competence observed in Ex oocytes in this study is consistent with results previously obtained in our laboratory (Alm & Hinrichs 1996, Hinrichs & Williams 1997, Hinrichs & Schmidt 2000, Choi *et al.* 2002*b*, Love *et al.* 2003). Other laboratories have reported that Ex oocytes have the same maturation rate as do Cp oocytes (Del Campo *et al.* 1995, Dell'Aquila *et al.* 1997*b*, 2001, Choi *et al.* 2000). This difference may arise from handling of ovaries (time from slaughter to oocyte collection), selection of follicle size for oocyte collection or classification of cumulus morphology. Oocytes collected immediately after slaughter have increased rates of meiotic resumption, especially Cp oocytes (Hinrichs *et al.* 2002). The maturation rate of Ex oocytes is significantly higher than that of Cp oocytes for follicles 1–20 mm in diameter, but not for follicles more than 20 mm in diameter (Hinrichs & Schmidt 2000). In the present study, we collected oocytes from all visible follicles; with this method we expect 43%, 35%, 19% and 3% of oocytes to originate from follicles 1–5 mm, 6–10 mm, 11–20 mm and >20 mm respectively (Hinrichs & Schmidt 2000). We categorize oocytes as Ex if there are any signs of expansion of either cumulus cells or granulosa cells. Other methods of classification may yield different results; for example, Dell'Aquila *et al.* (2001) classified 69% of oocytes as Cp (vs 31% in this study); they found that maturation rates of Ex and Cp oocytes, from follicles over 10 mm, were similar.

The increased meiotic competence of Ex oocytes is likely to be related to changes undergone by the oocyte during early follicle atresia (Hinrichs & Williams 1997). Our results suggest that these changes may also affect the developmental competence of Ex oocytes. The proportion of Ex oocytes cleaving normally in this study was significantly higher for oocytes matured 24 h than for those matured 30 or 42 h (Table 2); in contrast, Cp oocytes matured for 30 h had a significantly higher rate of morphologic cleavage than did the other two maturation times, and also the highest rate of normal cleavage among the three maturation time periods.

While Ex oocytes at 24 h had higher rates of normal cleavage than did Cp oocytes at any time period (Table 2), the extent of embryonic development achieved was greater for Cp than for Ex oocytes in both experiment 1 and experiment 2. Because Ex oocytes undergo prematurational changes within the follicle, they may be 'aged' relative to Cp oocytes and thus may be more easily activated, as has been shown for *in vitro* aged oocytes in cattle (Presicce & Yang 1994). This may be reflected in the increased rate of cleavage after ICSI seen in Ex oocytes in the present study. However, Ex oocytes may have deficiencies in cytoplasmic competence related to follicle degeneration and/or oocyte aging, which would explain

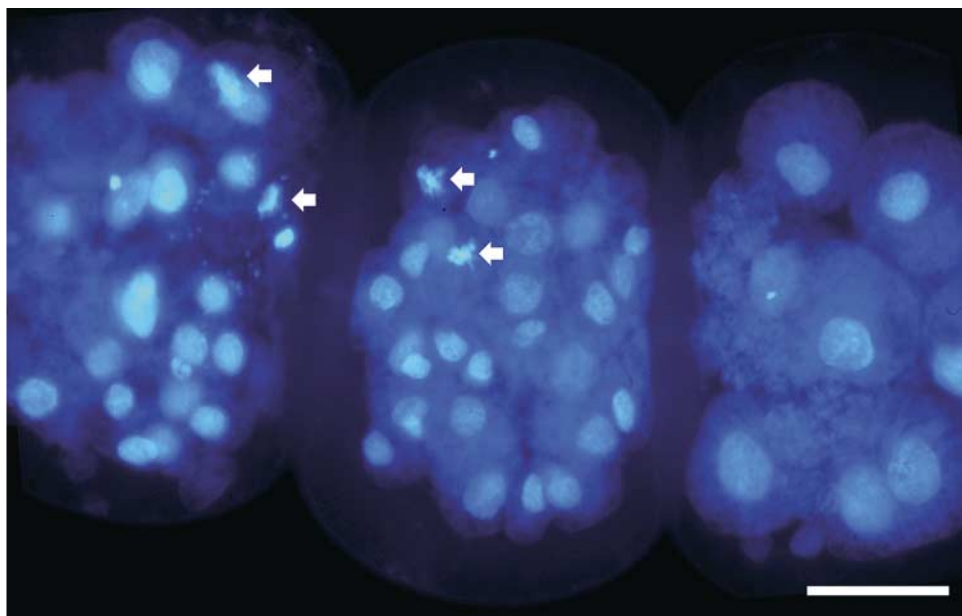

Figure 2 Three embryos originating from oocytes with compact (Cp) cumulus, fertilized by intracytoplasmic sperm injection at room temperature and cultured in CZB-C with 5.5 mM glucose for 96 h *in vitro*, showing 24, 30 and 8 nuclei including mitotic figures (arrows). Bar represents 50 µm.

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the relatively high proportion of embryos having delayed development (experiments 1 and 2). In cattle, oocytes with pronounced cumulus expansion had decreased developmental competence (Blondin & Sirard 1995).

The medium used for oocyte maturation in this study is relatively simple (M199 with FSH and FBS). Maturation medium may affect developmental competence; however, in the horse, no difference in rates of nuclear maturation, cleavage, or development to compact morulae or blastocysts has been found between relatively simple media and those supplemented with epidermal growth factor (EGF), insulin-like growth factor (IGF)-I, and/or additional hormones (luteinizing hormone (LH), E_2 and P_4) (Galli *et al.* 2002*b*, Choi *et al.* 2003*b*).

High glucose concentration (5.5 mM) has been reported to be detrimental to the development of equine embryos produced by fertilization of zona-dissected oocytes (Azuma *et al.* 1995). However, in the present study, high glucose did not inhibit development of ICSI embryos from Ex or Cp oocytes. Instead, the development of embryos from Cp oocytes was improved by high glucose, as reflected in significantly higher morphologic cleavage rates in this group. In addition, the rate of normal cleavage and the average nucleus number for Cp oocytes in the 5.5 mM glucose treatment were not depressed, being higher (though not significantly so) than the corresponding rates for Cp oocytes cultured in the low-glucose treatment. The average number of nuclei in embryos from Ex oocytes at 96 h in the present study, for both glucose treatments (6.6–8.6), is similar to that which we have reported for Ex oocytes previously (6.5–7.7) (Choi *et al.* 2002*a*, 2003*b*). The average nucleus number of embryos from Cp oocytes, especially those from the high-glucose treatments (11.6 and 12.0) is notably higher than in our previous findings. This beneficial effect of glucose on embryos from Cp oocytes was not anticipated. Early embryos in other species have typically been shown to have better development in low glucose (Bavister 1995, Lane 2001), or no difference has been seen (Devreker *et al.* 2000). Further investigation on the effect of glucose during equine embryo development is warranted.

We were interested in evaluating the effect of temperature during manipulation for ICSI, as this may have an effect on the integrity of the meiotic spindle. Microtubules are extremely temperature-sensitive; in human oocytes, temperatures of even 3–4 °C less than 37 °C during manipulation significantly decreased the proportion of oocytes with identifiable spindles, and also fertilization and pregnancy rates (Wang *et al.* 2002). Low temperatures have been used during ICSI with the piezo drill to minimize oocyte lysis (Wakayama & Yanagimachi 1998*a*,*b*). However, in the present study, there was no difference in survival rate of oocytes subjected to ICSI at low and high temperature (97% and 95% respectively). While there was no apparent detrimental effect of low temperature on embryo development, if microtubule disorganization results in chromosomal anomalies, the effect may not be seen until further stages of development.

In conclusion, Ex oocytes had higher meiotic competence than did Cp oocytes at all durations of maturation tested. Normal cleavage rates were highest for Ex oocytes when matured for 24 h, but they were highest for Cp oocytes when matured for 30 h. Expanded oocytes matured for 24 h had a higher rate of normal cleavage after ICSI than did Cp oocytes at any duration of maturation; however, the embryo development of Cp oocytes 48 or 96 h after ICSI was significantly advanced over that for Ex oocytes. High glucose concentration in culture medium improved cleavage rates of Cp oocytes, but did not affect Ex oocytes. The piezo drill may be used for ICSI of equine oocytes at 37 °C without increasing oocyte lysis, thus allowing manipulation at a temperature that supports maintenance of the meiotic spindle.

Acknowledgements

This research was supported by the Link Equine Research Endowment Fund, Texas A&M University and Bio-Arts Research Corporation.

References

Alm H & Hinrichs K 1996 Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology. *Journal of Reproduction and Fertility* **107** 215–220.

Azuma T, **Choi YH**, **Hochi S & Oguri N** 1995 Effect of glucose in the culture medium on development of horse oocytes matured and microfertilized *in vitro*. *Reproduction, Fertility and Development* **7** 1067–1071.

Bavister BD 1995 Culture of preimplantation embryos: facts and artefacts. *Human Reproduction Update* **1** 91–148.

Blondin P & Sirard MA 1995 Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Molecular Reproduction and Development* **41** 54–62.

Chatot CL, **Ziomek CA**, **Bavister BD**, **Lewis JL & Torres I** 1989 An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **86** 679–688.

Choi YH, **Hochi S**, **Braun J**, **Sato K & Oguri N** 1993 *In vitro* maturation of equine oocytes collected by follicle aspiration and by the slicing of ovaries. *Theriogenology* **40** 959–966.

Choi YH, **Chung YG & Squires EL** 2000 Culture of equine embryos in trophoblast-conditioned medium. *Theriogenology* **53** 291.

Choi YH, **Love CC**, **Love LB**, **Varner DD**, **Brinsko S & Hinrichs K** 2002*a* Developmental competence *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. *Reproduction* **123** 455–465.

Choi YH, **Shin T**, **Love CC**, **Johnson C**, **Varner DD**, **Westhusin ME & Hinrichs K** 2002*b* Effect of co-culture with theca interna on nuclear maturation of horse oocytes with low meiotic competence, and subsequent fusion and activation rates after nuclear transfer. *Theriogenology* **57** 1005–1011.

Choi YH, **Love CC**, **Love LB**, **Varner DD**, **Brinsko S & Hinrichs K** 2003*a* Effects of gas conditions, time of medium change, and ratio of medium to embryo on *in vitro* development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219–1229.

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Choi YH, **Chung YG**, **Walker SC**, **Westhusin ME & Hinrichs K** 2003*b* *In vitro* development of equine nuclear transfer embryos: effects of oocyte maturation media and amino acid composition during embryo culture. *Zygote* **11** 77–86.

Cochran R, **Meintjes M**, **Reggio B**, **Hylan D**, **Carter J**, **Pinto C**, **Paccamonti D & Godke RA** 1998 Live foals produced from sperm-injected oocytes derived from pregnant mares. *Journal of Equine Veterinary Science* **18** 736–740.

Del Campo MR, **Donoso X**, **Parrish JJ & Ginther OJ** 1995 Selection of follicles, preculture oocyte evaluation, and duration of culture for *in vitro* maturation of equine oocytes. *Theriogenology* **43** 1141–1153.

Dell'Aquila ME, **Cho YS**, **Minoia P**, **Traina V**, **Lacalandra GM & Maritato F** 1997*a* Effects of follicular fluid supplementation of *in-vitro* maturation medium on the fertilization and development of equine oocytes after *in-vitro* fertilization or intracytoplasmic sperm injection. *Human Reproduction* **12** 2766–2772.

Dell'Aquila ME, **Cho YS**, **Minoia P**, **Traina V**, **Fusco S**, **Lacalandra GM & Maritato F** 1997*b* Intracytoplasmic sperm injection (ICSI) versus conventional IVF on abattoir-derived and *in vitro*-matured equine oocytes. *Theriogenology* **47** 1139–1156.

Dell'Aquila ME, **Masterson M**, **Maritato F & Hinrichs K** 2001 Influence of oocyte collection technique on initial chromatin configuration, meiotic competence and male pronucleus formation after intracytoplasmic sperm injection (ICSI) of equine oocytes. *Molecular Reproduction and Development* **60** 79–88.

Devreker F, **Hardy K**, **Van den Bergh M**, **Winston J**, **Biramane J & Englert Y** 2000 Noninvasive assessment of glucose and pyruvate uptake by human embryos after intracytoplasmic sperm injection and during the formation of pronuclei. *Fertility and Sterility* **73** 947–954.

Franz LC, **Choi YH**, **Squires EL & Hinrichs K** 2003 Effects of roscovitine on maintenance of the germinal vesicle in horse oocytes, subsequent nuclear maturation, and cleavage rates after intracytoplasmic sperm injection. *Reproduction* **125** 693–700.

Fulka J & Okolski A 1981 Culture of horse oocytes *in vitro*. *Journal of Reproduction and Fertility* **61** 213–215.

Galli C, **Crotti G**, **Turini P**, **Duchi R**, **Mari G**, **Zavaglia G**, **Duchamp G**, **Daels P & Lazzari G** 2002*a* Frozen-thawed embryos produced by ovum pickup of immature oocytes and ICSI are capable to establish pregnancies in the horse. *Theriogenology* **58** 705–708.

Galli C, **Maclellan LJ**, **Crotti G**, **Turini P**, **Ponderato N**, **Duchi R**, **Mari G**, **Merlo B**, **Barbacini S & Lazzari G** 2002*b* Development of equine oocytes matured *in vitro* in different media and fertilised by ICSI. *Theriogenology* **57** 719.

Goudet G, **Bézard J**, **Belin F**, **Duchamp G**, **Palmer E & Gérard N** 1998 Oocyte competence for *in vitro* maturation is associated with histone H1 kinase activity and is influenced by estrous cycle stage in the mare. *Biology of Reproduction* **59** 456–462.

Guignot F, **Ottogalli M**, **Yvon JM & Magistrini M** 1998 Preliminary observations in *in vitro* development of equine embryo after ICSI. *Reproduction, Nutrition and Development* **38** 653–663.

Hinrichs K 1998 Production of embryos by assisted reproduction in the horse. *Theriogenology* **49** 13–21.

Hinrichs K & Schmidt AL 2000 Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biology of Reproduction* **62** 1402–1408.

Hinrichs K & Williams KA 1997 Relationships among oocyte-cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. *Biology of Reproduction* **57** 377–384.

Hinrichs K, **Schmidt AL**, **Friedman PP**, **Selgrath JP & Martin MG** 1993 *In vitro* maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biology of Reproduction* **48** 363–370.

Hinrichs K, **Schmidt AL & Selgrath JP** 1995 Activation of horse oocytes. *Biology of Reproduction Monograph Series* **1** 319–324.

Hinrichs K, **Choi YH**, **Love LB**, **Love CC**, **Varner DD & Ingram LA** 2002 Effects of holding time and media on meiotic and developmental competence of horse oocytes. *Theriogenology* **58** 675–678.

Kimura Y & Yanagimachi R 1995 Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* **52** 709–720.

Lane M 2001 Mechanisms for managing cellular and homeostatic stress *in vitro*. *Theriogenology* **55** 225–236.

Li X, **Morris LHA & Allen WR** 2000 Effects of different activation treatments on fertilization of horse oocytes by intracytoplasmic sperm injection. *Journal of Reproduction and Fertility* **119** 253–260.

Li X, **Morris LHA & Allen WR** 2001 Influence of co-culture during maturation on the developmental potential of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI). *Reproduction* **121** 925–932.

Love LB, **Choi YH**, **Love CC**, **Varner DD & Hinrichs K** 2003 Effect of ovary storage and oocyte transport method on maturation rate of horse oocytes. *Theriogenology* **59** 765–774.

Parrish JJ, **Susko-Parrish J**, **Winer MA & First NL** 1988 Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38** 1171–1180.

Presicce GA & Yang X 1994 Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Molecular Reproduction and Development* **37** 61–68.

Squires EL 1996 Maturation and fertilization of equine oocytes. *Veterinary Clinics of North America: Equine Practice* **12** 31–45.

Tremoleda JL, **Schoevers EJ**, **Stout TAE**, **Colenbrander B & Bevers MM** 2001 Organisation of the cytoskeleton during *in vitro* maturation of horse oocytes. *Molecular Reproduction and Development* **60** 260–269.

Wakayama T & Yanagimachi R 1998*a* Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nature Biotechnology* **16** 639–641.

Wakayama T & Yanagimachi R 1998*b* The first polar body can be used for the production of normal offspring in mice. *Biology of Reproduction* **59** 100–104.

Wang WH, **Meng L**, **Hackett RJ**, **Oldenbourg R & Keefe DL** 2002 Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. *Fertility and Sterility* **77** 1274–1277.

Willis P, **Caudle AB & Fayrer-Hosken RA** 1991 Equine oocyte *in vitro* maturation: influences of sera, time and hormones. *Molecular Reproduction and Development* **30** 360–368.

Zhang JJ, **Boyle MS**, **Allen WR & Galli C** 1989 Recent studies on *in vivo* fertilisation of *in vitro* matured horse oocytes. *Equine Veterinary Journal* **8** (Suppl) 101–104.

Received 20 June 2003
First decision 28 August 2003
Revised manuscript received 1 October 2003
Accepted 27 October 2003

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