Molecular Reproduction & Development 77:651–661 (2010)

# The Equine Oocyte: Factors Affecting Meiotic and Developmental Competence

KATRIN HINRICHS*

Departments of Veterinary Physiology and Pharmacology and Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas

## SUMMARY

There is currently much interest in assisted reproduction techniques in the horse, however, many aspects of oocyte maturation, fertilization, and embryo development in the horse differ from those in other species. Because of the close attachment of the equine oocyte to the follicle wall, scraping of the follicle is the most effective method for oocyte recovery. A notable feature of equine oocytes is that those with expanded cumuli (Ex oocytes), which originate from atretic follicles, have higher meiotic competence (ability to mature to metaphase II in vitro) than do oocytes with compact cumuli (Cp oocytes). Cp oocytes originate in viable follicles but are largely juvenile. Recovery and culture of equine oocytes immediately after slaughter yields a higher maturation rate than that obtained from oocytes after ovary storage; this is related to damage to chromatin in Cp oocytes during storage. In contrast, developmental competence (rate of blastocyst development in vitro) is higher in oocytes recovered from the ovary after a delay. The optimum duration of maturation varies based on cumulus morphology and time of recovery from the ovary, but there is no difference in developmental competence between Ex and Cp oocytes. Because standard in vitro fertilization is not repeatable in the horse, oocyte transfer (surgical transfer of oocytes to the oviducts of inseminated mares) has been developed to allow fertilization of isolated oocytes. Fertilization in vitro may be achieved using intracytoplasmic sperm injection; culture of injected oocytes in a medium with high glucose can yield over 30% blastocyst development.

*[The] meiotic and developmental competence [of horse oocytes] appears to be more dependent upon oocyte, follicle, and processing factors than those of other species.*

* Corresponding author:
TAMU 4466
College Station, TX 77843-4466.
E-mail: khinrichs@cvm.tamu.edu

*Mol. Reprod. Dev. 77: 651–661, 2010.* © *2010 Wiley-Liss, Inc.*

Received 31 October 2009; Accepted 8 March 2010

Published online 30 April 2010 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.21186

## INTRODUCTION

The physiology of follicle development and ovulation in the horse shows some interesting differences from those of other domestic species. The precise hormonal stimulation that induces ovulation in the mare is unknown. Unlike females of most other species, the mare does not experience a distinct luteinizing hormone (LH) peak; instead, LH levels rise gradually during estrus and reach their peak after ovulation, over a span of 8–10 days (Evans and Irvine, 1975; Geschwind et al., 1975). The stage of maturation of the equine oocyte at the time of ovulation was unclear for many years. In one of the first accounts of early embryonic development in the horse, Hamilton and Day (1945) reported on six recently ovulated, unfertilized oocytes found in the oviducts of pony and horse mares at slaughter. These authors

**Abbreviations:** CC, condensed chromatin; Ex/Cp oocyte, oocyte with an expanded/compacted cumulus; FN, fluorescent nucleus [configuration]; ICSI, intracytoplasmic sperm injection; IVM, in vitro maturation; LH, luteinizing hormone; MI/II, metaphase I/II of meiosis.

© 2010 WILEY-LISS, INC.

failed to find a polar body in the oocytes and suggested that the horse, like the dog, may ovulate primary oocytes. This suggestion was perpetuated until it became stated as a fact in textbooks (Hafez, 1974; Roberts, 1975). The situation was clarified by the report of Palmer et al. (1987) in which 15/19 oocytes recovered from the preovulatory follicles of mares 36 hr after administration of hCG or equine pituitary gonadotropins were found to be in metaphase II (MII); the remaining 4 were in metaphase I. It is possible that the post-ovulatory oocytes examined by Hamilton and Day (1945) had undergone polar body degeneration; this occurs in the horse as early as 96 hr after onset of maturation in vitro (Gable and Woods, 2001).

Unfertilized oocytes are retained within the oviduct in the mare (Van Niekerk and Gerneke, 1966). Betteridge et al. (1979) demonstrated that transport of the fertilized oocyte through the mare oviduct was dependant on cleavage. Equine embryos descend into the uterus on late day 5 (Freeman et al., 1991; Day 0 = the day that ovulation is detected). Equine embryos at this stage secrete prostaglandin E2 (Weber et al., 1991a) and this is hypothesized to be the signal for embryo transport into the uterus (Weber et al., 1991b).

Research on horse oocyte maturation, fertilization, and early embryonic development has been hampered by the difficulty in obtaining meaningful numbers of horse oocytes. The mare ovulates only one or, rarely, two follicles per 21-day cycle, and cannot be superovulated to any meaningful extent (McCue et al., 2007). The growing follicle in the horse is large, 39–45 mm at the time of ovulation (Palmer and Driancourt, 1980; Pierson and Ginther, 1985), and the number of visible antral follicles per ovary is low, averaging 6.5 follicles per ovary (Hinrichs, 1991). The supply of tissue from slaughterhouses is limited; notably, all horse slaughterhouses in the United States were closed in 2007. Recovery of horse oocytes from immature follicles in the mare, either from excised ovaries or from live mares, is complicated by the closer and stronger attachment of the equine oocyte–cumulus complex to the follicle wall. Comparison of the morphology of equine and bovine oocyte–cumulus complexes in situ showed that in the horse, the oocyte lies closer to the basement membrane, the cumulus hillock has a more obtuse angle with the mural granulosa and lacks fenestrations, and the cumulus cells have processes that extend into an edematous pad within the underlying theca, essentially anchoring the cumulus in place (Fig. 1; Hawley et al., 1995). As a result, needle aspiration of follicular fluid from follicles results in a low oocyte recovery rate and in preferential aspiration of oocytes from atretic follicles; oocytes that are recovered on aspiration are typically partially or completely denuded of cumulus (Hinrichs, 1991; Dell'Aquila et al., 2001). In contrast, scraping of the granulosa cell layer of opened follicles, followed by location of the oocyte within the recovered granulosa tissue, enables high recovery rates of oocytes with intact cumului (Hinrichs and DiGiorgio, 1991). This method also has the advantage that individual follicles may be evaluated in relation to the quality of the oocyte recovered, as the cumulus and granulosa cells associated with each collected oocyte are examined at the time of oocyte identification.

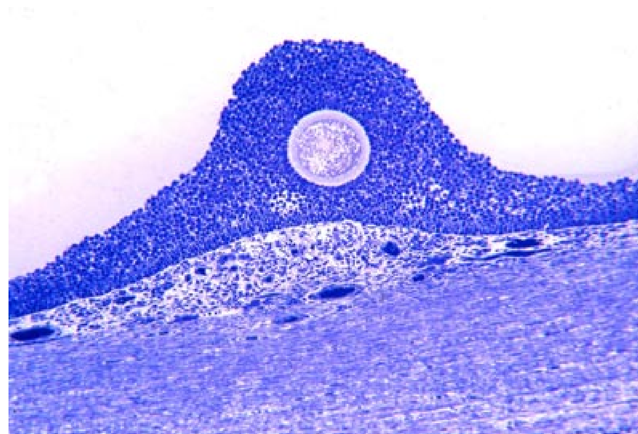

**Figure 1.** Horse oocyte within the follicle in situ, showing the few layers of granulosa between the oocyte and the follicle wall, the broad attachment of the cumulus hillock to the mural granulosa, without fenestrations, and the thecal pad underlying the oocyte, into which extend processes from the granulosa cells. From Hawley et al. (1995).

The tight attachment of the equine oocyte to the follicle wall also affects recovery rates on transvaginal follicle aspiration in live mares. These rates are commonly low when immature (i.e., not preovulatory) follicles are aspirated (12–25%; Brück et al., 1992; Cook et al., 1993; Duchamp et al., 1995; Mari et al., 2005). Relatively high oocyte recovery rates from immature equine follicles (45–60%) have been reported with use of aspiration techniques that include flushing of each follicle with medium up to eight times via a double-lumen aspiration needle with separate in- and outflow channels (Meintjes et al., 1995; Bøgh et al., 2002; Colleoni et al., 2007). In contrast, recovery rates of oocytes aspirated from the gonadotropin-stimulated dominant preovulatory follicle, within 12 hr of ovulation, are typically high (65–85%; Hinrichs et al., 1990; Carnevale and Ginther, 1995; Hinrichs et al., 2000; Carnevale et al., 2005). These higher recovery rates are due to cumulus expansion and loosening of the cumulus–oocyte complex from the follicle wall in preparation for ovulation.

## NUCLEAR MATURATION

In vitro maturation (IVM) of horse oocytes was first reported by Fulka and Okolski (1981); these authors achieved 68% maturation to MII. It is commonly stated that horse oocytes have a low rate of maturation in vitro, however, the meiotic competence of horse oocytes is dependent upon many follicular and technical factors, the most important of which are presented below. It should be remembered when comparing maturation rates of horse oocytes to those of other species such as cattle, that in other species strict selection of oocytes is performed before maturation is begun. In contrast, in horses, the low numbers of available oocytes tend to encourage culture of all oocytes that appear to be viable. In addition, some laboratories have selected horse oocytes for culture based on criteria used for selection

of bovine oocytes; as described below, this actually selects for viable, but meiotically incompetent, horse oocytes. Unfortunately, because of the failure of IVF in the horse, (review, Hinrichs et al., 2002a), factors affecting the developmental competence of in vitro-matured oocytes were difficult to define; these could not be efficiently determined until an effective method for fertilization, intracytoplasmic sperm injection (ICSI), was established (see the Developmental Competence Section).

An early perplexing finding regarding maturation of equine oocytes was that those having expanded cumuli at the time of recovery from the follicle (Ex oocytes) had higher rates of maturation than did oocytes having compact cumuli at the time of recovery (Cp oocytes). If oocytes found to be degenerating after culture were disregarded, maturation rates for the two oocyte types appeared to be similar (Hinrichs et al., 1993). However, we found that 65% of Cp oocytes degenerated during maturation culture, whereas only 13% of Ex oocytes degenerated (Alm and Hinrichs, 1996). This was reflected in the actual rates of maturation to MII per cultured oocyte—63% for Ex oocytes, but only 21% for Cp oocytes (Alm and Hinrichs, 1996). In cattle, oocytes with expanded cumuli are selected against, based on the finding that these oocytes originate from atretic follicles (Rajakoski, 1960; Blondin and Sirard, 1995). Similar selection would eliminate the majority of meiotically competent oocytes in the horse.

The chromatin configuration within the germinal vesicle also differed between Ex and Cp horse oocytes. We found that the majority of Cp oocytes had diffuse fluorescence within the germinal vesicle, whereas the majority of Ex oocytes had chromatin condensed into one small dense mass, which we designated the condensed chromatin (CC) configuration (Hinrichs et al., 1993; Fig. 2). While oocytes of other species also show condensation of chromatin around the nucleolus, associated with acquisition of meiotic competence (Daguet, 1980; Gougeon and Testart, 1986; Mattson and Albertini, 1990), the condensation seen in horse oocytes is more extreme, forming one dense mass around an apparent nucleolus, and appears to be similar to that reported in the human (Gougeon and Testart, 1986; Parfenov et al., 1989). The CC configuration has led to confusion as some reports state that at the time of collection from the follicle, the majority of Ex oocytes are in diakinesis (King et al., 1990), resumption of meiosis (Torner and Alm, 1995), or MI (Zhang et al., 1989). However, centrifugation of oocytes to allow visualization of the nuclear membrane indicates that oocytes having the CC configuration (Fig. 2) also have an intact germinal vesicle (Hinrichs et al., 1993). For comparison, photomicrographs of maturing horse oocytes are presented in Figure 3.

Ex oocytes not only mature in higher proportions than do Cp oocytes, they also resume meioisis more quickly and reach MII in a shorter period of time (Hinrichs et al., 1993; Alm and Hinrichs, 1996; Fig. 4). To understand the reasons behind the superior performance of Ex oocytes, we explored the relationship of cumulus and chromatin configuration to follicle atresia. Oocytes were recovered from individual follicles after part of the follicle wall had been sectioned for histological examination (Hinrichs and Williams, 1997).

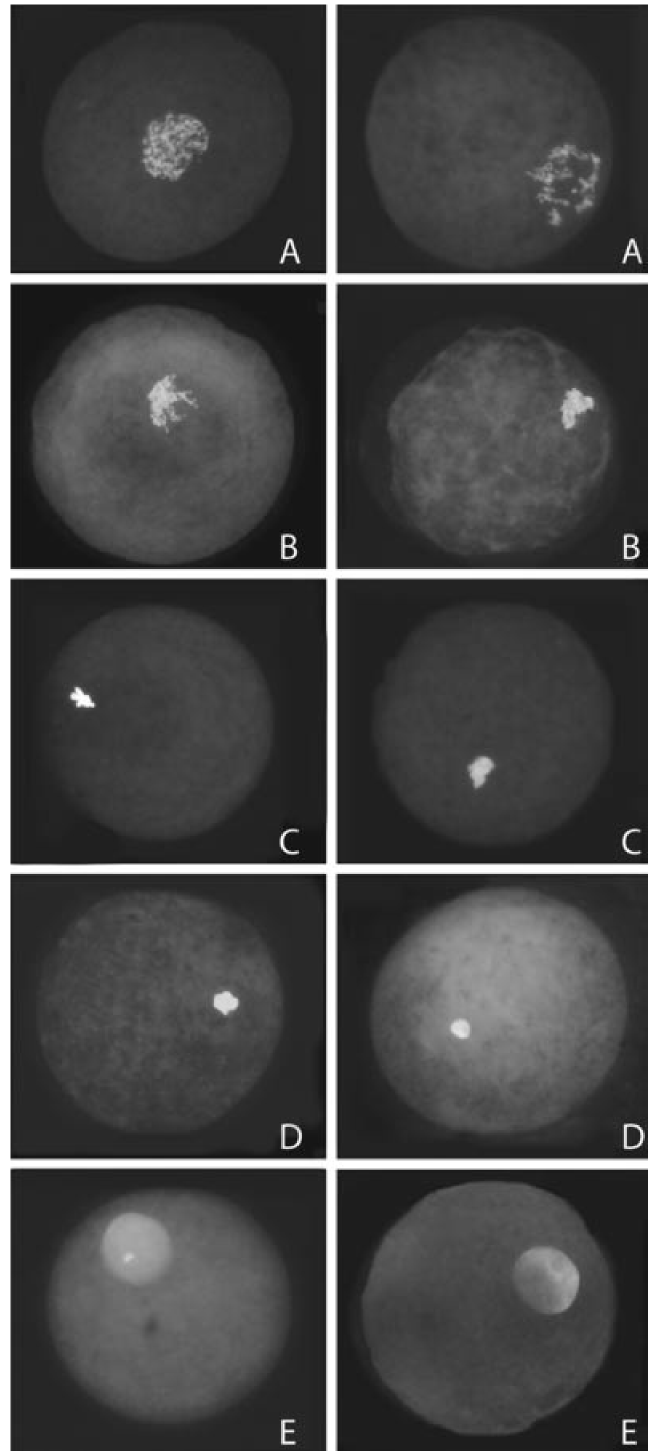


**Figure 2.** Depictions of entire horse oocytes stained with Hoechst 33258 and evaluated under fluorescence microscopy, demonstrating (**A**) fibrillar, (**B**) intermediate, (**C**) condensed chromatin (loose) (**D**) condensed chromatin (tight), and (**E**) fluorescent nucleus (FN) chromatin configurations. Two examples are shown of each configuration. The condensed chromatin configurations are associated with high meiotic competence; fibrillar and intermediate configurations may degrade during ovary storage to the FN configuration, which is not seen in oocytes recovered from the ovary immediately after slaughter, and which degenerates in culture.

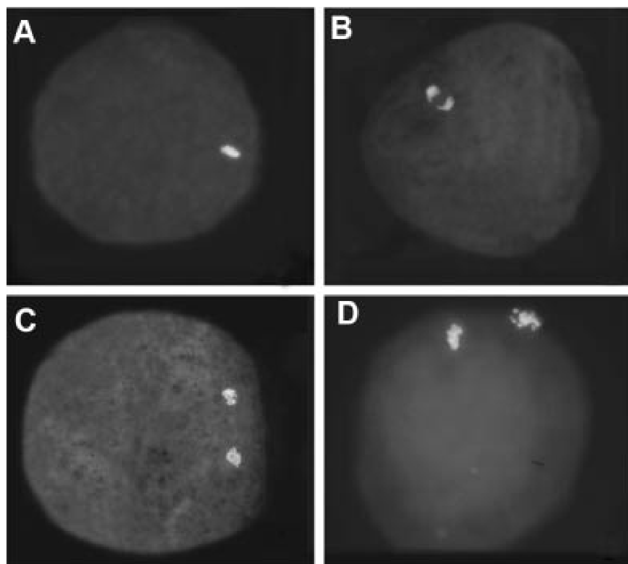


Figure 3. Depictions of maturing horse oocytes stained with Hoechst 33258 and evaluated under fluorescence microscopy. A: Metaphase I, (B) anaphase I, (C) telophase I, and (D) metaphase II.

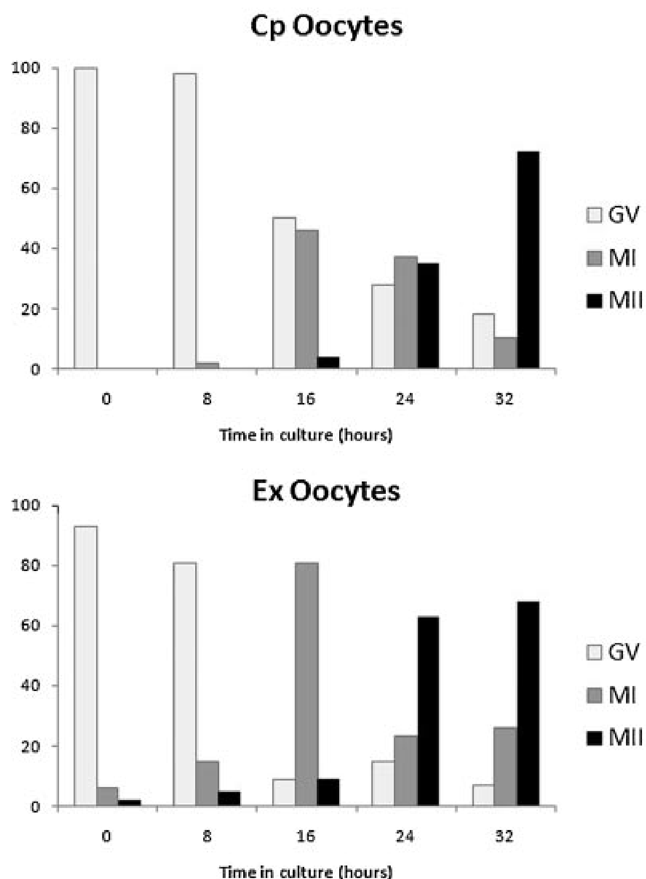


Figure 4. Kinetics of in vitro oocyte maturation in equine oocytes having compact cumuli (Cp oocytes) and expanded cumuli (Ex oocytes) at the time of recovery from the follicle. Only non-degenerating oocytes are included (data from Hinrichs et al., 1993).

Oocytes from viable follicles, as determined on histological section, had compact cumuli, a homogenous cytoplasm, diffusely fluorescent chromatin, and low rates of maturation in vitro, whereas oocytes from atretic follicles had expanded cumuli, separation of light and dark areas of the cytoplasm, CC, and matured in high proportions. The greatest maturation rate was obtained in oocytes from follicles in primary atresia (86%); however, even oocytes from follicles in tertiary atresia (end-stage atresia with degeneration of the granulosa cell layer and formation of a hyaline membrane) had higher rates of maturation in vitro than did oocytes from viable follicles (50% and 23%, respectively; Hinrichs and Williams, 1997). Direct examination of oocytes for chromatin configuration, followed by maturation, later confirmed the association of the CC configuration with meiotic competence (Hinrichs et al., 2005).

Examination of chromatin and cumulus parameters in relationship to follicle size revealed that Cp oocytes recovered from follicles $\leq$20 mm in diameter had mostly diffuse fluorescence within the GV, and low rates of maturation in vitro (30–43%), however, Cp oocytes recovered from follicles >20 mm diameter were mostly (80%) in the CC configuration, and 60% of these oocytes matured in culture. It appears that oocytes from viable follicles <20 mm diameter in the horse may be compared to oocytes from follicles <3 mm diameter in cattle—these oocytes are viable, but are juvenile and lack meiotic competence. Condensation of chromatin occurs as follicles reach the size at which deviation of the preovulatory follicle occurs in the mare (21–23 mm; Gastal et al., 1997). Unfortunately, fewer than 5% of all follicles processed during oocyte recovery were >20 mm diameter (Hinrichs and Schmidt, 2000). Thus, chromatin condensation into the dense CC configuration appears to be a physiological phenomenon associated with meiotic competence, seen in viable follicles as they reach preovulatory size, and in atretic follicles. In the latter group, the acquisition of meiotic competence associated with early atresia indicates that the early degenerating follicle first loses its ability to suppress the acquisition of meiotic competence in the enclosed oocyte; later in atresia, the follicle loses its ability to suppress meiosis itself and the oocyte resumes meiosis, as has been reported in other species (Rajakoski, 1960; Gougeon and Testart, 1986).

Meiotic competence of Ex oocytes also increased with increasing follicle size, however even Ex oocytes from the smallest follicles (1–5 mm) had higher maturation rates than did Cp oocytes until the Cp oocytes reached a follicle diameter over 20 mm (Hinrichs and Schmidt, 2000). In that study, we also examined the effect of season on recovery and maturation of horse oocytes; unexpectedly, season had no effect on the number of follicles processed per ovary, although the sizes of the follicles decreased during the nonbreeding season. When the effect of size on meiotic competence was taken into consideration, there was no effect of season on maturation rate. Thus, oocyte recovery and maturation from excised horse ovaries may be conducted without regard to season.

Examination of the cytoplasm of oocytes from atretic and viable follicles showed that oocytes from atretic follicles had uneven distribution of light and dark (presumably lipid) areas within the cytoplasm, whereas oocytes from viable follicles, having low meiotic competence, had homogenous cytoplasm (Hinrichs and Williams, 1997). Thus, to attain maximum rates of maturation in vitro, selection of horse oocytes should be based on presence of an expanded cumulus and an unevenly colored cytoplasm—criteria that are diametrically opposed to those used for selection of bovine oocytes in most laboratories.

In our laboratory, based on the above findings, we select for oocytes from early to mid-stage atresia by stringently categorizing any oocyte showing *any* expansion of either the granulosa or of the cumulus as Ex. The proportions of the different categories of oocytes, and their maturation rates, has been remarkably reproducible across studies: typically 60% of recovered oocytes are classified as Ex, 30% as Cp, and 10% as degenerating (Choi et al., 2002b, 2004a; Love et al., 2003; Hinrichs et al., 2005). These proportions have been replicated by our personnel in numerous settings. When proportions of Ex and Cp oocytes differ from the above in reports from other laboratories, typically in that the proportion of Cp oocytes increases to 50% or more, it can be inferred that oocytes having slight expansion of the cumulus or granulosa (and therefore would be categorized in our laboratory as Ex) are being included by the laboratory in question in the "compact" category. As oocytes with the mildest cumulus expansion represent those from early atretic follicles, that have the highest meiotic competence (Hinrichs and Williams, 1997), the category into which these oocytes are placed will greatly affect the results reported for the different cumulus categories.

The maturation rates in our laboratory are typically 65% for Ex oocytes and 20% for Cp oocytes (Choi et al., 2002b, 2004a; Love et al., 2003; Hinrichs et al., 2005). However, other laboratories have reported much higher rates of maturation of Cp oocytes (88%; Dell'Aquila et al., 1996; 63%; Carneiro et al., 2001). This variation could be related in part, to the criteria used for cumulus classification or to size of follicles processed, but this did not appear to totally explain the differences in maturation reported. Similarly, the proportion of Cp oocytes having diffuse fluorescence throughout the germinal vesicle (the fluorescent nucleus (FN) configuration) was repeatable over many studies in our laboratory, and has been reported by others (Pedersen et al., 2004), some laboratories have reported oocytes to have fibrillar chromatin throughout the GV (Goudet et al., 1997; Dell'Aquila et al., 2001) and have not reported the FN configuration. Interestingly, these laboratories recovered oocytes with little to no delay (recovered oocytes by transvaginal follicle aspiration of live mares, or had a limited transport period of ovaries from the abattoir before processing). The results detailed above in our laboratory stemmed from ovaries transported and held during processing so that there was a delay of 3–9 hr from death of the mare to oocyte recovery. Pedersen et al. (2004) reported an increased prevalence of the FN configuration when ovaries were held for extended periods before oocyte collection.

This led us to explore the effect of time for oocyte recovery (immediately after slaughter or delayed) on initial chromatin configuration and on maturation rate. We found that chromatin configuration and meiotic competence of horse oocytes changed during ovary storage. Compact-cumulus oocytes collected immediately after slaughter were in a fibrillar or intermediate chromatin configuration (Fig. 2). Oocytes collected after a delay had a significantly lower proportion of these configurations, and a significantly higher proportion in the FN configuration (Hinrichs et al., 2005). The FN configuration was found only in oocytes recovered from ovaries after a delay, and thus appeared to be an artifact of ovary storage. There was no effect of storage on the proportion of oocytes having the CC configuration, suggesting that the CC configuration was resistant to change during ovary storage, whereas the fibrillar and some intermediate configurations were labile and underwent degradation over time.

Maturation rates were significantly higher in Cp oocytes when these oocytes were recovered and placed into culture immediately versus after a delay (66% vs. 22%, Table 1; Hinrichs et al., 2005). Evaluation of meiotic competence of imaged oocytes showed that oocytes in the fibrillar configuration had low meiotic competence, but those in the intermediate configuration resumed meiosis at rates similar to that seen in oocytes with CC (Hinrichs et al., 2005). Our

**TABLE 1. Proportion of Ex and Cp Oocytes, Recovered From the Ovary Either Within 45 min of Slaughter or After a Delay, Reaching MI and MII After Maturation Culture for 24, 30, or 36 hr**

| Oocyte type | Treatment | Culture (hr) | n | MI | MII (%) | MI + II (%) |
|---|---|---|---|---|---|---|
| Cp | Immediate | 24 | 100 | 37 (37%)[a] | 25 (25%)[a] | 62 (62%)[a] |
| | | 30 | 53 | 5 (9%)[b] | 28 (53%)[b] | 33 (62%)[a] |
| | | 36 | 47 | 4 (9%)[b] | 31 (66%)[b] | 35 (74%)[a] |
| | Delay | 24 | 119 | 6 (5%)[b] | 34 (29%)[a] | 40 (34%)[b] |
| | | 30 | 158 | 5 (3%)[b] | 33 (21%)[a] | 38 (24%)[b] |
| | | 36 | 136 | 5 (4%)[b] | 43 (32%)[a] | 48 (35%)[b] |
| Ex | Immediate | 24 | 227 | 110 (48%)[d] | 74 (33%)[d] | 184 (81%)[d,e,f] |
| | | 30 | 123 | 18 (15%)[e] | 89 (72%)[e,f] | 107 (87%)[d] |
| | | 36 | 128 | 6 (5%)[f,g] | 102 (80%)[e] | 108 (84%)[d,e] |
| | Delay | 24 | 114 | 9 (8%)[e,f] | 77 (68%)[f] | 86 (75%)[e,f] |
| | | 30 | 115 | 4 (3%)[f,g] | 82 (71%)[e,f] | 86 (75%)[e,f] |
| | | 36 | 149 | 1 (1%)[g] | 108 (72%)[e,f] | 109 (73%)[f] |

From Hinrichs et al. (2005). Within columns within oocyte type, values with different superscripts differ significantly ($P < 0.05$).

conclusions were that intermediate configuration oocytes have high meiotic competence if cultured immediately, but appear to degrade during ovary storage, leading to a lower maturation rate if oocytes are collected from ovaries after a delay. The time of recovery also changed the kinetics of meiosis of the oocytes; oocytes which were recovered and cultured immediately post-mortem exhibited a significantly lower proportion of MII oocytes and a larger proportion in MI at 24 hr culture than did oocytes recovered after a delay. This suggests that prematuration changes may occur in horse oocytes held within the ovary, which speed maturation of the oocytes once they are placed in culture. However, maturation does not start during this ovary holding period, as oocytes collected from ovaries after a delay may be held in the germinal vesicle stage for an additional 18 hr by various methods (Choi et al., 2006a).

Overall, the above findings indicate that meiotic competence of horse oocytes is dependent upon initial cumulus configuration, size of the follicle from which the oocyte was recovered, and the period of time the oocyte is in the ovary before recovery. The duration of culture required for maximum rates of maturation is also dependent upon cumulus morphology and the period of time within the ovary. This helps to explain the variation in reported rates of equine oocyte maturation among laboratories; those using oocytes cultured immediately after recovery from the live mare may have very different findings from those working with oocytes from ovaries transported for 5 hr before recovery.

Equine oocytes may be obtained from the preovulatory follicles of mares after gonadotropin stimulation; in this case essentially 100% of recovered oocytes are meiotically competent. Oocytes recovered from the preovulatory follicle 24 hr after gonadotropin stimulation were in MI, and those recovered 35 hr after gonadotropin stimulation were in MII (Bézard et al., 1997).

Some information is available on molecular aspects of equine oocyte maturation. The period of protein synthesis required before the onset of maturation appears to be 8 hr in Ex oocytes and 12 hr in Cp oocytes (Alm and Hinrichs, 1996). Similar to other species, maturation promoting factor content and activity increase during IVM in the horse (Goudet et al., 1998a,b). MAP kinase is present in maturing equine oocytes, but remains in its non-phosporylated form in meiotically incompetent oocytes (Goudet et al., 1998a). Histone kinase activity is higher in oocytes that reach MII in vivo than in those that are matured in vitro (Goudet et al., 1998a). Studies on microfilament and microtubular anatomy of both in vivo and in vitro-matured oocytes have been conducted (Goudet et al., 1997; Tremoleda et al., 2001; Siddiqui et al., 2009); changes in these structures during maturation mirror those found in other mammalian species. The spindle of MII in vivo-matured oocytes appears to differ from that of other mammals in that it is not perpendicular to the oolemma (Siddiqui et al., 2009). Micro-opioid receptor proteins were detected in equine oocytes, cumulus, and granulosa cells, and appeared to vary with season (Dell'Aquila et al., 2008). Calcium-sensing receptor mRNA and protein have been identified in equine oocytes and cumulus cells, and stimulation of the receptor may influence nuclear maturation of Ex oocytes, although in this study, maturation of control Ex oocytes were low (38%; De Santis et al., 2009). Growth hormone receptor mRNA was detected in equine oocytes and cumulus cells, and addition of growth hormone during maturation culture appeared to increase maturation rates in serum-free medium (Marchal et al., 2003). These authors also detected connexin43 protein in equine cumulus cells. Oct-4 protein is present in both immature and mature horse oocytes (Choi et al., 2009). Oct-4 tends to accumulate in the nucleus of immature oocytes, but is not associated with chromatin at this stage.

## DEVELOPMENTAL COMPETENCE

It is only within the last few years that evaluation of the developmental potential of horse oocytes has been feasible. Conventional in vitro fertilization has not been repeatably successful, although recent findings on the induction of hyperactivated motility to achieve fertilization may provide a mechanism for this in the future (McPartlin et al., 2009). Because of this, methods for achieving in vivo fertilization after transfer of oocytes to the oviducts of inseminated recipient mares have been developed, and these have been used to explore the physiology of fertilization in this species (Carnevale and Ginther, 1995; Carnevale et al., 2001; Maclellan et al., 2002).

However, it was not until the development of effective methods for ICSI that in vitro evaluation of oocyte developmental competence could be performed. The first reports of fertilization after ICSI in the horse were published in short communications in 1995 (Cho et al., 1995; Dell'Aquila et al., 1995). The first successful pregnancy, after surgical oviductal transfer of sperm-injected oocytes, was reported by Squires et al. (1996) and the first in vitro produced blastocyst reported by Dell'Aquila et al. (1997). Subsequent studies reported fertilization rates after ICSI of around 50% (Grondahl et al., 1997; Dell'Aquila et al., 1999, 2001), increasing when chemical activation stimulus was applied (Li et al., 2000), and rates of cleavage of 16–65% were reported (Grondahl et al., 1997; Cochran et al., 1998; Guignot et al., 1998; Li et al., 2000, 2001). Multiple pregnancies and birth of foals was reported after surgical oviductal transfer of early ICSI-produced embryos (McKinnon et al., 2000). However, reproducible high rates of embryonic development (>75%) were not reported until use of the Piezo drill for equine ICSI, around 2002 (Choi et al., 2002a; Galli et al., 2002; Lazzari et al., 2002). Efficient in vitro blastocyst production (>30%) was first reported using the Piezo drill and a high-glucose medium for embryo culture, in 2005 (Hinrichs et al., 2005)

We used IVM, ICSI, and in vitro culture to determine the developmental potential of different classes of oocytes (Hinrichs et al., 2005). Blastocyst development varied with cumulus morphology, the time of collection of the oocyte from the ovary (immediate or delayed) and the duration of IVM. Table 2 shows the inter-related effects of these parameters; one notable finding is that oocytes collected from the ovary immediately after slaughter required a longer duration of maturation for optimum developmental competence, and yet still had lower developmental competence, than did oocytes recovered after a delay (Hinrichs et al., 2005).

**TABLE 2. Embryo Development at 7.5 Days After ICSI of Oocytes Recovered From the Ovary Either Immediately After Slaughter or After a Delay and Matured for 24, 30, or 36 hr Before ICSI**

| Oocyte type | Treatment | Culture (hr) | n | Cleavage rate | Morulae (%) | Blastocysts (%) | Morulae + Blastocysts (%) |
|---|---|---|---|---|---|---|---|
| Cp | Immediate | 24 | 23 | 19 (83%) | 3 (13%) | 2 (9%)[a] | 5 (22%) |
| | | 30 | 28 | 25 (89%) | 1 (4%) | 5 (18%)[a,b,c] | 6 (21%) |
| | | 36 | 31 | 25 (81%) | 1 (3%) | 7 (23%)[a,b,c] | 8 (26%) |
| | Delay | 24 | 32 | 22 (69%) | 1 (3%) | 4 (13%)[a,b] | 5 (16%) |
| | | 30 | 31 | 24 (77%) | 1 (3%) | 11 (35%)[b,c] | 12 (39%) |
| | | 36 | 37 | 33 (89%) | 0 | 14 (38%)[c] | 14 (38%) |
| Ex | Immediate | 24 | 73 | 60 (82%) | 8 (11%) | 8 (11%)[d] | 16 (22%) |
| | | 30 | 87 | 69 (79%) | 4 (5%) | 15 (17%)[d,e] | 19 (22%) |
| | | 36 | 91 | 76 (84%) | 3 (3%) | 16 (18%)[d,e] | 19 (21%) |
| | Delay | 24 | 75 | 64 (85%) | 7 (9%) | 22 (29%)[e,f] | 29 (39%) |
| | | 30 | 81 | 69 (85%) | 8 (10%) | 26 (32%)[f] | 34 (42%) |
| | | 36 | 105 | 88 (84%) | 8 (8%) | 28 (27%)[e,f] | 36 (34%) |

From Hinrichs et al. (2005). Within columns within oocyte type, values with different superscripts differ significantly ($P < 0.05$).

Holding of ovaries before oocyte recovery has been shown to increase blastocyst formation after IVM/IVF in cattle (Blondin et al., 1997). There was no difference in developmental competence (rate of blastocyst development) between Cp and Ex oocytes. These findings relate not only to collection of oocytes from slaughterhouse tissue for research, but also to handling of oocytes in a commercial situation, both of immature oocytes recovered ex vivo, for example, by transvaginal ultrasound-guided follicle aspiration, and of ovaries recovered from valuable mares after untimely death. Results from our slaughterhouse study suggest that immediate placement of these oocytes into maturation culture could result in lower developmental competence than that which might be obtained if there was a delay before culture. More research examining these possibilities is needed.

After collection, equine oocytes may be held in meiotic arrest for up to 18 hr in a simple medium (EH medium; modified M199 with 20% serum) at room temperature before placing them into maturation culture, with no detrimental effect on maturation rate or blastocyst development rate after ICSI (Choi et al., 2006a). This is a great aid in scheduling the onset of maturation, and therefore subsequent manipulations such as ICSI or nuclear transfer. Culture in the presence of the meiotic inhibitors cycloheximide, roscovitine, or butyrolactone can also suppress maturation (Alm and Hinrichs, 1996; Hinrichs et al., 2002b; Franz et al., 2003), but appear to have a detrimental effect on subsequent developmental competence (Choi et al., 2006a,b).

The finding that maturation rate, maturation kinetics, and oocyte developmental competence vary with initial cumulus morphology (which in itself may depend upon the criteria used for classification), time of collection of oocytes from the ovary, and duration of maturation make it difficult to compare maturation and developmental results among laboratories, or to interpret studies that examine the effects of specific technical factors, such as makeup of maturation media, on developmental competence. In addition, to our knowledge only five laboratories have reported effective rates (>15%) of equine blastocyst development in vitro (Li et al., 2001; Hinrichs et al., 2005; Galli et al., 2007; Matsukawa et al., 2007; Altermatt et al., 2009). This has greatly limited the information available on specific media factors affecting developmental competence of horse oocytes. Different laboratories employ different maturation media, typically based on M199 but with various additives; for example, in one report, maturation medium was M199 with added bicarbonate, glutamine, sodium pyruvate, calcium-L-lactate penthahydrate, FSH, LH, and estradiol, with 20% fetal bovine serum (Lange Consiglio et al., 2009). Galli et al. (2007) reported a higher blastocyst development rate in their laboratory for oocytes matured in modified DMEM/F-12 medium than in oocytes matured in modified M199 (26% vs. 12%, respectively). However, currently the highest published rates of in vitro blastocyst production (38–42%) from in vitro-matured oocytes have resulted from oocytes matured in a relatively straightforward medium of M199 with 10% fetal bovine serum and 5 mU/ml bovine FSH (Hinrichs et al., 2005; Choi et al., 2007; Ribeiro et al., 2008).

The influence of specific medium factors on nuclear maturation and developmental competence of horse oocytes is not well elucidated. Addition of IGF-1 appeared to increase embryonic development after parthenogenetic activation (Carneiro et al., 2001). The effect of EGF on oocyte nuclear maturation was equivocal (Goudet et al., 2000). Interleukin-1 appeared to induce meiosis and ovulation when injected into the preovulatory follicle in vivo, but depressed pregnancy rates (Caillaud et al., 2005). Similarly, interleukin-1 depressed fertilization rates and embryo development in vitro (Caillaud et al., 2008). Addition of cysteamine to maturation medium did not affect glutathione content of oocytes or rate of oocyte maturation, and maturation rate was not associated with glutathione content (Luciano et al., 2006). Addition of naloxone had a differential effect on in vitro oocyte maturation dependent upon season and the concentration of naloxone (Dell'Aquila et al., 2008). Culture in the presence of leptin appeared to increase maturation rates; however, rates in control Ex oocytes in this study were low (44%; Lange Consiglio et al., 2009). None of the above studies evaluated development of embryos to the blastocyst stage, thus more work is needed to determine what medium factors during oocyte maturation have a significant effect on developmental competence.

Oocytes recovered ex vivo from the preovulatory follicle ≥24 hr after gonadotropin stimulation (administration of hCG or a GnRH analog) may be cultured in medium without gonadotropins until the expected time of ovulation (i.e., 40 hr post-gonadotropin administration) without apparent loss of viability; oocytes so treated provide a ≥75% pregnancy rate after transfer to the oviducts of inseminated mares (Carnevale and Ginther, 1995; Hinrichs et al., 1998), and this rate was not different from that achieved after insemination of ovulating mares (Carnevale et al., 2004). There was no difference in pregnancy rate between oocytes recovered from the dominant preovulatory follicle 24 hr after gonadotropin administration and cultured for 12 hr before transfer and those recovered at 35 hr and cultured for 1 hr before transfer (Hinrichs et al., 2000). Similar oviductal transfer of IVM oocytes, originating from immature oocytes recovered either by transvaginal follicle aspiration ex vivo or from slaughterhouse tissue, yielded a <20% pregnancy rate (Scott et al., 2001; Preis et al., 2004), although the rate per fertilizable (MII) oocyte may be up to 100% higher in actuality because the maturation status of the oocytes at the time of transfer was unknown.

The difference in pregnancy rate after transfer to the oviduct demonstrates the lower developmental competence of IVM equine oocytes in comparison to those from the stimulated preovulatory follicle (in vivo-matured). In contrast, blastocyst rates after ICSI and in vitro culture do not appear to be markedly higher for preovulatory (in vivo-matured) oocytes (29%, Altermatt et al., 2009; 41%, Jacobson et al., 2010) than for IVM oocytes (25–35%, Hinrichs et al., 2005; Choi et al., 2007; Ribeiro et al., 2008; Jacobson et al., 2010). Transfer of in vitro-matured, sperm-injected oocytes to the oviduct of recipient mares yielded 39% blastocyst development (Choi et al., 2004b). These findings suggest that IVM oocytes are achieving near-optimal developmental ability in the current in vitro embryo production systems, whereas in vivo-matured oocytes are achieving only half of their potential. This is an area that needs further investigation.

Rates of blastocyst development are also, of course, dependent upon the culture system used. Early ICSI reports used culture in the oviducts of sheep to obtain blastocyst formation (Galli et al., 2002; Lazzari et al., 2002); blastocyst development in vitro was disappointing (Li et al., 2001; Lazzari et al., 2002; Choi et al., 2003). However, in 2004 we found that the equine embryo appears to require high concentrations of glucose in culture. Blastocyst development rates were significantly higher in embryos cultured in DMEM/F-12 medium that contains 17 mM glucose than in G1/G2 medium that contain 0.5/5.5 mM glucose (Choi et al., 2004b). Use of a mixed-gas environment (5% $O_2$, 5%$CO_2$, and 90% $N_2$) allowed development of >30% of sperm-injected oocytes into blastocysts in DMEM/F-12 medium with 10% serum (Hinrichs et al., 2005). Culture in a modified SOF until Day 5, followed by transfer to DMEM/F-12, also supports blastocyst development (C. Galli, personal communication, 2009).

Transfer of in vitro-produced embryos yielded a normal pregnancy rate (15/18 (83%), Colleoni et al., 2007; 5/10 (50%), Hinrichs et al., 2007), but the rate of early embryo loss was high in these reports (33–40%). This may resolve with improvements in embryo handling; recently we achieved a pregnancy rate of 12/17 (71%) after transfer of in vitro-produced embryos, and all but one pregnancy progressed normally to the heartbeat stage (Hinrichs and Choi, unpublished work).

In summary, horse oocytes are difficult to obtain in large numbers. Their meiotic and developmental competence appears to be more dependent upon oocyte, follicle, and processing factors than those of other species. In vitro fertilization is not yet reliably replicated, and thus in vitro studies of developmental competence must be performed after ICSI, limiting the number of laboratories able to work in this area. If horse oocytes are effectively selected and handled (e.g., Ex oocytes obtained from ovaries after a delay), maturation rates of 70% and blastocyst development rates of 30% or more may be achieved (Hinrichs et al., 2005). Interest in this area clinically should lead to new findings and improved methods for in vitro equine embryo production in the near future.

## ACKNOWLEDGMENTS

The work in the author's laboratory was supported in part by the Link Equine Research Fund, Texas A&M University.

## REFERENCES

Alm H, Hinrichs K. 1996. Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology. J Reprod Fertil 107:215–220.

Altermatt JL, Suh TK, Stokes JE, Carnevale EM. 2009. Effects of age and equine follicle-stimulating hormone (eFSH) on collection and viability of equine oocytes assessed by morphology and developmental competency after intracytoplasmic sperm injection (ICSI). Reprod Fertil Dev 21:615–623.

Betteridge KJ, Eaglesome MD, Flood PF. 1979. Embryo transport through the mare's oviduct depends upon cleavage and is independent of the ipsilateral corpus luteum. J Reprod Fertil (Suppl) 27:387–394.

Bézard J, Mekarska A, Goudet G, Duchamp G, Palmer E. 1997. Timing of in vivo maturation of equine preovulatory oocytes and competence for in vitro maturation of immature oocytes collected simultaneously. Equine Vet J (Suppl) 25:33–37.

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 41:54–62.

Blondin P, Coenen K, Guilbault LA, Sirard MA. 1997. In vitro production of bovine embryos: Developmental competence is acquired before maturation. Theriogenology 47:1061–1075.

Bøgh IB, Bézard J, Duchamp G, Baltsen M, Gérard N, Daels P, Greve T. 2002. Pure preovulatory follicular fluid promotes in vitro maturation of in vivo aspirated equine oocytes. Theriogenology 57:1765–1779.

Brück I, Raun K, Synnestvedt B, Greve T. 1992. Follicle aspiration in the mare using a transvaginal ultrasound-guided technique (short communication). Equine Vet J 24:58–59.

Caillaud M, Duchamp G, Gérard N. 2005. In vivo effect of interleukin-1beta and interleukin-1RA on oocyte cytoplasmic maturation, ovulation, and early embryonic development in the mare. Reprod Biol Endocrinol 3:26.

Caillaud M, Dell'Aquila ME, De Santis T, Nicassio M, Lacalandra GM, Goudet G, Gérard N. 2008. In vitro equine oocyte maturation in pure follicular fluid plus interleukin-1 and fertilization following ICSI. Anim Reprod Sci 106:431–439.

Carneiro G, Lorenzo P, Pimentel C, Pegoraro L, Bertolini M, Ball B, Anderson G, Liu I. 2001. Influence of insulin-like growth factor-1 and its interaction with gonadotropins, estradiol, and fetal calf serum on in vitro maturation and parthenogenetic development in equine oocytes. Biol Reprod 65:899–905.

Carnevale EM, Ginther OJ. 1995. Defective oocytes as a cause of subfertility in old mares. Biol Reprod Monograph 1:209–214.

Carnevale EM, Maclellan LJ, Coutinho da Silva MA, Checura CM, Scoggin CF, Squires EL. 2001. Equine sperm-oocyte interaction: Results after intraoviductal and intrauterine inseminations of recipients for oocyte transfer. Anim Reprod Sci 68:305–314.

Carnevale EM, Coutinho da Silva MA, Maclellan LJ, Seidel GE, Jr., Squires EL. 2004. Use of parentage testing to determine optimum insemination time and culture media for oocyte transfer in mares. Reproduction 128:623–628.

Carnevale EM, Coutinho da Silva MA, Panzani D, Stokes JE, Squires EL. 2005. Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. Theriogenology 64:519–527.

Cho YS, Dell'Aquila ME, Minoia P, Traina V. 1995. Intracytoplasmic sperm injection (ICSI) of in vitro matured equine oocytes. J Assist Reprod Genet 12:181S.

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

Choi YH, Shin T, Love CC, Johnson CA, Varner DD, Westhusin ME, Hinrichs K. 2002b. 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, Varner DD, Love LB, Hinrichs K. 2003. 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.

Choi YH, Love LB, Varner DD, Hinrichs K. 2004a. Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. Reproduction 127:187–194.

Choi YH, Roasa LM, Love CC, Varner DD, Brinsko SP, Hinrichs K. 2004b. Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. Biol Reprod 70:1231–1238.

Choi YH, Love LB, Varner DD, Hinrichs K. 2006a. Holding immature equine oocytes in the absence of meiotic inhibitors: Effect on germinal vesicle chromatin and blastocyst development after intracytoplasmic sperm injection. Theriogenology 66:955–963.

Choi YH, Love LB, Varner DD, Hinrichs K. 2006b. Blastocyst development in equine oocytes with low meiotic competence after suppression of meiosis with roscovitine prior to in vitro maturation. Zygote 14:1–8.

Choi YH, Love LB, Varner DD, Hinrichs K. 2007. Effect of holding technique and culture drop size in individual or group culture on blastocyst development after ICSI of equine oocytes with low meiotic competence. Anim Reprod Sci 102: 38–47.

Choi YH, Harding HD, Hartman DL, Obermiller AD, Kurosaka S, McLaughlin KJ, Hinrichs K. 2009. The uterine environment modulates trophectodermal POU5F1 levels in equine blastocysts. Reproduction 138:589–599.

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. J Equine Vet Sci 18:736–740.

Colleoni S, Barbacini S, Necci D, Duchi R, Lazzari G, Galli C. 2007. Application of ovum pick-up, intracytoplasmic sperm injection and embryo culture in equine practice. Proc Am Assoc Equine Pract 53:554–559.

Cook NL, Squires EL, Ray BS, Cook VM, Jasko DJ. 1993. Transvaginal ultrasound-guided follicular aspiration of equine oocytes. Equine Vet J (Suppl) 15:71–74.

Daguet MCY. 1980. In vivo change in the germinal vesicle of the sow oocyte during the follicular phase before the ovulatory LH surge. Reprod Nutr Dev 20:673–680.

De Santis T, Casavola V, Reshkin SJ, Guerra L, Ambruosi B, Fiandanese N, Dalbies-Tran R, Goudet G, Dell'Aquila ME. 2009. The extracellular calcium-sensing receptor is expressed in the cumulus-oocyte complex in mammals and modulates oocyte meiotic maturation. Reproduction 138:439–452.

Dell'Aquila ME, Cho YS, Fusco S, Lacalandra GM, Maritato F, Minoia P, Traina V. 1995. Fertilization rates of in vitro matured equine oocytes: In vitro fertilization vs. intracytoplasmic sperm injection. In: Enne G, Greppi GF, Lauria A, editors. Reproduction and animal breeding: Advances and strategy. Paris:Elsevier, pp 365–366.

Dell'Aquila ME, Fusco S, Lacalandra GM, Maritato F. 1996. In vitro maturation and fertilization of equine oocytes recovered during the breeding season. Theriogenology 45:547–560.

Dell'Aquila ME, Cho YS, Minoia P, Traina V, Lacalandra GM, Maritato F. 1997. 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. Hum Reprod 12:2766–2772.

Dell'Aquila ME, De Felici M, Massari S, Maritato F, Minoia P. 1999. Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured in vitro. Biol Reprod 61: 533–540.

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. Mol Reprod Dev 60:79–88.

Dell'Aquila ME, Albrizio M, Guaricci AC, De Santis T, Maritato F, Tremoleda JL, Colenbrander B, Guerra L, Casavola V, Minoia P. 2008. Expression and localization of the mu-opioid receptor (MOR) in the equine cumulus-oocyte complex and its involvement in the seasonal regulation of oocyte meiotic competence. Mol Reprod Dev 75:1229–1246.

Duchamp G, Bézard J, Palmer E. 1995. Oocyte yield and the consequences of puncture of all follicles larger than 8 millimetres in mares. Biol Reprod Mono 1:233–241.

Evans MJ, Irvine CHG. 1975. Serum concentratrions of FSH, LH and progesterone during the oestrous cycle and early pregnancy in the mare. J Reprod Fertil (Suppl) 23:193–200.

Franz LC, Choi YH, Squires EL, Seidel GE, 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.

Freeman DA, Weber JA, Geary RT, Woods GL. 1991. Time of embryo transport through the mare oviduct. Theriogenology 36:823–830.

Fulka J, Okolski A. 1981. Culture of horse oocytes in vitro. J Reprod Fertil 61:213–215.

Gable TL, Woods GL. 2001. Increasing culture time from 48 to 96 or 144 hours increases the proportions of equine cumulus oocyte complexes with negative or fragmented nucleus morphology. Theriogenology 55:1549–1560.

Galli C, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, Duchamp G, Daels P, Lazzari G. 2002. 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, Colleoni S, Duchi R, Lagutina I, Lazzari G. 2007. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim Reprod Sci 98:39–55.

Gastal EL, Gastal MO, Bergfelt DR, Ginther OJ. 1997. Role of diameter differences among follicles in selection of a future dominant follicle in mares. Biol Reprod 57:1320–1327.

Geschwind II, Dewey R, Hughes JP, Evans JW. 1975. Plasma LH levels in the mare during the oestrous cycle. J Reprod Fertil (Suppl) 23:207–212.

Goudet G, Bézard J, Duchamp G, Gérard N, Palmer E. 1997. Equine oocyte competence for nuclear and cytoplasmic in vitro maturation: Effect of follicle size and hormonal environment. Biol Reprod 57:232–245.

Goudet G, Belin F, Bézard J, Gérard N. 1998a. Maturation-promoting factor (MPF) and mitogen activated protein kinase (MAPK) expression in relation to oocyte competence for in-vitro maturation in the mare. Mol Hum Reprod 4:563–570.

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

Goudet G, Belin F, Mlodawska W, Bézard J. 2000. Influence of epidermal growth factor on in vitro maturation of equine oocytes. J Reprod Fertil (Suppl) 56:483–492.

Gougeon A, Testart J. 1986. Germinal vesicle breakdown in oocytes of human atretic follicles during the menstrual cycle. J Reprod Fertil 78:389–401.

Grondahl C, Hansen TH, Hossaini A, Heinze I, Greve T, Hyttel P. 1997. Intracytoplasmic sperm injection of in vitro-matured equine oocytes. Biol Reprod 57:1495–1501.

Guignot F, Ottogalli M, Yvon JM, Magistrini M. 1998. Preliminary observations in in vitro development of equine embryo after ICSI. Reprod Nutr Dev 38:653–663.

Hafez ESE. 1974. Reproduction in farm animals. Philadelphia, PA:Lea and Farbiger.

Hamilton WJ, Day FT. 1945. Cleavage stages of the ova of the horse, with notes on ovulation. J Anat 79:127–130.

Hawley LR, Enders AC, Hinrichs K. 1995. Comparison of equine and bovine oocyte-cumulus morphology within the ovarian follicle. Biol Reprod Mono 1:243–252.

Hinrichs K. 1991. The relationship of follicle atresia to follicle size, oocyte recovery rate on aspiration, and oocyte morphology in the mare. Theriogenology 36:157–168.

Hinrichs K, DiGiorgio LM. 1991. Embryonic development after intrafollicular transfer of equine oocytes. J Reprod Fertil (Suppl) 44:369–374.

Hinrichs K, Schmidt AL. 2000. Meiotic competence in horse oocytes: Interactions among chromatin configuration, follicle size, cumulus morphology, and season. Biol Reprod 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. Biol Reprod 57:377–384.

Hinrichs K, Kenney DF, Kenney RM. 1990. Aspiration of oocytes from mature and immature preovulatory follicles in the mare. Theriogenology 34:107–112.

Hinrichs K, Schmidt AL, Friedman PP, Selgrath JP, Martin MG. 1993. In vitro maturation of horse oocytes: Characterization of chromatin configuration using fluorescence microscopy. Biol Reprod 48:363–370.

Hinrichs K, Matthews GL, Freeman DA, Torello EM. 1998. Oocyte transfer in mares. J Am Vet Med Assoc 212:982–986.

Hinrichs K, Betschart RW, McCue PM, Squires EL. 2000. Effect of timing of follicle aspiration on pregnancy rate after oocyte transfer in mares. J Reprod Fertil (Suppl) 56:493–498.

Hinrichs K, Love CC, Brinsko SP, Choi YH, Varner DD. 2002a. In vitro fertilization of in vitro-matured equine oocytes: Effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization in vivo after oviductal transfer. Biol Reprod 67:256–262.

Hinrichs K, Love CC, Choi YH, Varner DD, Wiggins CN, Reinoehl C. 2002b. Suppression of meiosis by inhibitors of m-phase proteins in horse oocytes with low meiotic competence. Zygote 10:37–45.

Hinrichs K, Choi YH, Love LB, Varner DD, Love CC, Walckenaer BE. 2005. Chromatin configuration within the germinal vesicle of horse oocytes: Changes post mortem and relationship to meiotic and developmental competence. Biol Reprod 72:1142–1150.

Hinrichs K, Choi YH, Walckenaer BE, Varner DD, Hartman DL. 2007. In vitro-produced equine embryos: Production of foals after transfer, assessment by differential staining, and effect of medium calcium concentrations during culture. Theriogenology 68:521–529.

Jacobson CC, Choi YH, Hayden SS, Hinrichs K. 2010. Recovery of mare oocytes on a fixed biweekly schedule, and resulting blastocyst formation after intracytoplasmic sperm injection. Theriogenology 73:1116–1126.

King WA, Desjardins M, Xu KP, Bousquet D. 1990. Chromosome analysis of horse oocytes cultured in vitro. Genet Sel Evol 22:151–160.

Lange Consiglio A, Dell'Aquila ME, Fiandanese N, Ambruosi B, Cho YS, Bosi G, Arrighi S, Lacalandra GM, Cremonesi F. 2009. Effects of leptin on in vitro maturation, fertilization and embryonic cleavage after ICSI and early developmental expression of leptin (Ob) and leptin receptor (ObR) proteins in the horse. Reprod Biol Endocrinol 7:113.

Lazzari G, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, Barbacini S, Galli C. 2002. Equine embryos at the compacted morula and

blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. Theriogenology 58:709–712.

Li X, Morris LH, Allen WR. 2000. Effects of different activation treatments on fertilization of horse oocytes by intracytoplasmic sperm injection. J Reprod Fertil 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.

Luciano AM, Goudet G, Perazzoli F, Lahuec C, Gérard N. 2006. Glutathione content and glutathione peroxidase expression in in vivo and in vitro matured equine oocytes. Mol Reprod Dev 73:658–666.

Maclellan LJ, Carnevale EM, Coutinho da Silva MA, Scoggin CF, Bruemmer JE, Squires EL. 2002. Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology 58:911–919.

Marchal R, Caillaud M, Martoriati A, Gérard N, Mermillod P, Goudet G. 2003. Effect of growth hormone (GH) on in vitro nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. Biol Reprod 69:1013–1022.

Mari G, Merlo B, Iacono E, Belluzzi S. 2005. Fertility in the mare after repeated transvaginal ultrasound-guided aspirations. Anim Reprod Sci 88:299–308.

Matsukawa K, Akagi S, Adachi N, Sato F, Hasegawa T, Takahashi S. 2007. In vitro development of equine oocytes from preserved ovaries after intracytoplasmic sperm injection. J Reprod Dev 53:877–885.

Mattson BA, Albertini DF. 1990. Oogenesis: Chromatin and microtubule dynamics during meiotic prophase. Mol Reprod Dev 25:374–383.

McCue PM, LeBlanc MM, Squires EL. 2007. eFSH in clinical equine practice. Theriogenology 68:429–433.

McKinnon AO, Lacham-Kaplan O, Trounson AO. 2000. Pregnancies produced from fertile and infertile stallions by intracytoplasmic sperm injection (ICSI) of single frozen-thawed spermatozoa into in vivo matured mare oocytes. J Reprod Fertil (Suppl) 56:513–517.

McPartlin LA, Suarez SS, Czaya CA, Hinrichs K, Bedford-Guaus SJ. 2009. Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. Biol Reprod 81:199–206.

Meintjes M, Bellow MS, Paul JB, Broussard JR, Li LY, Paccamonti D, Eilts BE, Godke RA. 1995. Transvaginal ultrasound-guided oocyte retrieval from cyclic and pregnant horse and pony mares for in vitro fertilization. Biol Reprod Monograph 1:281–292.

Palmer E, Driancourt MA. 1980. Use of ultrasonic echography in equine gynecology. Theriogenology 13:203–216.

Palmer E, Duchamp G, Bézard J, Magistrini M, King WA, Bousquet D, Betteridge KJ. 1987. Non-surgical recovery of follicular fluid and oocytes of mares. J Reprod Fertil (Suppl) 35:689–690.

Parfenov V, Potchukalina G, Dudina L, Kostyuchek D, Gruzova M. 1989. Human antral follicles: Oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data). Gamete Res 22:219–231.

Pedersen HG, Watson ED, Telfer EE. 2004. Effect of ovary holding temperature and time on equine granulosa cell apoptosis, oocyte chromatin configuration and cumulus morphology. Theriogenology 62:468–480.

Pierson RA, Ginther OJ. 1985. Ultrasonic evaluation of the preovulatory follicle in the mare. Theriogenology 24:359–368.

Preis KA, Carnevale EM, Coutinho da Silva MA, Caracciolo di Brienza V, Gomes GM, Maclellan LJ, Squires EL. 2004. In vitro maturation and transfer of equine oocytes after transport of ovaries at 12 or 22°C. Theriogenology 61:1215–1223.

Rajakoski E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. Acta Endocrinol (Suppl) 52:21–27.

Ribeiro BI, Love LB, Choi YH, Hinrichs K. 2008. Transport of equine ovaries for assisted reproduction. Anim Reprod Sci 108:171–179.

Roberts SJ. 1975. Veterinary obstetrics and genital diseases. Ann Arbor, MI:Edwards Bros.

Scott TJ, Carnevale EM, Maclellan LJ, Scoggin CF, Squires EL. 2001. Embryo development rates after transfer of oocytes matured in vivo, in vitro, or within oviducts of mares. Theriogenology 55:705–715.

Siddiqui MA, Gastal EL, Ju JC, Gastal MO, Beg MA, Ginther OJ. 2009. Nuclear configuration, spindle morphology and cytoskeletal organization of in vivo maturing horse oocytes. Reprod Domest Anim 44:435–440.

Squires EL, Wilson JM, Kato H, Blaszczyk A. 1996. A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. Theriogenology 45:306.

Torner H, Alm H. 1995. Meiotic configuration of horse oocytes in relation to the morphology of the cumulus-oocyte complex. Biol Reprod Monograph 1:253–259.

Tremoleda JL, Schoevers EJ, Stout TAE, Colenbrander B, Bevers MM. 2001. Organization of the cytoskeleton during in vitro maturation of horse oocytes. Mol Reprod Dev 60:260–269.

Van Niekerk CH, Gerneke WH. 1966. Persistance and parthenogenetic cleavage of tubal ova in the mare. Onderstepoort J Vet Res 31:195–232.

Weber JA, Freeman DA, Vanderwall DK, Woods GL. 1991a. Prostaglandin E2 secretion by oviductal transport-stage equine embryos. Biol Reprod 45:540–543.

Weber JA, Freeman DA, Vanderwall DK, Woods GL. 1991b. Prostaglandin E 2 hastens oviductal transport of equine embryos. Biol Reprod 45:544–546.

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