Effect of collection–maturation interval time and pregnancy status of donor mares on oocyte developmental competence in horse cloning[1]

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ABSTRACT: The current limitations for obtaining ovaries from slaughterhouses and the low efficiency of in vivo follicular aspiration necessitate a complete understanding of the variables that affect oocyte developmental competence in the equine. For this reason, we assessed the effect on equine oocyte meiotic competence and the subsequent in vitro cloned embryo development of 1) the time interval between ovary collection and the onset of oocyte in vitro maturation (collection–maturation interval time) and 2) the pregnancy status of the donor mares. To define the collection–maturation interval time, collected oocytes were classified according to the slaughtering time and the pregnancy status of the mare. Maturation rate was recorded and some matured oocytes of each group were used to reconstruct zona free cloned embryos. Nuclear maturation rates were lower when the collection–maturation interval time exceeded 10 h as compared to 4 h (32/83 vs. 76/136, respectively; $P = 0.0128$) and when the donor mare was pregnant as compared to nonpregnant (53/146 vs. 177/329, respectively; $P = 0.0004$). Low rates of cleaved embryos were observed when the collection–maturation interval time exceeded 10 h as compared to 6 to 10 h (11/27 vs. 33/44, respectively; $P = 0.0056$), but the pregnancy status of donor mares did not affect cloned equine blastocyst development (3/49 vs. 1/27 for blastocyst rates of nonpregnant and pregnant groups, respectively; $P = 1.00$). These results indicate that, to apply assisted reproductive technologies in horses, oocytes should be harvested within approximately 10 h after ovary collection. Also, even though ovaries from pregnant mares are a potential source of oocytes, they should be processed at the end of the collection routine due to the lower collection and maturation rate in this group.

Key words: assisted reproductive technology, horse, maturation, meiotic, oocyte developmental competence, pregnancy

INTRODUCTION

In vitro maturation (**IVM**) of horse oocytes was first described by Fulka and Okolski (1981). Several studies followed, and in 1989 the successful embryo transfer of in vitro matured horse oocytes was recorded (Zhang et al., 1989). An intracytoplasmatic sperm injection (**ICSI**) pregnancy and a cloned foal were achieved for the first time in 1996 and 2003, respectively (Squires et al., 1996; Woods et al., 2003; Galli et al., 2003), and in 2010 and 2011 healthy cloned foals were also produced in our laboratory (Gambini et al., 2012). To carry out these assisted reproductive techniques (**ART**), matured oocytes are needed; however, some difficulties in horse oocyte recovery may be encountered due to the tight attachment of the cumulus–oocyte complexes (**COC**) to the follicle wall (Hawley et al., 1995). Therefore, it is imperative to be aware of the variables that hinder equine oocyte developmental competence.

Some of the variables that affect developmental competence were studied (reviewed by Carnevale and Maclellan, 2006; Hinrichs, 2010). Accordingly, Goudet et al. (1997) reported a positive relationship between the maturation rate and the follicle size.

Oocytes with an expanded cumulus had greater maturation rates compared to those with compact cumulus (Alm and Hinrichs, 1996); however, no differences were observed in embryo development (Lagutina et al., 2005; Galli et al., 2007). A study concluded that ovaries should be held at 35 to 37°C for no longer than 2 h to prevent deleterious changes in cumulus morphology, and to avoid chromatin changes, oocytes should be stored for less than 6 h (Pedersen et al., 2004). Additionally, embryo development of equine ICSI embryo seems to be affected by the storage of equine ovaries for only 7 h (Ribeiro et al., 2008).

This study sought to evaluate the effect on oocyte meiotic competence and the subsequent in vitro cloned embryo development of 1) the time interval between ovary collection and the onset of the oocyte IVM (collection–maturation interval time) and 2) the pregnancy status of the donor mares.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Oocyte Collection and In Vitro Maturation

Experiment 1: Evaluation of the Effect of Time Interval between Ovary Collection and Oocyte In Vitro Maturation (Collection–Maturation Interval Time) on Oocyte Developmental Competence. A total of 254 ovaries of slaughtered mares were collected during the breeding season (Buenos Aires, Argentina; 34°00′ S, 64°00′ W). They were packaged every half hour in plastic bags and processed separately after arriving at the laboratory. The time of slaughtering was registered on each bag to determine the collection–maturation interval time. The temperature of the ovaries at arrival was 27 to 29°C. Cumulus–oocyte complexes were recovered by a combination of scraping and flushing of all visible follicles using an 18-gauge needle and a syringe filled with a minimal volume of Dulbecco's modified Eagle's Medium (**DMEM**; 11885; Gibco, Grand Island, NY) supplemented with 1 m*M* sodium pyruvate (P2256) and 15 IU/mL heparin (H3149-50KU). Those oocytes that were denuded with an evident membrane damage or cytoplasm fragmentation were excluded from the study at the time of collection. Maturation of COC was conducted in 100 µL microdroplets of bicarbonate-buffered TCM-199 (31100-035; Gibco) containing 10% (vol/vol) fetal bovine serum (**FBS**; 10499-044; Gibco), 1 µL/mL insulin-transferrin-selenium (**ITS**; 51300-044, Gibco), 1 m*M* sodium pyruvate

(P2256), 100 m*M* cysteamine (M-9768), 0.1 mg/mL of FSH (NIH-FSH-P1; Folltropin; Bioniche, Belleville, ON, Canada), and 2% antibiotic–antimycotic (**ATB**; 15240-096; Gibco) under mineral oil (M8410) in 6.5% carbon dioxide (CO_2) in humidified air at 39°C. Cumulus–oocyte complexes were matured for 24 to 26 h in 5 groups according to the collection–maturation interval time: 4 (group I), 6 (group II), 8 (group III), 10 (group IV), and 12 h (group V). Matured oocytes of these groups were used for nuclear transfer (**NT**).

Experiment 2: Evaluation of the Effect of Pregnancy Status of Donor Mares on Oocyte Developmental Competence. By using the same conditions mentioned above, a total of 234 ovaries (4 replications) were packaged every half hour in 2 different groups according to the pregnancy status of the donor mare—nonpregnant (group I) or pregnant (group II)—and processed separately after arriving at the laboratory in an insulated container. Pregnancy status was established by looking and palpating at the asymmetrical uterus of the donor mare before the removal of the ovaries; however, early pregnancies (less than 30 d) could not be detected. Cumulus–oocyte complexes were recovered and matured as described previously. In this experiment, the collection–maturation interval time was the same for both groups (approximately 7 h). Matured oocytes of both groups were used for NT.

Preparation of Oocytes for Nuclear Transfer

Removal of Cumulus and Zona Pellucida. The cumulus was removed by pipeting the oocytes in 0.05% Trypsin-EDTA (25300; Gibco) and then vortexing them for 2 min in hyaluronidase (H-4272: 1 mg/mL in HEPES-buffered Tyrodes medium containing albumin, lactate, and pyruvate (**TALP-H**; Bavister and Yanagimachi, 1977). After rinsing in TALP-H, oocytes were observed individually under stereoscopic microscopy for the presence of the first polar body, which confirmed nuclear maturation.

Metaphase II oocytes were incubated in 1.5 mg/mL pronase (P-8811) in TALP-H for 3 to 6 min on a warm plate to remove the zona pellucida. Zona-free oocytes (**ZF oocytes**) were washed in TALP-H and placed in a microdrop of Synthetic Oviductal Fluid (**SOF**; Tervit et al., 1972; Holm et al., 1999) supplemented with 2.5% FBS and 1% ATB until enucleation.

Enucleation Procedure. Before enucleation, ZF oocytes were incubated for 5 min in a SOF microdrop containing 1 µg/mL Hoechst bisbenzimide 33342 (H33342) and 0.5 µg/mL of cytochalasin B (C6762). The metaphase plate was aspirated using a blunt pipette under UV light and a closed holding pipette to support the oocyte during enucleation. Zona-free enucleated oocytes (**ZFE oocytes**) were kept in a SOF microdrop until NT.

Nuclear Transfer and Embryo Reconstruction. Zona-free enucleated oocytes were individually transferred to 50 μL drops of 1 mg/mL phytohemagglutinin (L8754) dissolved in TCM-199 for a few seconds. Then they were quickly dropped over a single donor cell resting on the bottom of a 100 μL TALP-H drop; consequently, these 2 structures were attached. Afterward, the couplets were placed in fusion medium (0.3 M mannitol, 0.1 mM MgSO$_4$, 0.05 mM CaCl$_2$, and 1 mg/mL Poly(vinyl alcohol) for 2 to 3 min and then moved to a fusion chamber containing 2 mL of warm fusion medium. Fusion was performed with a double direct current pulse of 1.2 kV/cm, each pulse for 30 μs, 0.1 s apart. Later, the couplets were individually placed in a 10 μL drop of SOF medium supplemented with 2.5% FBS and incubated under mineral oil at 39°C in 5% CO$_2$ in air. Thirty to 50 min after the pulse, couplets were individually observed to confirm fusion. Nonfused couplets were refused. Two hours after fusion zona-free reconstructed embryos (**ZFRE**) were activated.

Activation. Two different protocols were tested for Exp. 1: In protocol A, ZFRE were treated with 5 mM of ionomycin (I24222; Invitrogen, Carlsbad, CA) in TALP-H for 4 min followed by an individual of 1.9 mM 6-dimethylaminopurine (D2629) in a 5 μL drop of SOF for 4 h. In protocol B, ZFRE were treated with 8.7 mM of ionomycin in TALP-H for 4 min followed by an individual culture in a combination of 1 mM 6-dimethylaminopurine and 10 μg/mL cycloheximide (C7698) in a 5 μL drop of SOF for 4 h. For Exp. 2, activation protocol A was used.

Somatic Cell Culture

Adult fibroblasts were obtained through a culture of minced tissue from tail biopsies from the skin of a Criollo horse. They were cultured in DMEM with 10% FBS, 1% ATB, and 1 μL/mL of ITS in 5% CO$_2$ in humidified air at 39°C. After the primary culture was established, fibroblasts were either subcultured every 4 to 6 d or expanded and frozen in DMEM with 20% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Donor cells were induced into quiescence by growth to confluency for 3 to 5 d before NT. Cells were prepared by trypsinization 30 min before NT and then washed and resuspended in DMEM.

In Vitro Embryo Culture. A slightly modified Well of the Well system (Vajta et al., 2000) was used for ZFRE culture. These microwells were produced using a heated glass capillary slightly pressed to the bottom of a 35 by 10 mm petri dish. Microwells were covered with a 50 μL microdrop of SOF medium (16 microwells per microdrop) and then 1 ZFRE was introduced into each microwell.

For Exp. 1, ZFRE activated with protocol A were cultured in 3 experimental groups according the collection–maturation interval time—4 to 6 h for group I, 6 to 10 h for group II, and 10 to 12 h for group III—whereas for ZFRE activated with the protocol B, 2 experimental groups were formed: 4 to 7 h for group I and 7 to 10 h for group II.

For Exp. 2, ZFRE activated with protocol A were cultured in 2 different experimental groups: group I (nonpregnant) and group II (pregnant).

Culture conditions for all the experiments were 5% CO$_2$ and 5% O$_2$ in humidified air at 38.5°C. Half of the medium was renewed on Day 3, with DMEM: Nutrient Mixture F-12 (DMEM/F-12), 10% FBS, and 1% ATB. Cleavage was assessed 72 h after activation, and blastocyst formation was recorded at Day 8.

Statistical Analysis. Differences among treatments in each experiment were determined using Statistix software 0.8 version (Statistix Analytical Software. Tallahassee, FL 32317, USA). Maturation, cleavage, and blastocyst rates were analyzed by chi-square or Fisher's exact test accordingly. A probability of $P \leq 0.05$ indicated that a difference was significant.

RESULTS

Effect of the Collection–Maturation Interval Time on Oocyte Developmental Competence (Experiment 1)

A total of 286 out of 608 oocytes (47.0%) were considered mature due to the presence of the first polar body (4 replications). Maturation rates were recorded for all experimental groups (Table 1). Nuclear maturation was adversely affected when the collection–maturation interval time exceeded 10 h (group I vs. group V, $P = 0.0128$). The proportion of degenerated oocytes increased significantly when the interval time exceeded 4 h (group I vs. group II, $P = 0.0097$).

In a total of 4 replications, 184 out of 367 oocytes (50.1%) were considered mature because of the presence of the first polar body and were subjected to zona free cloning procedure using activation protocol A. Cleavage and blastocyst rates were recorded for all experimental groups (Table 2). Cleavage was negatively affected when the interval between ovary collection and the onset of oocyte IVM exceeded 10 h (group II vs. group III, $P = 0.0039$). Cloned equine blastocysts were only achieved in experimental groups with an interval time lower than 10 h.

In a total of 2 replications, 82 out of 167 oocytes (49.1%) were considered mature because of the presence of the first polar body and were subjected to zona free cloning procedure using activation protocol B. Cleavage and blastocyst rates were recorded for all

Table 1. Effect on nuclear maturation of the collection–maturation interval time

Experimental group[1]	No. of oocytes	No. of mature oocyte, (%)	No. of immature oocyte, (%)	No. of degenerated oocyte, (%)
I: 4 h	136	76 (55, 9)[2a]	34 (25, 0)	26 (19, 1)[a]
II: 6 h	77	32 (41, 6)[b]	18 (23, 8)	27 (35, 0)[b]
III: 8 h	199	94 (47, 2)[ab]	43 (21, 6)	62 (31, 2)[b]
IV: 10 h	113	52 (46, 0)[ab]	24 (21, 2)	38 (33, 7)[b]
V: 12 h	83	32 (38, 6)[b]	25 (30, 1)	26 (31, 3)[b]
Total	608	286 (47.00)	144 (23.60)	179 (29.40)

[a,b]Within a column, means without a common superscript differ (chi-square test, $P < 0.05$).

[1]4, 6, 8, 10, and 12 h are hours of collection–maturation interval time.

Table 2. Effect of the collection–maturation interval time on equine cloned embryo development (activation protocol A)

Experimental group[1]	No. of oocytes	No. of mature oocytes, (%)	No. of ZFRE[2]	No. of cleaved, (%)	No. of blastocysts, (%)
I: 4 to 6 h	135	73 (54.07)[3a]	53	36 (67.92)[ab]	3 (5.66)
II: 6 to 10 h	138	73 (52.89)[ab]	44	33 (75.00)[a]	1 (2.27)
III: 10 to 12 h	94	38 (40.42)[b]	27	11 (40.74)[b]	0 (0)
Total	367	184 (50.01)	108	80 (74.07)	4 (3.70)

[a,b]Within a column, means without a common superscript differ (Fisher's exact test, $P < 0.05$).

[1]4 to 6, 6 to 10, and 10 to 12 h are hours of collection–maturation interval time.
[2]ZFRE = zona free reconstructed embryos.

experimental groups (Table 3). Despite the fact that no statistical differences were found between groups, a tendency to a better blastocyst embryo development was observed in the experimental group II (group I vs. group II, $P = 0.0859$).

Effect of the Pregnancy Status of the Donor Mare on Equine Oocyte Developmental Competence (Experiment 2)

A total of 230 out of 475 oocytes (48.42%) were considered mature based on the presence of the first polar body. Maturation rates were recorded for the 2 experimental groups (Table 4). Nuclear maturation was adversely affected in oocytes recovered from ovaries that were retrieved from pregnant mares ($P = 0.0003$). The proportion of degenerated oocytes also increased significantly in this group ($P = 0.0001$).

In a total of 2 replications, 76 ZFRE were produced to test if the pregnancy status of the donor mare affected the development of embryos cloned in vitro. Cleavage (43/49 or 87.8% vs. 23/27 or 85.2%) at $P = 0.7368$ and blastocyst rates (3/49 or 6.1% vs. 1/27 or 3.7%) at $P = 1.00$ were not significantly different between nonpregnant and pregnant groups when Fisher's exact test was performed. Matured oocytes of both groups were suitable for cloned equine embryo development.

DISCUSSION

This work addressed some of the variables that could affect oocyte quality and subsequent maturation and development of cloned equine embryos. It suggests new strategies on how ovaries should be selected for a more efficient cloned embryo production. To our knowledge, the present study is the first to evaluate the effect of different periods of equine ovary storage and the pregnancy status of donor mares on subsequent cloned equine blastocyst production in vitro.

Equine Oocyte Developmental Competence is Affected by the Interval Time between Ovary Collection and the Onset of In Vitro Oocyte Maturation

Our results show the importance of the time interval between the time of slaughtering of the mare and the onset of the IVM of oocytes retrieved from its ovaries on their nuclear maturation and the subsequent cloned embryo development. Nuclear maturation rate was lower when more than 10 h had elapsed after slaughter. In addition, cloned blastocysts only developed when matured oocytes were used from ovaries transported for less than 10 h. In our study, oocytes from ovaries with a collection–maturation interval time lower than 4 h showed the greatest proportion of matured oocytes and the lowest proportion of degenerated oocytes, suggesting that oocyte degeneration occurs after the first hours postmortem.

Although some studies have found no effect of ovary storage (Guignot et al., 1999), other reports have shown a negative effect on nuclear maturation of oocytes recovered from ovaries stored for more than 18 h at room temperature; the results have been worse when ovaries were stored at 4°C (Love et al., 2003). It has also been reported that when oocytes are collected after 5 to 9 h of ovary storage, normal chromatin configuration is altered and oocytes recovered immediately after slaughtering have a greater meiotic resumption (Hinrichs et al., 2005). Pederson et al. (2004) reported that an interval time lower than 6 h is needed to avoid chromatin changes; therefore, chromatin alterations are probably the reasons why oocyte viability could be affected after a period of postmortem time. In our work, 10 h seemed to be the time limit after which meiotic competence of equine oocytes was adversely affected. It is important to take this time limit into account when ovaries from dead mares are processed for the collection of oocytes for assisted reproduction. Conversely, other reports indicate that storage of equine ovaries for only 7 h may decrease blastocyst development and that longer storage reduces the rates of both oocyte maturation and blastocyst development (Ribeiro et al., 2008). In ad-

Table 3. Effect of the collection–maturation interval time on equine cloned embryo development (activation protocol B)[1]

Experimental group[2]	No. of oocytes	No. of mature oocytes, (%)	No. of ZFRE[3]	No. of cleaved, (%)	No.of blastocysts, (%)
I: 4 a 7 h	56	33 (58.9)[4]	22	20 (90.9)	1 (4.5)
II: 7 a 10 h	111	49 (44.5)	34	28 (82.3)	7 (20.6)
Total	167	82 (49.1)	56	48 (85.7)	8 (14.3)

[1]No statistical differences were found between experimental groups (Fisher's exact test, $P < 0.05$).

[2]4 to 7 and 10 to 12 h are hours of collection–maturation interval time.

[3]ZFRE = zona free reconstructed embryos.

Table 4. Effect on oocyte nuclear maturation of pregnancy status of the mare donor of ovaries

Experimental group	Collection rate (oocytes per ovary)	No. of oocytes	No. of mature oocytes, (%)	No. of immature oocytes, (%)	No. of degenerated oocytes, (%)
I: Nonpregnant	2.7[a]	329	177 (53.79)[1a]	39 (11.85)	113 (34.34)[a]
II: Pregnant	1.3[b]	146	53 (36.30)[b]	11 (7.53)	82 (56.16)[b]
Total	2	475	230 (48.42)	50 (10.52)	195 (41.05)

[a,b]Within a column, means without a common superscript differ (chi-square test, $P < 0.05$).

dition, equine oocytes can also suppress their maturation by their culture in the presence of the meiotic inhibitors but appear to have a detrimental effect on subsequent developmental competence (Choi et al., 2006).

Nuclear maturation was not the only parameter affected by this time interval. Cleavage rates of zona free cloned equine embryos were also negatively affected when oocytes were harvested after more than 10 h of ovary collection. No embryos from this group reached the blastocyst stage. While some of these oocytes could complete nuclear maturation, their capacity to support cloned equine embryo development was affected by this long time interval. In contrast, other studies have reported successful ICSI using ovaries that have been transported for more than 20 h (Preis et al., 2004; Matsukawa et al., 2007). Apparently, for embryo development after ICSI, oocytes can remain in the ovaries for a long period postmortem. These differences lead us to consider that for equines, a different grade of oocyte developmental competence is needed for ICSI as compared to the reprogramming of a somatic cell.

The greater cloned equine blastocyst rate was achieved with the activation protocol B and by using matured oocytes with 7 to 10 h of collection–maturation time. Even though no statistic differences were found between the experimental groups, there appears to be a positive effect on cloned equine embryo development when oocytes are held in ovaries for 7 to 10 h before the beginning of the in vitro embryo maturation. Similar observations were reported by Hinrichs et al. (2005) since oocytes recovered after a delay of 5 to 9 h had greater blastocyst development rates than did those collected immediately after slaughtering.

Oocyte competence also involves cytoplasmic changes that are known as cytoplasmic maturation. The volume of the Golgi complexes is reduced while the lipid component is increased, cortical granules align just inside the oocyte membrane, and the perivitelline space becomes enlarged (Hyttel et al., 1997). Failures in cytoplasmic maturation could be one of the reasons why matured equine oocytes are not able to support cloned embryo development.

Equine Oocyte Developmental Competence is Affected by the Pregnancy Status of the Donor Mare

The number of oocytes that could be collected from pregnant mares was significantly lower than from ovaries of nonpregnant animals. In addition, the maturation rate of pregnant mare oocytes was significantly lower while the degenerated oocyte rate increased in this experimental group.

Recently, a cloned foal was produced using oocytes recovered from live mares (Choi et al., 2013). Pregnant mares are a potential source of equine oocytes because transvaginal ultrasound-guided follicular aspiration can be performed in these animals (Purcell et al., 2007) even though the developmental competence of theses oocytes remains unclear. The physiology of gestation in the mare is unique. Between Days 36 and 130 of pregnancy, chorionic girdle cells invade the endometrium and produce equine chorionic gonadotropin (Goudet et al., 1998); this results in accessory corpora lutea that raise the levels of progesterone. Follicular dynamics in pregnant mares has also been studied; a marked increase in the number and diameter of follicles occurs during approximately Days 10 to 60 of gestation and then decreases by Days 180 to 200 (Squires et al., 1974; Ginther et al., 1986). It is unknown how the hormones that are present during gestation affect oocyte developmental competence in the mare.

The role of progesterone on in vitro oocyte maturation has been studied in the bovine and the presence of oocyte genomic and nongenomic progesterone receptors has been demonstrated. Physiological levels of progesterone seem to be necessary for the regulation of the differential expression of these receptors (Aparicio et al., 2011). Therefore, progestagen levels in pregnant mares could be one of the reasons for the lower oocyte maturation rate, but it remains interesting to clarify the effect of the hormones related to the pregnant mare on oocyte developmental competence.

Whereas a previous report that analyzed a total of 10 oocytes found that few oocytes mature when they are retrieved from pregnant mares (Del Campo et al., 1995), a total of 475 oocytes were used in our study to

demonstrate the negative effect of the pregnancy status on the oocyte meiotic competence. Purcell et al. (2007) reported that the ovaries of early pregnant mares yield a high number of oocyte collection when follicular aspiration is performed. In our hands, we observed a 50% reduction in the oocyte recovery rate from slaughtered pregnant mares.

Despite the fact that collection and maturation rates were adversely affected in oocytes derived from pregnant mares, embryo development of mature oocytes of this experimental group was similar to that of matured oocytes from nonpregnant mares; therefore, pregnant mares are a potential source of competent oocytes in horses. Since all ovaries from pregnant mares were included in the same experimental group, it remains unclear whether collection rates, oocyte meiotic competence, and embryo development could be differently affected by the stage of gestation.

Conclusions

To sum up, the data presented in this paper indicate that equine oocyte nuclear maturation is compromised by an increase in the collection–maturation interval time and also by the prospect pregnancy of the donor mare. In addition, a long collection–maturation interval time negatively affects cloned equine embryo development although this variable seems to be independent of the pregnancy status of the donor mares. These results are important to take into account for the strategic processing of equines ovaries for ART. If possible, ovaries derived from pregnant mares should be processed at the end of the collection routine and at the same time, oocytes should be harvested from ovaries as soon as possible within an approximate limit of 10 h before oocyte developmental competence is adversely affected.

LITERATURE CITED

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

Aparicio, I. M., M. Garcia-Herreros, L. C. O'Shea, C. Hensey, P. Lonergan, and T. Fair. 2011. Expression, regulation, and function of progesterone receptors in bovine cumulus oocyte complexes during in vitro maturation. Biol. Reprod. 84:910–921.

Bavister, B., and R. Yanagimachi. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol. Reprod. 16:228–237.

Carnevale, E. M., and L. J. Maclellan. 2006. Collection, evaluation, and use of oocytes in equine assisted reproduction. Vet. Clin. North Am. Equine Pract. 22:843–856.

Choi, Y. H., L. B. Love, D. D. Varner, and K. Hinrichs. 2006. 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, Y. H., J. D. Norris, I. C. Velez, C. C. Jacobson, D. L. Hartman, and K. Hinrichs. 2013. A viable foal obtained by equine somatic cell nuclear transfer using oocytes recovered from immature follicles of live mares. Theriogenology 79:791–796.

Del Campo, M. R., X. Donoso, J. J. Parrish, and O. J. Ginther. 1995. Selection of follicles, preculture oocyte evaluation and duration of culture for in vitro maturation of equine oocytes. Theriogenology 43:1141–1153.

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

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

Galli, C., I. Lagutina, G. Crotti, S. Colleoni, P. Turini, N. Ponderato, R. Duchi, and G. Lazzari. 2003. Pregnancy: A cloned horse born to its dam twin. Nature 424:635.

Gambini A., J. Jarazo, R. Olivera, and D. F. Salamone. 2012. Equine cloning: In vitro and in vivo development of aggregated embryos. Biol. Reprod. 87:15, 1–9.

Ginther, O. J. 1986. Ultrasonic imaging and reproductive events in the mare. Equiservices Inc., Cross Plains, WI.

Goudet, G., J. Bezard, G. Duchamp, N. Gerard, and E. Palmer. 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., L. Leclercq, J. Bézard, G. Duchamp, D. Guillaume, and E. Palmer. 1998. Chorionic gonadotropin secretion is associated with an inhibition of follicular growth and an improvement in oocyte competence for in vitro maturation in the mare. Biol. Reprod. 58:760-768.

Guignot, F., J. Bezard, and E. Palmer. 1999. Effect of time during transport of excised mare ovaries on oocyte recovery rate and quality after in vitro maturation. Theriogenology 52:757–766.

Hawley, L. R., A. C. Enders, and K. Hinrichs. 1995. Comparison of equine and bovine oocyte-cumulus morphology within the ovarian follicle. Biol. Reprod. 1:243–252.

Hinrichs, K. 2010. The equine oocyte: Factors affecting meiotic and developmental competence. Mol. Reprod. Dev. 77:651–661.

Hinrichs, K., Y. H. Choi, L. B. Love, D. D. Varner, C. C. Love, and B. E. Walckenaer. 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.

Holm, P., P. J. Booth, M. H. Schmidt, T. Greve, and H. Callesen. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo–inositol with or without serum-proteins. Theriogenology 52:683–700.

Hyttel, P., T. Fair, H. Callesen, and T. Greve. 1997. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 47:23–32.

Lagutina, I., G. Lazzari, R. Duchi, S. Colleoni, N. Ponderato, P. Turini, G. Crotti, and C. Galli. 2005. Somatic cell nuclear transfer in horses: Effect of oocyte morphology, embryo reconstruction method and donor cell type. Reproduction 130:559–567.

Love, L. B., Y. H. Choi, C. C. Love, D. D. Varner, and K. Hinrichs. 2003. Effect of ovary storage and oocyte transport method on maturation rate of horse oocytes. Theriogenology 59:765–774.

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

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

Preis, K. A., E. M. Carnevale, M. A. Coutinho da Silva, V. Caracciolo di Brienza, G. M. Gomes, L. J. Maclellan, and E. L. Squires. 2004. In vitro maturation and transfer of equine oocytes after transport of ovaries at 12 or 22 degrees C. Theriogenology 61:1215–1223.

Purcell, S. H., G. E. Seidel, P. M. McCue, and E. L. Squires. 2007. Aspiration of oocytes from transitional, cycling, and pregnant mares. Anim. Reprod. Sci. 100:291–300.

Ribeiro, B. I., L. B. Love, Y. H. Choi, and K. Hinrichs. 2008. Transport of equine ovaries for assisted reproduction. Anim. Reprod. Sci. 108:171–179.

Squires, E. L., R. H. Douglas, W. P. Steffenhagen, and O. J. Ginther. 1974. Ovarian changes during the estrous cycle and pregnancy in mares. J. Anim. Sci. 38:330–338.

Squires, E. L., J. M. Wilson, H. Kato, and A. Blaszczyk. 1996. A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. Theriogenology 45:306.

Tervit, H. R., D. G. Whittingham, and L. E. Rowson. 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30:493–497.

Vajta, G., T. T. Peura, P. Holm, A. Paldi, T. Greve, A. O. Trouson, and H. Callesen. 2000. New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. Mol. Reprod. Dev. 55:256–264.

Woods, G. L., K. L. White, D. K. Vanderwall, G. P. Li, K. I. Aston, T. D. Bunch, L. N. Meerdo, and B. J. Pate. 2003. A mule cloned from fetal cells by nuclear transfer. Science 301:1063.

Zhang, J. J., M. S. Boyle, W. R. Allen, and C. Galli. 1989. Recent studies on in vivo fertilization of in vitro matured horse oocytes. Equine Vet. J. 8:101–104.