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In Vitro Production of Equine Embryos: State of the Art

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Contents

In vitro embryo production is possible in the horse both clinically and for research applications. Oocytes may be collected from excised ovaries post-mortem, or from either immature follicles or stimulated pre-ovulatory follicles in the live mare. *In vitro* maturation of immature oocytes typically yields approximately 60% mature oocytes. As standard *in vitro* fertilization is not yet repeatable in the horse, fertilization is performed by intracytoplasmic sperm injection. Embryo culture requires medium with high glucose, at least during blastocyst development, and rates of blastocyst development similar to those for cattle (25% to 35%) may be obtained. Pregnancy rates after transfer of *in vitro*-produced blastocysts are similar to those for embryos recovered *ex vivo*.

Introduction

In the last 10 years, assisted reproduction in the horse has seen remarkable progress. Methods for oocyte maturation, fertilization *in vitro* by intracytoplasmic sperm injection and culture to the blastocyst stage for transcervical transfer have been developed and are now starting to be used for clinical production of foals (Colleoni et al. 2007).

Progress in these areas was initially slow, because of the failure of standard *in vitro* fertilization to be repeatably successful in the horse. Thus, there was no fertilization method available to test the developmental competence of different oocyte classes or methods of oocyte maturation and no source of fertilized oocytes with which to test procedures for embryo culture *in vitro*. The development of effective and repeatable methods for fertilization and early embryo production using intracytoplasmic sperm injection (ICSI) with the Piezo drill (Choi et al. 2002; Lazzari et al. 2002) set the stage for investigation into *in vitro* blastocyst production in the horse.

Oocyte Collection From Excised Ovaries

For research purposes, oocytes may be obtained from slaughterhouse tissue. Because of the tight attachment of the oocyte to the follicle wall (Hawley et al. 1995), aspiration of follicles to recover oocytes results in a low recovery rate and preferential recovery of oocytes from atretic follicles (Dell'Aquila et al. 2001). Oocytes may be effectively recovered by opening follicles with a scalpel blade and scraping the granulosa cell layer from the follicle using a bone curette (Hinrichs and DiGiorgio 1991). The oocyte is located in the recovered cells using a dissection microscope.

The method of follicle scraping is time- and labour-intensive, hence in some cases (e.g. if a large number of ovaries are to be processed with minimal personnel available), aspiration may be preferred, although the recovery rate may be only half that obtained via scraping (Alm et al. 1997; Dell'Aquila et al. 2001). Aspiration may be performed with a needle and syringe, or with a needle attached to a vacuum pump. Aspiration of follicles is associated with stripping of the majority of cumulus from the oocytes (Fig. 1e), making it difficult to classify oocytes according to cumulus morphology, as described later.

If collected by follicle scraping, oocytes may be classified by their cumulus morphology as compact (Cp) or expanded (Ex; Fig. 1). Interestingly, equine Cp oocytes come from viable follicles, but are largely from follicles so juvenile that the oocytes are not competent to mature *in vitro*. In contrast, Ex oocytes originate in atretic follicles, but have a high meiotic competence (Hinrichs and Williams 1997). In our laboratory, we classify oocytes stringently, so that only those oocytes having both compact granulosa and a compact cumulus are classified as Cp. The character of the granulosa is best evaluated by looking at the edge of the cumulus hillock (Fig. 1c, d, arrows) or the edge of a fold of granulosa. Compact granulosa shows a tight, clean line on the edge. Any slight puffiness of the cumulus or granulosa results in a classification of Ex. The oocyte itself may appear to be misshapen on examination at low power, such as in Fig 1a; however, examination on higher power is needed to determine if this is in fact misshapen (e.g. Fig. 1f), resulting in a classification of degenerating, or whether the apparent irregularity is because of the segregation of the light and dark areas of the cytoplasm (Fig 1b). Such a heterogeneous cytoplasm as in Fig 1b, commonly seen in Ex oocytes, is associated with high meiotic competence in horse oocytes (Hinrichs and Williams 1997). Oocytes classified as Cp are more likely to have a homogeneous cytoplasm (Fig. 1e); this is associated with low meiotic competence (Hinrichs and Williams 1997). The oocytes in Fig. 1e were obtained by follicle aspiration, resulting in the majority of the cumulus being stripped off and leaving only the corona radiata present, or in partial denudation to the zona pellucida. It is not possible to classify these oocytes as Ex or Cp, as the corona radiata is the last portion of the cumulus to show expansion (as seen in the degenerating oocyte in Fig. 1f).

Using these methods for classification, on scraping follicles from slaughterhouse-derived tissue, we typically classify approximately 60% of the recovered oocytes as Ex, 30% as Cp and 10% as degenerating. The *in vitro* maturation rates, as defined on staining of oocytes after maturation or by extrusion of the first polar body, are

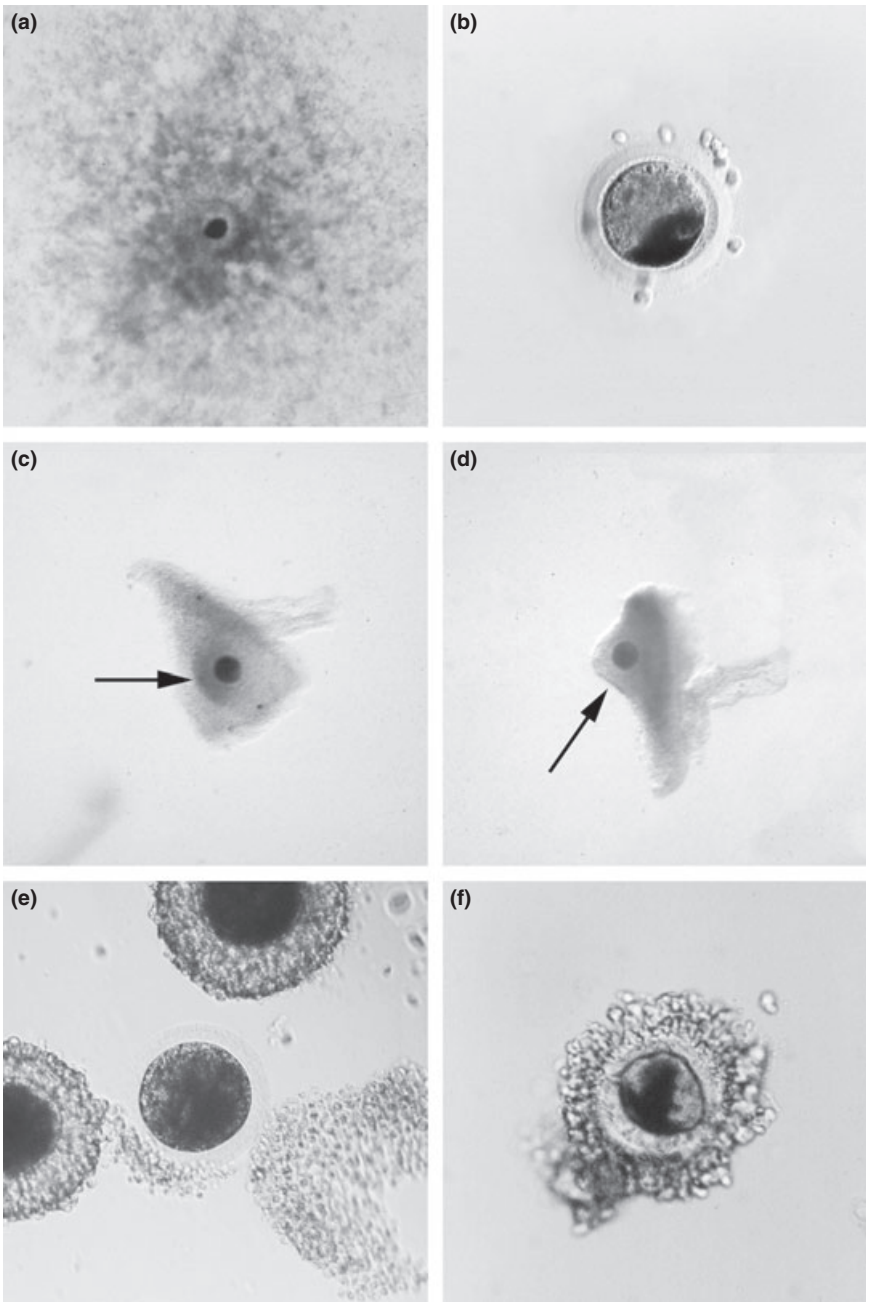

Fig. 1. Photomicrographs of equine oocytes. (a) Immature oocyte showing expanded cumulus (Ex) and apparent irregularity of the cytoplasm; this should be evaluated on higher power to determine whether it is actually misshapen (c.f. panel f), resulting in a classification of degenerating, or is because of segregation of light and dark areas of the cytoplasm in a viable oocyte (c.f. panel b). (b) Higher-power view of a denuded equine oocyte showing segregation of the light and dark areas of the cytoplasm, which is associated with high meiotic competence. (c) Immature oocyte showing compact cumulus (Cp); the arrow shows the smooth outline of the cumulus hillock. (d) Cumulus-enclosed oocyte turned on its side to outline the cumulus hillock; arrow shows the smooth outline of the cells resulting in a classification of Cp. (e) Oocytes recovered from the follicles of an excised ovary by follicle aspiration, showing the typical morphology of corona radiata only or partial denudation to the zona pellucida; these oocytes cannot be classified as Ex or Cp. These oocytes show a more homogenous cytoplasm, which is associated with low meiotic competence. (f) Oocyte with a shrunken and misshapen cytoplasm, resulting in a classification of degenerating

typically approximately 65% for Ex oocytes and 20% for Cp oocytes (Alm and Hinrichs 1996; Hinrichs and Williams 1997; Choi et al. 2004a; Hinrichs et al. 2005).

For *in vitro* embryo production to be used clinically, oocytes must be collected from the donor mare from whom the embryo is desired. Embryos can be produced post-mortem from valuable mares that die or are euthanized, by recovering oocytes from the mares' ovaries as described previously. Embryos may be obtained by transfer of the *in vitro*-matured oocytes to the oviducts of mares *in vivo* (Carnevale et al. 2004), but also by *in vitro* embryo production: In the past 4 years, we have produced 21 embryos from the ovaries of 16 client mares post-mortem, for 13 pregnancies (Hinrichs et al. 2010). The ovaries should be recovered from the mare as soon as possible post-mortem and held at room temperature during shipment to the laboratory. The ability to produce embryos or viable pregnancies with the recovered oocytes decreases with time after approximately 6 h post-mortem (Carnevale et al. 2004; Ribeiro et al. 2008).

Oocyte Collection From Live Mares

There are two main approaches to the recovery of oocytes from live mares: (i) Recovery of the *in vivo*-maturing oocyte from the pre-ovulatory follicle after stimulation with hCG or a GnRH analogue and (ii) recovery of immature oocytes from all visible follicles on the ovary, followed by *in vitro* maturation.

Aspiration of the gonadotropin-stimulated dominant pre-ovulatory follicle is commonly used to obtain

oocytes for oocyte transfer, and recovery rates from these follicles are typically high (65% to 80%; Hinrichs et al. 1990; Carnevale and Ginther 1995; Hinrichs et al. 1998; Carnevale et al. 2005). In these follicles, the oocyte–cumulus complex is expanding and loosening from the follicle wall in preparation for ovulation. A benefit to aspiration of the pre-ovulatory follicle is that, due its large size (typically >35 mm diameter), it can be easily recovered by using a needle placed through the flank while the ovary is manipulated per rectum (Vogelsang et al. 1988; Hinrichs et al. 1998). Recovery via flank aspiration is associated with reduced time, labour and equipment when compared to transvaginal ultrasound-guided follicle aspiration (TVA). Methods for aspiration of the pre-ovulatory follicle have recently been reviewed (Hinrichs 2010).

Because treatment for superovulation is still problematic in the horse, only one, or sometimes two, pre-ovulatory oocytes are available per cycle. Even when superstimulatory treatments are used, the large size of the ovary (because of the presence of multiple pre-ovulatory follicles) may cause aspiration of the follicles to be difficult when more than one or two are present (Maclellan et al. 2002).

Aspiration of immature oocytes from all follicles present on the ovaries, followed by *in vitro* oocyte maturation, is an alternative option to obtain oocytes for *in vitro* embryo production. Such follicles include those that are undergoing atresia as well as those that are growing, but the oocytes within the follicles are immature. In cattle, TVA has been utilized to collect immature oocytes since the 1980s (Pieterse et al. 1988). Recovery rates using this procedure in cows are high (typically approximately 60%) and follicles may be aspirated as often as every 3 to 4 days without reduction in follicle number. Unfortunately, recovery rates on aspiration of immature follicles in the mare have been low, typically <30% (Brück et al. 1992; Cook et al. 1993; Duchamp et al. 1995; Kanitz et al. 1995; Mari et al. 2005). Again, the low recovery rates are attributed to the closer and stronger attachment of the equine oocyte to the follicle wall, in comparison with that of the bovine oocyte (Hawley et al. 1995). Recovery rates are higher for small immature follicles than for larger immature follicles (Kanitz et al. 1995; Bøgh et al. 2002), a difference attributed to the smaller surface area of the smaller follicles, thus the increased likelihood of dislodging the oocyte during aspiration.

Relatively high recovery rates from immature follicles (43% to 58%) have been reported by some laboratories implementing aspiration techniques that include flushing of each follicle up to eight times (Meintjes et al. 1995; Bøgh et al. 2002; Colleoni et al. 2007; Jacobson et al. 2010) via a double-lumen aspiration needle with separate in- and outflow channels. In the report of Colleoni et al. (2007), the number of aspirated follicles was high (mean of 17 per mare), and this effective recovery rate resulted in a mean of 10 immature oocytes recovered per mare per aspiration session. Methods for TVA have recently been reviewed (Hinrichs 2010).

Care must be taken regarding the interval between aspirations in the mare. Aspiration of follicles on a weekly basis was associated with a decrease in follicle number: the average number of follicles within the ovary decreased from 8.7 to 4.7 follicles per mare when mares were subjected to follicle aspiration once every 7 days for a period of 11 weeks (Duchamp et al. 1995). In addition, the maturation rate of oocytes recovered by TVA 8 days after a previous aspiration was low (Bøgh et al. 2002). It is possible that follicles are juvenile at this time, as the proportion of compact cumulus in oocytes recovered from this population was high.

Repeated aspiration of follicles in individual mares over a long period of time is possible. Bøgh and coworkers reported on four mares that had TVA performed repeatedly over an 8-year period. A mean of 19 puncture sessions were performed and 122 follicles were punctured per mare; the oocyte recovery was not given (Bøgh et al. 2003). The mares continued to have normal oestrus cycles; however, evaluation of the ovaries on removal showed one mare to have ovarian abscesses. We recently reported the results of TVA of mares every 14 days throughout the breeding season; mares underwent 8 aspiration sessions each for an average of 72 follicle punctures each (Jacobson et al. 2010). The number of follicles aspirated per session did not change over time (7.3 to 10.3 follicles per session; Jacobson et al. 2010), nor did oocyte recovery (48% for the first session vs 54% for the last (Jacobson, Hinrichs et al., unpublished data).

When immature oocytes are collected by TVA, they must be matured *in vitro*. Expected maturation rates are approximately 60% (Bøgh et al. 2002; Colleoni et al. 2007; Jacobson et al. 2010).

The choice of approach (aspiration of the pre-ovulatory follicle or aspiration of all immature follicles) to use clinically is still unclear: While there is only one pre-ovulatory oocyte available per cycle, the recovery rate on aspiration is high, essentially all recovered oocytes are mature, and the oocytes have maximum developmental competence. In contrast, while numerous immature follicles are available for aspiration, this number varies (Warmblood mares may bring up more follicles than do other breeds), the recovery rate is generally low and varies greatly with the operator, only a proportion of recovered oocytes will mature and the developmental competence of these oocytes after *in vitro* maturation may be low. In a recent study on ICSI in our laboratory (Jacobson et al. 2010), 72 oocytes were recovered after 19 TVA aspiration sessions (3.8 oocytes per mare per session). Of these oocytes, 37 (51%) matured *in vitro* and 33% of these formed blastocysts after ICSI, resulting in a 63% blastocyst production per aspiration session. In contrast, when aspiration of the stimulated pre-ovulatory was performed, the maximum oocyte recovery rate was 0.8 oocytes per mare per session, and the blastocyst development after ICSI was 41%, for an estimated 33% blastocyst development per mare per aspiration (Jacobson et al. 2010). It is remarkable that the blastocyst development from pre-ovulatory, *in vivo*-matured oocytes was not notably higher than that for *in vitro*-matured oocytes. Colleoni et al. (2007) recovered 10 oocytes per mare by TVA of immature follicles, of which 66% matured *in vitro*; after ICSI, 40/313 (13%) of injected oocytes formed blastocysts, for an 85% blastocyst rate per aspiration session. It is almost not possible

to equal this blastocyst production per aspiration session using aspiration of the one mature follicle, followed by ICSI and *in vitro* culture.

Maturation of Oocytes *In Vitro*

In vitro oocyte maturation may be simply performed by placing immature oocytes into culture. However, many variables affect both the rate of nuclear maturation and the acquisition of cytoplasmic maturation as shown by the oocyte's developmental competence (ability to form blastocysts *in vitro*) after fertilization. As noted previously, Ex oocytes have a significantly higher rate of maturation to metaphase II (MII) than do Cp oocytes. For oocytes recovered from excised ovaries, the period of time that the oocyte spends within the ovary before it is recovered affects the duration of culture necessary for both nuclear maturation and cytoplasmic maturation or development competence (Hinrichs et al. 2005). Oocytes collected from the ovary immediately after slaughter required a longer duration of culture, and had lower developmental competence, than did oocytes recovered after a delay of 5 to 9 h (Hinrichs et al. 2005). Holding of ovaries before oocyte recovery has been shown to increase blastocyst formation after IVM/IVF in cattle also (Blondin et al. 1997). The optimum duration of maturation was between 24 and 30 h for Ex oocytes and between 30 and 36 h for Cp oocytes. Once matured, there was no difference in developmental competence (rate of blastocyst development) between Cp and Ex oocytes (Hinrichs et al. 2005). To aid scheduling of the onset of maturation, equine oocytes may be held overnight in a simple medium (EH medium) at room temperature before they are placed into maturation culture, with no effect on maturation or blastocyst development after ICSI (Choi et al. 2006). EH (Earles-Hanks) medium is a mixture of 40% M199 with Hanks salts and 25 mM Hepes, 40% M199 with Earles salts and 20% certified, heat-inactivated FBS (all from Invitrogen, Carlsbad, CA, USA). Maturation may be performed effectively by using M199 with Earles salts, 10% fetal bovine serum and 5 mU/ml bovine follicle stimulating hormone in a humidified atmosphere of 5% CO_2 in air at 38.2°C. Although other media may also work for oocyte maturation, currently the highest reported *in vitro* blastocyst production rates have been achieved using oocytes matured in this medium (Hinrichs et al. 2005; Choi et al. 2007; Ribeiro et al. 2008). Evaluation of oocytes after *in vitro* maturation requires removal of the cumulus; this is more difficult in horses than it is in many other species. The transzonal processes of the equine cumulus are extensive and denuding requires repeated pipetting with smaller and smaller-bore pipettes. Hyaluronidase is helpful in mature oocytes; denuding of immature oocytes is greatly aided by pipetting in a solution of trypsin/EDTA in a calcium- and magnesium-free medium.

Fertilization and Embryo Culture

Standard IVF has not been repeatably successful in the horse. Recent data indicate that good fertilization rates may be achieved after treatment of sperm with procaine to induce hyperactivation (McPartlin et al. 2009), but advanced embryo development after this treatment has not yet been demonstrated. Further work in this area may produce an effective method for equine IVF. Currently, however, fertilization in the horse is achieved using ICSI. While fertilization and early embryonic development has been reported by groups using standard micromanipulation techniques for ICSI, good blastocyst rates *in vitro* appear to be associated with the use of the Piezo drill to perform the sperm injection (Hinrichs et al. 2005; Galli et al. 2007). The drill may work by ensuring that the oocyte membrane is completely disrupted before injection and that the sperm plasma membrane is eliminated after injection, as sperm are subjected to pulses while within the pipette that may aid in membrane disruption. No exogenous activation treatment is needed after sperm injection. Methods used in our lab for ICSI have been given in detail (Choi et al. 2006).

Little work has been performed on the effect of specific medium factors on development of equine embryos *in vitro*. Good development to 4 days (8- to 16-cell stage) can be obtained in a variety of media; however, embryo culture media as used in other species, such as G1/G2, do not support acceptable rates of equine blastocyst formation (Choi et al. 2003a,b, 2004a,b). Good rates of blastocyst development (25% to 35% of injected oocytes) have been achieved using culture of embryos in DMEM/F-12 medium with 10% fetal bovine serum, at a ratio of 1 μl medium per embryo, in a mixed gas atmosphere of 5% O_2, 5% CO_2 and 90% N_2, at 38.2°C (Hinrichs et al. 2005). Although the specific factors in DMEM/F-12 that support equine blastocyst development have not been investigated, this medium contains 17 mM glucose. Development of equine embryos to morula and blastocyst was greater in a modified SOF supplemented with 19 mM glucose than in the same medium with 1.5 mM glucose (Herrera et al. 2008). Equine blastocyst production can be obtained by initial culture in a modified SOF, followed by transfer to DMEM/F-12 at approximately day 5 (C. Galli, personal communication, 2009).

Equine embryos show signs of development to blastocyst starting at 7 days of culture; these include a mild degree of expansion and the formation of an organized outer layer of cells, presumably trophoblast (Fig. 2; Hinrichs et al. 2007). Vivid expansion of the blastocoele with a thin trophoblast layer and formation of an inner cell mass, as seen in bovine embryos, does not occur *in vitro* in the horse. Embryos should be transferred to recipient mares as soon as any signs of blastocyst development are seen. Embryos may start to develop to the blastocyst stage at any time between day 7 and day 10 of culture, and we have seen no difference in pregnancy rates or live foal rates related to the age of the embryo at the time of blastocyst development.

Pregnancy rates after transfer of *in vitro*-produced blastocysts in our laboratory are 70% to 80%. While our early studies showed a tendency for pregnancies to develop trophoblast only (no embryo proper or heartbeat seen; Hinrichs et al. 2007), a subsequent study yielded 13 pregnancies after transfer of 17 embryos (76%) with 12 of 13 pregnancies developing normally

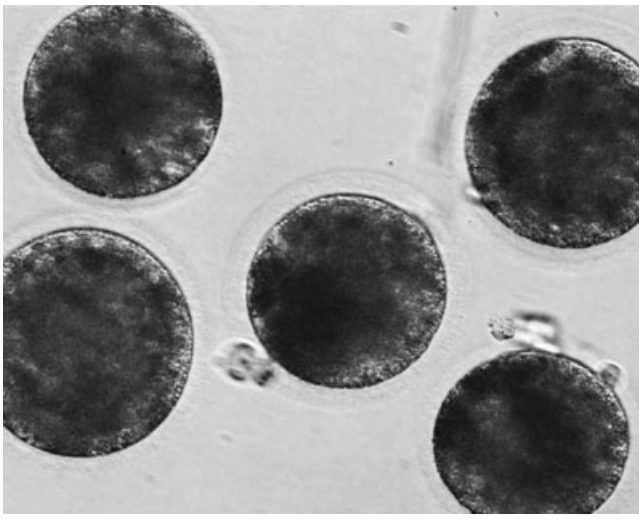

Fig. 2. *In vitro*-produced equine blastocysts. Note the very mild blastocoele expansion; blastocysts are recognized by the formation of a presumptive trophoblast layer immediately adjacent to the zona pellucida

(foetus with heartbeat; Hinrichs and Choi, unpublished data). The reason for the improved viability may be related to ensuring consistent culture conditions.

Conclusions

Progress in *in vitro* embryo production in the horse has been rapid over the last decade, and this procedure is currently effective enough to be offered clinically. The equine oocyte and embryo present many species-specific attributes that affect collection, classification and culture. More work is needed to establish optimal systems for *in vitro* embryo culture in the horse, especially to improve embryo development after fertilization of oocytes recovered from pre-ovulatory follicle *in vivo*.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

The author (KH) devised and wrote this review.

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