Biotechnologies in the horse reproduction

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INTRODUCTION

We recently presented a review on biotechnologies in horses (Deleuze 2012). The present paper is an updated version of this review, hilighting the progress that has been made in biotechnologies for the horse breeding industry over the last few years.

Producing a live foal is along and complex process. The oocyte that remained quiescent in the ovary must resume its maturation and reach a stage where it can be fertilized. Then the ovulation can occur. The matured oocyte is released from the follicle and will migrate into the ampulla of the oviduc where fusion with a sperm cell will take place. The fertilized oocyte will then progress throughout the oviduct and reach the uterine lumen about 6 days after the ovulation. The young embryon signals its presence and inhibits luteolysis. The implantation and placentation then take place and the foetus further develops to term. Any defect at any of these numerous steps may compromise the whole process and prevent the birth of a healthy foal.

Assisted Reproductive Technologies (ARTs) for the horse developed steadily over the last decades. These techniques range from simple procedures such as artificial insemination to the complex production of clones. These technologies now enable salvation of otherwise lost genetics from either subfertile or even dead animals both male and female.

Further use and development of these techniques highly depends on availability of oocytes and their manipulation. These embryotechnologies were first developed in the bovine. Thanks to the virtually unlimited availability of bovine abattoir oocytes and the econolical benefice for the bovine industry, research rapidly led to efficacious *in vitro* maturation (IVM) of oocytes and their subsequent *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the early embryos produced, allowing *in vitro* embryo production.

The limited interest of the horse industry for these technologies as well as the scarcity of abattoir oocytes available for research and the technical difficulties for their recovery from

follicles have seriously impeded development of ARTs in this species. This review will focus on the clinical aspects of the oocyte recovery, their assessment and their use in assisted reproduction in the mare.

This paper reviews the state-of-the-art in the different techniques using oocytes for in vitro horse embryo production.

EMBRYO TRANSFER

The first report on embryo transfer in he horse was published in 1972 (for review see (Kraemer 2013)). Embryo transfer enables production of more than one foal for one season. It is also an option to produce foals from performing mares without having to interrupt their sports career. And last but not least it may be the only to obtain a foal from infertile mares with uterine disorders that keep them from bringing a pregnancy to term. The early embryo reaches the tip of the uterine horn by day 6 after ovulation. The embryo collection is routinely performed at day 7 or 8 post-ovulation. Collection is performed by uterine lavage: a single or double lumen cuffed-catheter is passed through the cervix and the uterine cavity is flushed several times with 1 to 2 liters of Lactate Ringer, PBS or some specific collection medium (eg Emcare Embryo Flush medium®, Bodinco, The Netherlands). Oxytocin is routinely administered during the last flush as it improves liquid retrieval and increases collection rate. The flush is filtered and embryos are searched for under a stereomicroscope. The size of the embryo at that stage averages 300µM. The embryo is washed, its quality is assessed and it is loaded in an AI straw in a drop of embryo medium. The embryo is transferred trans-cervically into the uterine body of a synchronized recipient mare. The ovulation of the recipient is planned to occur between 1 day before to 4 days after that of the donnor (Allen 2005). The ideal candidates as recipients should be young as they usually have intact uteruses, and docile because of the numerous manipulations and ultrasound exams involved.

The expected embryo collection rate is approximately 50%. The pregnancy rate after transfer of a good quality embryo is roughly 75% leading to an overall embryo transfer success rate of 35% per cycle (Squires 2005). Use of fresh semen as opposed to chilled or frozen semen is associated with a higher recovery rate (Stout 2006, Panzani et al. 2014). Risks and difficulties associated with embryo transfer program are the same as those of an AI center plus the aspects of the mares'synchronization and the actual transfers. By comparison to the other ARTs that will be discussed here, embryo transfer is technically rather accessible but still suffers from a lack of efficient and reproductible superovulation in the mare. Moreover, the

permanent need of available synchronised mares requires a large size herd of recipient mares and involves a severe workload for their daily follow up, which is economically difficult to bear for small centers. This limitation is partly reduced by the recent and progressive introduction of cryopreservation or cooling techniques. Embryos can be cooled and transported at 5°C for up to 24h to be transferred into a recipient (Carnevale et al. 1987, Carney et al. 1991). Over the last decades, highly specialized centers with such a herd have started offering embryo transfer services in America and in Europe. Ultimately, successful cryopreservation of embryos would be the key as it would remove the need to synchronize recipients, allow genetic banking. Reasonable success (approx. 50%) with either slow frozen (Lascombes and Pashen 2000) or vitrified (Eldridge-Panuska et al. 2005) embryos smaller than 300 μm have been reported. However, small embryos are difficult to obtain as equine embryos are usually signifcantly larger at the day of embryo collection. The current approach is to puncture the large embryos and aspirate blastocoelic fluid prior to vitrification (Choi et al. 2011).

OOCYTE COLLECTION, EVALUATION AND MATURATION

Handling and transportation of ovaries

No study directly compares techniques, media and transportation conditions of ovaries from abattoirs to the laboratories. Media used for this purpose are crystalloids supplemented with antibiotics, phosphate buffered solution (PBS), a complete medium (TCM-199) or commercial embryo flush solutions (Hinrichs et al. 1993, Alm and Torner 1994, Del Campo et al. 1995, Franz et al. 2003, Preis et al. 2004).

Storage periods for shipment ranging from 1 to 24 hours have been studied and it has been shown that storage up to 15 hours does not affect oocyte meiotic competence (Shabpareh et al. 1993, Del Campo et al. 1995, Guignot et al. 1999). However, high maturation rates reported by labs where transportation duration was minimal suggest that storage time for shipment could influence oocytes quality and their subsequent resuming of meiosis (Love et al. 2003, Hinrichs et al. 2005).

Transportation temperature has also been investigated. Based on a morphological study of the cumulus and the apoptosis of its cells, it has been suggested that ovaries transported between 20°C - 30°C and 35°C - 37°C, should ideally be processed within 3 hours and 2 hours

respectively (Pedersen et al. 2004). Another study showed that maturation rates (Metaphase I+II) after 24 hours were higher for oocytes collected immediately than for those retrieved after further storage of the ovaries for 15 to 18 hours at 4°C or at room temperature (Love et al. 2003). This study also suggests that 4°C is probably too low a temperature for an optimal storage of ovaries except when delay between ovaries collection and oocytes retrieval cannot be avoided. However, recent studies have shown that COCs can be stored overnight at room temperature either in follicular fluid (Choi et al. 2016) or in embryo holding medium (Dini et al. 2016).

The optimal solution might be found in harvesting oocytes at the slaughterhouse (Love et al. 2003). The oocytes can then be placed under maturation conditions in a portable incubator and undergo IVM during their transport to the lab. Due to its technical difficulties (collection facilities in the abattoir, transportation under controlled temperature and atmosphere), this method is not widely spread but could potentially optimize maturation rates for abattoir oocytes (Caillaud et al. 2008).

Oocyte collection *ex vivo*

Oocyte harvesting can be technically challenging in the equine. In order to preserve oocytes quality, each step requires a constant concern for conditions of hygiene, temperature, pH and medium quality. Oocytes can be aspirated (Desjardins et al. 1985, Shabpareh et al. 1993) with a 18G needle and an aspiration system (+/- 1 bar) or a vacuum pump (100-150mm Hg). The ovary can be sliced to gain access to more follicles. The equine oocyte is tightly fixed on a short, broad-based cumulus hillock with cumulus cell expansions into the underlying thecal pad (Hawley et al. 1995). They are therefore difficult to detach from the follicular wall. Consequently, recovering of equine oocytes requires incision of the follicles, scraping of the follicle wall with curette and flushing to detach the oocyte and its surrounding cells. Highest collection rates, approximating 80%, have been achieved using this technique (Del Campo et al. 1995). Most of the oocytes collected by this technique maintain cells immediately surrounding the oocyte intact and are imbedded in sheets of granulosa cells, forming Oocyte-Cumulus Complexes (COCs) (Hinrichs et al. 1993). Conversely, aspirated oocytes often only maintain immediately surrounding cells (Hinrichs 1991). The ovary is then sliced in small pieces to gain access to the follicles deeper in the ovarian stroma. Alternatively, the follicles can be aspirated and scraped simultaneously using the tip of an injection needle. Despite the

scraping and the flushing, oocyte recovery remains rather low (3-5 oocytes/ovary), as average number of antral follicles present on an equine ovary is 6 (Hinrichs 1991, Del Campo et al. 1995). This also reflects in a ten-fold increase in required time and personnel for harvesting of equine versus bovine oocytes (Galli et al. 2007). All these technical difficulties and the scarce availability of abattoir ovaries represent major limitations for fundamental research on equine oocyte and consequently for all the technologies that depend on its success. The recent reduction of horsemeat comsumption in many European countries has closed down nay slaughterhouses and ovum pick up (OPU) also called transvaginal aspiration (TVA) has become the main source of equine oocytes that remains.

Oocyte collection by ovum pick up (*in vivo*)

Ovaries recovered from slaughterhouses provide a material with an obvious lack of repeatability, delay in time between collection and placement in culture medium and lack of information about the stage of cycle and follicular growth and the age of the donor mare.

Embryo technologies in species such as cattle and pigs rapidly developed to the point of commercial application. By contrast, their development and application in the equine is mainly only accessible to a minority of performing horses. Consequently, abattoir derived oocytes are almost exclusively of interest for research purposes.

Most oocytes that are transferred for clinical purposes are collected *in vivo* from preovulatory follicles. Oocytes collections are scheduled based on the induction of the ovulation. This can be achieved by administration of hCG or a GnRH analog to the donor (Carnevale 2004). Crude Equine Gonadotropin (CEG), which is not commercially available, can also be used for research purposes (Duchamp et al. 1987).

Follicles have been punctured and oocytes collected by laparotomy (Vogelsang et al. 1988), colpotomy (Hinrichs and Kenney 1987), and flank puncture (Vogelsang et al. 1983, Palmer et al. 1986). The surgical approaches were rapidly considered too invasive and flank or ultrasound guided punctures became the most common methods of oocyte collection. Flank puncture is done by manual palpation of the ovary without the aid of an ultrasound machine. The ovary is manipulated per rectum and gently pulled against the flank wall. A trocard is

placed through the flank musculature against the preovulatory follicle. A large gauge needle is inserted through the canula into the antral cavity. The follicular fluid is aspirated and the follicular antrum is lavaged several times with PBS supplemented with heparin. As there is no visual control, this technique can only be successfully used for large follicles.

Transvaginal ultrasound-guided aspiration as performed in the bovine (McKinnon et al. 1988, Bruck et al. 1992), was modified for the equine (Bracher et al. 1993, Cook et al. 1993, Duchamp et al. 1994, Carnevale and Ginther 1995, Kanitz et al. 1995, Meintjes et al. 1995, Hinrichs et al. 1998) and is now yielding collection rates ranging from 51 to 86% (Cook et al. 1993, Bezard et al. 1995, Meintjes et al. 1995, Goudet et al. 1997a, Scott et al. 2001). The technique has been recently adapted for jennies with similar collection rates (Goudet et al. 2016).

Practically, the donor mare is sedated and a 7.5 MHZ sector transducer is positioned within the anterior vagina, lateral to the posterior cervix (fornix). The ovary is manipulated transrectally and positioned against the transducer. A needle is advanced through a needle guide and the vaginal and follicular walls are punctured. Follicular fluid is aspirated and the antral cavity is flushed several times with heparinised PBS or an embryo flush solution. Scraping of the follicular wall can be achieved by rotating the needle during the aspiration. COCs are searched in follicular fluid and flushes under stereomicroscope. Follicles larger than 25mm can be punctured using a single lumen needle. Follicles smaller than 25mm can be punctured with a double lumen needle allowing a continuous flush of their antral cavity as described by Duchamp and collaborators (Duchamp et al. 1995).

Oocyte Evaluation

Ex vivo or *in vivo* collected oocytes can be denuded. However they are usually surrounded by a multilayered cumulus investment. Granulosa and cumulus cells are similar in texture. The innermost cumulus cells have numerous projections penetrating the zona pellucida and establishing close contact with the oolemma (Grondahl et al. 1995). COCs can be characterized based on their degree of expansion (Hinrichs et al. 1993, Goudet et al. 1997a, Hinrichs and Williams 1997), which is correlated to the nuclear maturation of the oocyte (Zhang et al. 1989, Goudet et al. 1997a, Gable and Woods 2001). In general, immature

oocytes are associated with a compact cumulus. Expanded cumuluses are usually associated with atretic follicles or the preovulatory stages. Oocytes from atretic follicles are known to have a greater ability to resume meiosis and progress from germinal vesicle (GV) stage to metaphase of the second meiotic division (MII) (Hinrichs 1991). It is well established that follicles are responsible for meiotic arrest of the oocyte (Pincus and Enzemann 1935) and this has also been confirmed in the horse (Hinrichs et al. 1995). As they undergo atresia, follicles lose their capacity to maintain that meiotic arrest (Gougeon and Testart 1986, Blondin and Sirard 1995). This explains the higher maturation rates for expanded than compact COCs obtained in several studies (Hinrichs and Williams 1997, Hinrichs and Schmidt 2000, Galli et al. 2007).

The cumulus cells directly surrounding the oocyte are called the corona radiata. They are often last to expand resulting in a typical image of a cellular ring around the oocyte. Compared to the COC, the oocyte is small and very often difficult to image as its surrounding cumulus cells effectively prevent its detailed observation. These cells play an essential role in oocyte maturation and early embryonic development. Except for the Intra Cytoplasmic Sperm Injection (ICSI), their presence is required for all the techniques involving oocyte transfer, making morphological assessment of the oocyte difficult to perform. However, it has been shown that ooplasm aspect is correlated to follicular viability assessed by histological appearance of the mural granulosa. Oocytes with a homogeneous ooplasm are significantly associated with viable follicles and compact COCs. Conversely, oocytes with a granulous, polarized ooplasm, corresponding to an uneven distribution of lipid droplets and organelles, are associated with expanded COCs and slightly or moderately atretic follicles (Hinrichs and Williams 1997).

Oocytes in vitro Maturation (IVM)

Oocyte maturation involves nuclear and cytoplasmic events by which the oocyte acquires its developmental competence. The final maturation of the oocyte has been associated with 6 consecutive stages of nuclear and cytoplasmic changes. These changes have been described by Grondahl and co-workers (Grondahl et al. 1995) and can be summarized as : (1) the central spherical nucleus stage ; (2) presence of a spherical nucleus located at the periphery of the ooplasm ; (3) a peripheral oocyte nucleus in stage II ; (4) oocyte nuclear breakdown also termed Germinal Vesicle Break Down (GVBD) ; (5) metaphase I ; and (6) metaphase II with

metaphase chromosome peripherally located and a polar body within the perivitelline space. Observation of the polar body under the stereomicroscope is difficult but it can occasionally be seen in the perivitelline space. A specific DNA staining, such as Hoechst 33258 can be used on decoronised oocytes allowing chromatin examination and classification into: (1) Germinal Vesicle (GV), (2) Dense chromatin, (3) Metaphase I, (4) Metaphase II, (5) Degenerated (Hinrichs et al. 1993, Goudet et al. 1998b).

Most IVM media used for horse oocyte are derived from those used in the bovine species. Since the first equine oocyte IVM (Fulka and Okolski 1981), a variety of media comprising synthetic tissue culture media such as TCM-199 (Willis et al. 1991, Dell'Aquila et al. 1997b, Galli et al. 2001), or Ham's F10 (Okolski et al. 1991, Willis et al. 1991, Shabpareh et al. 1993) have been investigated. Most media now are based on TCM-199, equine maturation medium I (EMMI) and with addition of foetal calf serum, various hormones such as LH, FSH, oestradiol (Carnevale et al. 2004b) or EGF (Goudet et al. 1998a, Lorenzo et al. 2002). A few studies have investigated the effects of co-cultures for IVM (Hinrichs et al. 1995, Li et al. 2001, Choi et al. 2002b, Tremoleda et al. 2003). These gave a better understanding of the control mechanisms of the meiotic arrest within the follicle but failed to show a significant improvement in oocyte maturation.

IVM duration has also been extensively investigated (Fulka and Okolski 1981, Zhang et al. 1989, Del Campo et al. 1990, Willis et al. 1991, Del Campo et al. 1995, Bezard et al. 1997). After 30 hours of maturation under a controlled atmosphere (39°C and 5% CO2), 50% to 80% oocytes reach the metaphase II stage (Scott et al. 2001). It has also been shown that further duration of IVM does not improve maturation rates (Shabpareh et al. 1993). Optimum maturation duration depends on the initial degree of maturation of the oocytes, which is correlated to the aspect of their COC. Maximum maturation rates for oocytes retrieved from expanded and compact COCs were obtained after 24h and 32h respectively for Hinrichs and co-workers (Hinrichs et al. 1993) and 24h and 30h for Zhang and collaborators (Zhang et al. 1989). Donkey oocytes seem to have an optimal IVM maturation duration of 34 hours and no improvement of metaphase II rates was observed when IVM was continued after 48 hours (Goudet et al. 2016).

Fertilization of an oocyte requires both its nuclear and cytoplasmic maturation. Nuclear maturation can resume spontaneaously when oocytes are removed from their inhibitory follicular environment. Nuclear maturation is achieved when metaphase II stage of meiosis is

reached. This can be readily evaluated by specific DNA staining. Conversely, to date there is no reliable marker of cytoplasmic maturation. The ultimate criterion of oocyte competence is its ability to be fertilized and develop into a viable embryo. The lack of an efficient *in vitro* embryo production (IVP) system has limited oocyte maturation assessment to the nuclear maturation. Although some studies investigated cytoplasmic changes observed during nuclear maturation, such as migration of cortical granules or mitochondria (Grondahl et al. 1995, Goudet et al. 1997a, Aguilar et al. 2002, Carneiro et al. 2002), they did not measure their significance to developmental competence of the oocyte. Cytoplasmic maturation depends on the accumulation of proteins and mRNA (Grondahl et al. 1993). As already mentioned, once oocytes are removed from their follicular environment they are relieved of its meiotic inhibitory effect and they condense their chromatin. This stops transcription and subsequent protein production. Consequently, proteins and mRNA storage should obviously be complete before meiotic resumption and germinal vesicle breakdown is induced (Sirard 2001). Based on this observation, bovine oocytes have been cultured under conditions that temporally maintain meiotic arrest but allow cytoplasmic maturation, which translated into an improved developmental competence (Fouladi Nashta et al. 1998, Hashimoto et al. 2002). A similar approach has been considered in the horse (Franz et al. 2003) but the effect on cytoplasmic maturation has only been studied on small numbers (Hinrichs et al. 2006, Hinrichs et al. 2007). A better understanding of the control mechanisms of the meiotic arrest might certainly lead to new IVM strategies based on a better synchronisation of nuclear and cytoplasmic maturation events *in vitro*.

USE OF EQUINE OOCYTES IN ASSISTED REPRODUCTION

Development of ARTs in the horse has been slow compared to other species, but significantly progressed over the last decade. However, the techniques involved require specific equipment and expertise, which are most often only accessible in specialised centres.

Conventional IVF failed to give consistent results in the equine and reports of successes with IVF are very few. There are only two live foals reported to be born following conventional IVF and both derived from *in vivo* matured oocytes (Palmer et al. 1991, Bezard et al. 1992). Failure of IVF is probably related to inefficient sperm capacitation (Alm et al. 2001), changes occurring with the zona pellucida (Dell'Aquila et al. 1999, Hinrichs et al. 2002) or to incomplete or imperfect *in vitro* oocytes maturation (Li et al. 2001). To date, calcium ionophore A23187 is the most efficient sperm capacitation treatment, inducing acrosome reaction and sperm penetration (Zhang et al. 1990, Alm et al. 2001, Hinrichs et al. 2002) although heparin was shown to give similar results in one study (Alm et al. 2001) and an other paper reports even higher penetration rates (60%) after capacitation with modified Whittens and hyperactivation with procaine (McPartlin et al. 2009) . However, recent studies have shown that procaine treatment in fact induces parthenogenesis of the equine (Leemans et al. 2015) and the donkey (Goudet et al. 2016) oocytes. Work on conventional IVF is still going on, as it should be the easiest way to produce embryo in vitro, like in the bovine where heparin capacitation is efficient for most bulls. In order to overcome the barrier of inefficient IVF, research focused on either in vivo or in vitro techniques to produce embryos such as intra-follicular and intra-oviductal oocyte transfer, and ICSI.

Intra-Follicular Oocyte Transfer (IFOT)

IFOT is based on the injection of one or more oocytes into the preovulatory follicle of a recipient mare where the oocytes can find the conditions to complete their maturation. When ovulation occurs, transferred oocytes are ovulated at the same time as the recipient's preovulatory oocyte. IFOT has been first unsuccessfully attempted in the baboon, bovine and porcine, probably due to damage caused to the follicle during manipulations (Fleming et al.

1985, Hinrichs and Digiorgio 1991). Thanks to the large size of the ovary, its specific anatomy with a peripheral medulla and a surrounding fibrous tunique, the mare appears to be a unique model for the study of IFOT. Technically, the procedure is similar to that of the ovum pick up. The recipient mare is sedated and a sector transducer is positioned within the anterior vagina, lateral to the posterior cervix. The ovary is manipulated transrectally and positioned against the transducer. A needle is advanced through a needle guide and the vaginal and follicular walls are punctured. A few millilitres of follicular fluid are aspirated, the oocytes are injected into the follicle and the needle is flushed with some follicular fluid. Hinrichs and Di Giorgio (Hinrichs and Digiorgio 1991) developed the technique and transferred of immature oocytes into a preovulatory follicle. The same technique has been used for a study of the follicular fluid contents during the oocyte maturation but there was no attempt to produce embryos (Goudet et al. 1997b). More recently, embryos were obtained following IFOT of *in vitro* matured oocytes and (Deleuze et al. 2009). Embryo production rates, following IFOT vary from 8.9% (12 embryos in excess/ 135 transferred of oocytes) (Hinrichs and Digiorgio 1991) to 12.6% (6 embryos in excess/ 45 transferred of oocytes) (Deleuze et al. 2009). IFOT seems to increase the incidence of anovulatory follicles but it could still be a viable option to increase the number of embryos of selected mares (Deleuze et al. 2009) as superovulation treatments are yet not very efficient in the mare (Dippert and Squires, 1994; Bezard *et al.*, 1995; Alvarenga *et al.*, 2001b).

Intra-oviductal Oocyte Transfer (OT)

The technique involves surgical transfer of one or more oocytes from a donor mare into the oviduct of an inseminated recipient. Subsequent fertilization and early embryo development occur within the recipient's oviduct.

Alternatively, sperm and oocyte can be transferred together into the recipient's oviduct. This technique called: « Gamete Intra-Fallopian Transfer » (GIFT) has been shown to give pregnancy rates similar to those obtained by OT (Carnevale and Ginther 1995, Coutinho da Silva et al. 2002b). However, fresh sperm appears to be more efficient than frozen-thawed semen for GIFT (Coutinho da Silva et al. 2002a). Oocyte transfer and GIFT are essentially used for research purposes and for mares affected with oviductal, uterine or

cervical pathologies that preclude embryo transfer as an option to obtain a live foal from these mares. Cyclic and non-cyclic mares have been used as recipients with similar pregnancy rates (Carnevale et al. 2005) and xenogenous transfer into sheep oviducts has also been reported (Wirtu et al. 2004). However, most oocyte transfers involve injection of oocytes into the oviduct of a cyclic recipient mare, whose preovulatory oocyte has been aspirated. This a major limitation of the technique as a risk of producing a foal from the recipient's oocyte should always be considered when it has not been successfully punctured from her preovulatory follicle. However, this risk is very low if the follicle has been thoroughly aspirated (follicle fully collapsed and presence of blood in the collection tubing system). When the aspiration of the recipient's preovulatory follicle in incomplete, the risk of producing a foal from her own oocyte is approximately 30% and she should not be used as a recipient (Hinrichs et al. 2000). Oocyte transfer in the horse was first described by Mac Kinnon and co-workers (McKinnon et al. 1988), but their blastocyst rate and those from the subsequent studies (Zhang et al. 1989, Ray et al. 1994) remained low. Carnevale and Ginther (Carnevale and Ginther 1995) first reported higher embryo production rates following transfer of *in vivo* matured oocytes. More recent studies transferring a single oocyte collected from a preovulatory follicle 24 hours after an injection of hCG and matured *in vitro* for another 16 to 20 hours before transfer also showed high embryo production rates reaching approximately 85% (Hinrichs et al. 1997) and commercial programmes using OT for mares with reproductive abnormalities are now available (Carnevale et al. 2001). Intra-oviductal oocyte transfer can nowadays be considered a viable clinical alternative to embryo transfer in selected mares (Hinrichs et al. 2002, Carnevale 2004), especially those suffering from uterine disorders. Another clinical application of the technique is the production of foals following sudden and unexpected death of a valuable mare. The ovaries of the mare can be collected rapidly after the death of the animal and should be transported to the lab as soon as possible, where the oocytes could be matured *in vitro* and transferred into a recipient's oviduct. Birth of a foal following transfer of *in vitro* matured oocytes collected from ovaries after the death of the donor mare was first reported in 2003 (Carnevale et al. 2003). The same authors reported embryo development rates of 15% following transfer of oocytes (n=191) recovered from commercial mares post-mortem (Carnevale et al. 2004a).

Reported embryo yields following intra-oviductal transfer of *in vitro* matured oocytes vary: 7% (2/29) (Scott et al. 2001), 15% (11/73), 18% (13/73) (Preis et al. 2004), 7% (2/29) (Zhang et al. 1989). These results remain lower than those obtained after transfer of preovulatory, *in*

vivo matured oocytes, that range from 73% to 83% (Carnevale and Ginther 1995, Hinrichs et al. 1998, Scott et al. 2001, Carnevale et al. 2004b). Age of the donor is a major factor affecting success of OT (Carnevale 2004). More morphological abnormalities were observed in oocytes from older mares (Carnevale et al. 1999) and significantly more oocytes collected from young donor mares can develop into embryonic vesicles (Carnevale and Ginther 1995).

Intra-Cytoplasmic Sperm Injection (ICSI)

ICSI was developed as an alternative to bypass the inadequacy conventional IVF in the equine and fertilize equine oocytes *in vitro* (Squires et al. 2003, Hinrichs 2005, Galli et al. 2007). Since the first report of a pregnancy established after ICSI in the horse (Squires et al. 1996), it has produced several foals after injection of both *in vivo* and *in vitro* matured oocytes (Mc Kinnon et al. 2000, Li et al. 2001, Galli et al. 2002). It is now considered the reference technique for IVF as conventional IVF has not been established due to the poor capacitation results achieved.

Briefly, a single motile spermatozoon is immobilized by crushing its tail. It is then injected into the cytoplasm of a denuded metaphase II oocyte. This overcomes the barrier of capacitation and acrosome reaction and even permits the use of sperm with poor motility and performances *in vivo* (Lazzari et al. 2002). Early studies rapidly reported high fertilization rates (40% vs. 5% for conventional IVF) (Dell'Aquila et al. 1997a, Dell'Aquila et al. 1997b, Grondahl et al. 1997). Embryo yields following ICSI with either frozen-thawed or fresh semen have been shown to be similar (Choi et al. 2002a) as long as a motile sperm is selected (Lazzari et al. 2002). In another study, oocytes successfully developed to blastocysts after being injected with immotile spermatozoa isolated from semen that had undergone two freeze-thaw cycles (Choi et al. 2006a). Enhancement of further development of the zygote to the blastocyst stage with oocyte activators has been tested but most only increased parthenogenesis (Li et al. 2000).

The culture of 2-cell embryo to the blastocyst stage when they can be transferred into a recipient's uterus is another major limiting step in IVP. Many culture systems for early cleavage stage embryos have been investigated, including: co-cultures with oviduct cells (Battut et al. 1991), Vero cells (Dell'Aquila et al. 1997b), cumulus cells (Li et al. 2001),

granulosa cells (Rosati et al. 2002), and culture in defined media : G1.2 (Choi et al. 2002a), DMEM-F12 and CZB (Choi et al. 2004), and modified SOF (Galli et al. 2002). In most of these systems, however, the percentage of 2-cell embryos developing to blastocysts remained rather low, ranging from 4 to 16%. ICSI early embryos can alternatively be transferred into the oviduct of a recipient mare (Squires et al. 1996) or a temporary recipient sheep, which remains the most successful system for producing equine blastocysts with a blastocyst rate of 45% (Galli et al. 2002, Lazzari et al. 2002). However, Choi and collaborators (Choi et al. 2006c, Choi et al. 2006b) recently developed a culture system using DMEMF/F-12 medium and achieved blastocyst development rates (27-38%) similar to those obtained after transfer in both the mare and the sheep (approximately 36%) (Galli et al. 2007). In vitro produced embryos can be cryopreserved at a smaller size than their in vivo retrieved conterparts. This increases post-thaw survival and pregnancy rate exceeding 60% (Galli et al. 2007).

Somatic Cell Nuclear transfer (SCNT or Cloning)

The first cloned foal was born in Italy in 2003 using the nucleus of a fibroblast (Galli et al. 2003a) and additional cloned foals have been documented since (Lagutina et al. 2005, Hinrichs et al. 2006, Hinrichs et al. 2007). Basically, good quality matured oocytes are enucleated before the donor cell is fused or its DNA is injected into the enucleated oocyte and the reconstructed embryo is activated. Following activation, developing embryos must be cultured to the blastocyst stage so they can be transferred trans-cervically into a recipient's uterus (Galli et al. 2003b). Overall, blastocyst rates remain lower than 10% (Lagutina et al. 2005). Cloned embryo transfer rates are similar to classical embryo transfers (+/- 60%) but approximately 50 to 70% of obtained pregnancies are lost before term. Cloned foals have a higher risk of developing neonatal maladaptation syndrome, angular deformities but seem to be normal after 2 weeks of age (Johnson et al. 2010). The female horse produced by Galli in 2003 (Galli et al. 2003a) has since produced a healthy foal (Galli et al. 2008) and the male produced by Lagutina (Lagutina et al. 2005) has been shown to be fertile, suggesting that cloned animals are reproductively normal. The mean rate of transferrable embryos per used oocytes is 3.55% (Lagutina et al. 2005). With an overall success rate that remains low, nuclear transfer remains a challenge, as it demands high numbers of oocytes, which are difficult to obtain.

Cloning an animal must be seen as a way to salvage some genetic potential rather than to reproduce a specific individual. The obvious candidates for cloning are geldings that turn out to be very good performers but from which no foal had been produced before castration.

As already mentioned, clones are prone to early life disorders that may interfere with their further sports career. Moreover, clones will not express the genetic material they received from the donor in an identical way (epigenesis), explaining why they can be phenotypically different. The environment, the training and many other external factors will also affect the clone's performance. The clone and the donor have the same nuclear DNA. However, after the injection the embryo develops with the mitochondria and their DNA from the enucleated oocyte. The actual impact of the mitochondrial DNA on phenotype and performances of cloned horses still needs to be elucidated.

A serious reduction of the genetic pool may also be a concern with cloning horses. Long-term semen conservation and artificial insemination have already extended and increased the distribution and use of a stallion when compared to natural breeding. With cloning, there is technically no time limitation in the use of one individual's genetic material, possibly leading to a selection cul-de-sac. However, looking at the number of clones that have been produced since the birth of the first one in 2003, it seems that equine clone production will remain a niche activity restricted to highly selected animals.

CONCLUSION

In the equine, most often based on performances but sometimes even for emotional reasons, some individuals' value is such that high-tech ARTs can be considered to salvage those individuals' genetic potential. ARTs can nowadays optimize the reproduction of a mare by increasing the number of foals she can produce without necessarily interrupting her sports career e.a. with embryo transfer. ARTs allow births of foals from infertile mares that could no longer produce an offspring with classical breeding techniques. These assisted reproductive techniques require great expertise and very often, highly specialized and expansive equipement. However, they have undergone tremendous development over the last decade. Some difficulties such as establishing a consistent procedure for embryo or oocyte cryopreservation or a simple conventional IVF technique as ICSI is limited to a few centers

are still hard to overcome. However, despite the scarcity of experimental material with the closure of many slaughterhouses, new approaches are being explored like the vitrification of oocytes (Curcio Bda et al. 2014) and research is going one worldwide to establish conventional IVF, finalize embryo cryopreservation and develop new assisted reproductive technologies. Use of ARTs will probably also be extended to salvage local breeds or wild equids where numbers are growing concerningly low, thus compromising genetic and biodiversity.

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