Current status and clinical potential of IVF, ICSI and cloning in horses

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Introduction

Progress in assisted reproduction in the horse was slow in starting, but has been rapid over the last decade. While the first recovery of oocytes from mares in vivo was reported in 1983 [1], it was not until 1994 that an efficient method for obtaining foals from these isolated oocytes was presented (oocyte transfer, [2]). One main reason for the delay in utilizing isolated horse oocytes was the failure of in vitro fertilization (IVF) to be repeatably successful. In vitro fertilization is used clinically in many other species. In cattle, oocytes are recovered from all visible follicles of cows in vivo by transvaginal ultrasound-guided follicular puncture; this procedure may be performed twice a week in each cow. The recovered oocytes are subjected to in vitro maturation (IVM) then fertilized in vitro, and the resulting embryos are cultured in vitro (in vitro culture, IVC) to the blastocyst stage, at which point they are transferred transcervically to the uteri of recipient cows. The procedure of IVM/IVF/IVC is commercially successful and allows progeny to be obtained from cows that cannot themselves provide embryos for transfer.

While the clinical demand for similar protocols in the horse is high, equine in vitro embryo production has not been successful. The oocyte recovery rate from small (non- preovulatory) follicles in the horse is lower than that in cattle, because of the tight connection of the horse oocyte to the follicle wall [3]. Horse follicles grow more slowly than do those of cattle, and oocyte recovery procedures must be spaced 10 or more days apart or follicle numbers are lower with each successive puncture [4]. Because of the low average number of visible follicles per mare ovary, and the low recovery rate, only an average of 1 to 3 oocytes are obtained per mare per aspiration attempt [4-6]. Only about 50% of the collected oocytes will mature in vitro, and in vitro matured oocytes have a lower developmental competence than do in vivo matured oocytes (10% pregnancy rate after oocyte transfer vs. 82% for in vivo matured oocytes [7]). Therefore, most people working in assisted reproduction in the horse obtain oocytes preferentially from the mature preovulatory follicle, but our inability to superovulate the mare limits such oocyte recovery to one (or perhaps two) oocytes per cycle. Timing of follicle aspiration is determined by administration of hCG or deslorelin to induce ovulation, and aspiration 1 to 12 hours before the anticipated time of ovulation, with the duration of maturation completed in vitro. The oocyte recovery rate on aspiration of a mature preovulatory follicle is 60 to 80% [2, 8, 9].

IVF and ICSI

While the above aspects of oocyte recovery and maturation reduce our ability to obtain large numbers of mature, competent oocytes, a major barrier to use of IVM/IVF/IVC in the horse has been the inability to efficiently perform IVF. Only 2 foals

have been born from IVF, both of these from oocytes matured in vivo [10, 11]. Reported fertilization rates after standard IVF have ranged from 4% [12] to 33% [13] but the higher rates have not been shown to be repeatable[14, 15].

The major difficulty associated with IVF in the horse appears to be penetration of the zona pellucida, as fertilization rates may be increased by zona dissection or zona drilling, and in fact polyspermy results if this is done [12, 16]. The barrier of the zona pellucida may be overcome by directly injecting one sperm through the zona into the cytoplasm of the oocyte via micromanipulation (intracytoplasmic sperm injection, ICSI). The first successful report of ICSI in the horse was that of Squires et al., in 1996 [17]; these workers injected 4 in vitro-matured oocytes with sperm, and transferred them to the oviducts of recipient mares. They obtained one pregnancy which went on to term. However, subsequent studies on ICSI showed that this success rate was difficult to repeat.

After sperm is injected into the oocyte, factors in the sperm induce the oocyte to activate. Oocyte activation involves post-fertilization events including completion of oocyte metaphase II and extrusion of the second polar body, decondensation of both sperm and oocyte chromatin to form the male and female pronuclei, dissolution of the nuclear membranes, combination of the male and female chromosomes, and onset of mitosis resulting in cleavage. Some laboratories working with ICSI in the horse have had difficulty in achieving good activation rates after sperm injection, and therefore have used a chemical activation method (such as calcium ionophore) after ICSI. However, other laboratories achieved similar activation and cleavage rates without additional activation treatments. Reported percentages of male pronucleus formation of oocytes after ICSI in the horse have varied from 21 to 68%, and cleavage rates have ranged from 20-65% [14, 15, 18-23].

Recently our laboratory reported results of equine ICSI using the Piezo drill. This device causes minute vibrations in the injection pipette which allow it to be drilled through the zona pellucida, rather than pushed through it as with standard injection. Several pulses of the drill were applied to sperm before injection, to damage the sperm plasma membrane. It is thought that damage to the sperm plasma membrane is necessary to allow release of the sperm factor necessary for activating the oocyte after the sperm is injected. In standard injection systems, this is done by rolling the sperm tail against the bottom of the Petri dish with the pipette. Using the Piezo drill to perform ICSI on in vitro-matured oocytes, we achieved up to 84% cleavage, and 72% cleavage with normal nucleus formation, with an average of over 8 cells per cleaved embryo at 96h in vitro culture [24]. Similarly, other workers have achieved over 80% cleavage with ICSI using the Piezo drill [25]. The drill probably increases success by ensuring that the cell membrane of the oocyte is punctured (thus assuring that the sperm is deposited within the oocyte cytoplasm, rather than outside of the membrane) and by inducing consistent changes in the sperm plasma membrane to allow release of sperm factors. The success of this procedure now makes ICSI a possible technique for use in clinical assisted reproduction in the horse.

Currently, however, the potential for clinical application of ICSI in the horse is limited. Our embryo culture systems are not adequate to culture the resulting embryos to a stage at which they may be transferred directly to the uterus without a loss in viability. Because of this, embryos produced by ICSI must be transferred directly to the oviduct after injection in order to achieve optimum pregnancy rates. Transfer of ICSI-oocytes to the oviducts after even 24 hours of in vitro culture results in lower pregnancy rates than that for oocytes transferred immediately after injection [22]. There are only two reports of transfer of ICSI-derived embryos, cultured in vitro, directly to the uterus [23, 25]. In the report of Li et al., 20% of ICSI-oocytes developed to blastocysts, but only 6/13 (46%) were of transferable quality. After transfer of 6 blastocysts to the uterus, 2 normal and 2 abnormal pregnancies resulted ([23]; X. Li, personal communication 2001), thus, the normal pregnancy rate per injected oocyte was approximately 3%. In the report of Galli et al., 13% of injected oocytes developed to morulae or blastocysts during in vitro culture. Two embryos were frozen, thawed and transferred to the same recipient. Two embryonic vesicles were seen in the recipient but the outcome of the pregnancy was not given. Interestingly, these same workers have cultured equine ICSI embryos by transferring them to the ligated oviducts of sheep; in this case 50% of injected oocytes developed to morulae or blastocysts, but the pregnancy rate after transfer of frozen-thawed embryos was only 2/10, or a pregnancy rate of 10% per injected oocyte. In contrast, transfer of ICSI embryos to the mare oviduct immediately after injection has resulted blastocyst recovery of up to 40% (Choi and Hinrichs, unpublished data) and pregnancy rates of 25 to 38% [17, 22].

If ICSI-oocytes must be transferred directly to the oviduct, then there is little benefit to performing ICSI over simply performing oocyte transfer. Oocyte transfer, i.e., transfer of mature oocytes to the oviduct of inseminated recipient mares, is an efficient procedure that has been used clinically now for over 5 years [26, 27]. Because oocyte transfer is an effective method for achieving pregnancy with isolated oocytes, currently the main use for ICSI is in the case of poor semen quality, in which fertilization rates after standard insemination are expected to be low. Gamete intrafallopian transfer (GIFT, transfer of both sperm and oocyte to the oviduct) also holds promise for achieving pregnancy with low numbers of sperm, but so far has been successful only with fresh, and not cooled or frozen sperm, thus limiting its application [28].

We have performed ICSI clinically at Texas A&M in cases in which only poor quality semen was available from the desired stallion. In these cases, the semen was frozen, with low post-thaw motility, and was associated with poor conception rates in vivo. Fresh and frozen-thawed semen give equivalent embryo development after ICSI [24], therefore we utilize frozen-thawed semen because of its ease of use. Performing ICSI for clinical embryo production does have some advantages over standard oocyte transfer. Because frozen semen is used, no arrangements for semen shipment need to be made. The recipient mare is not inseminated, therefore there is minimal risk of post-breeding endometritis. Post-breeding endometritis is common in oocyte recipient mares and can limit pregnancy rates after oocyte transfer [29]. There is no concern over the recipient mare's oocyte -- she simply needs to ovulate at about the time of oviductal transfer. In contrast, for oocyte transfer, since the recipient mare is inseminated, the

recipient oocyte must be removed by follicle aspiration, or a non-ovulatory, hormone-treated recipient must be used. These differences make mare management for ICSI simpler than that for oocyte transfer. However, the inefficiencies associated with the ICSI procedure, including the 1 to 2 hours needed for micromanipulator set-up, injection, and take-down, the risk of lysis of the oocyte during the injection procedure, and the reduced rate of embryo cleavage in comparison with in vivo fertilization, render ICSI a less efficient method than oocyte transfer when semen of normal fertility is used.

Development of effective methods for culture of equine embryos will greatly increase the clinical usefulness of the ICSI procedure. If embryos can be grown successfully to the stage that they may be transferred directly to the uterus, then we can in fact achieve an IVM/IVF/IVC system of embryo production similar to that used in cattle. This should be a major thrust of research in equine assisted reproduction in the next few years.

Nuclear Transfer (Cloning)

Research in nuclear transfer (NT) in the horse is still in its infancy. Few laboratories are working in this area; again, the low supply of horse oocytes makes research on NT in this species problematic. To perform NT, oocytes are matured in vitro (or collected after maturation in vivo). Using micromanipulation, the area of cytoplasm containing the chromatin of the oocyte is removed, thus creating an enucleated oocyte or cytoplast. Somatic cells from the genetic donor are grown in vitro from a tissue sample such as a skin biopsy. One somatic cell from the donor is selected and recombined with the cytoplast, either by injecting the donor cell beneath the zona of the cytoplast and fusing the two cells by electric pulse, or by breaking the donor cell membrane and injecting the cell directly into the cytoplasm of the oocyte. The recombined oocyte, now containing the nucleus of the genetic donor, is then artificially activated. The transferred nucleus decondenses and the oocyte starts embryonic development.

The first equine NT embryos produced were reported in brief communications in 2000 [30, 31]. Fusion rates were low and cleavage rates even lower (less than 15%). However, when horse somatic cells were transferred to enucleated bovine oocytes, the cleavage rate was over 50% [30, 32], indicating that the equine oocyte was the major source of inefficiency in this system.

Subsequently, two full papers have been published on equine NT, reporting increased fusion and cleavage rates of NT oocytes using injection of inactivated Sendai virus (which changes the properties of the plasma membrane to promote fusion) with electrofusion [33] or by use of the Piezo drill for direct injection of somatic cells into the cytoplast [34]. Cleavage rates of NT-oocytes were improved to around 50% in the best treatments [33, 34]. Li et al. reported production of NT blastocysts after culture in vitro, but at a very low rate (2% of recombined oocytes). Transfer of these blastocysts to the uteri of recipient mares did not result in pregnancy (W.R. Allen, personal communication, 2001). In a recent report, several hundred oocytes were recovered from mature preovulatory follicles in vivo and were transferred to the oviducts of recipient

mares immediately after NT; 7 embryonic vesicles were detected on ultrasonography of the recipient mares, one of which formed an embryo proper, but was lost before 100 days gestation [35].

These results show that equine cloning is far from being clinically applicable at this time; however, advances in this field are occurring rapidly. It is likely that pregnancies and birth of live cloned foals will be achieved in the next few years. At that point, producers and researchers will be able to assess the usefulness of cloning as a clinical technique. Nuclear transfer has already provided the opportunity for us to gain vast amounts of knowledge regarding the processes of oocyte meiotic and developmental competence, chromatin remodeling, oocyte activation, and early embryo development. There are also myriad applications for cloning in research, in assessing the relative effects of genetics and environment on physical and behavioral traits and establishing genetically identical herds for research trials. Clinically, cloning will allow the preservation of valuable genetics, from the old mare who is the "last of her line" to that of endangered equids. Production of cloned foals may allow reproduction of currently infertile animals; for example, a gelding that has proven to be an exceptional individual may be used as a donor to produce a colt with the ability to pass on the original gelding's genetics. Mares that are too old to reproduce may be used as genetic donors and the resulting filly may carry on the original mare's reproductive capacity. The genetics of exceptional individuals that die before being able to reproduce could also be saved. It is unlikely that nuclear transfer will become a common clinical procedure, but it will widen the possibilities available to us as clinicians when we are faced with many problems that are currently insurmountable.

While cloning is not yet clinically feasible, "banking" of tissue from animals, including horses, with valuable genetics is currently being done commercially by a number of companies. A skin or lip biopsy is taken from the animal and sent to the company in a specially made transportation package. At the laboratory, cells are grown from the tissue in culture and the company stores the cells in liquid nitrogen until the technology for nuclear transfer becomes available. It is possible to obtain cells from animals hours to days post-mortem (especially if tissue has been cooled but not frozen) and still allow successful tissue culture. Thus, tissue banking is an option that we can currently offer clients that are extremely concerned about the loss of genetic potential when a horse dies or becomes infertile; the decision on whether to use the cells to produce a foal can be made at some time in the future.

The possibility of cloning opens up many new areas for study, many clinical options and many ethical questions. However, it should be understood that a cloned foal will not be an exact duplicate of the original horse. There are three major mechanisms by which the phenotype of the cloned individual may differ from the genetic donor.

First, the NT embryo will have the nuclear DNA of the genetic donor, but the mitochondrial DNA of the recipient oocyte. A small number of mitochondria from the donor cell are also present within the recombined oocyte, but proportionately few. The impact of the source of mitochondria, or a mixture of mitochondria, on the traits of the

progeny is currently unknown. It can be hypothesized that, since mitochondria are the source of energy for the cell, differences in mitochondria could have an effect on speed, stamina or other physical or behavioral traits. The presence of heterogeneous mitochondria may be avoided by using oocytes from the genetic donor's female relatives, but collecting enough familial oocytes to produce a cloned offspring, given the current inefficiency of nuclear transfer procedures, would be difficult.

The heterogeneous mitochondria present in a filly produced by nuclear transfer will be passed down to the filly's offspring, as they will be present in her oocytes. However, while sperm from a colt produced by nuclear transfer will carry the heterogeneous mitochondria, the contribution of the male mitochondria to the embryo after fertilization is minimal and the cloned colt could probably be considered to produce the same progeny that its genetic donor would have produced.

A second factor in the phenotype of the new foal is the environment – both within the uterus and postnatally. We know from observing naturally-occurring twins and the few split-embryo identical twins produced that the intrauterine environment affects not only the size of the foal at birth, but also the adult size and phenotype (W.R. Allen, personal communication 1996). Thus identical cloned embryos placed in different mares, or having different quality placentae, may produce progeny of different size and proportion at adulthood. The milk production of the dam and nutritional and exercise programs to which the foal is exposed will also influence it as an adult. These are factors in any transferred embryo; however, the effects will be more noticeable in a cloned individual that may be compared directly with its expected phenotype.

The third major factor in the development of the cloned foal is the presence of epigenetic effects. We know that within the genome certain genes are "turned off" while the transcription of others is enhanced. This is controlled largely by methylation and demethylation of the DNA. The methylation status of the genes changes throughout embryonic and fetal life and also depending upon which tissue the cell is in. The synchrony of the methylation status of genes with the requirements of the fetus during gestation is essential to normal development.

In nuclear transfer, the donor nucleus is that of a somatic cell; genes important to the somatic cell are turned on and many non-essential genes are turned off. The DNA of the transferred cell must be reprogrammed to an embryonic state by the oocyte in order to produce a normal embryo. We know from success in producing nuclear transfer offspring that such reprogramming does occur. However, the exact state of activity of the DNA during fetal life may affect the phenotype of the animal at birth, again providing a means for the cloned offspring to differ from the genetic donor. Epigenetic effects may also influence the phenotype of the animal after birth; e.g. an animal with growth factor genes programmed "on" may grow larger than its cloned sibling with less active genes, even though the actual number and makeup of the genes are the same.

Cloning is a procedure which offers amazing opportunities, but which also has many limitations. Books and movies in which cloned humans retain all of the memories

and abilities of their genetic donors have greatly influenced the public's perception of this technique. It is important that we inform clients who are interested in cloning that the resulting foal will not be the original genetic donor brought back to life, but a new individual. The foal will have the same genetic makeup as the donor, but will also have differences in looks, ability, and behavior due both to physiology and to environment. A good comparison is to identical twins, nature's own way of "cloning".

Summary

It has been an exciting 10 years in assisted reproduction in the horse. Development of the techniques of oocyte transfer, GIFT, ICSI, and nuclear transfer have led to a major increase in our knowledge of the physiology of fertilization and early embryo development in the horse, while offering the practitioner clinical avenues that were only dreamed of a few years ago. Clinical utilization of oocyte transfer, GIFT and ICSI is already occurring, and it is likely that nuclear transfer will be available in the near future. It will be fascinating to see what the next 10 years will yield.

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