



Update on equine ICSI and cloning

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Abstract

Intracytoplasmic sperm injection (ICSI) has recently become efficient enough to be considered for clinical use. With ICSI, one spermatozoa is injected into a mature oocyte. Harvesting of an oocyte *ex vivo*, followed by ICSI and transfer of the fertilized oocyte to the oviduct, may be applicable when semen quality is insufficient for standard insemination. Sperm injection, followed by *in vitro* embryo culture to the blastocyst stage, may be used in cases where multiple oocytes are to be fertilized (e.g. when oocytes are collected post-mortem). Nuclear transfer (cloning) of horses is possible but still inefficient; however, commercial companies currently will culture and store cells from privately owned animals for a reasonable fee. Horse owners are beginning to realize the potential of cloning for salvaging valuable equine genetics that may otherwise be lost.

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1. Introduction

Progress in assisted reproduction in the horse has been rapid over the last decade. Efficient methods for oocyte recovery from live mares have engendered clinical interest in methods for fertilization *in vitro*. Standard *in vitro* fertilization, that is, culture of oocytes with capacitated sperm, is not efficient in the horse. However, efficient fertilization of horse oocytes *in vitro* is possible using intracytoplasmic sperm injection (ICSI). Fertilized equine oocytes, produced by ICSI, are being used to develop effective equine embryo culture systems. Equine embryos produced by *in vitro* oocyte maturation, ICSI and *in vitro* embryo

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culture have been transferred transcervically to recipient mares, resulting in the birth of normal foals.

Nuclear transfer (cloning) allows production of a foal having the same genetics as the donor animal, and serves as a way to preserve the genetics of valuable individuals. A major application of cloning is with geldings that have proven to be excellent competitors. A colt produced from the gelding's cells by nuclear transfer could be left intact, and as a stallion would produce sperm that could pass on the traits of the original gelding. Similarly, valuable mares or stallions from whom the owner desires more foals could serve as cell donors to produce individuals that would carry on their genetics. The equine industry has started to change its attitude toward cloning from one of unease (at the imagined prospect of multiple identical horses transforming equine competition), to a more realistic one, i.e. using the procedure to preserve the genetics of very valuable individual horses.

2. Intracytoplasmic sperm injection

Intracytoplasmic sperm injection requires a mature oocyte, that is, an oocyte in metaphase II. Mature oocytes may be obtained *ex vivo* by aspirating the preovulatory follicle of a mare after gonadotropin stimulation, or may be obtained by *in vitro* maturation of oocytes collected from small immature follicles, either *ex vivo* or post-mortem. Spermatozoa used for ICSI may be either fresh or frozen-thawed; there is no significant difference in embryo development between these semen types [1]. Under micromanipulation, one spermatozoa is picked up in a micropipette and its sperm membrane broken to assure that it is immotile and that sperm cytosolic factors important in oocyte activation will be released. The sperm is then injected into the cytoplasm of the mature oocyte.

The first successful report of ICSI in the horse was that of Squires et al. [2]; these workers injected four *in vitro*-matured oocytes with sperm, and transferred them to the oviducts of recipient mares. They obtained one pregnancy, that was carried to term. However, subsequent studies on ICSI showed that this success rate was difficult to repeat. Initially, laboratories working with ICSI in the horse had difficulty in achieving good rates of embryo development after sperm injection. However, in about 2000, use of the Piezo drill for ICSI was reported to increase both activation and cleavage rates [1,3]. This device causes minute vibrations in the injection pipette; these not only facilitate penetration of the zona pellucida but also ensure breakage of the sperm and oocyte plasma membranes. Using the Piezo drill to perform ICSI on *in vitro*-matured oocytes resulted in over 80% cleavage, with an average of >8 cells per cleaved embryo at 96 h of *in vitro* culture [1].

While the success of the ICSI procedure provided a technique for fertilization, it was initially of limited clinical use because methods were not established for culture of fertilized equine oocytes to the blastocyst stage, at which they may be transferred directly to the uterus. However, transfer of ICSI-derived embryos (produced from *in vitro*-matured oocytes) to the mare oviduct immediately after injection resulted in a blastocyst recovery rate of 36% [4], showing that ICSI can result in efficient embryo production if embryos are cultured in an optimal environment.

If ICSI-oocytes are transferred directly to the oviduct, there would seem to be little benefit to performing ICSI over simply performing oocyte transfer (transfer of an immature

oocyte to a recipient mare inseminated with semen from the desired stallion). However, even if surgery for oviductal transfer of the injected oocyte is performed, ICSI does have some advantages over standard oocyte transfer for clinical embryo production. Poor quality semen or semen with low numbers of spermatozoa, which would be unsuitable for insemination of a recipient for oocyte transfer, may be used successfully for ICSI. Because frozen-thawed semen may be used, no arrangements for semen shipment on a given day need to be made. Because the oocyte is fertilized before transfer, the recipient mare does not need to be inseminated, and thus there is minimal risk of post-breeding endometritis. In that regard, endometritis is common in standard oocyte recipient mares and can limit pregnancy rates after oocyte transfer [5]. In addition, with ICSI, there is no concern regarding the recipient mare's oocyte—the recipient simply needs to ovulate at about the time of the 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 time 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 one oocyte (as would collected from the preovulatory follicle of a mare *ex vivo*) and semen of normal fertility is used.

A major advantage of ICSI as a clinical procedure rests with culture of the fertilized oocyte *in vitro* to a stage at which it may be transferred transcervically to a recipient mare. For this to be commercially viable, blastocyst development rates must be acceptable; in cattle, typically 25–35% of fertilized oocytes develop to blastocysts *in vitro*. Much of the work with *in vitro* culture of equine embryos has been disappointing; reported blastocyst development has typically been <10%, even if co-culture systems are used [6–9]. However, we recently found that culture of ICSI-produced equine embryos in the general cell culture medium DMEM/F-12, in a mixed-gas environment, can support >35% blastocyst development [10]. We have found the blastocyst development rate to be repeatable within our laboratory; results of separate studies with various oocyte treatments range from 23 to 44% blastocyst development using this system (Choi YH and Hinrichs K, unpublished data). We currently have a 50% pregnancy rate after transfer of *in vitro*-produced embryos, although half of these are lost in early gestation [11]. Hopefully, the incidence of embryo loss will decrease as culture methods are optimized. All three pregnancies from *in vitro*-produced embryos that went beyond 30 days resulted in the birth of live foals (unpublished data).

Establishment of an effective method for embryo culture greatly increases the practical application of ICSI. Perhaps the most promising application is to obtain embryos from mares post-mortem, that is, when a mare dies and the owner wishes to attempt to obtain foals from the mare's oocytes. In this case, multiple immature oocytes are recovered from the follicles present in the ovaries, and are matured *in vitro*. Use of ICSI under these circumstances has a major advantage over oocyte transfer; with ICSI, every oocyte that is capable of making a blastocyst has a chance to produce a foal. After the oocytes have been collected and matured *in vitro*, those in metaphase II are fertilized by ICSI and placed into embryo culture. Blastocysts are identified after 7–8 d of *in vitro* culture, and each blastocyst may be transferred separately to a recipient mare by transcervical transfer. In

contrast, if oocyte transfer is to be performed, the number of transfers is limited by the amount the client wants to spend. If 10 oocytes are available for transfer after maturation, but the client only wishes to pay for two transfers, then each recipient mare would get five oocytes. It is possible that one recipient mare may become pregnant with two or more vesicles, while another does not establish pregnancy [12]. In this case, the extra embryos must be crushed, and these potential foals are lost to the client; in addition, a non-productive surgery was performed. These problems may be avoided when ICSI and embryo culture are utilized.

3. Nuclear transfer (NT; cloning)

To perform NT, oocytes (oocyte donor genetics are not important) 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 (e.g. a skin biopsy). One somatic cell from the donor is selected and combined with the cytoplast, either by 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 [13,14]. Fusion rates were low and cleavage rates even lower (<15%). However, work continued and in 2003, three cloned mule foals and one cloned horse foal were born [15,16]. Three more cloned horse foals were born in 2005 (one in Dr. Cesare Galli's laboratory in Italy (E. Palmer, personal communication 2005) and two at Texas A&M (unpublished data)).

One of the most exciting aspects of the birth of these cloned equids is that all four foals were born without assistance, and have been essentially normal from birth. However, we must await the delivery of more cloned foals before we have a good understanding of the proportion of cloned equine pregnancies that are viable. In the case of the mule project, many pregnancies were lost early in gestation, although all pregnancies that were viable after 60 days continued uneventfully to term [15]. However, the mule project used several techniques that have poor clinical applicability. The cells used for cloning were obtained from a mule fetus, rather than an adult mule, which may have increased the likelihood of live birth. In addition, the oocytes used were recovered by follicle aspiration from the dominant preovulatory follicles of mares *ex vivo*, which provides oocytes with optimal ability to reprogram the transferred nucleus, but is expensive in terms of mares, animal care and maintenance and labor. Lastly, the recombined oocytes were transferred immediately to the oviducts of recipient mares via flank laparotomy, again an expensive and laborious approach, given that only a small proportion of the transferred oocytes would be expected to produce a pregnancy. This same group reported establishment of seven pregnancies from transfer of 62 oocytes subjected to adult somatic cell nuclear transfer, but all pregnancies were lost before 80 days of gestation [17].

The cloned horse foal born in Italy in 2003 [16] was produced using adult fibroblasts, *in vitro*-matured oocytes and embryo culture to the blastocyst stage before transcervical

transfer to the uterus of the recipient mare; this was similar to the methods used for cloning in other species. In that report, 841 recombined oocytes were cultured, and 22 blastocysts developed (3%). Seventeen blastocysts were transferred, and four pregnancies resulted (24% pregnancy rate after transfer). Of the four pregnancies, two were lost around 30 days, one was lost at 6 mo of gestation and one was carried to term for a normal birth.

During production of the foal born in Italy in 2005, a news report stated that the laboratory cultured 200 recombined oocytes, resulting in 34 embryos (17%) and three pregnancies (9% pregnancy rate), of which one produced a viable foal. At A&M in 2005, in our first study, 423 recombined oocytes resulted in 6 blastocysts (1.4%), and one pregnancy (17% pregnancy rate), which produced a viable foal; in our second study, 144 recombined oocytes resulted in 8 blastocysts (6%); 5 were transferred for 3 pregnancies (60% pregnancy rate), of which one produced a viable foal (unpublished data).

From these data, it is apparent that the *in vitro* blastocyst development rate for cloned embryos is much lower than that for ICSI embryos (1–17% versus 25–35%). While the pregnancy rate after transfer of cloned embryos is variable, this is a relatively high pregnancy rate given the nature of the embryo. The proportion of cloned horse pregnancies that are carried to late gestation or term is not clear at this point; it appears to vary greatly among laboratories. The cloned equid foals reported have all been healthy at birth and have developed normally after birth. The situation should become clearer in the near future, as more pregnancies and births are reported.

The increasing efficiency of equine cloning makes it potentially clinically applicable at this time, and there is at least one commercial company that is attempting equine cloning for clients. Producers and researchers will now be able to assess the usefulness of cloning as a clinical technique. Clinically, cloning will allow the preservation of valuable genetics, from the old mare that is the “last of her line” to that of endangered equids. A mare that is too old to reproduce may be used as a genetic donor and the resulting filly may carry on the original mare’s reproductive capacity. The genetics of exceptional individuals that have been castrated, or 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 problems that are currently insurmountable.

While cloning is not yet clinically efficient, “banking” of tissue from animals, including horses, is currently being done commercially by a number of companies at a reasonable cost. A skin or lip biopsy is taken from the animal and sent to the company in a transportation package (supplied by the company). At the laboratory, cells are grown from the tissue in culture and the company stores the cells in liquid nitrogen. It is possible to obtain tissue from animals hours to days post-mortem (especially if tissue has been cooled but not frozen) and still support successful tissue culture. Thus, cell 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 cloned foal can be made 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:

1. 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. 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, in the case of a colt, the mitochondria present in the sperm do not contribute to the mitochondria of the embryo after fertilization, thus the cloned colt could be considered to produce the same progeny that its genetic donor would have produced.
2. The phenotype of the new foal will be affected by the environment, both within the uterus and postnatally. Based on spontaneous twins and the few split-embryo identical twins produced, the intrauterine environment affects not only the size of the foal at birth, but also the adult size and phenotype [18; Allen WR, personal communication, 1996]. Thus, identical cloned embryos placed in different mares may produce progeny of different size and proportions 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. Although these are factors in any transferred embryo, the effects will be more noticeable in a cloned individual because its expected phenotype is known.
3. The development of the cloned foal will be influenced by epigenetic effects. While the genetic makeup of the foal will be exactly that of the donor, within the genome certain genes may be "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 the tissue in which the cell resides. 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. Such reprogramming may not be perfect in the cloned fetus, and the state of activity of the DNA during fetal life may affect the phenotype of the animal at birth and after birth. However, while the phenotype of cloned foals may vary from the original donor, the offspring of the clones should be essentially the same as would have been produced by the donor, as reprogramming in the gametes of cloned animals appears to proceed normally.

It is essential that we inform clients interested in cloning that the resulting foal will not be the original genetic donor brought back to life, but a new individual. Although the foal will have the same genetic makeup as the donor, it will have differences in appearance, ability and behavior due both to physiology and to environment. A good comparison is identical twins, nature's own way of "cloning".

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