Intracytoplasmic sperm injection-produced equine embryos: transport, thawing, and transfer

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Abstract

Intracytoplasmic sperm injection (ICSI)-produced equine embryos are transferred at the early blastocyst stage, similar to a day 6.5 in vivo-produced embryo and will benefit from a recipient mare that ovulated 3 - 5 days before transfer. Frozen-thawed and vitrified-thawed embryos may have an even narrower window of synchrony with recipients for best results. Day of blastocyst formation is a useful indicator of embryo quality and potential for establishing a successful pregnancy; early embryonic loss is higher for blastocysts that formed on days 9 and 10 after ICSI than those formed earlier.

Keywords: Intracytoplasmic sperm injection, equine embryo, embryo transfer

Introduction

Production of equine embryos via intracytoplasmic sperm injection (ICSI) and their subsequent transfer is becoming more important for commercial horse production. Many of these embryos are potentially valuable and contain genetics prized by their owners, so it is in the best interest of veterinarians and clients to handle and transfer these embryos as optimally as possible. Intracytoplasmic sperm injection-produced embryos (hereafter referred to as ICSI embryos) differ from in vivo-produced embryos. Although they can be handled similarly, some adjustments are necessary. Careful selection of appropriate recipient mares, embryo handling, and timing of transfer are necessary to optimize results.

Comparison of in vivo and intracytoplasmic sperm injection-produced embryos

Equine ICSI embryos differ from their in vivo counterparts morphologically and metabolically.[1] In vitro culture conditions can be successful for viable embryo production; however, conditions are not similar to those that exist in utero (uterus or oviduct), leading to many differences. One of the more prominent morphological differences of ICSI embryos is the lack of development of an embryonic capsule. The capsule, a mucin-like glycoprotein, is normally produced from secretions from the trophoblast cells, and forms between them and the zona pellucida. A very strong, elastic structure, it presumably provides protection for the developing blastocyst after shedding zona pellucida. A capsule does not form on ICSI embryos in vitro; however, it develops after embryos are transferred into a recipient uterus. Due to the injection-induced breach in the zona, there is a short interval after transfer when ICSI embryos may be directly exposed to the uterine immune system and therefore inflammation.

The discrete inner cell mass that develops in the early blastocyst in vivo is not observed in ICSI embryos, another result of in vitro culture. The delay in the aggregation of inner cell mass cells may contribute to the higher rate of monozygotic twinning after transfer of equine ICSI embryos.

Zona pellucida appears to be somewhat hardened by in vitro culture. This has a role when the early blastocyst starts expanding and is limited in its expansion by the zona. During in vivo development, the zona thins as the blastocyst expands and then, after the capsule forms, thins enough until it flakes away. During in vitro, the zona does thin; however frequently the trophoblast layer will herniate through the ICSI-generated hole in the zona. This, when the embryo is transferred, would further expose the otherwise unprotected blastocyst to the uterine environment. Herniation of trophoblast and blastocoele might also predispose to monozygotic twinning, forming a separate blastocyst outside the zona pellucida.

An ICSI embryo, growing in culture instead in an oviduct, is subjected to physiologic stress throughout the culture period. Without the conditions provided by oviductal epithelium, the various cytokines and other 'embryo-friendly' compounds, embryos do grow; however, they are probably compromised by the stress of adapting to in vitro culture conditions. There are differences in gene activation of the ICSI embryo compared to in vivo embryos.[2] Problems with chromosomal segregation are more common in vitro leading to embryos with ploidy issues, i.e., too many or too few chromosomes in daughter cells.[3] All of these factors contribute to the fact that at our current state of knowledge, ICSI embryos, on average, are less likely to establish a pregnancy and then maintain a viable pregnancy to term than their in vivo counterparts. Author's practice advises clients to expect a 10% lower pregnancy

rate with ICSI embryos versus in vivo embryos, and approximately twice the rate of early embryonic loss.

Transfer of ICSI embryos

Transfer of ICSI embryos is usually done at the early blastocyst stage as it is starting to expand but before much expansion occurs. This permits transfer of a uterine stage embryo that has not had trophoblast cells herniate through the breach in the zona. Currently, developmental status of the ICSI embryo is roughly equivalent to that of a day 6.5 in vivo embryo, although the embryo may be anywhere from 6 to 10 days after fertilization by ICSI.

Synchrony

Selection of a recipient mare to receive an ICSI embryo should consider the developmental stage of the embryo. Preferred synchrony for in vivo days 7 and 8 embryos is usually considered ideal at -1 or -2 (a recipient that is on day 5 or 6 after ovulation), but with a window of +1 to -3 (recipient days 8 - 4). This suggests that the preferred recipient for an ICSI early blastocyst would be a recipient that was days 4 or 5 postovulation. Reports on the success of ICSI embryo transfer have suggested that the window of synchrony for transfer is narrower for ICSI embryos than their in vivo counterparts, although these reports utilized frozen-thawed embryos.[4,5] Author's clinical practice has not identified a narrower window of acceptable synchrony for embryos that have not been frozen. Data from 926 transfers (Table 1) suggested a trend toward higher pregnancy rates with day 3, 4, and 5 recipient mares and no statistical difference with days 2 through 7 recipients.

Table 1. Pregnancy by day after recipient ovulation

Recipient day	Pregnant/transferred	Pregnant (%)*
Noncyclic	21/25	84.0 [a]
7	45/58	77.6 [a]
6	146/181	80.6 [a]
5	225/274	82.1 [a]
4	229/278	82.3 [a]
3	86/105	81.9 [a]
2	3/5	60.0 [a]
Total	755/926	81.5

*Within a column, percentages without a common superscript differed ($p < 0.05$)

Day of development

Day of development, representing the number of days following ICSI that blastocyst formation occurs, has a distinct effect on results of embryo transfer. As expected, on average, the faster an embryo reaches the early blastocyst stage the healthier it is and the more likely to produce a viable pregnancy. Data (Table 2) from the same group of 926 transfers were compared by day of blastocyst development and pregnancy rate. A fairly consistent trend of decreasing pregnancy rate with increasing days of development was evident; however, the difference was not significant until days 9 and 10.

Table 2. Pregnancy by day of blastocyst development

Day	Pregnant/transferred	Pregnant (%)*
6	36/39	92.3 [a]
7	288/348	82.7 [a]
8	232/278	83.5 [a]
9	147/185	79.5 [a,b]
10	52/76	68.4 [b]
Total	755/926	81.5

[a,b]Within a column, percentages without a common superscript differed ($p < 0.05$)

Early embryonic loss

Early embryonic loss is more common with ICSI embryos than with in vivo embryos; author's practice informs the clients to expect ~ double the rate of embryonic loss. Most of these losses occur before day 30 of pregnancy and are usually preceded by a period of slower than expected embryonic growth or they appear as an empty trophoblastic vesicle. There is a smaller group that appear normal at the heartbeat stage that are lost by 45 days, and an even smaller group that is lost by 60 days. Early embryonic losses for the group of 926 transfers (discussed previously) by recipient mare day postovulation are summarized (Table 3). The overall total loss rate was 17.7% with no significant differences related to synchrony. Table 4 has the same group classified by day of blastocyst development. A consistent trend in increased loss with increased day of development was noticed, becoming significant by days 9 and 10 post-ICSI.

Table 3. Early embryonic loss by day of recipient cycle

Recipient day	Pregnant/transferred	%*	Losses	%*
Noncyclic	21/25	84.0[a]	2/21	9.5[a]
7	45/58	77.6[a]	10/45	22.2[a]
6	146/181	80.6[a]	24/146	16.4[a]
5	225/274	82.1[a]	46/225	20.4[a]
4	229/278	82.3[a]	35/229	15.2[a]
3	85/105	81.9[a]	14/86	16.2[a]
2	3/5	60.0[a]	3/3	100[a]
Total	755/926	81.5	134/755	17.7

*Within a column, percentages without a common superscript differed ($p < 0.05$)

Table 4. Early embryonic loss by day of blastocyst development

Day	Pregnant/transferred	%	Loss	%
6	36/39	92.3[a]	4/36	11.1[a]
7	288/348	82.7[a]	34/288	11.8[a]
8	232/278	83.5[a]	35/232	15.1[a]
9	147/185	79.5[a,b]	41/147	27.8[b]
10	52/76	68.4[b]	20/52	38.5[b,c]
Total	755/926	81.5	134/755	17.7

[a-c]Within a column, percentages without a common superscript differed ($p < 0.05$)

Grading ICSI embryos

Traditionally embryos have been graded by evaluating morphological characteristics at a single point in time with the

goal of providing useful information concerning the estimated ability of a given embryo transfer to result in an ongoing pregnancy. Similar morphologic evaluation of ICSI embryos generally does not give the same ability to estimate the pregnancy potential of a given embryo. ICSI embryos are transferred at an early blastocyst stage that will appear remarkably similar for vigorous healthy embryos and those that are not so vigorous and healthy. Day of blastocyst formation/transfer has a higher correlation to pregnancy than morphology. Tables 4 and 5 suggest that early embryonic loss and pregnancy rate decreased with increasing day of blastocyst formation. Since owners and veterinarians alike want to know the grade of an individual embryo, author's practice grades embryos based on day of blastocyst formation, adjusted for morphologic abnormalities. Typically, days 6 and 7 blastocysts are graded as Grade 1, day 8 blastocysts are graded as Grade 1.5, Grade 2 for day 9, and Grade 2.5 for day 10. History noted during the culture period may also be used to adjust grades. Blastocyst formation normally occurs 2 days after morula compaction and if this period is longer, the embryo will be graded lower.

Table 5. Pregnancy for vitrified/thawed embryos by day of recipient cycle

Recipient day	Pregnant/transferred	Pregnant (%)
Noncyclic	11/15	73.3 [a]
7	4/6	66.6 [a]
6	5/12	41.7 [a]
5	62/81	76.5 [a]
4	115/157	73.2 [a]
3	23/28	82.1 [a]
Total	220/299	73.5

*Within a column, percentages without a common superscript differed ($p < 0.05$)

Monozygotic twins

Occurrence of monozygotic twins is more likely following transfer of ICSI embryos than in vivo embryos with an incidence of ~ 1.5%.[6] These twin pregnancies occur following the transfer of a single embryo and are typically monochorionic, so that only a single vesicle is seen on initial pregnancy examinations. A diagnosis for twin pregnancy is not performed until more than 1 embryo-proper is visible in the vesicle. Twin reduction of monozygotic twins by cranio-cervical dislocation is possible but generally not successful,[7] so it is often prudent to consider this a pregnancy loss and abort the pregnancy. It is interesting to note that monozygotic twinning in human IVF embryos occurs ~ 1.7 - 2.5%,[8] the lower rate from transfers on day 2 or 3 with the higher rate from transfers on day 5 or 6. This is an indication that it is the culture environment that leads over time to an increased incidence of twinning. This may be from ineffective migration of inner cell mass cells leading to the formation of two inner cell masses. Twin production via herniation of trophoblast with some inner cell mass cells through the Piezo hole in the zona is a less likely explanation as 2 embryonic vesicles should be formed in this manner leading to a dichorionic twin pregnancy.

Shipping of ICSI embryos

Since ICSI laboratories are often geographically distant from recipient mares, shipping becomes necessary. The ICSI embryo, being produced in only an imitation of its natural environment, may be considered stressed, so additional stress should be avoided. Stresses to consider are temperature, duration of transport, and selection of culture/holding medium providing pH maintenance and embryo nutrition. Temperature or osmotic stress are also a possibility, if the embryo is moved to a different medium (after shipping) before transfer.

ICSI embryos are generally transported in passive heating/cooling devices, such as the EquOcyte (Hamilton Biovet, Ipswich, MA), or portable incubators capable of active heating and cooling (e.g., Micro Q [Micro Q Technologies, Scottsdale, AZ]). Equine ICSI embryos can tolerate several hours at room temperature, but pregnancy rates decreased as the interval at room temperature increased.[7] Addition of nutritional support in the form of 10 mM glucose can increased the survival time, up to 50% pregnancy rate at 48 hours.[7] Currently, author's practice utilizes a modified culture medium for shipping in an incubator (Micro Q) at 38°C. The shipping medium can be used for transfer of the embryo so that temperature and osmotic changes are avoided, and an attempt is made to minimize transport time as much as possible.

Thawing and transfer of vitrified embryos

Vitrified ICSI embryos can be thawed and transferred in most situations and practices that can transfer fresh embryos. Due to the number of embryos produced in the nonbreeding season and surplus embryos produced during the breeding season, many are vitrified. Vitrification gives an opportunity not only for storage but also for shipping and transfer into a specific recipient or even back into the donor. Author's practice has utilized thawing into a 0.3 M sucrose solution with timed stepwise reduction in sucrose concentration for several years, but have utilized a single step procedure for the last 2 years.[9] This procedure consists of plunging the device containing the embryo (e.g., Cryolock [Bio Tech Inc., Alphareta, GA]) or open pulled straw, directly from liquid nitrogen into a petri dish containing the thawing solution warmed to 38°C. This thawing solution is GMOPS Plus (Vitrolife, Goteborg, Sweden) in the author's practice but commercial embryo holding medium can also be used. Embryo is held in the thawing solution on a warm stage for 5 minutes and then either prepared for transfer or placed in culture. If a specific recipient mare is to be used, directly transferring the embryo may be preferable, but if multiple recipients are available, culture for a short interval may be preferable as it allows evaluation of embryo re-expansion. Embryo re-expansion will take place rather quickly, 2 - 3 hours, in most instances but other embryos that may not have tolerated the freezing as well may take a day or even 2. This can drastically affect embryo-recipient synchrony, so culturing improves recipient selection in those instances.

There is also a difference in timing between embryo re-expansion and resumption of growth. Embryonic vesicles detectable via ultrasonography on the first day of pregnancy are generally smaller or even a day later than expected from transfer of fresh ICSI embryos. This delay in resumption of growth may

explain the narrower window of synchrony reported for transfer of frozen-thawed ICSI embryos.[4,5] Results (Table 5) from transfer of vitrified-thawed embryos in the author's practice broken down by recipient mare's day of cycle. There is a trend toward higher pregnancy rates from days 3, 4, and 5, but it does not reach the level of significance. This may be since the majority of these embryos were cultured until re-expansion versus directly transferred into recipients.

Conflict of interest

None to declare.

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