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Intracytoplasmic sperm injection in the horse: the ultimate blind date

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ABSTRACT

Background: Intracytoplasmic sperm injection (ICSI) has become a useful technology to produce foals when availability of semen is limited or when *in-vitro* fertilization is desired, as is the need for subfertile mares. However, its application into clinical practice is challenging. The purpose of this review was to discuss some fundamental molecular aspects of oocyte maturation that should be considered when performing ICSI and to report factors of age and subfertility affecting the success of a commercial ICSI program.

Review: The molecular synchrony of oocyte maturation included nuclear, epigenetic and cytoplasmic maturation. Oocyte developmental competence was found to be dependent on the ability to remain in meiotic arrest until the initiation of final maturation, requiring the adequate timing involved with follicular maturation prior to ovulation. Studies performed in cattle and humans have demonstrated that *in-vivo* oocyte maturation results in high pregnancy rates per oocyte fertilized. Therefore, determining precise maturation of the oocyte would be valuable for the timing of ICSI and subsequent embryo development. Reproductive aging in the mare was characterized by a decline in fertility. Using RT-PCR, quantitative and temporal differences were found in mRNA content of key regulatory maturation genes in granulosa and *cumulus* cells and in oocytes during *in vivo* maturation in young and old mares. These results suggested premature oocyte maturation in aged mares that potentially could result in subfertility. Consequently, the timing of oocyte retrival after gonadotropin administration should be carefully evaluated when performing ICSI. In a commercial program, equine patients were classified into normal mares (2.5 to 15 years), problem mares (15-23 years that had not been producing embryos or pregnancies) and old mares (>24 years). Old mares were assessed for endocrine, physical and nutritional imbalances. Follicular and oocyte maturation were induced with a dominant follicle >30 mm in diameter after a normal growth and blood flow and uterine edema with a combination of hCG and GnRH. Transvaginal oocyte retrieval was performed 20 hours after administration of gonadotropins. Oocytes were further cultured *in vitro* for 12 to 20 hours. Frozen semen was used for all sperm injections. Injected oocytes were further cultured *in vitro* for at least 24 hours. Embryos were then transferred surgically into oviducts of synchronized recipients. Oocyte recovery rate was 94% (523/557 cycles), cycles per month were 3.3, 2 and 1.3 for young, problem and old mares respectively. Cleavage rates were different ($p<0.05$) between young (82 %), problem (70 %) and old (52%) mares. Pregnancy rates at day 60 were also different ($p<0.05$) for young (68 %), problem (50 %) and old (23%) mares. Number of pregnancies obtained from a single straw of frozen semen ranged from 2 to 12. Reproductive senescence was observed in 10% of old mares. In addition, Cushing's disease and elevated diestrus FSH were observed in 80% of the old mares. Foaling rates were evaluated in 55 pregnancies; 5% were lost in the last trimester and the remaining foals have shown no apparent abnormalities.

Conclusion: More studies are needed to further elucidate the mechanisms of oocyte maturation and activation in the horse, as well as more objective methods to determine oocyte maturity and quality. A clinical ICSI program required an understanding of gamete physiology and detailed mare reproductive management. Our clinical data demonstrated that aging affects fertility profoundly in ways that may be difficult to address with current technology; nonetheless, ICSI has provided the equine industry an alternative to produce offspring from valuable mares and stallions that are subfertile.

Keywords: equine, ICSI, equine, oocyte, maturation

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I. INTRODUCTION

Assisted reproductive technologies (ART) for the horse have been fundamental for preservation of valuable genetics. Intracytoplasmic sperm injection (ICSI) has become a useful technology to produce foals when availability of semen is limited including semen which has been sex sorted, semen from sub-fertile or deceased stallions, or epididymal harvested sperm, or when *in-vitro* fertilization is desired, as is the need for many subfertile mares. In many ICSI cases, a combination of both mare and stallion subfertility are the circumstances. However, since the first successful pregnancy obtained by ICSI in 1996 [27], relatively few facilities in the world currently offer ICSI commercially. Some of the obstacles that impede the dissemination of the technology into veterinary clinical practice include technically challenging procedures such as *in vivo* oocyte collections, sperm injection and lack of training in areas such as clinical embryology and laboratory quality control. At present, the ICSI procedure remains a high-class dating service, required to match egg and sperm, essentially of unknown quality, with only vague awareness of their good character. Do we really know what a healthy, viable oocyte looks like, aside from a nice image? Likewise, is the appearance of a motile, seemingly normal sperm a good indicator of its virility? Studies have addressed these questions to some extent, including *cumulus* cell expansion grades and subsequent blastocysts and sperm head logorhythms and association with DNA integrity [2,9,28-29,33]. However, more scientific information is needed in the area of equine oocyte maturation including understanding molecular events that help clinicians more precisely manage the reproductive cycle of the patient.

The purpose of this review is to discuss some molecular aspects of oocyte maturation that need to be considered when performing ICSI and to report factors of age and subfertility affecting the success of a commercial ICSI program.

II. OOCYTE MATURATION

During ICSI a single sperm is mechanically introduced into a metaphase II oocyte. Both, *in vitro* [15,17] and *in vivo* matured [5,10-11] oocytes have been used with variable results. However, the goal is to inject an oocyte with high developmental competence which involves the ability to resume meiosis, cleave after fertilization, reach the blastocyst stage, establish pregnancy, and ultimately result in normal offspring [26]. Clearly, these events require strict molecular synchrony, beginning with oocyte maturation. The study of molecular synchrony of oocyte maturation can be dissected into three categories: 1) nuclear maturation 2) epigenetic maturation and 3) cytoplasmic maturation.

Nuclear maturation includes the reinitiation and completion of meiosis I and arrest in metaphase II, ultimately, reducing the number of chromosomes from diploid to haploid [22]. Oocytes resume meiosis in response to the preovulatory rise in LH. Oocytes at prophase I have an intact nuclear membrane called the germinal vesicle (GV). Resumption of meiosis is characterized by the breakdown of the GV, chromosomal condensation, assembly of the metaphase I spindle and extrusion of the first polar body resulting in a haploid cell. Then, a second meiotic spindle is formed; the oocyte enters metaphase II and arrests until activation by fertilization [25]. In mares, MII stage is completed at the time of ovulation.

Epigenetic maturation is defined as stable and heritable chromatin modifications that influence gene expression without changing DNA sequence [30]. During oogenesis germ cells undergo epigenetic modifications that confer to the genome a genomic imprint that can be maternal or paternal. The timing of epigenetic processes of imprint establishment and maintenance during oogenesis may alter the events of oocyte maturation [12]. In addition, environmental conditions can potentially alter imprinting during iatrogenic manipulation and management of the oocyte. Completion of imprinting is essential for the developmental competence of the oocyte to ultimately establish a normal pregnancy.

Cytoplasmic maturation occurs when the oocyte has completed its RNA and protein synthesis which includes nucleolus condensation and depletion of ribosomes [26]. Other cytoplasmic changes in preparation for oocyte activation by sperm at fertilization involve re-distribution of mitochondria, cortical granules and smooth endoplasmic reticulum and increasing receptor (IP3) sensitivity for calcium release [13].

The presence of redundant systems makes dissecting these maturation events in the oocyte very difficult. However, oocyte viability is dependent on the ability to remain in meiotic arrest until the initiation of final maturation, requiring the adequate timing involved with follicular maturation prior to ovulation. *In-vitro* maturation of equine oocytes suggests an inefficient system [17]. A cascade of cellular events that take days during the follicular phase are artificially induced in a short (30 h) period of culture time in media (TCM 199) designed for somatic cells and supplemented with undefined fetal calf serum. Furthermore, studies performed in cattle [3,20,32] and humans [21,24] have robustly demonstrated at the level of gene expression and developmental competence that *in-vivo* oocyte maturation results in higher pregnancy rates per oocyte fertilized.

III. THE AGED MARE

The aging process is a complex and multifactorial phenomenon that affects reproductive efficiency. Reproductive aging in the mare is characterized by a decline in fertility, and more abnormal reproductive cycles occur with advancing age. Old mares (e" 20 y) had longer follicular phases and slower growth of the preovulatory follicle [6]. In addition, circulating progesterone increased sooner after ovulation in old than young mares, suggesting premature luteinization of the follicle [6]. Oocytes collected from the preovulatory follicles of young (6-10 y) and old (20-26 y) mares and transferred into oviducts of young, inseminated recipients resulted in pregnancy rates of 92% and 31%, respectively, suggesting intrinsic deficiencies in older mare oocyte quality [8]. In addition, embryos collected from the oviducts of old mares (e" 20 years) *versus* young mares (2-9 y) were delayed in development and had abnormal morphology [7]. In a recent study [4] using RT-PCR, quantitative and temporal differences were found in mRNA content of the LH receptor (LHR), and key regulatory maturation genes: amphiregulin (AREG) and epiregulin (EREG) in granulosa cells; phosphodiesterase (PDE) 4D in *cumulus* cells; and PDE3A, G protein coupled receptor (GPR) 3, growth and differentiation factor (GDF) 9, bone morphogenetic protein (BMP) 15, and mitochondrial DNA (mtDNA) in oocytes during *in vivo* maturation in young (3-12 yr) and old (>20 yr) mares. These results suggest premature oocyte maturation and potentially, a degradation of oocyte mtDNA in the old mare that could result in subfertility and compromised synchrony of communication between the oocyte with follicular cells. In addition, these findings support the idea that the follicular environment in old mares could be suboptimal. Consequently, the timing of oocyte retrival after LH administration should be carefully evaluated when performing ICSI.

IV. ICSI IN CLINICAL PRACTICE

During the 2009-2010 breeding seasons, our ICSI program in the USA and Argentina comprised a team of experienced clinicians, embryologists and lab technicians. Quality control in the laboratory and individual clinical assessment of patients were prioritized. Equine patients (Polo, Arabian and Warmblood mares) were classified into normal mares (2.5 to 15 years, n=40), problem mares (15-23 years that had not been producing embryos or pregnancies, n=18) and old mares (>24 years, n=12). Old mares were assessed for endocrine (Cushing's, insulin resistance and diestrus FSH profiles), physical (laminitis, lameness, etc.) and nutritional imbalances. Based on the implications of oocyte developmental competence described in the previous section, we performed *in-vivo* oocyte maturation and collection.

Follicular and oocyte maturation were induced during the follicular phases of mares with a dominant follicle >30 mm in diameter after a normal growth of 3-5 mm/day and blood flow [1,16] and uterine edema with a combination of Deslorelin(1.5 mg) and hCG (2000 U). Transvaginal oocyte retrieval was performed approximately 20 hours after administration of gonadotropins. Oocytes were further cultured in a semi-defined equine maturation media (EqMat) supplemented with BSA for 12 to 20 hours. Frozen semen was used for all sperm injections with a post-thaw motility between 1 and 55 %. Injected oocytes were cultured in semi-defined equine culture media (EqC) supplemented with BSA for at least 24 hours. Embryos were then transferred surgically into oviducts of synchronized recipients (3 to 12 years old).

V. RESULTS

Oocyte recovery rate was 94% (523/557 cycles), cycles per month were 3.3 (9 day interval), 2 (15 day interval) and 1.3 (22 day interval) for young, problem and old mares respectively. Cleavage rates after blocking for stallion effects were different ($p<0.05$) between young (82 %), problem (70 %) and old (52%) mares. Pregnancy rates at day 60 were also different ($p<0.05$) for young (68 %), problem (50 %) and old (23%) mares. Efficiencies of obtaining a pregnancy per cycle (oocyte recovery rate x cleavage rate x pregnancy rate) were 52, 33 and 12 % for young, problem and old mares, respectively. Number of pregnancies obtained from a single straw of frozen semen (200×10^6 cells/ml) ranged from 2 to 12. Reproductive senescence was observed in 10% of old mares which failed to cycle in two consecutive breeding seasons. In addition, Cushing's disease and elevated diestrus FSH were observed in 80% of the old mares. Foaling rates were evaluated in 55 pregnancies; three pregnancies (5%) were lost in the last trimester and the remaining foals have shown no apparent abnormalities.

VI. DISCUSSION

Indeed, choosing who you want to set up for a date is not a simple task. Quality candidates are ideal for a successful matchmaking, and the technical expertise involved with ICSI is only as good as the oocyte and sperm to be injected. Once oocytes are collected, a standardized and functional grading system for oocyte competence and maturation, based on *cumulus* cell expansion, ooplasm appearance and other morphologic characteristics are subjective, not to mention the void of physiologic assessments or molecular markers [31]. It also helps the dating cause if the candidates are mature, and for a change, we mainly refer to the female. Determining precise maturation of the oocyte in a grade fashion would be valuable for the timing of sperm injection. Because true maturation of the oocyte is a compilation of nuclear, epigenetic and cytoplasmic components, determination of the point of maturation would include an assessment of each of these aspects. However, currently, oocytes are simply assessed for the 1) degree of *cumulus* cell expansion, which may not indicate cytoplasmic maturation and 2) the presence of an extruded polar body, which is generally indicative of nuclear maturation, however, this extrusion occurs spontaneously once severed from the attachment to the follicle wall [25].

Regarding the contribution of the stallion, circumstances tend to be more forgiving than those surrounding the single oocyte. Limited sperm reserves is a primary indication for ICSI and an attractive alternative to traditional or low-dose insemination approaches in terms of conserving numbers. Even with a few frozen straws, multiple pregnancies can be obtained. Given our results, even with an overall post-thaw motility of 1%, a single viable sperm can be obtained and used to produce pregnancies. The single requirement of a sperm for ICSI is intact DNA [19] .

There are a plethora of variables to consider when scrutinizing the success of an ICSI program. The population of mares and, to a lesser extent, stallions selected for ICSI is likely the most crucial. Ideal situations may include young mares selected for use with limited amounts of frozen semen from currently subfertile or deceased stallions. Unfortunately, unlike other assisted reproduction modalities such as embryo transfer, many of the patients are chosen for ICSI as a "last resort," when multiple, previous attempts have failed and when fertility is most compromised, true causes of which likely remain unknown. Fortunately, many mares unmysteriously benefit from ICSI when the obvious cervical and uterine deterrents or even less obvious oviductal anomalies obstruct fertility. A percentage of problem mares are as such because of abnormal cycles and follicles. These abnormalities are likely due to an aberrancy in the hormonal axis preventing normal maturation of the follicle and oocyte, whether by an imbalance in gonadotropin, or as yet undeterimined hormone, or receptor or both. Deciphering the viability of these follicles is difficult prior to oocyte collection. Once an oocyte is collected, the ovulatory outcome of the follicle remains

unknown, whether normal, regressing, or anovulatory, and the possibility of thus rescuing an oocyte otherwise destined for atresia is quite real. Fortunately, color Doppler blood flow assessment of these follicles has proven valuable [1,16,23] but more work needs to be done associating blood flow with oocyte viability. Many ICSI candidate mares are beyond 20 years and affected in varying degrees by the aging process. Addressing the basic health needs of these mares to encourage normal reproductive *status* is time-consuming (it may encompass half the breeding season), challenging and often unrewarding. Cycling of these mares, as demonstrated by their increased inter-estrus intervals, is often abnormal and produces oocytes of very questionable viability. In these cases, it may be necessary to not only treat the mare, but the oocyte as well, as new advances such as ooplasm transfer are adapted from the human technology [14]. Maturation media will also likely have to be supplemented with as yet undetermined additives for oocytes from aged mares, once the gene expression and molecular differences are better elucidated.

VII. CONCLUSION

A successful clinical ICSI program requires a team of professionals, an understanding of in-depth gamete physiology and crucial mare reproductive management. From the academic realm, studies are needed to further elucidate the mechanisms of oocyte maturation and activation in the horse, as well as methods to determine oocyte maturity and quality. To further complicate the ICSI equation, the number of aged patients is increasing as the life expectancy and sentimental attachment to horses are changing around the world. Like other investigators [5], our clinical data demonstrates that aging affects fertility profoundly in ways that may be difficult to address even with the most advanced procedures. Without more scientific data to decipher these variables, ICSI in the horse is still the ultimate blind date between the oocyte and the sperm.

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