Current Therapy in

EQUINE REPRODUCTION

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CHAPTER 47

Intracytoplasmic Sperm Injection

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An examination of the procedures, opportunities, and some of the ethical issues of human assisted reproductive techniques (ART) would appear relevant to this discussion of equine intracytoplasmic sperm injection (ICSI). The reasons for use of human ART often are different from those in domestic animals, and especially ICSI. For instance, the most common reason for human ICSI is male factor infertility, because standard in vitro fertilization (IVF) works well with normal fertile men. In horses, ICSI is used primarily to obtain embryos from mares that fail to provide them in routine reproductive procedures or as a research tool for embryo production. In the future, equine ICSI use is expected to parallel the developments seen in the human field. This is likely to result in similar technical and even ethical issues. Human ART and ICSI rely on multiple oocyte collection and transfer, as well as embryo culture and cryopreservation. Equine ICSI is just starting to address these issues, and currently the best pregnancy rates are reported after in vivo–matured single oocyte collection and ICSI coupled with immediate embryo transfer performed before confirmation of the fertilization result (Figure 47-1).[1]

HUMAN STUDIES AND ISSUES

Collection and Preparation of Gametes

Oocytes

Human ICSI commonly involves collection of multiple in vivo matured oocytes after ovarian superovulation induced by pituitary gonadotropins. Stimulation protocols typically involve either gonadotropin-releasing hormone (GnRH) agonists or antagonists to suppress release of luteinizing hormone (LH), which can cause premature ovulation. Follicle-stimulating hormone (FSH) is used to stimulate multiple follicles, and human chorionic gonadotropin (hCG) may be used for final maturation of oocytes. The drug, dose and timing of gonadotropin administration will vary according to individual decisions of the clinician and the specifics of the patient's case. For instance, some women are over suppressed by long duration administration of the GnRH agonists, and others may respond rapidly to initial stimulation. Some women respond better to LH suppression with antagonists of GnRH, rather than agonists. In addition, approximately 0.5% to 2% of superovulation treatments result in **ovarian hyperstimulation syndrome**, which may be very painful for the patient.[2]

Most commonly, multiple in vivo–matured oocytes are utilized. Collection is facilitated by either ultrasonographically guided transvaginal aspiration or laparoscopic retrieval. "Ultrasonically guided follicular aspiration is shown to be superior to laparoscopic oocyte recovery as far as ovarian accessibility and complication rate are concerned."[3]

Spermatozoa

If spermatozoa cannot be harvested by ejaculation, then surgical extraction must be performed. Spermatozoa blocked by obstructive azoospermia can be removed by various microsurgical approaches, such as testicular sperm extraction (TESE). TESE is the most common technique to diagnose the cause of azoospermia and to obtain sufficient tissue for sperm extraction to be used either fresh or as a cryopreserved specimen. It involves one or multiple small biopsies of testis tissue and therefore is called **testicular fine needle aspiration** (TFNA). When a needle and syringe are used to puncture the skin to aspirate a sperm specimen, the procedure is called **percutaneous epididymal sperm aspiration** (PESA). PESA is popular because it can be performed repeatedly at low cost. PESA, like TFNA, can be completed without a surgical incision; however, it may not yield a suitable sample. **Microsurgical epididymal sperm aspiration** (MESA) involves direct retrieval of sperm from individual epididymal tubules and is performed under a microscope. It is completed by isolating the tubes and then aspirating the fluid. MESA limits damage to the epididymis while avoiding blood contamination of its fluid and yields high quantities of motile sperm that can be readily frozen and thawed for subsequent IVF treatments.

> *There no longer seem to be any categories of male factor infertility that cannot be treated with ICSI. Even for men with azoospermia caused either by obstruction or by germinal failure, ICSI may be performed successfully. The only failures will be in azoospermic men who have neither spermatozoa nor spermatids retrievable from the testis, but these men comprise a small percentage of the cases with severe male factor. The source of the spermatozoa and the*

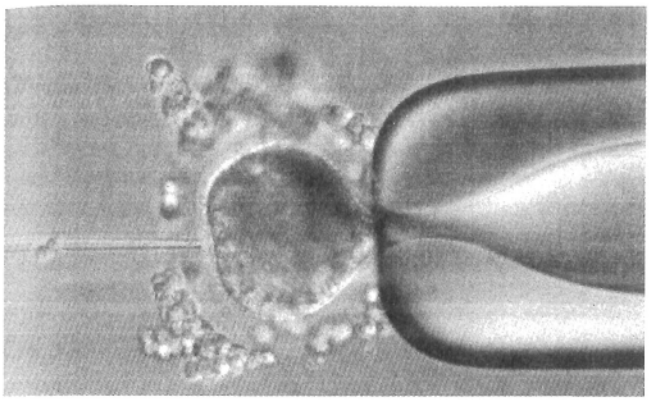

Figure 47-1 Injection of the spermatozoa into an in vivo–matured equine oocyte.

cause of the sperm defect appear to have no effect on the success of the procedure, whether the spermatozoon is epididymal, fresh or frozen, testicular, ejaculated, or from the testicles of men with severe defects in spermatogenesis. Maturation arrest, Sertoli cell-only, cryptorchidism, chemotherapy and mumps do not appear to have a major impact on the pregnancy rate. Of all the factors studied in couples where the male is severely infertile or azoospermic, the only factor that seems to matter (as long as spermatozoa are retrieved) is the age of the wife and, to a considerably lesser extent, her ovarian reserve. Extensive genetic and paediatric follow-up studies of ICSI pregnancies have revealed no increased risk of congenital malformation (2.6%), no increased risk of de-novo autosomal abnormalities, and a 1.0% risk of sex chromosomal abnormalities.[4]

Fertilization

Fertilization can be achieved through a number of means, including "classical" IVF, gamete intrafallopian transfer (GIFT), intracytoplasmic sperm injection (ICSI), and various other methods of micro-manipulation.

Classical IVF relies on laboratory fertilization, and GIFT relies on normal physiologic fertilization in vivo. With both techniques, normal spermatozoa with the ability to swim, penetrate, decondense, and fertilize the oocyte are needed. Techniques of zona manipulation—zona drilling, zona dissection, and subzonal injection (SUZI)—all carry the risk of poorer embryonic development and/or the potential for multiple sperm fertilization of the one oocyte (polyspermy) but were developed to address the poor results with IVF and GIFT in cases of severe male factor infertility.[5]

ICSI was developed by Gianpiero Palermo in Belgium,[6] resulting in the birth of the first child in 1992.[7] Subsequently, a large amount of experimental and clinical data have been amassed. ICSI involves injection of a single spermatozoon into the cytoplasm of the oocyte. The oocyte is held in position by suction through a fine, rounded glass pipette. A single spermatozoon is immobilized by tail crushing or chemical retardation before aspiration into an extremely fine, sharp glass pipette and then insertion though the zona pellucida. Concerns were expressed initially about the potential to produce offspring from "inferior" sperm and the opportunity to transmit genetic abnormalities.

There is an ongoing discussion regarding conflicting data on malformation rate in children born after intracytoplasmic sperm injection (ICSI). A prospective, multicentric, control cohort study was done in Germany. Fifty-nine centres prospectively recruited pregnancies before the 16th week of gestation, which were included in the study if they were ongoing beyond this time. Children were examined according to a standardized procedure. A control cohort of children conceived spontaneously was taken from a prospective birth registry (Mainzer Modell), where children were examined according to the exact same criteria as the ICSI cohort. Major malformation rate was calculated, based on data of all liveborn and stillborn children, as well as on all spontaneous and induced abortions, beginning with the 16th week of gestation. In the ICSI cohort, 8.6% of infants (291/3372), and in the control cohort 6.9% of infants (2140/30940), had a major malformation. This resulted in a crude relative risk (RR) of 1.25 (95% confidence interval 1.11-1.40). There was no influence of sperm origin on major malformation rate in children born after ICSI. There is an increased risk for a child born after ICSI to have a major malformation compared with a child that has been spontaneously conceived. Based on knowledge of the early developmental steps following ICSI, as well as on data of conventional IVF in general, it is assumed that this increased risk is due to parental factors causing the infertility, which has led to ICSI in the first place.[8]

Other studies have not supported these findings and are discussed later in this chapter.

The growing popularity of this technique reflects an ability to increase the control over and success rates for fertilization. ICSI, unlike standard IVF, guarantees the entrance of a single sperm directly into a single egg.

After micro-manipulation (i.e., ICSI and other techniques), the oocytes are cultured in vitro using defined medium and observed for early embryonic development. Developing embryos are graded, sorted, and transferred either fresh or frozen, or discarded.

The use of cytoplasmic transfer as an assisted reproductive technique has generated much attention and criticism. The technique involves the injection of cytoplasm from a healthy oocyte into a recipient oocyte considered unviable and includes the transfer of donor mitochondria. The consequences are the possible transmission of two mitochondrial (mt)DNA populations to the offspring. This pattern of inheritance is in contrast with the strictly maternal manner in which mtDNA is transmitted after natural fertilization and ICSI.[9]

Embryo Culture

With human ART, embryo culture and cryopreservation have resulted in an increased efficiency and flexibility. Commonly early-cleavage-stage embryos are transferred into the uterus despite the recognition that the embryo would not be expected in the uterine environment for at least 2 or 3 more days. This may result in a uterine environment that is not ideal for the embryo, perhaps associated with poor uterine clearance or an abnormal hormonal milieu. In addition, synchrony between the embryo and the uterus is poor. Oocytes fertilized and then cultured in vitro may be subjected to culture conditions that have affected the ability of the embryo to grow, and the technique also assumes that all oocytes, fertilized and in vitro–matured, have a similar ability to develop.

Pregnancy rates are three times higher when in vivo–derived blastocysts (obtained by uterine flushes in fertile women) are transferred, compared with in vitro–derived early-cleavage-stage embryos (as with IVF for infertile women).[10] Culturing embryos to the blastocyst stage in vitro has recently been associated with significant advantages. First, embryo morphology at the blastocyst stage is a predictor of pregnancy rates.[10] Second, the most viable embryo within the group of cultured embryos may be selected.[10] Third, the problems of multiple pregnancies may be addressed by examination of the probability that a single embryo will be transferred successfully before the transfer is performed. It also has been hypothesized that the uterine environment may not be as hostile to a blastocyst as to an earlier-cleavage-stage embryo.[10]

Furthermore, as the score of the blastocysts obtained using sequential media is directly related to implantation and pregnancy rates, it is possible to determine which patients should be offered a single blastocyst transfer, thereby addressing the issue of twins conceived through ART.[11]

Transfer

Embryos commonly are transferred at the 4- to 8-cell stage (day 2 or 3 after fertilization), although a current trend is for embryo culture and transfer at the blastocyst stage[11] because this approach could be expected to improve implantation rates. In addition, embryo grade is correlated with pregnancy rates.[12]

Embryos are mostly transferred through the vagina in a non–embryo-toxic catheter, through the cervix and into the uterus. The number of embryos transferred depends on embryo quality and age of the woman. More embryos are transferred into older women, with less success.[13-15]

Preimplantation genetic diagnosis (PGD) to identify chromosomally normal embryos has recently become a development applied to embryos before transfer. This technique involves the biopsy of a blastomere from an 8- to 16-cell embryo. Gender, genetic disorders, and chromosomal number and structure are commonly evaluated.

Despite its novelty, preimplantation genetic diagnosis has become an alternative to traditional prenatal diagnosis, allowing the establishment of only unaffected pregnancies and avoiding the risk of pregnancy termination. In addition, preimplantation genetic diagnosis is presently applied for much wider indications than prenatal diagnosis, including common diseases with genetic predisposition and preimplantation human leukocyte antigen typing, with the purpose of establishing potential donor progeny for stem cell treatment of siblings. Many hundreds of apparently healthy, unaffected children have been born after preimplantation genetic diagnosis, presenting evidence of its accuracy, reliability and safety. Preimplantation genetic diagnosis appears to be of special value for avoiding age-related aneuploidies in patients of advanced reproductive age, improving reproductive outcome, particularly obvious from their reproductive history, and is presently an extremely attractive option for carriers of balanced translocations to have unaffected children of their own.[16]

On occasion, zona-assisted hatching may be employed to help the embryo break out of the zona pellucida. Previous implantation failure in otherwise normal patients may suggest this problem. Chemicals, lasers, and mechanical manipulation all have been used to create a breach in the zona.[17]

Assisted hatching entails the opening or thinning of the zona pellucida before embryo transfer in order to improve the results of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The technique can be performed mechanically, chemically or with a laser beam. A piezoelectric method has also been described. Meta-analyses of randomised trials have shown that assisted hatching increases the clinical pregnancy, implantation and on-going pregnancy rates in patients with poor prognosis for IVF and ICSI, particularly those with repeated implantation failure. The technique is not without risks, and has been associated with an increased incidence of monozygotic twinning. Nevertheless, it remains an invaluable tool in assisted reproductive technology.[18]

This may be even more relevant in cases of in vitro maturation of oocytes.

Immature oocyte recovery followed by in-vitro oocyte maturation and in-vitro fertilization is a promising new technology for the treatment of human infertility. The technology is attractive to potential oocyte donors and infertile couples because of its reduced treatment intervention. Immature oocytes were recovered by ultrasound-guided transvaginal follicular aspiration. Oocytes were matured in vitro for 36-48 h followed by intracytoplasmic sperm injection (ICSI). Embryos were cultured in vitro for 3 or 5 days before replacement. Assisted hatching was performed on a day 5 blastocyst stage embryo. Embryo and uterine synchrony were potentially enhanced by luteinization of the dominant follicle at the time of immature oocyte recovery. Mature oocyte and embryo production from immature oocyte recovery were similar to the previous IVF results of the patients. A blastocyst stage embryo, produced as a result of in-vitro maturation, ICSI, in-vitro culture and assisted hatching, resulted in the birth of a healthy baby girl at 39 weeks of gestation.[19]

Pregnancy

Approximately 30% of IVF pregnancies produce twins, 5% triplets, and less than 1% more than three children. Multiple pregnancies carry high risks for the woman and the fetuses.

Multiple gestation pregnancy rates are high in assisted reproductive treatment cycles because of the perceived need to stimulate excess follicles and transfer excess embryos in order to achieve reasonable pregnancy rates. Perinatal mortality rates are, however, 4-fold higher for twins and 6-fold higher for triplets than for singletons. Since the goal of infertility therapy is a healthy child, and multiple gestation puts that goal at risk, multiple pregnancy must be regarded as a serious complication of assisted reproductive treatment cycles. The 1999 ESHRE Capri Workshop addressed the psychological, medical, social and financial implications of multiple pregnancy and discussed how it might be prevented. Multiple gestations are high risk pregnancies which may be complicated by prematurity, low birthweight, pre-eclampsia, anaemia, postpartum haemorrhage, intrauterine growth restriction, neonatal morbidity and high neonatal and infant mortality. Multiple gestation children may suffer long-term consequences of perinatal complications, including cerebral palsy and learning disabilities. Even when the babies are healthy they must share their parents' attention

and may experience slow language development and behavioural problems. Current data indicate that the average hospital cost per multiple gestation delivery is greater than the average cost of in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles. Prevention is the most important means of decreasing multiple gestation rates. Multiple gestation rates in ovulation induction and superovulation cycles can be reduced by using lower dosage gonadotrophin regimens. If there are more than three mature follicles, the cycle should be converted to an IVF cycle, or it should be cancelled and intercourse should be avoided. In IVF cycles two embryos can be transferred without reducing birth rates in most circumstances. Embryo reduction involves extremely difficult decisions for infertile couples and should be used only as a last resort. Assisted reproductive treatment centres and registries should express cycle results as the proportion of singleton live births; twin and triplet rates should be reported separately as complications of the procedures. Reducing the multiple gestation pregnancy rate should be a high priority for assisted reproductive treatment programmes, despite the pressure from some patients to transfer more embryos in order to improve success. If nothing is done, public concern may lead to legislation in many countries, a step that would be unnecessary if assisted reproductive treatment programmes and registries took suitable steps to reduce multiple pregnancy rates.[20]

There are data regarding the possible influences of extended embryo culture to the blastocyst stage as well as zona pellucida manipulation on the incidence of monozygotic multiples. This is interesting, as one aim of extended culture with embryo selection is to minimize the multiple pregnancy rate. We report, to our knowledge, on the first case of monozygotic twins and monozygotic triplets after ICSI and the transfer of two blastocysts. Monozygotic multiples after ICSI and blastocyst transfer and the resulting problems are another reason to encourage the transfer of only one blastocyst. In our opinion, the incidence of 5.9-8.9% monozygotic multiple occurrence after ICSI and blastocyst transfer reported in the literature requires that patients are informed of the uncertainties until this phenomenon and its risk factors are better understood.[21]

Current Status of Human Intracytoplasmic Sperm Injection

Since the publication of the first papers on the use of ICSI for oligozoospermia in 1992 and 1993, much scientific work has been applied to extending its application to virtually every type of male infertility.[4,7,22,23] In 1995 Nagy[24] confirmed that the most severe cases of oligoasthenoteratozoospermia produced the same pregnancy rates as for mild cases of male factor infertility, which were no different from those for men with normal spermatozoa undergoing conventional IVF. Then it was demonstrated that the way in which the spermatozoa are pretreated before ICSI is immaterial, and that any method for aspirating the spermatozoa into an injection pipette and transferring them into the oocytes is adequate.[25] It also was reported that fertilization failure was always related either to poor egg quality or to sperm nonviability.[26] It appeared that no matter how severe morphologic defect, or with the most severe motility defect or the smallest number of spermatozoa in the ejaculate (pseudoazoospermia), no negative effect on the pregnancy rate was observed with ICSI. Only absolute immotility of ejaculated or epididymal spermatozoa lowered the fertilization rate, and this was found to be not due to the immotility of the spermatozoon but rather to its nonviability. Completely nonmotile spermatozoa that were viable were still capable of normal fertilization and pregnancy rates.[27]

ICSI then took another leap forward with the development of sperm aspiration and extraction techniques, which allowed couples in which the male was absolutely azoospermic to achieve pregnancy rates no different from those in which the male had a normal sperm count.[28] The first successful attempts at sperm aspiration combined with ICSI were reported in 1994.[29] Conventional IVF with aspirated epididymal spermatozoa yielded a pregnancy rate of only 9% and a delivery rate of only 4.5%, whereas ICSI with aspirated epididymal spermatozoa in men with congenital absence of the vas deferens (CAVD) yielded a pregnancy rate of 47% and a delivery rate of 33%. Furthermore, no difference in pregnancy rates was found with use of epididymal spermatozoa retrieved for any cause of obstruction, whether it was failed vasoepididymostomy, CAVD, or simply irreparable obstruction.[30]

However, this breakthrough for men with CAVD brought with it a serious problem. It was soon discovered that CAVD is caused by mutations on the cystic fibrosis transmembrane conductance regular gene (CFTR) located on chromosome 7. Although now this is taken for granted, in 1992 it was a startling discovery. . . . This discovery meant that all patients and their wives undergoing sperm aspiration with ICSI for CAVD required careful genetic screening for cystic fibrosis, and if the wife was a carrier (4% risk of carrier status in the general population), then the embryos should undergo preimplantation genetic diagnosis using polymerase chain reaction, so that only healthy embryos would be replaced.[27]

The first case of successful preimplantation embryo biopsy for cystic fibrosis, on the embryo of a man who had undergone MESA and ICSI for CAVD, was reported in 1994.[31] "The use of MESA and ICSI for CAVD led to intense molecular study of the genetic mystery of how the condition of CAVD is transmitted via defects in the cystic fibrosis gene."[27]

Soon after the MESA-ICSI procedure was developed in 1994, it was discovered that testicular spermatozoa could fertilize as efficiently as ejaculated spermatozoa and also result in normal pregnancies.

In this study (May 1 until August 31, 1994) a total of 15 azoospermic patients suffering from testicular failure were treated with a combination of testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). Spermatozoa were available for ICSI in 13 of the patients. Out of 182 metaphase II injected oocytes, two-pronuclear fertilization was observed in 87 (47.80%); 57 embryos (65.51%) were obtained for either transfer or cryopreservation. Three ongoing pregnancies out of 12 replacements (25%) were established, including one singleton, one twin and one triplet gestation. The ongoing implantation rate was 18% (six fetal hearts out of 32 embryos replaced).[32]

TESE truly revolutionized the treatment of infertile couples with azoospermia. The development of TESE meant that even patients with zero motility of the epididymal spermatozoa or of ejaculated spermatozoa, or even men with no epididymis, could still have their own genetic child, so long as there was normal spermatogen-

esis. It also meant that surgeons could easily perform a testicle biopsy, enabling the retrieved spermatozoa to be used for ICSI without the need for the microsurgical expertise required to perform a conventional MESA procedure.[27]

It also was demonstrated that epididymal spermatozoa, despite fairly weak motility, could be frozen and, after thawing, yield pregnancy rates no different from those obtained with freshly retrieved epididymal spermatozoa.

> *In seven patients who did not become pregnant following microsurgical epididymal sperm aspiration (MESA) and intracytoplasmic sperm injection (ICSI), a subsequent ICSI was performed using previously cryopreserved supernumerary epididymal spermatozoa without re-operating on the husband. During the original MESA procedure a mean sperm concentration of 12.3 × 10(6)/ml was achieved. The supernumerary spermatozoa were cryopreserved for later use. After thawing frozen epididymal spermatozoa a mean concentration of 1.9 × 10(6) spermatozoa/ml was obtained in straws containing a total volume of sperm suspension of 250 microliters. From 68 intact oocytes injected with frozen-thawed epididymal spermatozoa, a two pronuclear fertilization rate of 45% and a cleavage rate of 82% were obtained. A total of 17 embryos were replaced in the seven patients, resulting in two ongoing singleton pregnancies and one twin delivery. Six embryos were cryopreserved. In conclusion, it would appear mandatory to cryopreserve supernumerary spermatozoa during a MESA in order to avoid subsequent further scrotal surgery.*[33]

Because of the remarkable success with the freezing of very-poor-quality epididymal spermatozoa for subsequent ICSI, couples did not have to time the MESA exactly with the partner's egg retrieval. This also meant that men about to undergo chemotherapy and/or radiotherapy for cancer could have a single ejaculate frozen, causing no delay in treatment of the cancer, and this one ejaculate would be sufficient for almost an infinite number of IVF-ICSI cycles.[27]

Finally, in a majority of patients with testicular failure (caused either by maturation arrest, Sertoli cell–only syndrome, cryptorchid testicular atrophy, post-chemotherapy azoospermia, or even Klinefelter's syndrome), a very tiny number of spermatozoa or spermatids usually can be extracted from extensive testicular biopsy specimens and used for ICSI.[32,34]

> *Early studies of the kinetics of spermatogenesis in the testicle demonstrated that often a tiny amount of spermatogenesis was present if one examined quantitatively and carefully the testicle biopsy of men who were azoospermic from non-obstructive testicular failure. However, the significance of this 'threshold' phenomenon was not appreciated until the era of ICSI, when it was realized that these spermatids could be harvested, and normal pregnancy rates achieved, in the ~60% of such patients who possessed this minuscule degree of spermatogenesis in otherwise completely deficient testicles.*[27]
>
> *There has been some excitement generated over the possibility of using 'round cells' derived from testicular tissue (or even from the ejaculate), which are presumably early spermatids, for ICSI in the absence of elongated spermatozoa. Infertility clinics around the world are now endeavoring to use this technique, which may have unfortunate implications. . . . The problems with this approach are: (i) in human spermatogenesis, it is widely known that maturation arrest is a problem associated with meiosis, and not with sperm maturation. Wherever there are early round spermatids, there will also be elongated mature spermatids with a tail; and (ii) clinics not aware of this reality are tempted to inject 'round cells' when they cannot retrieve spermatozoa. The truth is, that if those round cells were genuinely spermatids, then a better search would have revealed the presence of mature spermatozoa. On the other hand, it is extremely difficult, using Hoffman optics, to distinguish with certainty a round spermatid from a Sertoli cell nucleus with its prominent nucleolus, or even from some spermatocytes. Where there are truly no spermatozoa, there may be many 'round cells' either with Sertoli cell only, or with maturation arrest, that are not round spermatids.*[27]

The current ability of most men to father a child, regardless of the quality of the sperm count, even if there are apparently no mature spermatozoa at all, is dramatic. It appears that the results of ICSI are not related to the source of the spermatozoa (whether ejaculated, testicular, or epididymal), or to the quality of the spermatozoa (with regard to morphology or motility). ICSI results are not influenced by whether the spermatozoon has been frozen or is fresh, whether it is retrieved with ease (as in the case of normal spermatogenesis or with ejaculated spermatozoa), or whether the spermatozoon was extracted directly from the Sertoli cell after hours of painstaking searching of a testis sample.[27] With regard to the infertile or azoospermic male and ICSI, none of these factors has had any significant influence on fertilization, cleavage, or pregnancy rate. In fact, the only significant factor in the success of ICSI appears to be the age of the female partner.[27,30]

Are the Offspring Normal? The Genetics of Intracytoplasmic Sperm injection

A major development in 1996 was the detailed follow-up study of ICSI-conceived children and the information it provided about the genetics of infertility.

> *A prospective follow-up study of 877 children born after ICSI was carried out. The aim of this study was to compile data on karyotypes, congenital malformations, growth parameters and developmental milestones so as to evaluate the safety of this new technique. The follow-up study included agreement to genetic counselling and prenatal diagnosis and was based on a physical examination at the Centre for Medical Genetics (Dutch-speaking Brussels Free University, Brussels, Belgium) at 2 months, 1 year and 2 years, when major and minor malformations and a psychomotor evolution were recorded. Between April 1991 and July 1995, 904 pregnancies obtained after intracytoplasmic sperm injection (ICSI) led to the birth of 877 children (465 singletons, 379 twins and 33 triplets). Prenatal diagnosis determined a total of 486 karyotypes, of which six were abnormal (1.2%) and six (1.2%) were familial structural aberrations, all transmitted from the father. This slight increase in de-novo chromosomal aberrations and the higher frequency of transmitted chromosomal aberrations are probably linked directly to the characteristics of the infertile men treated rather than to the ICSI procedure itself. In all, 23 (2.6%) major malformations were observed in the children born, defined as those causing functional impairment or requiring surgical correction. No particular malformation was disproportionately frequent. Compared with most registers of children born after assisted reproduction*

and with registers of malformation in the general population, the figure of 2.6% was within the expected range. These observations should be further completed by others and by collaborative efforts. In the meantime, patients should be counselled about the available data before any treatment: the risk of transmitted chromosomal aberrations, the risk of de-novo, mainly sex chromosomal, aberrations and the risk of transmitting fertility problems to the offspring. Patients should also be reassured that there seems to be no higher incidence of congenital malformations in children born after ICSI.[35]

Since the introduction of ICSI in 1991, medical outcome studies on ICSI children have been performed, but few have addressed developmental outcome. Hence, this outcome was assessed by performing a standard developmental test on children born after ICSI as compared with children born after IVF, at the age of 2 years.

METHODS: In a prospective study, the medical and developmental outcomes of 439 children born after ICSI (378 singletons, 61 twins) were compared with those of 207 children born after IVF (138 singletons, 69 twins), at the age of 24-28 months. These children were part of a cohort of children followed since birth. Of children reaching the age of 24-28 months between May 1995 and March 2002, 44.3% (2375/5356) were examined by a paediatrician who was unaware of the type of treatment used for each couple. Of all the children born, 12.2% (439/3618) in the ICSI group and 11.9% (207/1738) in the IVF group underwent a formal developmental assessment using the Bayley Scale of Infant Development (mental scale) by a paediatrician blinded to the type of treatment. RESULTS: There was no significant difference in maternal educational level, maternal age, gestational age, parity, birthweight, neonatal complication rate or malformation rate at 2 years between ICSI and IVF singletons, or between ICSI and IVF twins. No significant difference was observed in the developmental outcome using the Bayley scale at the age of 24-28 months (raw scores or test age) between ICSI children or IVF children. A multivariate regression analysis for the singleton children indicated that parity, sex (boys had lower scores than girls) and age had a significant influence on the test result, but that the fertility procedure (ICSI versus IVF) did not influence the test result. ICSI children from fathers with low sperm concentration, low sperm motility or poor morphology had a similar developmental outcome to that of children from fathers with normal sperm parameters. There were no significant differences between the initial cohort and the group lost to follow-up, nor between the psychologically tested and the non-tested group for a number of variables such as maternal educational level, birthweight in singletons and neonatal malformation rate. Although only some of the cohort of ICSI children were evaluated, a representative sample of both ICSI and IVF children was compared. CONCLUSIONS: There is no indication that ICSI children have a lower psychomotor development than IVF children. Paternal risk factors associated with male-factor infertility were found not to play a role in developmental outcome.[36]

Genetic Basis for Infertility

There is now abundant evidence in a wide range of mammalian and non-mammalian species to show that the relative size of the testis and the morphology of the spermatozoa are infallible predictors of the mating system. Species with the largest testis/body weight ratios and the best spermatozoa have a multi-male or promiscuous mating system in which sperm competition operates. Judged by these criteria, men were not designed to be promiscuous. There is increasing evidence in humans to show that most spontaneous mutations of the germ line occur in the testis. Because these provide the variability on which natural selection can operate, the testis holds the key to evolution. Genes on the Y chromosome that control male fertility are particularly prone to mutations, perhaps because of the mutagenic metabolites produced by the metabolically active testis. Testicular descent into a scrotum, and cooling by countercurrent heat exchange between the spermatic artery and vein may have evolved as a way of holding the mutation rate in check. The hormones secreted by the testis, which control libido and aggression, ensure that these male mutations are disseminated as widely as possible throughout the population.[37,38]

This led us to speculate that perhaps deficient spermatogenesis, despite a variety of so-called causes is in most cases genetically determined (excluding, of course, mumps, orchitis, post-cryptorchidism testicular atrophy, postchemotherapy azoospermia, etc.).[39]

The first phase of this genetic control of male fertility came from Professor Roger Short's analysis of infertility in the three types of great apes, i.e., gorilla, orangutan and chimpanzee. Chimpanzees travel together in troops of about 40 males and females, and whenever anyone of the females in the troop goes into heat, every single male in the troop has intercourse with her. Consequently, one would expect that the male with the highest sperm count and the best quality sperm is most likely to be the father of any child born to any female who has had sex with multiple partners. The extensive sperm competition among the sperm of all the chimpanzees who have mounted her results in male offspring that have inherited the best sperm count. Therefore, chimpanzees would be expected to have high sperm counts because of their promiscuous mating behavior if we assume that sperm production, both quantitative and qualitative, is genetically transmitted. In fact, this is the case. Chimpanzees, who are extremely promiscuous, have gigantic testicles and huge sperm counts, and gorillas who are faithful to their mates, and in whom there is no sperm competition because the female is only having sex with one given mate for her entire lifetime, have very small testicles and extremely low sperm counts. Professor Short concluded from this that male fertility was a genetically transmitted trait.[39]

The next suggestion from studies of exotic species came with the cheetah. All cheetahs in the world are so genetically inbred and identical that even skin grafts from one cheetah to another never reject. This inbreeding has resulted in all male cheetahs having an extremely poor sperm count with 90% abnormal forms and very low numbers. The infertility of the inbred male cheetah clearly demonstrates a genetic cause of the very same types of semen abnormalities that we see in the infertile human. Therefore, we had speculated for the last eleven years that there may be a genetic cause for the majority of cases of oligospermia and azoospermia.[39]

The common domestic cat is an important research model for endangered felids, as well as for studying genetic dysfunctions, infectious diseases and infertility in humans. Especially significant is the trait of teratospermia (ejaculation of <40% morphologically normal spermatozoa) that commonly occurs in about 70% of the felid species or subspecies studied to date. Teratospermia, discovered more than two decades ago in the cheetah, is important: (i) for understanding the significance of sperm form and function; and (ii) because this condition is common in human males. It is apparent from IVF that deformed spermatozoa from teratospermic felids do not fertilize oocytes. However, the

inability of spermatozoa from teratospermic males to bind, penetrate and decondense in the cytoplasm of the oocyte is not limited to malformed cells alone. Normal shaped spermatozoa from teratospermic males have reduced functional capacity. IVF results have consistently revealed a direct correlation between teratospermia and compromised sperm function across felid species and populations. The most significant differences between normospermic (>60% normal spermatozoa per ejaculate) and teratospermic felids include: (i) the time required for sperm capacitation and the acrosome reaction to occur in vitro; (ii) culture media requirements for capacitation in vitro; (iii) phosphorylation patterns of tyrosine residues on sperm membrane proteins during capacitation; (iv) susceptibility to chilling-induced sperm membrane damage; (v) sensitivity to osmotic stress; (vi) stability of sperm DNA; (vii) sperm protamine composition; and (viii) fertilizing ability after intracytoplasmic sperm injection. In conclusion, (i) the felids (including wild species) are valuable for studying the functional significance of both pleiomorphic and normally formed spermatozoa from teratospermic donors, and (ii) the impact of teratospermia is expressed at both macrocellular and subcellular levels. [39,40]

Now, in a collaborative study with the human genome project and the Massachusetts Institute of Technology, we have indeed found that about 11% of men with either azoospermia or severe oligospermia consistently are found to have deletions in the Y chromosome in a specific region we have called the AZF loci (short for "azoospermia factor"), and the first specific gene candidate localized to this area has been called DAZ (deleted in azoospermia). Deletion I of this DAZ gene is always associated with azoospermia or severe oligospermia, and thus far no normal fertile men have been found to have a deletion of this gene candidate. Furthermore, there is a homolog copy of this DAZ gene not only on the Y chromosome in men, but on chromosome 3 in both men and women, indicating it may even tell us something about female infertility. There are undoubtedly many other genes and loci that affect sperm production, and we have only just begun the search. Furthermore, we know that about 1.3% of azoospermic or severely oligospermic men have chromosomal abnormalities, usually balanced translocation, that can give us other clues and doorways to explore with our molecular tools many other possibilities for genetic causes of male infertility.[39]

HORSE STUDIES

Live Foals from Horse ART

Only two foals have been reported from conventional IVF in the horse.[41]

The results of in vitro fertilization (IVF) in the mare are less advanced than in most of the other domestic mammals. The different steps which have contributed to the success of IVF with the birth of two foals are shown: the preparation of oocytes and capacitated spermatozoa, the fertilization technique, the embryo culture and transfer. After induction of ovulation in the mare with equine pituitary extract, the preovulatory follicles are punctured just before ovulation. The oocytes matured in vivo are in vitro inseminated with fresh semen treated by Ionophore A23187. The embryos are cultured until their transfer to the oviduct of a recipient mare by a surgical technique. The development of in vitro maturation of immature oocytes from slaughtered ovaries will give a larger number of oocytes for IVF attempts. The development of the embryo culture to obtain the blastocyst stage will allow a non surgical transfer in the uterus of a mare.[41]

Both foals were born from fertilization of in vivo matured oocytes. Despite much interest and some promising results,[42] further success has not been forthcoming, casting doubt on the usefulness of the technique as an infertility treatment.

The reason for such poor results of IVF in the horse is unknown but has been suggested to be inability of the horse oocyte to allow penetration of spermatozoa. This was not associated with zona pellucida hardening (ZPH).

In vitro fertilization (IVF) has had poor success in the horse, a situation related to low rates of sperm penetration through the zona pellucida (ZP). Zona pellucida hardening (ZPH) is seen in mouse and rat oocytes cultured in serum-free medium. The hardened ZP is refractory to sperm penetration. Fetuin, a component of fetal calf serum, inhibits ZPH and allows normal fertilization rates in oocytes cultured in the absence of serum. We evaluated whether fetuin is present in horse serum and follicular fluid (FF) and whether fetuin could inhibit ZPH in equine oocytes matured in vitro, thus increasing sperm penetration during IVF. The presence of fetuin in equine serum and FF was confirmed by immunoblotting. Oocytes submitted to in vitro maturation (IVM) in medium containing fetuin were used for ZPH assay or IVF. Intracytoplasmic sperm injection (ICSI) was carried out as a control procedure, The presence of fetuin during IVM did not affect the rate of maturation to metaphase II. Maturation of oocytes in the presence of fetuin reduced ZPH in a dose-dependent manner. After both IVF and ICSI, there was no significant difference in oocyte fertilization between fetuin-treated and untreated oocytes. The fertilization rate was significantly higher after ICSI than after IVF, both in fetuin-treated and in untreated oocytes. In conclusion, fetuin reduced ZPH in equine oocytes but did not improve sperm penetration during IVF. This implies that, in the horse, "spontaneous" ZPH is unlikely to be the major factor responsible for inhibiting sperm penetration in vitro.[43]

The birth of the first ICSI foal was reported in 1996.[44] A technique relevant to a commercial application was reported with birth of live foals from in vivo–matured oocytes using ICSI with frozen spermatozoa from fertile and infertile stallions.[1] The study examined the use of ICSI for in vitro fertilization of equine oocytes and their developmental potential when transferred to the fallopian tubes of synchronized mares. Oocytes were aspirated from mature follicles 39 hours after a GnRH analogue injection and transported 190 kilometers at 39°C. Semen from both a fertile and an infertile stallion was frozen and later prepared for injection. Successfully injected oocytes were surgically transferred into the ampulla of the fallopian tube either (1) 4 to 8 hours after injection or (2) were cultured for 24 to 48 hours before transfer. Twenty-six oocytes were treated by ICSI. Three oocytes fragmented after injection (12%). Eight oocytes were returned for immediate transfer to recipient mares. Of the 15 cultured oocytes, 8 (53%) had two polar bodies and cleaved to 2 cells at 24 hours after injection. Pregnancies were identified on days 12 to14 after transfer in 4 of the 16 (25%) recipients that had either transferred embryos (1/8) or freshly injected oocytes (3/8). Two of the four pregnancies did not progress beyond 30 days, and two

mares had foals. There was no apparent difference in the establishment of pregnancies from oocytes injected with frozen/thawed sperm of the fertile (3/14) and the infertile stallion (1/2).[1]

Subsequently, foals were born from follicles aspirated from pregnant mares.[45] These oocytes, as in the work of Squires,[44] were matured in vitro. A total of 263 follicles were punctured in 20 aspiration procedures, and 174 oocytes were retrieved in one of two experiments. Eighty-six (49%) were classified as mature after culture in TCM-199. A cleavage rate of 55% (47/86) occurred after ICSI. A total of 31 embryos were transferred into the oviducts of recipient mares, resulting in the birth of two foals. Four pregnancies in total were established (one pregnancy per eight oocytes transferred); however,two underwent early embryonic death, and two subsequently foaled.[45]

An important development that increased the applicability of equine ICSI to clinical situations was the report of live foals from embryos cultured to the blastocyst stage.[46] Success with this technique means that oocytes and semen can be shipped to remote locations that are skilled in ICSI techniques and then transferred nonsurgically after demonstration of normal blastocyst development.

The influence of coculture with either oviduct epithelial cells or fetal fibroblast cells on in vitro maturation of equine oocytes and their potential for development to blastocysts and fetuses after intracytoplasmic sperm injection (ICSI) was investigated. The oocytes were obtained from ovaries from abattoirs and were matured in vitro for 2-30 h in TCM199 only, or in TCM199 coculture with oviduct epithelial cells or fetal fibroblast cells. Metaphase II oocytes were subjected to ICSI with an ionomycin treated spermatozoon. The injected oocytes were cultured for 79 days in Dulbecco's modified Eagle's medium. Morphologically normal early blastocysts were transferred to the uteri of recipient mares. Nuclear maturation rates and the rates of cleavage to the two cell stage for injected oocytes were similar in the groups of oocytes that were matured in TCM 199 (49 and 63%), in coculture with oviduct epithelial cells (53 and 65%) or in coculture with fetal fibroblasts (51 and 57%). There were no significant differences in the proportions of blastocysts that developed from the two cell embryos derived from oocytes matured by coculture with either oviduct epithelial cells (30%) or fetal fibroblasts (17%). However, significantly higher proportions of blastocysts were produced from both these coculture groups than from the groups of oocytes matured in TCM199 only ($P < 0.05$). Six of the blastocysts that had developed from oocytes cocultured with oviduct epithelial cells were transferred into recipient mares and four pregnancies resulted. These results demonstrate a beneficial influence of coculture with either oviduct epithelial cells or fetal fibroblasts for maturation of oocytes in vitro.[46] A report from Italy highlighted the production of pregnancies from embryos that were frozen and thawed and successfully transferred into recipients. The technique combined transvaginal collection of immature oocytes, in vitro maturation, ICSI and culture of the early embryos in vitro or in vivo (sheep oviduct). The efficiency of the procedure was higher when in vivo culture was used.[47]

Sperm/Oocyte Interactions

Classically it has been suggested that sperm need to activate the oocyte after ICSI. This would appear to be less important for human ICSI than for bovine ICSI.[48]

In the human and the mouse, intracytoplasmic sperm injection (ICSI) apparently triggers normal fertilization and may result in offspring. In the bovine, injection of spermatozoa must be accompanied by artificial methods of oocyte activation in order to achieve normal fertilization events (e.g., pronuclear formation). In this study, different methods of oocyte activation were tested following ICSI of in vitro-matured bovine oocytes. Bovine oocytes were centrifuged to facilitate sperm injection, and spermatozoa were pretreated with 5 mM dithiothreitol (DTT) to promote decondensation. Sperm-injected or sham-injected oocytes were activated with 5 mu M ionomycin (A23187). Three hours after activation, oocytes with second polar bodies were selected and treated with 1.9 mM 6-dimethylaminopurine (DMAP). The cleavage rate of sperm-injected oocytes treated with ionomycin and DMAP was higher than with ionomycin alone (62 vs 27%, P less than or equal to 0.05). Blastocysts (2 of 41 cleaved) were obtained only from the sperm-injected, ionomycin + DMAP-treated oocytes. Upon examination 16 h after ICSI, pronuclear formation was observed in 33 of 47 (70%) DMAP-treated oocytes. Two pronuclei were present in 18 of 33 (55%), while 1 and 3 pronuclei were seen in 8 of 33 (24%) and 7 of 33 (21%) oocytes, respectively. In sham-injected oocytes, pronuclear formation was observed in 15 of 38 (39%) with 9 (60%) having 2 pronuclei. As a single calcium stimulation was insufficient and DMAP treatment could result in triploidy, activation by multiple calcium stimulations was tested. Three calcium stimulations (5 mu M ionomycin) were given at 30-min intervals following ICSI. Two pronuclei were found in 12 of 41 (29%) injected oocytes. Increasing the concentration of ionomycin from 5 to 50 mu M resulted in a higher rate of activation (41 vs 26%). The rate of metaphase III arrest was lower while the rate of pronuclear formation and cleavage development was higher in sperm-injected than sham-injected oocytes, suggesting that spermatozoa contribute to the activation process. Further improvements in oocyte activation following ICSI in the bovine are necessary.[48]

The need for horse oocyte activation is less apparent. Conflicting results are reported with use of fresh, chilled, or frozen semen, suggesting that the type of sperm preparation influences the need to activate the oocyte.[49]

Studies involving frozen spermatozoa and ICSI reported good cleavage rates without sperm activation.[1,43,50-53]

The effects of four reagents on the activation and subsequent fertilization of equine oocytes, and the development of these after intracytoplasmic sperm injection, were investigated. Cumulus-oocyte complexes collected from equine ovaries obtained from an abattoir were matured in vitro for 40-44 h in TCM199 medium before being injected, when in metaphase II, with an immobilized stallion spermatozoon. The cumulus-oocyte complexes were then subjected to one of five activation treatments: (a) 10 mu mol ionomycin l(-1) for 10 min; Co) 7% (v/v) ethanol for 10 min; (c) 100 mu mol thimerosal l(-1) for 10 min; (d) 250 mu mol inositol 1,4,5-triphosphate l(-1) injection; and (e) no treatment (control). After 18-20 h further culture, the cumulus-oocyte complexes were assessed for activation by observing whether they had progressed through second anaphase-telophase and had formed a female pronucleus. The proportions of oocytes activated after each treatment were: 16/27 (59%) for ionomycin; 14/25 (56%) for ethanol; 22/28 (79%) for thimerosal; 15/27 (56%) for inositol 1,4,5-triphosphate; and 0/20 (0%) for the untreated controls. Thus, significantly more oocytes ($P < 0.05$) were acti-

vated by treatment with thimerosal than by the other four treatments. The proportions of oocytes that cleaved to the two-cell stage at 24-30 h after sperm injection in the groups treated with ionomycin, ethanol and thimerosal were 7/20 (35%), 5/19 (26%) and 11/ 23 (48%), respectively. No cleavage was observed in any of the control oocytes or those treated with inositol 1,4,5-triphosphate, Furthermore, evidence of normal fertilization was observed in 2/7 (29%), 2/5 (40%) and 7/11 (64%) of the oocytes treated with ionomycin, ethanol and thimerosal, respectively. These results demonstrated that: (a) it is possible to activate equine oocytes with the chemical stimulants, ionomycin, ethanol, thimerosal and inositol 1,4,5-triphosphate; (b) thimerosal is more effective than the other three reagents in facilitating both meiotic activation and normal fertilization of equine oocytes; and (c) chemical activation may also stimulate parthenogenetic cleavage of oocytes without concurrent changes in the head of the spermatozoon.[54]

Intracytoplasmic sperm injection (ICSI) is the method of choice for fertilizing horse oocytes in vitro. Nevertheless, for reasons that are not yet clear, embryo development rates are low. The aims of this study were to examine cytoskeletal and chromatin reorganization in horse oocytes fertilized by ICSI or activated parthenogenetically. Additional oocytes were injected with a sperm labeled with a mitochondrion-specific vital dye to help identify the contribution of the sperm to zygotic structures, in particular the centrosome. Oocytes were fixed at set intervals after sperm injection and examined by confocal laser scanning microscopy. In unfertilized oocytes, microtubules were present only in the metaphase-arrested second meiotic spindle and the first polar body. After sperm injection, an aster of microtubules formed adjacent to the sperm head and subsequently enlarged such that at the time of pronucleus migration and apposition it filled the entire cytoplasm. During syngamy, the microtubule matrix reorganized to form a mitotic spindle on which the chromatin of both parents aligned. Finally, after nuclear and cellular cleavage were complete, the microtubule asters dispersed into the interphase daughter cells. Sham injection induced parthenogenetic activation of 76% of oocytes, marked by the formation of multiple cytoplasmic microtubular foci that later developed into a dense microtubule network surrounding the female pronucleus. The finding that a parthenote alone can produce a microtubule aster, whereas the aster invariably forms at the base of the sperm head during normal fertilization, indicates that both gametes contribute to the formation of the zygotic centrosome in the horse. Finally, 25% of sperm-injected oocytes failed to complete fertilization, mostly due to absence of oocyte activation (65%), which was often accompanied by failure of sperm decondensation. In conclusion, this study demonstrated that union of the parental genomes in horse zygotes is accompanied by a series of integrated cytoskeleton-mediated events, failure of which results in developmental arrest.[55]

In oocytes from all mammalian species studied to date, fertilization by a spermatozoon induces intracellular calcium [Ca^{2+}]i oscillations that are crucial for appropriate oocyte activation and embryonic development. Such patterns are species-specific and have not yet been elucidated in horses; it is also not known whether equine oocytes respond with transient [Ca^{2+}]i oscillations when fertilized or treated with parthenogenetic agents. Therefore, the aims of this study were: (i) to characterize the activity of equine sperm extracts microinjected into mouse oocytes; (ii) to ascertain in horse oocytes the [Ca^{2+}]i -releasing activity and activating capacity of equine sperm extracts corresponding to the activity present in a single stallion spermatozoon; and (iii) to determine whether equine oocytes respond with [Ca^{2+}]i transients and activation when fertilized using the intracytoplasmic sperm injection (ICSI) procedure. The results of this study indicate that equine sperm extracts are able to induce [Ca^{2+}]i oscillations, activation and embryo development in mouse oocytes. Furthermore, in horse oocytes, injection of sperm extracts induced persistent [Ca^{2+}]i oscillations that lasted for >60 min and initiated oocyte activation. Nevertheless, injection of a single stallion spermatozoon did not consistently initiate [Ca^{2+}]i oscillations in horse oocytes. It is concluded that stallion sperm extracts can efficiently induce [Ca^{2+}]i responses and parthenogenesis in horse oocytes, and can be used to elucidate the signalling mechanism of fertilization in horses. Conversely, the inconsistent [Ca^{2+}]i responses obtained with sperm injection in horse oocytes may explain, at least in part, the low developmental success obtained using ICSI in large animal species.[56]

In all species studied, fertilization induces intracellular Ca2+ [Ca^{2+}]i oscillations required for oocyte activation and embryonic development. This species-specific pattern has not been studied in the equine, partly due to the difficulties linked to in vitro fertilization in this species. Therefore, the objective of this study was to use intracytoplasmic sperm injection (ICSI) to investigate fertilization-induced [Ca^{2+}]i signaling and, possibly, ascertain problems linked to the success of this technology in the horse. In vivo- and in vitro-matured mare oocytes were injected with a single motile stallion sperm. Few oocytes displayed [Ca^{2+}]i responses regardless of oocyte source and we hypothesized that this may result from insufficient release of the sperm-borne active molecule (sperm factor) into the oocyte. However, permeabilization of sperm membranes with Triton-X or by sonication did not alleviate the deficient [Ca^{2+}]i responses in mare oocytes. Thus, we hypothesized that a step downstream of release, possibly required for sperm factor function, is not appropriately accomplished in horse oocytes. To test this, ICSI-fertilized horse oocytes were fused to unfertilized mouse oocytes, which are known to respond with [Ca^{2+}]i oscillations to injection of stallion sperm, and [Ca2+]i monitoring was performed. Such pairs consistently displayed [Ca^{2+}]i responses demonstrating that the sperm factor is appropriately released into the ooplasm of horse oocytes, but that these are unable to activate and/or provide the appropriate substrate that is required for the sperm factor delivered by ICSI to initiate oscillations. These findings may have implications to improve the success of ICSI in the equine and other livestock species.[57]

ICSI Used for Studying Developmental Competence of In Vivo and In Vitro Matured Oocytes

In vitro versus in vivo oocyte maturation usually has resulted in reduced embryonic development and lower pregnancy rates after ICSI and culture and/or embryo transfer. This difference also has been demonstrated with in vitro culture systems for embryonic development compared with in vivo systems (e.g., the sheep oviduct), regardless of method of oocyte collection.

Blastocyst formation rates during horse embryo in vitro production (IVP) are disappointing, and embryos that blastulate in culture fail to produce the characteristic and vital glycoprotein capsule. The aim of this study was to evaluate the impact of IVP on horse embryo development and capsule formation. IVP embryos were produced by intracytoplasmic sperm injection of in vitro matured oocytes and either culture in synthetic oviduct fluid (SOF) or temporary

transfer to the oviduct of a ewe. Control embryos were flushed from the uterus of mares 6-9 days after ovulation. Embryo morphology was evaluated with light microscopy, and multiphoton scanning confocal microscopy was used to examine the distribution of microfilaments (AlexaFluor-Phalloidin stained) and the rate of apoptosis (cells with fragmented or terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive nuclei). To examine the influence of culture on capsule formation, conceptuses were stained with a monoclonal antibody specific for capsular glycoproteins (OC-1). The blastocyst rate was higher for zygotes transferred to a sheep's oviduct (16%) than for those cultured in SOF (6.3%). Day 7 IVP embryos were small and compact with relatively few cells, little or no blastocoele, and an indistinct inner cell mass. IVP embryos had high percentages of apoptotic cells (10% versus 0.3% for in vivo embryos) and irregularly distributed microfilaments. Although they secreted capsular glycoproteins, the latter did not form a normal capsule but instead permeated into the zona pellucida or remained in patches on the trophectodermal surface. These results demonstrate that the initial layer of capsule is composed of OC-1-reactive glycoproteins and that embryo development ex vivo is retarded and aberrant, with capsule formation failing as a result of failed glycoprotein aggregation.[58]

This study was conducted to evaluate in vivo and in vitro development of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. Oocytes were collected from slaughterhouse-derived ovaries, matured in vitro, and injected with frozen-thawed stallion sperm. In vivo development was assessed after transfer of injected oocytes to the oviducts of recipient mares. Mares were killed 7.5-8.5 days after transfer and the uterus and oviducts flushed for embryo recovery. Of 132 injected oocytes transferred, 69 (52%) were recovered; of these, 25 (36%) were blastocysts with a blastocoele and capsule. In vitro development was assessed in three culture systems. Culture of zygotes in modified Chatot, Ziomek, Bavister medium with BSA containing either 5.5 mM glucose for 7.5 days or 0.55 mM glucose for 3 days, followed by 3 mM glucose for 2 days, then 4.3 mM glucose for 2.5 days, did not result in blastocyst formation. Culture of zygotes in Dulbecco modified Eagle medium (DMEM)/F-12 with 10% fetal bovine serum with and without coculture with equine oviductal epithelial explants yielded 16% and 15% blastocyst development, respectively. Development to blastocyst was significantly lower in G1.3/2.3/BSA than in DMEM/F-12/BSA or in either medium with 10% added serum (2% vs. 18%, 18% or 20%; $P < 0.05$), suggesting that requirements for equine embryo development differ from those for other species. These results indicate that in vitro-matured equine oocytes are sufficiently competent to form 36% blastocysts in an optimal environment (in vivo). While we identified an in vitro culture system that provided repeatable blastocyst development without coculture, this yielded only half the rate of development achieved in vivo.[59]

Follicle atresia and granulosa cell apoptosis may be related to oocyte meiotic and developmental competence. We analyzed the relationships among granulosa cell apoptosis, initial cumulus morphology, oocyte nuclear maturation in vitro, and pronucleus formation after intracytoplasmic sperm injection (ICSI) in the horse. For each follicle, the size was measured and granulosa cells were used for DNA laddering analysis. Oocytes were evaluated for cumulus morphology, cultured for in vitro maturation, and submitted to ICSI. Apoptosis was categorized as absent, intermediate, or advanced according to the relative concentrations of two DNA fragments at 900 and 360 base pairs (bp). In 98 oocyte-follicle pairs, 52 oocytes were classified as expanded (Exp), 39 as compact (Cp), and 7 as having a partial (P) cumulus. Advanced apoptosis was detected in 55% (54/98) of follicles; 37% (36/98) of follicles showed an intermediate level of apoptosis; and 8 follicles (8%) were nonapoptotic. Follicle size was not significantly correlated with granulosa cell apoptosis ($P > 0.05$). Significantly more Exp than Cp oocytes originated from follicles with advanced apoptosis ($P < 0.001$). The proportion of oocytes maturing in vitro was significantly higher in oocytes issuing from apoptotic follicles than in oocytes issuing from healthy follicles ($P < 0.05$). The proportion of normally (two pronuclei) or abnormally fertilized oocytes (one or greater than two pronuclei, or partially decondensed sperm) did not differ in relation to granulosa cell apoptosis. We conclude that, in the mare, granulosa cell apoptosis is related to cumulus expansion and an increase in oocyte meiotic competence but has no effect on the proportion of meiotically competent oocytes that activate after ICSI. These results provide selection criteria for horse oocytes used in assisted reproductive techniques so that embryo production may be maximized.[60]

This study was conducted to evaluate the effect of initial cumulus morphology (expanded or compact) and duration of in vitro maturation (24, 30 or 42 h) on the developmental competence of equine oocytes after intracytoplasmic sperm injection (ICSI). The effect of manipulation temperature (room temperature vs 37 degrees C) at the time of ICSI and concentration of glucose (0.55 vs 5.5 mM) during embryo culture was also investigated. The nuclear maturation rates of expanded (Ex) oocytes were significantly ($P < 0.001$) higher than those of compact (Cp) oocytes at all maturation times (61-72 vs 23-25% respectively). Forty-eight hours after ICSI of mature Ex oocytes, the rate of cleavage with normal nuclei was significantly ($P < 0.05$) higher for oocytes matured for 24 h than for those matured for 30 or 42 h (73 vs 57-59% respectively). For Cp oocytes, the morphologic cleavage rates for oocytes matured for 30 h were significantly higher ($P < 0.05$) than for those matured for 24 or 42 h (86 vs 55-61% respectively). The overall proportion of embryos having more than four normal nuclei at 48 h culture was significantly higher ($P < 0.05$) for Cp than for Ex oocytes. Manipulation temperature did not affect development of embryos from Ex or Cp oocytes at 96 h after ICSI. Culture in high-glucose medium significantly increased morphologic cleavage of Cp, but not Ex, oocytes ($P < 0.05$). Embryos from Cp oocytes had a significantly higher average nucleus number after 96-h culture than did embryos from Ex oocytes. These data indicate that developmental competence differs between Ex and Cp equine oocytes, and is differentially affected by the duration of maturation and by composition of embryo culture media.[61]

The foregoing studies emphasize the importance of media capable of optimal culture conditions for both nuclear and cytoplasmic maturation of any oocytes to be used in ART. To date in vivo matured and ICSI injected equine oocytes have been better at providing normal development (per embryo) and live foal rate (per embryo transferred). Techniques to culture in vitro–matured and ICSI-fertilized oocytes to the blastocyst stage and resultant pregnancy, however, have provided much encouragement for continued development of co-culture with oviductal epithelial cells.

The future of equine ICSI from a commercial standpoint will revolve around the ability to consistently

provide embryos that can develop to an age that they can be transferred nonsurgically into the uterus—from morula to expanding blastocyst.

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