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Integration of future biotechnologies into the equine industry☆

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Abstract

There has and will continue to be reproductive techniques available that have a positive impact upon the equine breeding industry. This review focuses on semen technologies that have been developed or are in the process of being developed. The use of fluorescent dyes and flow cytometry has provided the researcher and clinician with powerful tools to evaluate several sperm attributes. These procedures have been utilized to evaluate sperm viability, acrosome status, mitochondrial status, DNA integrity and stages of capacitation. Flow cytometry allows several sperm attributes to be evaluated on thousands of spermatozoa in a matter of seconds. Development of procedures for insemination of mares with relatively small numbers of spermatozoa has the potential to change how stallions and their semen are managed. This review discusses the use of insemination of fresh, frozen and sex-sorted spermatozoa in relatively small numbers compared with conventional insemination technologies. The recent acceptance of frozen-thawed semen by many of the major breed registries has stimulated an increase in research on frozen semen. Many of the studies have focused on identifying damage during the freezing and thawing process. Numerous studies also have been conducted to modify freezing extenders so that the sperm are protected during the freezing and thawing process. The production of in vitro-produced embryos is extremely limited in the horse due to the failure of in vitro fertilization. However, intracytoplasmic sperm injection (ICSI) has been used for the production of foals from stallions that have less than typical sperm numbers or from stallions that have died and a limited quantity of frozen semen is available. This technique has been used by several laboratories to produce embryos in vitro.

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The breeder and veterinarian now have access to techniques that allow assessment of semen quality, improvement of procedures for freezing and thawing and insemination of mares with fewer numbers of spermatozoa. It is likely that the next decade will also produce tremendous advances in semen technologies that can be utilized in the horse industry.

Keywords: Reproductive techniques; Sperm; Equine

1. Introduction

During the past decade, we have witnessed an explosion in new reproductive technologies. Some of these technologies already have been incorporated into the breeding industry, while others will be incorporated more slowly and some may never be incorporated. How quickly technologies are accepted and utilized in the equine breeding industry depends upon the success of the technology, the attitude of the breeders and veterinarians, and the cost compared with the benefit of the technology. This review will focus only on semen technologies, even though there are numerous other assisted reproductive techniques that have been developed for mares. The author is unable to provide an in-depth discussion on each technology considering the scope of this review, but hopes to provide the reader with an overview and stimulate the reader to focus on their area of interest. Some of the specific technologies are the subject of other manuscripts within these proceedings.

2. Flow-cytometric evaluation of equine sperm

Ideally, one would like to have laboratory tests that could evaluate several attributes of the sperm simultaneously. The use of fluorescent dyes and flow cytometry has provided the researcher and clinician with a powerful tool to evaluate several sperm attributes. With this technology, a large number of sperm can be evaluated in a relatively short period of time. However, the limitations of flow cytometry include expense of the equipment, as well as technical training necessary to properly operate the equipment. This technique is extremely useful for evaluating the effects of various cooling and freezing treatments on sperm damage. Based on evaluation of various sperm attributes, it may be possible to use flow cytometry techniques to identify stallions that have sperm that cools and freezes poorly and eliminate them from consideration for commercial breeding programs using these technologies, or perhaps be used to predict the fertility of a sperm sample.

Numerous tests have been developed to evaluate sperm viability, acrosomal status, mitochondrial status, DNA integrity and stages of capacitation. Christensen et al. (2004, 2005) reported on the incorporation of flow cytometry in commercial boar and bull sperm production. These authors used a simple and reliable flow-cytometric method for simultaneous determination of sperm concentration and viability. SYBR-14, in combination with propidium iodide (PI), allows for estimation of the proportion of live sperm and an internal standard of fluorescent microspheres makes it possible to determine the sperm concentration

during the same analysis. They demonstrated that sperm viability, based on SYBR-14 and PI was more closely related to litter size of sows than traditionally used motility variables. They also demonstrated that variation in numbers of motile sperm per insemination dose could be reduced significantly if flow cytometry was used instead of spectrophotometers for determination of sperm concentration.

These authors conducted a similar experiment in four Danish bull studs (Christensen et al., 2005). Each of these bull studs had purchased a desktop flow cytometer and hired technicians. The aim was to assess if flow-cytometric determination of sperm concentration could result in a more uniform production of semen doses. Both sperm concentration and sperm viability were determined with high precision. In one experiment, frozen semen was exchanged between participating bull studs and was analyzed by flow cytometry, as well as traditional microscopic assessment by eight technicians. The average correlations between technicians were 0.38 for motility assessments, while correlations between technicians for flow-cytometric agreement were greater (0.86 for sperm viability and 0.92 for sperm concentration). They concluded that flow-cytometric determination of sperm concentration and viability can be used to improve semen assessments by bull and boar AI studs and result in more precise quality control. As these flow cytometers become less expensive, equine freezing stations may utilize this technology as well.

Traditionally, quality of equine sperm has been determined by estimation of total and progressive motility. This can be done either visually or with computer-assisted analysis (CASA). Most research on evaluation of sperm quality has included CASA analysis, as well as other attributes of the sperm function. Unfortunately, motility of the spermatozoa is poorly correlated with fertility. This seems reasonable because motility is only one attribute of the sperm. Thus, attempts have been made to evaluate several sperm attributes as a means of determining semen quality. Love et al. (2003) evaluated the relationship between sperm motility and sperm viability using the fluorochromes, SYBR-14/PI or SYBR-14/PI and the mitochondrial membrane probe, JC-1. They evaluated samples immediately after collection or after 24 h storage at 5 °C. There was a high correlation ($r = 0.98$) between membrane integrity and total sperm motility. This raises the question whether assessment of viability adds enough information to justify the cost.

The sperm chromatin structure assay (SCSA), an assay that measures the stability of DNA within the sperm nucleus, was used to evaluate the cause of subfertility in stallions, as well as to assess the damage that occurs during cooling and/or freezing and thawing. Love and Kenney (1994) reported on the relationship between sperm chromatin and fertility in the stallion. They recorded the mean and standard deviation of COMP αT, which reflects the degree of chromatin denaturization, and the number of cells outside the main population. They reported that COMP αT was most negatively correlated with pregnancy rate per cycle. The sperm chromatin assay has been used numerous times to evaluate the effects of cooling and freezing, as well as other seminal treatments. Love et al. (2005) evaluated the relationship between seminal plasma and extender type on maintenance of DNA integrity and sperm motility in cooled-stored equine semen. Three seminal plasma concentrations (0, 10 and 20%) and two extender types were evaluated (Kenney type or Kenney-modified Tyrode's [KMT]). Sperm motion characteristics were determined by CASA and DNA integrity (% COMP αT) evaluated by the sperm chromatin structure assay prior to cooling and after 24 and 48 h cooled storage at 5 °C. After 48 h of storage at 5 °C, extenders with 0% seminal

plasma maintained the integrity of the DNA. Progressive sperm motility was greatest after 48 h of storage for samples with 10% seminal plasma in Kenney extender, despite a reduction in DNA integrity. Regardless of extender type, addition of 20% seminal plasma after centrifugation resulted in a nearly two-fold increase in % COMP αT. They suggested that the sperm chromatin structure assay may be extremely useful, because motility and DNA integrity may respond independently to environmental conditions.

Another technique for evaluating sperm chromatin structure was recently published by Madrid-Bury et al. (2005). They evaluated chromatin structural stability by treating sperm with chelating agents, such as EDTA. The aim was to evaluate frozen bull sperm chromatin condensation and stability by perturbating the chromatin with EDTA and SDS and determining its relationship to non-return rates of cows. Chromatin stability was greater in those bulls that had greater fertility. They concluded that sperm chromatin stability assay may be a valuable tool to complement the routine microscopic evaluation of cryopreserved bull sperm.

Investigators in The Netherlands have used various fluorochromes to evaluate membrane damage during the freeze-thaw process for equine sperm (Neild et al., 2003). The objective of this study was to determine at what stage of the freeze-thaw process membrane changes occurred and whether evaluation by chlorotetracycline (CTC) stain could predict the freezability of sperm from a given stallion. Sperm viability and capacitation state were simultaneously evaluated using CTC and the dye, Hoechst 333258. Membrane function was also evaluated using the hypoosmotic swelling test. Sperm were evaluated immediately after collection, after dilution and centrifugation, after re-dilution and equilibration at room temperature, after cooling to 5 °C, after super-cooling to −15 °C and after thawing. The most pronounced damage to sperm occurred after thawing. A unique aspect of this study was the ability to evaluate capacitation and acrosomal integrity in conjunction with viability. This allowed separation of acrosomal changes in dead sperm from those of viable sperm that were undergoing capacitation and the acrosome reaction. These tests could be helpful in determining the beneficial effect of various freezing and thawing procedures.

With the recent acceptance of frozen semen by major breed association in the United States, there is an increased interest and demand for frozen equine semen. Several studies have attempted to use laboratory tests to predict the fertility of cryopreserved stallion sperm. Wilhelm et al. (1996) conducted a study to evaluate the fertility of stallion spermatozoa frozen in several extenders and to determine if results from laboratory analyses of sperm samples were correlated with fertility. In this study, semen from five stallions was frozen in three different extenders or cooled for 24 h in skimmilk–egg yolk extender. Fertility was based on the percentage of embryos recovered 7 days post-ovulation. The percentage of motile spermatozoa immediately after thawing was not different due to extender or stallion. However, when spermatozoa were treated with PC-12 to induce the acrosome reaction, values differed among stallion (17–42%). The percentage of viable sperm and viable acrosome-intact sperm ranged from 30 to 57% and 27 to 48%, respectively, across stallions. The percentage of penetrated hamster oocytes ranged from 19 to 55%. Analysis of single-sperm parameters was not highly correlated with stallion fertility. However, with a statistical model that included data on percentage of viable sperm, percentage of motile spermatozoa and percentage of hamster oocytes penetrated, these tests were highly correlated with stallion fertility ($r = 0.85$).

In a subsequent study, Kirk et al. (in press) developed and validated flow-cytometric assays for viability, acrosome status and mitochondrial membrane potential of frozen-thawed sperm. They compared these analyses with visual and computer-assisted motility analysis and the results of these assays were correlated with stallion sperm fertility. Viability was assessed using propidium iodide and SYBR-14. Acrosomal integrity was assessed using FITC-PNA and a mitochondrial probe, JC-1, was used to measure mitochondrial membrane potential. The ultimate goal of multi-parametric sperm analysis was to be able to distinguish sperm samples that have potentially good fertilizing potential from those likely to have poor fertility. As a second part of this study, frozen semen was provided from stallions that had first-estrous cycle fertility rates of frozen-thawed sperm ranging from 0 to 69%. Progressive motility ranged from 22 to 83% and the percentage of live cells ranged from 41 to 84%. Integration models were developed utilizing all four-variables. The model which included motility at 90 min, straightness measured by CASA at 90 min, percentage of live sperm cells evaluated by PI and mitochondrial membrane potential accounted for the majority of variation in first-cycle fertility ($r^2 = 0.93$). Regardless of the model chosen, most three- and four-variable models included a measure of sperm motility at 90 min, a sperm velocity variable at 90 min and an organelle attribute measured by flow cytometry. The most practical model included visual motility at 0 and 90 min, perecentage live sperm and percentage live with the acrosome-intact. The four-variable model explained 79% of the difference in first-cycle fertility between stallions. Evaluation of only a single variable, did not adequately explain differences among stallions' fertility. However, combining results of assays that measured multiple sperm attributes improved the ability to evaluate fertilizing potential of frozen-thawed spermatozoa.

Although it is unlikely that most stallion semen collection processing and cryopreservation facilities would possess a flow cytometer to conduct the sperm analyses described, frozen-thawed sperm could be shipped to a central facility equipped with a flow cytometer for these analyses to be conducted. Colenbrander et al. (2003) emphasized the need for a multiple sperm functions test to predict a stallion's fertility. Although predicting stallion fertility using multiple laboratory tests may be somewhat problematic because of the many other factors that go into obtaining a pregnancy in a mare, these tests may prove useful in determining the cause of subfertility or infertility in a stallion, as well as identifying stallions that have sperm that is easily damaged during cooling and freezing and thawing.

3. Low-dose insemination—fresh, frozen, sex-sorted sperm

Numbers of spermatozoa per inseminate for fresh, cooled and frozen-thawed spermatozoa have generally been established. Mares are typically inseminated with 500 million progressively motile spermatozoa (PMS) immediately after collection or one billion PMS that have been cooled and stored for 24 h at 5 °C. The typical dose for mares inseminated with frozen-thawed spermatozoa varies from 400 to 800 million total spermatozoa. These numbers limit the number of mares that can be inseminated from a given stallion. There are occasions where semen is limited and, thus, insemination of fewer sperm numbers is advantageous. Many times, stallions become more popular with age and, thus, the demand for his semen increases. These older stallions typically have lesser sperm numbers. Other

reasons for low-dose insemination include a limited availability of frozen semen straws from a given stallion and the use of sex-sorted spermatozoa.

Low-dose insemination of equine sperm can be done either with a rectally guided approach or with the use of an endoscope. Rectally guided insemination requires only a long, flexible pipette, whereas a videoendoscope is required for endoscopic insemination. Morris et al. (2000) demonstrated that pregnancies could be achieved by hysteroscopic insemination of a dose with as few as one million PMS placed on the uterotubal papilla. Hysteroscopic insemination with small numbers of fresh or frozen-thawed spermatozoa is now being used clinically as a technique to obtain pregnancies of stallions whose semen is in short supply. Recently, Weems and Byers (2004) reported on the results of two breeding seasons in which mares were hysteroscopically inseminated with small numbers of sperm. The stallion was a 24-year-old Quarter Horse. In 2002, 136 mares were bred by standard AI techniques and a 42% pregnancy rate per estrous cycle was achieved. In 2003, 136 mares were bred by standard AI or low-dose hysteroscopic insemination and an overall 40.8% pregnancy rate per estrous cycle was achieved. Of the 158 estrous cycles of breeding by standard AI (500 million PMS), a 33% pregnancy rate per estrous cycle was obtained. Of mares bred at least once during an estrous cycle by low-dose, hysteroscopic AI, a 44.0% pregnancy rate/estrous cycle was achieved. These authors concluded that low-dose, hysteroscopic insemination allowed them to "meet the challenge of inseminating all mares each breeding day without decreasing the number of mares in the stallion's book".

There are occasions where the fertility of the stallion is excellent and insemination with small numbers of spermatozoa using a long, flexible catheter may result in excellent pregnancy rates. Brinsko et al. (2003) reported similar pregnancy rates for mares inseminated hysteroscopically with 5 million PMS compared to those inseminated with a rectally guided pipette. Petersen et al. (2002) compared embryo recovery rates in mares after daily insemination with either 50 million frozen-thawed PMS delivered by rectally guided, deep-horn AI or insemination of 500 million fresh or cooled PMS into the uterine body. Embryo recovery rates were not different for mares inseminated with fewer numbers of frozen-thawed sperm (7 of 11, 64%) and those inseminated with 500 million fresh and cooled PMS (4 or 11, 37%).

Practitioners are often faced with the decision whether to use hysteroscopic insemination or deep-horn insemination with a flexible catheter. Sieme et al. (2004) investigated the effects of different artificial insemination techniques and sperm dose on fertility of normal mares and mares with abnormal reproductive histories. They compared pregnancy rates of mares inseminated into the uterine body, deep into the uterine horn with a rectally guided pipette or hysteroscopic insemination onto the uterotubal junction (UTJ) ipsilateral to the preovulatory follicle. Mares were inseminated with either fresh or frozen-thawed sperm. They reported differences in pregnancy rates between normal and problem mares using different insemination techniques. With fresh semen, problem mares showed a significant decrease in pregnancy rates when inseminated hysteroscopically as compared with routine insemination into the uterine body. In normal mares, pregnancy rates were greater for hysteroscopic insemination.

Morris et al. (2000) inseminated mares with 25 million frozen-thawed, progressively motile spermatozoa using a hysteroscope and reported no advantage over uterine body insemination. However, when these authors used the same technique and only 5 million

frozen-thawed, progressively motile spermatozoa, insemination onto the UTJ resulted in greater pregnancy rates than insemination into the uterine body. Thus, it appears that the advantage of hysteroscopic insemination is clearly seen when fewer spermatozoa (5–20 million PMS) are used for insemination.

Rectally guided, deep-horn insemination may be the method of choice for stallion's with excellent fertility and sperm numbers greater than 25–50 million are used. If lesser numbers of sperm are used, and particularly if the sperm have been damaged by freeze/thaw or sex-sorting, then perhaps videoendoscopic insemination is justified.

Most of the early studies published on low-dose insemination, focused on development of techniques for insemination of sex-sorted spermatozoa. Buchanan et al. (2000) demonstrated that pregnancies could be obtained from insemination of sperm that had been separated into X- and Y-chromosome-bearing populations using a flow cytometer. However, to obtain a full insemination dose (500 million PMS), several days of sorting would be required. The current procedure is to sort sperm on the flow cytometer for approximately 2 h and obtain a dose of 5–20 million PMS. Insemination of 25 million sexed PMS with deep-horn insemination resulted in 8 of 20 mares becoming pregnant (40%). In a subsequent study (Lindsey et al., 2002a), a greater pregnancy rate was reported for mares inseminated with 5 million sex-sorted PMS using hysteroscopic techniques (5 of 10, 50%) than ultrasound-guided, deep-horn insemination (0 of 10, 0%). This same group (Lindsey et al., 2002b) later compared hysteroscopic and rectally guided, deep-horn insemination of spermatozoa stored 18 h at 5 or 15 °C prior to flow-cytometric sorting. Pregnancy rates were greater for mares hysteroscopically inseminated with 20 million sex-sorted PMS (18 of 25, 72%) compared with those inseminated with 20 million sex-sorted PMS by a rectally guided technique (9 of 24, 38%). This study demonstrated that relatively high pregnancy rates can be obtained with insemination of sex-sorted sperm using a hysteroscopic technique. This study also demonstrated that semen can be collected and stored for 18 h prior to flow-cytometric sorting and still result in reasonable pregnancy rates.

Although the technique of flow cytometry has been proven to be an accurate method for separating X- and Y-chromosome-bearing spermatozoa, its use in the horse industry has been minimal. Factors limiting the use of sexed semen in the horse industry include the greater cost of the equipment, as well as the license to use the equipment. Furthermore, the fertility of sex-sorted spermatozoa is highly stallion dependent and the logistics of having the mare, stallion and equipment in the same region is difficult.

4. Advances in freezing semen

The number of mares inseminated with frozen-thawed semen appears to be on the increase, particularly in the United States. This has stimulated an increase in research on frozen semen. Several studies have focused on identifying damage during freezing and thawing. There appear to be two major classes of sperm damage: oxidative and osmotic. Thus, most of the progress in improving survival of frozen-thawed spermatozoa centers on minimizing oxidative damage and decreasing osmotic stress.

Equine sperm are particularly susceptible to oxidative stress, relative to other species, since they contain high concentrations of unsaturated fatty acids. In addition to membrane

effects, lipid peroxidation can also damage DNA. Baumber et al. (2003) examined the effect of reactive oxygen species and cryopreservation on DNA of equine sperm. They demonstrated that reactive oxygen species and cryopreservation promotes DNA fragmentation in equine sperm. The addition of antioxidants to extenders has been used as a method to decrease lipid peroxidation. The effects of various antioxidants added to extenders used for cooling equine spermatozoa have been equivocal. Response is more than likely dependent upon the type of antioxidant and its concentration. Addition of the water-soluble vitamin E analog, Trolox (Peña et al., 2003), increased motility and mitochondrial activity in frozen-thawed boar spermatozoa. Butylated hydroxytoluene (BHT), a lipid-soluble antioxidant, also was shown to increase post-thaw sperm survival of boar sperm when added to the freezing extender. Furthermore, BHT improved the development of rate to blastocyst of embryos derived from sperm frozen in extender supplemented with BHT (Roca et al., 2004). Almeida and Ball (2005) compared the ability of α-tocopherol and a vitamin E ester, α-tocopherol succinate, to prevent lipid peroxidation and maintain motility of equine sperm during storage at 5 °C for 48 h. During short-term incubation in TALP-PVA, α-tocopherol succinate reduced membrane lipid peroxidation as determined by using the fluorescent probe C11 bodipy. In a prior study in this same laboratory, the addition of 1–4 mM of α-tocopherol did not improve maintenance of motility during liquid semen storage (Ball et al., 2001). The addition of 200 μg/ml of α-tocopherol in semen extenders protected boar spermatozoa against the oxidative stress associated with cryopreservation (Peña et al., 2003). It is likely that antioxidants at the appropriate concentrations will be identified that can be added to cooled and frozen semen extenders that will be useful in limiting the amount of oxidative stress that sperm undergo during the cooling and freezing process.

Attempts to minimize osmotic stress during cryopreservation have included step-wise dilution of cryoprotectants (Wessel and Ball, 2004), evaluation of alternative cryoprotectants to glycerol and increasing membrane stability by incorporating cholesterol-loaded cyclodextrins (CLC) in freezing diluents. Sperm membrane destabilization occurs when the membrane undergoes a phase transition from the fluid phase to the gel phase as temperatures decrease. Purdy and Graham (2004) studied the addition of cholesterol-loaded cyclodextrins to egg yolk diluents. After thawing, higher percentages of motile and viable sperm were obtained when 1.4 mg/ml of CLC was added to bull sperm prior to freezing. Similar studies were done with stallion sperm. Moore et al. (2005) reported a beneficial effect of CLC at a level of 1.5 mg/ml when added to stallion sperm prior to freezing.

An alternative to adding CLC to extenders would be to provide greater amounts of polyunsaturated fatty acids in the feed as a means of altering the sperm–lipid membrane profile. Studies in boars have shown an enhanced fertility in boars fed a nutriceutical containing greater amounts of polyunsaturated fatty acids. Brinsko et al. (2005) fed eight stallions a nutriceutical rich in docosahexaenoic acid (DHA). After 14 weeks of treatment, the nutriceutical resulted in a three-fold increase in semen DHA. After 48 h of cooled storage, an increase in total and progressive sperm motility was observed when stallions were fed the nutriceutical. In another study, supplementation with a nutriceutical containing greater amounts of DHA resulted in increased sperm output (Harris et al., 2005). This compound was particularly useful in those stallions having semen that cooled or froze poorly. Further studies are needed on large numbers of stallions to determine how consistently feeding products rich in DHA will improve variables related to semen quality of stallions.

Another area of extensive investigation has been evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Four amides have been evaluated as an alternative to glycerol as a cryoprotectant. These compounds are of lower molecular weight than glycerol and may penetrate the sperm plasma membrane more readily. Squires et al. (2004) demonstrated that methyl formamide (MF), dimethyl formamide (DMF) or ethylene glycol (EG) at 0.9 M protected stallion spermatozoa equally as well as glycerol. Vidament et al. (2002) reported that the combination of DMF and glycerol was more effective than either DMF or glycerol alone. However, pregnancy rates were similar for mares inseminated with sperm frozen in extenders containing 2% glycerol (46%), 3% glycerol (58%) and 2% DMF (50%). In another study, dimethyl acetamide (3%) and MF (5%) provided greater post-thaw motility than 5% glycerol (Medeiros et al., 2002). It would appear that MF, DMF or EG may be alternative cryoprotectants for individual stallions whose sperm has lesser post-thaw motility when frozen in glycerol.

Recently, cryoprotective effect of the amino acid L-glutamine on preserving motility of stallion spermatozoa during the freezing and thawing process have been evaluated (Khlifaoui et al., 2005). The addition of 50 mM glutamine plus 2.5% glycerol significantly improved sperm motility compared with freezing extenders containing glycerol only.

It is unlikely that any single ingredient added to a freezing extender will dramatically improve post-thaw motility and fertility. However, it seems reasonable that addition of antioxidants, amino acids and, perhaps, use of alternative cryoprotectants may improve the freezability of semen of some stallions.

The majority of equine semen is frozen in 0.5-ml straws at a concentration of 200–400 million sperm per ml. Cooling rates in the range of 10–50 °C/min are typically used, with relatively low concentrations of cryoprotectants. Limited studies are available on the effect of cooling rate on survival of stallion spermatozoa. Recently in our laboratory, Moore et al. (unpublished data) evaluated post-thaw motilities and viability of equine sperm cooled at several rates from −10 to −50 °C in two different freezing extenders. They found no difference in post-thaw motility or viability for sperm cooled at any of these cooling rates.

Other freezing procedures have been reported in the last several years. One method, termed multi-thermal gradient (MTG), has been reported (Arav et al., 2002). This freezing technology is based on directional freezing. The biological material is moved through a linear temperature gradient so that, theoretically, the cooling rate and ice front propagation are precisely controlled. Limited data are available on fertility of stallion spermatozoa frozen under these conditions. Another freezing method, unique freezing technology (UFT), was recently reported (Goolsby et al., 2004). This technology initially was used in freezing of foodstuffs. The UFT involves placing extended samples in a bath that "contains an organic fluid with a heat capacity similar to water with a freezing rate of −6.1 °C/min." In one experiment, the post-thaw motilities of four UFT treatments were similar to those for sperm frozen with traditional liquid nitrogen procedures. These authors did not provide any fertility data using this unique freezing technology. Further studies with larger numbers are needed to determine the usefulness of this technique for freezing equine sperm.

Álamo et al. (2005) reported on the use of ultra-freezers of −152 °C as a viable alternative to liquid nitrogen for freezing dog spermatozoa. Sperm motility recorded with the use of ultra-low temperature freezers at −152 °C was comparable to that obtained with liquid nitrogen. It is interesting that straws moved directly from 4 °C to the ultra-freezer at −152 °C

did not result in any greater loss of sperm motility to that observed with liquid nitrogen freezing techniques.

5. Intracytoplasmic sperm injection (ICSI)

The first foal born from intracytoplasmic sperm injection (ICSI) was in Colorado in 1996 (Squires et al., 1996). Since that time, several other researchers have successfully produced foals from ICSI. In fact, this technique is now being offered commercially in several laboratories around the world. The clinical use of ICSI is for production of foals from stallions that have very few sperm or perhaps stallions that have died and a limited quantity of frozen semen is available. Our laboratory (Carnevale, unpublished) has routinely produced foals from frozen-thawed semen of sub-fertile stallions using ICSI. One reason for the increased use of ICSI is the inability to produce embryos in vitro by standard IVF technology. Sperm injection circumvents the problem of having sperm bind to the oocyte, penetrate the oocyte and initiate fertilization. With ICSI, spermatozoa are injected directly into the oocyte, initiating fertilization.

Choi et al. (2002) used ICSI to evaluate the development of oocytes in vitro and in vivo after sperm injection with either fresh or frozen sperm. Research in Italy (Lazzari et al., 2002) evaluated the embryo development rate from oocytes injected with sperm from stallions of varying fertility. There was a 47% morula-blastocyst development rate for three of the four stallions. The fourth stallion had no motile sperm available for injection and no blastocysts were formed after ICSI.

Obviously, ICSI is the ultimate in low-dose insemination, because only a single spermatozoa is injected into the oocyte. Breeders are beginning to request that semen be frozen with few numbers per straw for subsequent sperm injection. Based on preliminary data in our laboratory, freezing of semen in 0.25-ml straws at a total dose of 200,000 to 500,000 sperm is adequate for sperm injection. Other studies are being conducted to determine the effect of thawing, re-dilution and re-freezing of semen on embryo development after ICSI. It seems possible that one straw containing 100 million spermatozoa may be thawed and re-frozen to provide nearly a thousand additional straws for subsequent sperm injection. McCue (unpublished) froze semen from six stallions in 0.5-ml straws at a concentration of 400 million per ml. Semen was thawed and either re-frozen at the same concentration, or diluted to 40 million, 4 million, 400,000 or 40,000 per ml with additional extender. Re-frozen semen was evaluated for motility visually and by CASA and stained with PI for determination of sperm viability. Total motility was 92% pre-freeze, 64% after first freeze, and 46% after second freeze. Dilution prior to re-freezing resulted in similar motility to those samples re-frozen without further dilution. Other possibilities include cutting a piece of the straw while under liquid nitrogen, thawing the semen, then refreezing the extra sperm that are not needed for the ICSI procedure. This technique would allow one to conserve genetic material for a long time period and extend the use of valuable semen several orders of magnitude compared with its use in conventional breeding methods.

Sperm injection is a powerful tool that can be used not only clinically to produce foals from subfertile stallions. It also is being used to evaluate in vitro maturation systems for oocytes, study fertilization and provide in vitro-produced embryos for subsequent studies.

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