New Reproductive Technologies in the Stallion

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Introduction

In the past decade, we have seen tremendous changes in how we handle semen and breed mares. These have included development of techniques for cooling and transporting semen, freezing semen, the use of sexed semen and low-dose insemination using videoendoscopic insemination or deep uterine horn insemination. Although frozen semen has been used for years in the equine industry, with the first foal from frozen semen being born in 1957, its application has been limited because of breed restrictions. Within the last several years, major breed associations within the United States, including the American Paint Horse Association and the American Quarter Horse Association, have accepted the use of frozen semen. Last year, the American Quarter Horse Association accepted the use of frozen semen even after the stallion has died. These changes have stimulated interest in breeding mares with frozen semen and have resulted in numerous studies being conducted to improve the fertility of frozen-thawed equine spermatozoa. This manuscript will review some of the recent changes in semen technology.

Sexed Semen

A safe and reliable method of pre-conceptual sex selection of offspring has been sought for decades in man, livestock and companion animals. Johnson et al. (1989) were the first to report a reliable method to predetermine sex by using DNA as a quantitative marker for X- and Y-chromosome-bearing spermatozoa and sorting spermatozoa via flow cytometry. Buchanan et al. (2000) and Schmid et al. (2000) were the first to report foals produced from mares inseminated with sex-sorted spermatozoa. The basis of sorting spermatozoa into X- and Y-chromosome-bearing cells is that the X chromosome contains 3.7% more DNA than the Y chromosome. Sperm are stained with a Hoechst dye, passed through the flow cytometer and excited with a laser. A computer then determines the amount of fluorescence so that the spermatozoa can be sorted into X- and Y-chromosome-bearing fractions. Accuracy of sorting spermatozoa can be extremely high (i.e. >95%). However, sorting speeds are relatively slow, such that obtaining 500 x 10^6 sex-sorted spermatozoa would take several days and the spermatozoa would be non-viable at the end of sorting. Thus, one is forced to inseminate mares with a minimal number of sex-sorted spermatozoa (i.e. 5 to 25 x 10^6 spermatozoa).

Sex-sorted spermatozoa (sorted sperm) have been inseminated either by deep uterine insemination or videoendoscopic insemination. Buchanan et al. (2000) inseminated 25 x 10^6 progressively motile, sorted spermatozoa by use of an ultrasound-guided method to direct a flexible insemination pipette into the tip of the uterine horn. A 40% pregnancy rate was obtained.

The ability to cryopreserve sorted spermatozoa would greatly increase the practicality of using flow-sorted spermatozoa in the horse industry. Stallion sperm do not survive for long periods after the sorting process. Thus, mares must be inseminated immediately after sperm sorting. However, if the sperm could be frozen following sorting, they could be used at a future time or location.

Lindsey et al. (2002a) determined the effects of flow-cytometric sorting and freezing on stallion sperm fertility. Mares were assigned to a 2 x 2 factorial design, the factors being fresh vs frozen and sorted vs non-sorted sperm. Mares were inseminated hysteroscopically with 5 x 10^6 motile spermatozoa in a volume of 230 µl. Pregnancy was determined ultrasonographically 16 days post-ovulation. No difference was found in the pregnancy rate for mares inseminated with fresh, non-sorted (4 of 10, 40%); fresh, flow-sorted (6 of 16, 37.5%); frozen-thawed, non-sorted (6 of 16, 37.5%); and frozen-thawed, flow-sorted spermatozoa (2 of 15, 13.3%). Pregnancy rates tended to be lower following insemination of frozen-thawed flow-sorted spermatozoa. Further studies are needed with a larger number of mares to determine if fertility of flow-sorted, frozen-thawed spermatozoa can be improved.

Although videoendoscopic insemination provides great visibility during the insemination process, it is time-consuming and requires expensive equipment. Thus, a subsequent study (Lindsey et al., 2002b) was conducted to compare pregnancy rates after insemination of 5 x 10^6 spermatozoa deep in the uterine horn with the aid of ultrasonography with rates obtained after deposition of the spermatozoa onto the papilla of the uterotubal junction

using hysteroscopy. Also compared were pregnancy rates of mares inseminated hysteroscopically with either 5 x 10^6 non-sorted or 5 x 10^6 flow-sorted spermatozoa. Mares were inseminated one time after administration of hCG. Pregnancy was determined ultrasonographically at 16 days post-ovulation. Hysteroscopic insemination resulted in more pregnancies (5 of 10, 50%) than did the ultrasound-guided technique (0 of 10, 0%). Pregnancy rates were not significantly lower when hysteroscopic insemination was used for sorted (5 of 20, 25%) and non-sorted (5 of 10, 50%) spermatozoa. These authors concluded that hysteroscopic insemination of low numbers of flow-sorted spermatozoa resulted in a reasonable pregnancy rate. The results of the deep-uterine insemination were somewhat confusing because Buchanan et al. in an earlier study (2000) had obtained a 40% pregnancy rate with insemination of 25 x 10^6 sorted spermatozoa.

Lindsey et al. (2002c) conducted another study to compare hysteroscopic or rectally-guided, deep uterine insemination of mares with sorted spermatozoa. Also in this study, they evaluated the ability to store spermatozoa for 18 hr prior to sorting. For commercialization of flow-sorted spermatozoa in the equine industry, it will be necessary to develop a method for shipment of stallion spermatozoa prior to sorting. Mares in this study were assigned to one of three treatment groups: 1) sperm that had been stored at 15EC then sorted and inseminated using the videoendoscopic technique, 2) sperm stored at 5EC then sorted and inseminated using the videoendoscopic technique, and 3) sperm stored at 5EC then sorted and inseminated using the rectally-guided technique. Mares were inseminated one time 30 hr post-hCG. Pregnancy rates did not differ among mares inseminated hysteroscopically with sperm stored at 15EC (72%) or 5EC (55%). There was a tendency for fewer mares to become pregnant following rectally-guided insemination (38%) compared to hysteroscopic insemination (55%) when stored, sorted sperm were inseminated. However, this study did demonstrate that excellent pregnancy rates could be obtained with hysteroscopic insemination of 18 hr-stored, flow-sorted spermatozoa. Pregnancy rates were higher for all three stallions when sperm were stored at 15 vs 5EC, although these differences were not significant. Further studies are needed to confirm whether storage at 15EC maintains more viable spermatozoa prior to sorting than storage at 5EC.

It is somewhat surprising that pregnancies can be obtained with stored, sorted sperm, since sperm are centrifuged, cooled, stored and sorted, during which time they are stained, passed through the flow cytometer at 60 mph, collected in a diluted state, centrifuged a second time and finally inseminated into the mare. It is likely that further refinements of semen handling prior to and after sorting will be developed which will improve the overall pregnancy rate with sex-sorted spermatozoa.

Low-Dose Insemination

It has been demonstrated numerous times that insemination at the uterotubal junction is a practical technique that can be used to decrease the number of spermatozoa needed for insemination. This technique is extremely useful for stallions with low sperm numbers or when the supply of frozen-thawed spermatozoa is extremely limited. Another potential advantage of low-dose insemination is to minimize post-breeding endometritis which is characteristic of most bred mares and is more severe in older mares. Post-breeding endometritis has been shown to be a result of a reaction to the spermatozoa. Thus, placing a small number of sperm at the uterotubal junction may decrease the incidence of post-breeding endometritis. As mentioned previously, most of our experience with low-dose insemination has resulted from the use of sex-sorted spermatozoa. There are essentially three methods of inseminating with low sperm numbers: 1) oviductal insemination, 2) deep uterine insemination using a rectally-guided or ultrasound-guided technique, or 3) videoendoscopic insemination.

McCue et al. (2000) achieved a 21% pregnancy rate by surgically depositing 50,000 progressively motile spermatozoa into the oviduct of a mare with a preovulatory follicle. Coutinho da Silva et al., in our laboratory (2002a,b), compared pregnancy rates from mares in which oocytes were transferred into the oviducts and insemination was made either into the oviduct with the oocyte (gamete intrafallopian tube transfer — GIFT) or insemination into the uterus (oocyte transfer). He reported similar pregnancy rates for oocyte transfer (65%) vs GIFT transfer (55%) when fresh semen was used. However, when frozen semen was placed into the oviduct, only an 8% pregnancy rate was obtained. Furthermore, when stored, cooled spermatozoa was placed into the oviduct, a reduced pregnancy rate (25%)was also obtained. The reasons for the reduced pregnancy rate with cooled and frozen semen when placed in the oviduct were not determined. It may be that the extenders used for cooling and freezing or the process itself affects the binding of spermatozoa to the oviduct. In any event, acceptable pregnancy rates can be obtained with oviductal insemination of fresh semen, but not cooled or frozen spermatozoa.

The videoendoscope has been shown to be a very accurate method of inseminating low numbers of spermatozoa onto the uterotubal junction (Morris et al., 2000). Insemination of as few as 5 x 10^6 motile spermatozoa onto the papilla of the uterotubal junction has resulted in pregnancy rates of 50 to 75%. This is 1/100th of the dose typically used for conventional uterine insemination. Alvarenga et al. (2002) reported on the use of videoendoscopic insemination with frozen-thawed spermatozoa. They obtained similar pregnancy rates with insemination of 10 x 10^6 motile spermatozoa vs 400 x 10^6 inseminated into the uterine body. It is likely that low-dose insemination of frozen-thawed spermatozoa may become a routine clinical procedure.

It is somewhat controversial whether rectally-guided deep uterine insemination results in similar pregnancy to those obtained with the videoendoscope. In two studies conducted at Colorado State University, deep uterine insemination resulted in lower fertility than that obtained with videoendoscopic insemination. This is in contrast to Rigby et al. (2001) who reported similar pregnancy rates between videoendoscopic and rectally-guided insemination when shipped semen was used. However, their results did show a 12 percentage point advantage for hysteroscopic insemination compared to deep uterine insemination.

In a very recent study in our laboratory (Squires et al., 2002), 200 x 10^6 frozen-thawed spermatozoa were placed either into the uterine body or into the uterine horn by a rectally-guided approach. We obtained a higher pregnancy rate with body insemination than deep uterine horn insemination. It may be that, if spermatozoa are sorted by flow cytometry or frozen/thawed, then videoendoscopic insemination may enhance fertility over deep uterine insemination. If spermatozoa are fresh or cooled, and have been relatively undamaged, then perhaps one would not see a difference in these two insemination techniques.

Frozen Semen Technology

There has been a renewed interest in further studies on frozen semen in the past several years. Some of these studies have centered on attempts to determine what part of the freezing process is causing the most damage. Ball et al. (2001) has reported that spermatozoa that are frozen and thawed may experience both osmotic and oxidative stresses. The osmotic stress is caused by movement of the cryoprotectant and water in and out of the cell. Oxidative stress is related to the production of reactive oxygen species during the time prior to freezing and after thawing. Certainly one area of active research in the future will be determining the degree of oxidative stress during the process of freezing and whether or not antioxidants and other additives can be added to minimize this stress. Although glycerol is the most common cryoprotectant used for freezing equine spermatozoa, other cryoprotectants, such as DMSO, ethylene glycol and several of the amides, have been tested.

Two recent papers have evaluated the use of dimethylformamide or methyl formamide as an alternative cryoprotectant to glycerol. Alvarenga et al. (2002) reported that stallions with a history of being poor “freezers” had higher fertility when their spermatozoa were frozen in extenders containing dimethylformamide. In contrast, Vidament et al. (2002) reported no increase in pregnancy rates for mares inseminated with equine spermatozoa frozen in extenders containing dimethylformamide.

It is known that a considerable amount of damage occurs during the thawing process. This is related to the difference in membrane permeability of glycerol vs water. If spermatozoa are placed into a solution with no glycerol, then water tends to rush into the cells and expands the volume of the spermatozoa. This is typically what would happen if sperm are placed directly into the mare’s uterus. Several studies have looked at multi-step removal of glycerol from the equine spermatozoa after thawing. Unfortunately, none of these studies have demonstrated a clear advantage to serially diluting out the glycerol prior to insemination. This is somewhat surprising since, theoretically, osmotic stress during thawing may be a major cause of damage. Certainly with embryos, it is quite common to do a multi-step removal of the cryoprotectant.

Besides studies that have centered on improving the techniques of freezing and thawing spermatozoa, other areas of research have focused on management of the mare for insemination with frozen-thawed spermatozoa. One area that has received considerable attention is breeding strategies with frozen semen. Barbacini (2000) has demonstrated that fertility of mares bred prior to ovulation vs within 6 hr after ovulation is quite similar. Furthermore, we have demonstrated in a very recent study (Squires et al., 2002) that insemination of the mare 24 and 40 hr after administration of hCG with a half-dose of semen (400 x 10^6 spermatozoa) resulted in similar fertility to that of mares inseminated within 8 hr post-ovulation with 800 x 10^6 spermatozoa. Those bred 24 and 40 hr after hCG administration were only palpated once daily.

Workers in France have also demonstrated excellent fertility with mares inseminated more than once in a cycle. Their approach is to inseminate mares with frozen-thawed spermatozoa much as one would inseminate mares with cooled semen. Palpations are required only once daily and mares are inseminated after the use of either hCG or GnRH. As mentioned previously, one trend in the industry is to inseminate mares with frozen-thawed spermatozoa deep into the uterine horn ipsilateral to the side of ovulation. In one study performed in our laboratory (citation, year), we found a disadvantage to deep uterine insemination vs body insemination. However, additional studies are needed to truly determine whether rectally-guided deep uterine insemination of frozen-thawed semen is a means of improving fertility and minimizing the number of spermatozoa that need to be inseminated.

Methods to Enhance Sperm Quality

Semen used for assisted reproductive techniques (e.g. GIFT, in vitro fertilization, ICSI) must be free of seminal plasma, debris and contaminants. In addition, a population with a high percentage of motile and morphologically normal sperm must be selected from the ejaculate. Methods frequently used to prepare semen for assisted reproductive procedures and to enhance percentage of motile sperm in other species include swim-up (Parrish and Foote, 1987), Percoll gradients (Saeki et al., 1991; Morales et al., 1991), Sephadex gel filtration (Ohashi et al., 1992) and glass-wool filtration (Paulson et al., 1979).

In the horse, several procedures have been used to select equine spermatozoa, including discontinuous albumin gradient (Pruit, 1985), glass-wool and Sephadex filtration (Samper et al., 1991) and discontinuous Percoll gradient (Del Campo et al., 1990; Leao et al., 2001). Percoll gradient is the most commonly used technique. Reasons for the use of Percoll include: 1) ease, 2) recovery of highly motile sperm population with minimum debris, and 3) 5- to 10-fold greater sperm recovery than swim-up (Avery and Greve, 1995). In addition, Percoll was as effective or superior to other techniques in selecting sperm with intact acrosomal membrane. Percoll has also been used to improve motility, longevity and viability of frozen-thawed stallion semen (Leao et al., 2001).

Because the use of Percoll gradient provides a sperm population with enhanced sperm quality, this technique could be beneficial to low-dose insemination techniques. Recent studies using Percoll gradient to prepare semen for hysteroscopic insemination obtained controversial results. Morris et al. (2000) obtained a 64% pregnancy rate after inseminating 1 x 10^6 fresh, Percoll-treated sperm hysteroscopically. They suggested that the higher pregnancy rate obtained in their experiment compared to previous studies resulted from the use of Percoll gradient. However, Alvarenga and Leao (2002) obtained similar results after hysteroscopic insemination of 10 x 10^6 frozen-thawed sperm, separated or not, through Percoll gradient. Therefore, the efficacy of Percoll to improve results with low-dose insemination techniques needs further investigation.

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