XVII Congresso Internazionale SIVE
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Palacongressi d’Abruzzo
Montesilvano (PE) - ITALY

4 - 6 Febbraio 2011
February 4th - 6th, 2011

ATTI
PROCEEDINGS
Due primarily to the fact that breeders select stallions for breeding based on performance, confirmation and pedigree with little regard for reproductive efficiency, the general stallion population includes numerous valuable stallions with poor reproductive efficiency. A stallion may be subfertile in a frozen or cooled semen program and exhibit normal fertility in fresh AI or natural mating conditions. Many of these subfertile stallions have inadequate production of functionally normal sperm or produce ejaculates which contain seminal plasma components that have a deleterious effect on sperm quality during storage. Processing techniques to reduce or remove seminal plasma and concentrate sperm include centrifugation using conventional or cushioned techniques. Sperm separation techniques such as motile sperm migration (swim up or swim down), sperm adherence (glass-wool, glass beads or Sephadex filtration) and density gradient centrifugation (BSA, Percoll, EquiPure) have been used to improve the quality of inseminates and select for an enriched population of normal sperm. Many of these techniques were developed for use in human assisted reproduction and IVF applications and have been used for more than 20 years. Recently, clinicians have used some of these techniques to improve pregnancy rates of subfertile stallions.

**SEmen COLLECTION**

Natural mating provides a “closed-loop system.” Spermatozoa are exposed to an appropriate environment from ejaculation, directly into the female reproductive tract, until fertilization in the oviduct. Because semen is ejaculated through the open cervix and into the uterus of an estrous mare, prolonged exposure of spermatozoa to seminal fluids is minimized. The stallion ejaculates in a series of five to eight jets or fractions. The composition of each fraction varies. The first secretions (presperm) contain no spermatozoa and are primarily from the bulbourethral glands. The next two or three jets contain most spermatozoa (sperm-rich fractions), and the final fractions contain few spermatozoa and are composed primarily of secretions from the seminal vesicles. Damage to sperm can be caused by prolonged exposure to components of seminal plasma and to byproducts of metabolism during incubation. Furthermore, seminal plasma from some stallions appears to be extremely toxic to sperm resulting in a rapid loss of motility after exposure. The practitioner’s goal should be to obtain a complete ejaculate with a single mount and minimal sexual stimulation of the stallion before collection of semen, optimizing the potential to obtain an ejaculate with relatively low volume and high sperm concentration. This type of ejaculate is preferable when semen preservation protocols are used. Ejaculate volume but not total sperm output is affected by the amount of sexual stimulation before ejaculation. One simple and practical technique to decrease the collected seminal volume and, conversely, increase sperm concentration is to void the presperm (bulbourethral gland secretions). This is done by diverting the stallion’s penis away from the AV for a few seconds after the stallion has mounted and while the presperm is emitted.
ROLE OF SEMINAL PLASMA

Seminal plasma has been shown to be detrimental to stallion spermatozoa during storage, and most protocols involve dilution or partial removal of seminal plasma before cooling. Recently, the role of seminal plasma in protecting spermatozoa in the mare’s reproductive tract and mediating the postbreeding inflammatory response has been elucidated. After the introduction into the mare’s reproductive tract, spermatozoa trigger an inflammatory response. In most mares, this is a normal physiologic response; however, in susceptible mares, the duration of this inflammatory response is prolonged and results in postbreeding endometritis. Seminal plasma modulates the duration of postbreeding endometritis and plays an important role in sperm transport and elimination of dead spermatozoa. Proteins in seminal plasma selectively protect live spermatozoa from binding and phagocytosis by polymorphonuclear neutrophils while promoting elimination of dead spermatozoa. In addition to the vital role of seminal plasma in the mare’s reproductive tract, the absence of seminal plasma during storage results in poor maintenance of sperm motility. Therefore, except in cases where toxic seminal plasma has been identified, protocols that involve removal of all seminal plasma should be avoided; generally, 5% to 20% of seminal plasma should be retained after centrifugation.

Love and coworkers from Texas A&M cooled spermatozoa in the presence of seminal plasma, however, and reported a reduction in DNA integrity, as measured by the sperm chromatin structure assay, although sperm motility was maintained. They concluded that sperm motility and DNA integrity are protected when semen is centrifuged to remove the seminal plasma and spermatozoa are resuspended in Kenney-type extender supplemented with high-potassium Tyrode’s medium. These researchers concluded that if seminal plasma is to be completely removed in cases of toxic seminal plasma or sperm enrichment techniques, this modified Kenney’s extender should be used.

CENTRIFUGATION “CUSHIONS”

Centrifugation of stallion semen can be harmful, especially when spermatozoa are packed tightly at the bottom of the tubes after vigorous centrifugation. Stallion semen is usually diluted and centrifuged for 10 to 15 minutes at between 400g and 600g to achieve a soft pellet. Total sperm recovery of approximately 75% is generally accomplished with this technique. Attempts to increase recovery rates by increasing centrifugation time or force usually result in a decrease in semen quality. The concept of layering a dense solution (0.25 mL) below the extended semen to provide a “cushion” for spermatozoa during centrifugation was introduced by Cochran and colleagues from Colorado State University in 1984; initially, a glucose-ethylenediaminetetraacetic acid (EDTA) buffer and, later, freezing extender containing egg yolk and 4% glycerol were used.

A higher percentage of motile spermatozoa were obtained after centrifugation using this cushion technique. In a 1997 report, Revell and coworkers described a cushion technique that involved layering a dense solution (60% wt/vol) of iodixanol in water (OptiPrep; Axis-Shield PoC AS, Oslo, Norway) below diluted stallion semen before centrifugation. The density of the solution (1.32 g/mL at 20°C) prevented spermatozoa from compacting at the bottom of the tube; therefore, more rigorous centrifugation could be used to increase sperm recovery. Varner and colleagues from Texas A&M reported using OptiPrep (30 mL) layered in the bottom of a specially designed glass centrifuge tube (“nipple tube”; Pesce Laboratory Sales, Kennett Square, Pennsylvania) before centrifugation at 400g for 20 minutes to achieve good sperm recovery from centrifuged semen. When using this technique, they simply resuspended the sperm pellet and OptiPrep in extender and the spermatozoa has been used to obtain excellent pregnancy rates. When using such a dense cushion, even after vigorous centrifugation, spermatozoa are suspended in a band below the supernatant and above the cushion material.
Recently, two proprietary products (Eqcellsire, now called MaxiFreeze IMV, L’Aigle, France; Cushion Fluid, Minitube, Landshut, Germany) were introduced to aid in obtaining higher sperm recovery after centrifugation of stallion semen. Both products are described as dense, inert, isotonic solutions that have similar functions. The original protocol for these products involved: 1) diluting semen approximately 1:1 in a skim milk based extender, 2) loading 40 ml of the extended semen into a 50 ml conical bottomed centrifuge tube, 3) layering 3.5 ml of the cushion material below the extended semen and 4) centrifugation at 1000 x g for 20 minutes. We have modified this technique in our laboratory and have had comparable results when using as little as 0.3 ml instead of the 3.5 ml of the cushion material in the bottom of the 50 ml centrifuge tube. Following centrifugation, approximately 90% of the supernatant is removed, leaving approximately 5% of the original seminal plasma. The cushion material is then aspirated using a small bore disposable transfer pipette from below the sperm band being careful not to aspirate any of the concentrated sperm. With this cushioned centrifugation technique, recovery rates of 90 to 95% can be achieved.

SPERM SEPARATION TECHNIQUES

The techniques discussed thus far have focused on separating spermatozoa, other cells, and debris from seminal plasma. These centrifugation techniques do little to improve the percentage of viable spermatozoa in the sample or to remove contaminants or other cells. Several techniques for sperm separation have been developed and for more than 20 years have been widely used in human and bovine ARTs. Semen separation techniques have been used for low-dose insemination of sexed and unsexed semen, intracytoplasmic sperm injection (ICSI), and gamete intrafallopian transfer (GIFT) in the horse. Of practical interest to the equine practitioner is the use of sperm separation techniques to enrich a semen sample with a higher percentage of viable spermatozoa before insemination of mares. This could pertain to poor-quality fresh ejaculates or to damaged spermatozoa after cooling or freezing.

The first successful separation of motile from nonmotile spermatozoa was done by filtering diluted semen through a column of small glass beads. Numerous techniques have since been used to separate spermatozoa. There are three basic approaches to differential sperm separation: (1) motile sperm migration (swim-up [SU], swim-down techniques); (2) sperm adherence techniques (eg, glass wool [GW], glass beads, Sephadex [FS] filtration); and (3) density gradient centrifugation (eg, bovine serum albumin [BSA]; Percoll [Sigma Chemical Company, St. Louis, Missouri]; PureSperm, Nidacon International AB, Goteborg, Sweden).

SPERM MIGRATION

The most simple method to separate motile spermatozoa from the other components of semen is a SU technique. Semen is carefully overlaid with culture medium. The sample is incubated, allowing the motile spermatozoa to swim from the semen layer into the culture medium. In the mare, only highly motile spermatozoa complete the migration through the uterotubal junction to populate the oviduct, and in theory, the SU technique mimics the selection of motile spermatozoa that occurs in the female reproductive tract. The standard SU technique has been modified for bovine and human spermatozoa by adding hyaluronic acid, a glycosaminoglycan found in the female reproductive tract, to the SU medium. The addition of hyaluronic acid resulted in a significantly higher recovery of motile and membrane-intact spermatozoa. Although this technique does provide a purified population of motile spermatozoa, the yield is extremely low (10%–20%); therefore, the SU procedure is most appropriate for harvesting small numbers of motile spermatozoa for techniques like in vitro fertilization (IVF) or ICSI and, possibly, low-dose hysteroscopic insemination. The SU technique has been used with semen from fertile stallions, with a significant in-
crease in progressive motility and significant decreases in sperm head and tail abnormalities. The SU technique has also been used with poor-quality frozen-thawed semen from subfertile stallions to obtain acceptable spermatozoa for ICSI. Because low yield and small processing volumes are associated with the SU technique, it is not appropriate for separating spermatozoa from whole ejaculates for cooling or freezing. The presence of dead or dying spermatozoa in an ejaculate has been reported to be detrimental to motility and fertility of the remaining spermatozoa. Therefore, removing dead or dying spermatozoa from the ejaculate could help to maximize the post processing quality of frozen and cooled semen. Techniques that could be used for processing poor quality ejaculates before artificial insemination or cryopreservation include filtration (sperm adherence) and density gradient centrifugation.

**SPERM ADHERENCE TECHNIQUES**

There is a tendency for dead and damaged spermatozoa to stick to glass surfaces and to be bound or trapped by hydrated FS (polysaccharide beads) filters. Acrosome and plasma membrane intact spermatozoa pass through columns of FS and GW. This technique has been used to remove dead spermatozoa from ejaculates of semen before cryopreservation, significantly increasing the motility and fertility of frozen-thawed spermatozoa from low-fertility bulls. Based on the different surface charges for dead and live spermatozoa, this technique was modified to include gels of positively and negatively charged celluloses and FS, referred to as Sephadex ion-exchange (FS + IE) filtration. GWS trapping of stallion spermatozoa with damaged acrosomes or plasma membranes was reported to be highly correlated with the fertility of frozen-thawed stallion semen. Columns of GWS are prepared by first packing a small amount of GW at the bottom of a plastic syringe with the plunger removed. Tubing is attached to the tip of the syringe and clamped before layering FS gel over the GW. The 20% FS gels are prepared by adding FS particles (20 g, G-10 or G-15; Sigma Chemical Company) to an appropriate buffer or semen extender, allowing the FS to swell during incubation for 0.5 to 1 hour. Extended semen is then layered over the FS gel and filtered through the GWS column. An enriched population of spermatozoa is recovered in the filtrate for further processing. FS + IE filter columns are prepared in a similar fashion, but the two (positive and negative charged) cellulose gels are layered above the FS gel before the addition of extended semen.

**DENSITY GRADIENT CENTRIFUGATION**

This technique is based on the separation of spermatozoa into subpopulations with different specific gravities. A suspension of colloidal silica particles coated with polyvinylpyrrolidone (PVP; Percoll) or covalently bound hydrophilic silane (PureSperm) is placed in a centrifuge tube as a continuous or multilayered discontinuous gradient. Often, a two-layer discontinuous gradient is prepared by layering a 40% to 45% solution over an 80% to 90% solution of Percoll or PureSperm. Semen is layered over the gradient, and the tubes are centrifuged. Density gradient centrifugation separates spermatozoa based on motility and morphology, because most of the normal spermatozoa pass through the gradients and are located in the pellet at the bottom of the tube. Most of the nonmotile or morphologically abnormal spermatozoa, premature germ cells, other cells, and extender components are found trapped at the interface between the gradients or suspended in one of the two gradients. Although Percoll has been the most widely used material for density gradient separation of spermatozoa, concerns over the potential toxicity of unbound PVP from Percoll and the possibility that it may contain endotoxins resulted in its removal from human clinical use. Subsequently, silanized silica products, such as PureSperm, were developed; these products were presumably safer. The new generation of gradients using silane-coated colloidal silica particles (PureSperm,
labeled as Equipure and BoviPure for species-specific applications) have been evaluated for enhancing the quality of stallion spermatozoa. Extended stallion semen was centrifuged through a two-layer (40% and 80%) discontinuous gradient of EquiPure, resulting in an increased percentage of total and progressively motile spermatozoa. In addition, the mean percentage of morphologically normal spermatozoa tended to be higher in the processed versus the unprocessed semen. The 40% and 80% Percoll gradients contained significantly more morphologically abnormal spermatozoa (proximal droplets, detached heads, abnormally shaped midpieces, bent or coiled tails, and premature germ cells) than the pelleted spermatozoa that had completely passed through the gradients. Because stallions are not selected based on fertility or semen quality, an application of particular interest to equine theriogenologists is the use of sperm selection techniques to improve the quality and potential for cryopreservation of asthenospermatozoaic (low-quality) ejaculates from stallions that would otherwise be unsuitable for cooled or frozen semen preservation. The aim of these techniques would be to concentrate while removing dead, dying, and abnormal sperm that may negatively affect fertility. More recently, both Equipure and a similar product (Androcoll-E, SLU, Sweden) have been used in a single layer centrifugation technique (SLC) to isolate an enriched population of good quality sperm. This modification of the density gradient technique involves using only a single layer of colloid particles and can be used to process larger volumes of semen than was previously done using the density gradient technique. With this technique, semen is diluted 1:1 with a skim milk extender and up to 15-20 ml is carefully layered over a single 20 ml layer of colloid in a 50 ml conical bottom centrifuge tube. To achieve the best separation it is critical that the interface between the colloid and extended semen is not disturbed therefore the semen must be layered very slowly. The semen is then centrifuged gently (approximately 300 x g) for 25 to 30 minutes. The supernatant and the colloid are carefully aspirated leaving a small sperm pellet of high quality sperm at the bottom of the tube. Numerous studies using both density gradient and single layer centrifugation techniques with these saline-coated colloidal silica particles have demonstrated the ability to produce a selected population of sperm that have a higher percentage of progressive motility, normal morphology, increased longevity, increased membrane integrity and increased chromatin integrity and are promising for processing semen from subfertile stallions or low quality ejaculates.