Techniques to enhance the use of suboptimal semen - a practitioner's approach
Brian S. Carroll
Oklahoma City Equine Clinic, Oklahoma City, OK

Abstract
The clinical use of semen processing techniques, including cushioned centrifugation and density
gradient centrifugation, is discussed. Case scenarios involving these techniques, coupled with fertility
trials to determine lowest effective insemination doses, are used to illustrate how semen processing can
increase breeding efficiency (pregnancy rates achieved per cycle or season; number of pregnancies
established) for subfertile stallions with high demand.

Keywords: Stallion, semen quality, cushioned centrifugation, density-gradient centrifugation, low dose
insemination

Introduction
Stallions are often offered to the public to breed mares without regard to their potential fertility.
This has led to demand for breeding of a significant number of stallions that, while they may produce
desirable performance traits in progeny, are subfertile (for a variety of reasons). Semen characteristics
may vary greatly among stallions in a given breed, particularly amongst those that are subfertile.1
Nevertheless, suboptimal semen quality and quantity is common in immature stallions recently retiring
from performance careers,2 as well as in aging stallions suffering from testicular dysfunction.3 There are
many mature stallions that have poor semen quality of undetermined cause.4 Due to the financial
incentive to book as many mares as possible to a stallion, owner/manager expectations for the number of
mares bred per year often exceeds the stallion’s spermatogenic potential. The common use of cooled
transported and frozen semen by many breeds, compounded by an oftentimes casual approach to semen
processing by many suppliers, can further hamper the ability to produce pregnancies in an efficient
manner. Consequently, the equine breeding industry abounds with stallions that actually achieve less-
than-desirable pregnancy rates.

Thus, there is a need for veterinary practitioners to assist stallion owners/managers to maximize
pregnancies achieved by subfertile stallions. In our practice area, this demand often occurs only after
routine breeding management procedures have failed. This communication discusses the clinical use of
previously described techniques involving advancements in centrifugation of semen, often incorporated
with deep-horn low-dose insemination techniques, to enhance pregnancy rates or the number of
pregnancies produced by stallions used in artificial insemination programs. Our primary goal has been to
improve the per-cycle pregnancy rate of a given stallion, while concurrently inseminating more mares per
ejaculate. Subsequently, we have been able to generate income for the veterinary practice, while
simultaneously producing a significant return on investment to stallion management, by securing more
breeding fees and increasing the number of pregnancies produced by a given stallion.

Stallion evaluation
The plan to maximize fertility of a given stallion begins with an evaluation of the complete
breeding process, in addition to a thorough breeding soundness examination.5 The entire semen-
collection and preparation processes should be evaluated, including cleaning and storage of reusable
labware, preparation of the artificial vagina, method of sperm concentration determination, semen volume
estimation, and motility estimations.1 Fertile horses that only breed a small numbers of mares may
tolerate unfavorable laboratory techniques or semen processing, while less fertile stallions in high demand
will not.

Breeding history is invaluable, but is sometimes difficult to obtain.6 Per-cycle and seasonal
pregnancy rates for prior seasons and pregnancy rates to-date in the current season should be calculated.
Pregnancy rates for different classes of mares (e.g., maiden, lactating, and barren mares) are helpful, if
available. Numbers of sperm per inseminate and pregnancy outcomes for breedings with fresh semen
used on-site and for transported semen used off-site should be compared to provide historical data for later comparison to pregnancy rates achieved following any subsequently instituted changes in semen processing, number of sperm inseminated, or method of insemination.

A complete breeding soundness examination should be performed to generate data to identify areas where altering breeding management strategies could possibly improve pregnancy rates. Predicted daily sperm output (DSO) determined from total testicular volume measurements are compared to actual total sperm in the ejaculate (at DSO) to estimate spermatogenic efficiency. Awareness of total sperm in the ejaculate at DSO, and typical volumes and concentrations for a complete ejaculate, of the given stallion is imperative for allowing judgments to be made regarding potential semen processing techniques that could be practical for use. Obtaining and processing multiple ejaculates are often required to allow such determinations to be made. Morphologic profiles determine the relative percentage of normal sperm to abnormal sperm and identify specific abnormalities that might be contributing to suboptimal fertility. Sperm chromatin quality of fresh raw semen is determined by the sperm chromatin structure assay. Percentage of total and progressive sperm motility in different extenders are determined for fresh semen and for extended-cooled semen following 24 and 48 hours of storage. These assessments provide baseline values of semen quality for comparison to those obtained by varying semen processing techniques that are used.

**Proficient semen collection technique**

While a “how to” on stallion collection is beyond the scope of this presentation, it is the author’s view that regardless of techniques used to process semen to potentially enhance fertility, the success of said efforts is dependent, to a large degree, on the collection process itself. All ejaculates are not equal. The volume of pre-ejaculatory secretions from the bulbourethral glands, secretions from the accessory sex glands (particularly gel from the seminal vesicles) and relative sperm concentration affect semen viscosity and therefore the sperm sedimentation rates that are achieved during centrifugation. Water soluble lubricant and other contaminants present in the ejaculate can alter the osmolarity and pH to a degree that adversely impacts sperm quality. The goal should be to obtain a complete clean ejaculate in a single mount with minimal sexual stimulation of the stallion before collection, all in order to obtain an ejaculate with relatively low volume and high sperm concentration. The importance of a skilled stallion handler and basic ground training of the stallion cannot be over emphasized in this regard.

**Cushioned centrifugation**

Centrifugation of semen is oftentimes indicated to concentrate dilute ejaculates and remove excess seminal plasma. Ideally, centrifugation should result in a 100% sperm recovery rate with no resulting damage to sperm quality. Loss of a significant portion of the ejaculate, leaving fewer sperm available for breeding purposes, could negate the benefit of concentrating the insemination dose. The main concern when attempting to maximize sperm recovery through centrifugation is the adverse effect that centrifugation can have on the integrity of sperm. Typically, an increase in centrifugation time or gravitational (g) force results in an increased sperm recovery rate, but it can also lead to decreased sperm motility or quality because of the mechanical forces associated with centrifugation and excessive packing of the sperm.

Recently, a cushioned centrifugation procedure has been applied to stallion semen to maximize sperm harvest without attendant injury to sperm. A non-ionic iodinated compound, iodixanol, was first reported for density-gradient cell fractionation and has since been used as either a density gradient or a cushion for centrifugation of sperm. Investigations regarding cushioned centrifugation of stallion semen with this product showed excellent yields of sperm that were undamaged by the centrifugation process, but an optically clear centrifugation medium was required to reduce sperm losses. Texas workers found that cushioned centrifugation of stallion semen in either 50 mL conical- bottom tubes containing 3.5 mL of iodixanol solution and centrifuged for 20 min at 1000 x g, or 45 mL nipple-bottom tubes containing 30 µL of iodixanol solution and centrifuged for 20 min at 400 x g, yielded a high sperm harvest while maintaining sperm function. They also noted an optically opaque extender, as is typically
used in the equine breeding industry, was suitable to achieve this goal. The nipple-bottom tubes are recommended over the 50-mL conical-bottom tubes for cushioned centrifugation when the sperm number in ejaculates is relatively low (i.e., less than 2–3 x 10^9 sperm) or when it is necessary to remove more seminal plasma from sperm after centrifugation than is possible with cushioned centrifugation in conical-bottom tubes. Recently, it has been demonstrated that the volume of iodixanol solution can be reduced from 3.5 to 1 mL in 50 mL conical-bottom tubes without impairing sperm harvest or semen quality.23

Three proprietary iodixanol products are available: 1) Otiprep™ (Sigma-Aldrich, St. Louis, MO; #D1556-250 mL, hypotonic, 170 mOsm/L); 2) Cushion Fluid™ (Minitüü, Tiefenbach, Germany; isotonic, 300 mOsm/L); and 3) Maxifreeze™ (IMV, L’Aigle, France; isotonic, 300 mOsm/L). The iodixanol products cushion the sperm during centrifugation to allow concentration of sperm for preparing deep horn insemination doses, prior to mixing semen with freezing extenders for cryopreservation, prior to final extension for cooled storage, prior to placing a small volume over density gradients, and perhaps to aid in removal of urine and urinary sediment when ejaculates are contaminated with urine.

Density-gradient centrifugation

Discontinuous density centrifugation to aid in separation of better-quality sperm has enjoyed broad clinical application in recent years. Density-gradient centrifugation is relatively simple to perform, and has been shown to effectively separate sperm with various morphological features in an ejaculate.

Centrifugation of equine semen through a silanated silica-particle solution (EquiPure™, Nidacon International AB, Mölndal, Sweden) has shown promise for selecting sperm with good motility, morphology, and chromatin quality24,25 and enhancing the fertility of selected subfertile stallions.8 Sperm recovery rate has been found to be higher when 2-4 mL of Equi Pure™ Bottom Layer is used in 15-mL capacity conical-bottom tubes25 than with double layer (Equi Pure™ Top Layer plus Equi Pure™ Bottom Layer). Centrifugation of semen through a silica-particle solution, such as EquiPure™, is not a logical approach for stallions with normal semen quality, because a relatively high percentage of the sperm population can be lost after centrifugation. Because this technique results in sperm separation based on sperm buoyancy or isopycnic point, its use is most justified when an ejaculate contains a high percentage of sperm with morphologic defects, particularly sperm with abnormal heads, abnormal midpieces, bent midpieces, bent tails, coiled tails, or premature (round) germ cells. However, it has been found that the technique sometimes improves chromatin quality in the recovered sperm population, regardless of sperm morphologic profile. The technique can also be used when more complete separation of seminal plasma from sperm is desired.26

Low dose insemination

The minimum effective number of sperm that can be used to inseminate mares to achieve commercially acceptable pregnancy rates is a subject of much debate in recent years.27 A single ideal number that can be applied in all cases eludes us. A more rational scenario would seem to indicate that the appropriate threshold number of sperm is related to the fertility of a given stallion and the insemination procedure that is applied (e.g., fresh or cooled-transported semen). Industry standards have typically been to inseminate 200-500 x 10^6 progressively motile sperm into the uterine body when fresh semen is used, or typically beginning with 1 x 10^9 progressively motile sperm prior to cooling when semen is to be chilled and transported.27 However, a recent report revealed no difference in pregnancy rates when 50 to 300 million fresh sperm from normal fertile stallions was used to inseminate reproductively normal mares.28

Rigby et al29 demonstrated that only 0.0007% of sperm that are deposited into the body of mare uteri actually gained access into the oviducts to be available for fertilization of an oocyte. They found that insemination in the tip of the uterine horn ipsilateral to an ovary containing a dominant follicle resulted in a greater percentage of oviductal sperm (77%) occupying that oviduct than with insemination in the uterine-body (54%). They concluded that more sperm gain access into the desired oviduct following deep-horn, compared to uterine-body, insemination.
This technique for low-dose insemination has been investigated in the research setting and applied clinically in recent years. Two techniques are commonly used to accomplish deposition of an insemination dose on, or near, the oviductal papilla: (1) use of an endoscope to visually confirm placement of semen on the papilla through a long catheter passed through the biopsy channel, and (2) use of a flexible pipette in which the tip is guided to a position near the oviductal papilla by manipulation per rectum, before deposition of the insemination dose. The optimal method for insemination of very low doses of sperm is a subject of some controversy. Hayden et al found no significant difference in pregnancy rates achieved between the two procedures when mares were inseminated with sperm numbers below the determined threshold required to achieve normal pregnancy rates (i.e., 0.5-1 x 10⁶ sperm from a stallion with known good fertility).

The value of low-dose insemination for improving fertility of subfertile stallions has been questioned, although apparently successful results with this breeding strategy exist. Contributing further to the controversy is whether low dose insemination using separated sperm (e.g., density gradient centrifugation, etc.) results in more normal sperm actually colonizing the uterotubal junction (UTJ) and oviduct, and actually improves fertility. Non-proponents for breeding with separated sperm argue that natural selection for normal sperm at the UTJ/oviduct precludes most abnormal sperm from participating in the fertilization process. Certainly, a much higher percentage of those sperm that actually colonize the UTJ are morphologically normal than are present in the entire inseminate. Proponents for breeding with separated sperm suggest that fewer uncompensable sperm defects will be present that might access the oviduct and lead to fertilization failure or early embryonic death. Obviously, more research in this area needs to be done. However, this author is convinced that the low dose insemination strategy is a valuable tool when combined with an appropriate centrifugation procedure for preparation of the insemination dose.

Case 1

A 12-yr old Quarter Horse stallion was presented on April 1, 2003, with a history of impregnating 35 of 100 mares bred with fresh semen on the farm. A review of breeding records revealed 14 pregnancies from 45 cycles of breeding in the month of March (31% pregnancy rate per cycle). Mares had been bred with at least a “shipping dose” (1 x10⁹ progressively motile sperm) based on sperm concentrations obtained with a densimeter (Animal Reproduction Systems, Chino, CA) and visual estimation of sperm motility (estimated typically to be approximately 60%). Mares bred per ejaculate ranged from one to five, most often being three or four. Total estimated number of sperm per insemination dose used ranged from 2-8 x 10⁹.

Total testicular volume of this stallion was determined to be 308 cc, resulting in a predicted DSO of 6.1 x 10⁹, assuming that the testes were producing sperm with normal efficiency. The stallion had been bred 35 times in the last 60 days (approximately every other day), and total sperm number obtained in an ejaculate was 13.87 x 10⁹, therefore, spermatogenic efficiency was considered to be normal. Sperm motility was estimated to be 50%/35% (total/progressive), with velocity estimated to be 3 of 5 (moderate). The percentage of morphologically normal sperm in the ejaculate was 18%. The most common morphological defects were abnormal heads (39%), abnormal midpieces (22%), bent tails (32%) and coiled tails 4%. The sperm chromatin structure assay yielded 30%COMP-α compared to 6% for semen from a control stallion of known good fertility, indicating a high percentage of sperm had unstable DNA.

With the morphological abnormalities and the degree of abnormal DNA present in the stallion’s sperm, it was unclear whether increasing the numbers of sperm inseminated would improve pregnancy rates. We reduced the number of mares bred per ejaculate to two, and achieved nine pregnancies from 14 cycles in seven mares (64% pregnancy rate per cycle). While it appeared that increasing sperm numbers per inseminate would improve fertility, the mare book would have to be reduced for the rest of the season. Pregnancy rates improved as increased numbers of sperm were inseminated into the uterine body, so the plan was to breed more mares per ejaculate by using deep-horn insemination to see if placing the sperm as near to the oviductal papilla as possible would still produce acceptable pregnancy rates with a
reduced (compared to uterine body inseminations) number of sperm. Management was changed so that four or five mares were bred per ejaculate, which resulted in insemination doses ranging from 1.28-2.6 x 10^9 in 10-20 mL extended semen, using a transrectally-guided deep horn insemination technique. By utilizing this technique, an additional 52 pregnancies were obtained from 117 cycles by July 10. End-of-season (total of all methods of breeding throughout the year) results for this stallion were: pregnancy rate per cycle 43.6% (106/243); seasonal pregnancy rate 96% (106/110).

The same breeding protocol was implemented from the beginning of the next (2004) breeding season, with an addition of a change in mare management. Mares were palpated daily and an ovulation-inducing agent was administered the day prior to breeding, to ensure most mares would be bred within 24 hours prior to ovulation. Pregnancy rate per cycle for mares ovulating within 24 hours after breeding was 61%, while pregnancy rate per cycle for mares ovulating between 24 and 48 hours after breeding was 44%. Overall, a 54% pregnancy rate per cycle was achieved, resulting in a seasonal pregnancy rate of 96% (115/120) by May 15 rather than July 10, despite no apparent differences in sperm output, morphological profile, or sperm chromatin quality in the stallion’s ejaculates.

The same management strategy produced similar results through mid-March of 2005 when a precipitous decline in pregnancy rate per cycle occurred (i.e., from 69% achieved during the first six weeks of the season to 39.4% (28/71) achieved from mid-March through the end of April). No obvious difference was detected in sperm motility estimates during this decline in fertility, but the percentage of morphologically normal sperm declined to 10% due primarily to an increase in abnormal heads. Sperm chromatin structure assay values were greatly elevated, demonstrating marked sperm DNA instability consistent with that of stallions that are extremely subfertile. Subsequent pregnancies were expected only to occur as random events unaffected by sperm numbers or insemination technique. The decision was made to try concentrating the semen using cushioned centrifugation, followed by deep-horn insemination. The ejaculates were concentrated and divided between two to five mares with 2-6 x 10^9 sperm in 5-8 mL volumes and mares were inseminated with a transrectally-guided deep horn technique. Pregnancy rate per cycle returned to 58% for the remainder of the season, resulting in an overall 92.4% (105/114) seasonal pregnancy rate.

Case 2

A 4-yr-old Quarter Horse stallion was presented to manage for breeding following an evaluation at the Texas Veterinary Medical Center for breeding soundness. The stallion had concluded his first season at stud where he achieved a 59% seasonal pregnancy rate when covering 165 mares. Approximately one-half of the mares had been bred with cool-transported semen. During that first season, progressive sperm motility was estimated to be 70% for 76 of 84 semen collections performed, with an average total sperm number in the 84 ejaculates of 5.349 x 10^9 sperm. The sperm concentration was estimated to be less than 100 x10^6 sperm/mL for 67 of 84 ejaculates collected.

Estimated testicular volume for the stallion was 225 ml. If testes were producing sperm at a normal rate, daily sperm output was expected to be 4.6 x 10^9 sperm. Four ejaculates were collected once daily from the stallion to stabilize extragonadal sperm reserves. Total sperm number in the fourth ejaculate was 3.67 x 10^9 sperm, suggesting spermatogenic efficiency (≤ 80%) was below normal. Among all four ejaculates, the percentage of progressively motile sperm averaged 27% and the percentage of morphologically normal sperm averaged 38%. The most common morphologic defects were abnormally shaped midpieces (30%) and bent tails (19%). Semen from three ejaculates was processed by cushioned centrifugation or cushioned centrifugation followed by density gradient centrifugation in EquiPure™ (Bottom Layer). Semen was evaluated for sperm motion characteristics immediately after each processing step and after 24 hours of cooled storage. The effect of seminal plasma was also evaluated. The sperm morphologic profiles of unprocessed (raw) semen and EquiPure™-processed semen were also compared. The percentage of morphologically normal sperm increased from 40% to 76% after density-gradient centrifugation, primarily due to reduced percentages of abnormal (irregular or bent) midpieces and bent tails. For this ejaculate and others evaluated from this stallion, sperm velocity was increased (approximately doubled) when seminal plasma was replaced with that obtained from a known fertile.
stallion. Sperm motility parameters in semen extended after density-gradient centrifugation and replacement of seminal plasma were maintained through 24 hours of cooled storage.

A fertility trial was conducted with semen from this stallion to determine if insemination of mares with EquiPure™-processed semen would yield commercially acceptable pregnancy rates. Ten reproductively normal mares were inseminated as follows: 1) five mares were inseminated once with 100 x 10⁶ total sperm, and 2) five mares were inseminated once with 200 x 10⁶ total sperm. The seminal plasma was replaced with that of a known fertile stallion. Seminal plasma from the donor stallion was procured by centrifuging raw semen at 1000 x g for 15 min, followed by filtration of supernate through tandem 5.0- and 1.2-µm pore-size nylon filters to remove any remaining sperm from the seminal plasma. One-mL aliquots of seminal plasma were frozen in vials at -80°C until used. Inseminate volumes ranged from 0.25 to 0.58 mL. A transrectally-guided deep horn insemination technique was used. The pregnancy rate per cycle was 100% (5/5) for mares inseminated with 100 x 10⁶ processed sperm and 80% (4/5) for mares inseminated with 200 x 10⁶ processed sperm. Two mares, one in each treatment group, experienced double ovulations, and each of these mares was diagnosed with twin pregnancies. As such, pregnancy rate per ovulation was 100% (6/6) for mares inseminated with 100 x 10⁶ processed sperm and 83% (5/6) for mares inseminated with 200 x 10⁶ processed sperm. Based on the post-density gradient centrifugation recovery rate of sperm in this trial, the stallion would have had produced sufficient processed sperm to breed 17 mares if mares were inseminated with 100 x 10⁶ processed sperm.

For the following breeding season, 212 mares were inseminated - primarily using fresh EquiPure™-processed semen with seminal plasma replaced with that from a fertile donor stallion. Chilled transported semen was not offered during this season because there were in excess of 60 re-breed contracts expected from the previous season, and demand for breedings on any given day early in the season would be high. Pregnancy rates per cycle and per season were 62% and 91%, respectively. Mares were inseminated with 0.5-2.0 mL doses of EquiPure™-treated semen with a transrectally guided deep-horn technique. The lowest dose contained 98 x 10⁶ sperm. The maximum number of mares bred with one ejaculate was 11.

This stallion has become a successful sire and has produced as many as 220 pregnancies in subsequent years. Chilled transported semen has been added to the program and he currently breeds more mares away from the farm than on-site. EquiPure™-processed semen and cushion centrifuged and re-extended semen are currently utilized to cover shipment orders on a day-to-day basis.

References
33. Hayden SS, Blanchard TL, Brinsko SP, et al: Pregnancy rates in mares inseminated with 0.5 or 1 x 10^6 sperm using hysteroscopic or transrectally guided deep-horn insemination techniques. Theriogenology 2012; Epub ahead of print.