

## **Improvements in equine semen processing techniques that aid optimal fertility**

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Technology is constantly changing in all aspects of our lives. Although developments in the equine breeding industry do not move as fast as those in computers or cell phone technology, there is nonetheless a slow and steady progress forward with respect to the diagnostic tools and equipment veterinarians have available, as well as improved protocols for semen processing and breeding management of the mare. Improved success with assisted reproductive technologies like embryo transfer and intracytoplasmic sperm injection (ICSI), have led to increased utilization by breeders in recent years, and frozen semen has allowed growth in the global genetics market. Some of the advances in semen processing techniques shall be reviewed here; however, whether these advances in technology have resulted in an overall improvement in fertility rate is tough to say. Aspects of semen processing are just a small piece of the puzzle when it comes to achieving a pregnancy in the mare; there are so many other variables that can impact the final result. Nonetheless, every little bit helps and only by optimizing all the pieces do we give ourselves the best opportunity for success. Therefore, it is in our best interest to utilize those semen processing techniques that are available in order to prepare a breeding dose that will provide the most optimal chances of conception.

### **Centrifugation cushion**

Centrifugation cushions, as their name suggests, provide a soft pillow at the bottom of the centrifugation tube upon which the sperm come to rest during the process of centrifugation. The use of the cushion allows longer centrifugation times at a higher g force, resulting in optimal sperm recovery. Whereas standard centrifugation protocols for semen processing result in recovery rates of 70-80%, the centrifugation cushion can result in recovery rates of 90-95%.

Although the use of a centrifugation cushion is unlikely to result in a direct impact on fertility, improved sperm recovery allows optimization of the ejaculate potentially increasing the number of breeding doses available. In addition, improved sperm motility due to reduced cellular stress during the centrifugation process may benefit sperm longevity during cooled transport or improve post-thaw motility after cryopreservation.

### **Sperm enrichment protocols**

One of the most significant applications for semen processing that may actually impact fertility has been the introduction of sperm enrichment protocols like density gradient centrifugation. Commercially available products include EquiPure™ (Nidacon International AB, Mölndal, Sweden) and AndroColl® (Minitube International, Tiefenbach, Germany). During centrifugation the density gradient selectively separates and enriches a population of sperm with improved morphology and motility. It has also been reported to remove bacteria and viruses. The cost of the product and the limited sperm recovery generally rule out routine application, and there is still some debate about whether the removal of dead or dying sperm from a normal ejaculate offers any benefit to fertility. However, the technique has particular application for those stallions with poor initial semen quality or sub-fertility. Sperm enrichment offers the possibility of transported cooled semen for those stallions whose sperm did not have the longevity of motility after standard processing protocols, or allows the option of semen cryopreservation. Semen processing with density gradient centrifugation has resulted in an improvement in pregnancy rates, primarily for those stallions with previous sub-fertility.

### **Sperm filter**

Developed by BotuPharma (Botucatu, Sao Paulo, Brazil) the SpermFilter™ is a new alternative for centrifugation. The filter is composed of a synthetic hydrophobic membrane that essentially separates sperm cells from seminal plasma or the dilution medium. Comparative studies with cushioned centrifugation have not demonstrated significant improvement in semen quality. However, it is a quick,

simple procedure that may offer benefit for breeding farms that do not have a centrifuge, for stallions whose semen does not respond well to centrifugation protocols or for low concentration samples where optimal recovery is desirable.

### **Changes in extender formulation**

Although numerous commercial extenders are available for diluting stallion semen for cooling, the majority of them are skim milk-sugar based with the most commonly used being a skim milk-glucose based extender known as the Kenney extender, named for its developer Dr. Bob Kenney. This extender along with slight variations of the original formula is used by many laboratories processing cooled semen today and is available under numerous commercial brands. More recently extenders that contain defined milk protein rather than complete milk powder have been introduced for diluting and cooling equine semen. These extenders were developed to allow for a more chemically defined media that contains only the native phosphocaseinates from milk which were determined to be the milk fraction that provides the most benefit to sperm survival.

Vendors may claim to have developed the latest and best semen extender, with new and improved formulas or additives that improve semen quality. However, one must look critically at the supporting literature to determine if appropriately controlled studies have been performed and if the increase in reported semen quality actually correlated with increased fertility. In our experience there are some individual stallions that benefit from use of these extenders over standard skim milk-glucose extenders and some that do not. It is therefore essential to test the longevity of sperm motility in split ejaculates for each stallion in a variety of available extenders to determine which one provides the best retention of sperm quality after storage for 24-48 hours.

There are many commercial extenders available for semen freezing, but most of these are variations on a few basic formulations. Most all freezing extenders have a buffered salt solution as a base, they may include non-fat dried skim milk powder or milk components, a combination of sugars and they almost always contain egg yolk and a cryoprotective agent (CPA) or agents. The most common CPA added to freezing extenders for sperm is glycerol and is typically included in extenders for stallion sperm at concentrations of 2 to 5%. Alternative CPA's including ethylene glycol, propylene glycol, DMSO and amides such as methyl formamide and dimethyl formamide have also been used to successfully to cryopreserve stallion sperm, as well as sugars like lactose and mannose that are non-membrane permeable CPAs.

A search of the literature will find many journal articles reporting the results of various trials related to extender composition, and/or the addition of different additives to the semen extender in the hopes of improving post-thaw semen quality and fertility. Many of these studies investigate the addition of antioxidants, like vitamin E and glutathione. There is a growing amount of evidence for a role of reactive oxygen species (ROS) and oxidative stress in the promotion of cellular damage that occurs during cryopreservation and thawing. Addition of antioxidants to semen extenders, as well as oral supplementation of antibiotics has been demonstrated to show improvements in semen quality for humans and food animal species. The reports for equine semen are variable, with possibly the same number of manuscripts reporting a positive effect as those that report no impact at all. Nonetheless, it is an active avenue for research.

It is important to remember that no one extender is going to provide an optimal result in every stallion. Each stallion is an individual and they likely have their own preference for a particular brand or extender formulation or freezing protocol. This is likely related to differences in their sperm membrane composition, components of their seminal plasma and their inherent ability to withstand cellular stress. The best way to determine the right protocol for your stallion is to perform a split-ejaculate test freeze with a selection of available extenders.

### **Improved accuracy in determination of cell concentration**

Most methods for determination of cell concentration are indirect and rely upon densitometry or photometry. The main disadvantage of these methods is that any contaminating debris in the ejaculate

contributes to the sperm count. In contrast, for sperm counting with the Nucleocounter SP-100 (Chemometec, Denmark) semen is diluted in a detergent solution and then loaded into a cassette through a microfluidic network containing the DNA stain, propidium iodide. Internal fluorescence detection by the software enables measurement of an accurate sperm count, as only the sperm heads are fluorescently labeled by the propidium iodide. Consequently, the sperm count obtained by the Nucleocounter may be 15-20% less than a traditional densimeter, because of the exclusion of debris. In some stallions known to have a lot of background debris in their ejaculates, we have observed the count on the Nucleocounter to be almost 50% less than the densimeter. This means that breeding doses can be more accurately proportioned. Despite being more expensive, the Nucleocounter SP-100 has been utilized by institutions, veterinary clinics and breeding farms alike throughout the equine industry for the reliable, repeatable and accurate determination of sperm cell concentration. Other advantages are that it is quick and easy to use, and that it can be used to measure sperm concentration when semen has been diluted into extender.

### **Stallion selection**

In the dairy industry young bulls are fertility tested by inseminating thousands of cows with their frozen semen to establish the level of fertility that can be expected before the frozen semen is sold on the commercial market. Furthermore, the difference between a low fertility bull and a high fertility bull due to genetic selection for fertility and “freezability” over many decades is just a few percentage points per cycle. This is vastly different from the situation for stallions where most will never breed “thousands” of mares over the course of a lifetime in order to demonstrate a reliable estimate of expected fertility, let alone before the semen is marketed. Also as pleasure and competition animals the selection for fertility is not as relevant to equines as it is for food animals, instead the temperament, pedigree or performance of an individual is more likely to influence his selection as a breeding stallion. Nonetheless, there is a growing research interest focused at developing better tools for the prediction of fertility in a breeding stallion or for success in a frozen semen breeding program.

We have limited assays available to measure semen quality in the average semen processing laboratory-primarily sperm motility, viability and morphology. Sperm motility is the sperm attribute most readily applied for the assessment of semen quality, and industry recommended minimums for a breeding dose of cooled semen (500 million progressively motile sperm (PMS)/dose at 24hr) or frozen semen (200 million PMS/dose and a minimum of 30% post-thaw progressive motility) are based upon the number of PMS in the breeding dose. However, motility by itself is an indicator of relative cell health but fertilization is a complex process that requires numerous functional attributes of both sperm and egg. There may be dysfunction of one of these attributes that renders the sperm incapable of fertilization even in the presence of “acceptable” motility.

It is unlikely that we will have the capability in the near future to measure all the components a sperm cell needs in order to effect fertilization, however access to better markers of sperm function, those that actually correlate with fertility, would be advantageous. Flow cytometry offers a wider range of semen analysis options, and the general practitioner can usually access these tools through university laboratories. One such test is the sperm chromatin structure assay (SCSA) that can detect sperm samples that have a high degree of DNA fragmentation (small breaks in the sperm chromosomes). Other flow cytometry tests available include assessment of sperm mitochondrial function, capacitation and the acrosome reaction, membrane fluidity and integrity, apoptosis, and measures of ROS production, oxidative stress and lipid peroxidation; although the application of many of these tests to select for or predict fertility has still to be determined. The SCSA is one of the few sperm analysis tools that has actually been correlated to fertility in humans, bulls, stallions and other species.

### **Genetic markers for fertility**

With the completion of the equine genome sequence in 2009 we now have new tools to determine the genetic components of complex conditions like disease resistance, athletic performance, or potentially fertility. Genetic screening panels are already available for the identification of multiple genetic disorders, for several coat color traits, and there are even genetic tests related to gene markers for speed

and gait. Genetic mechanisms have been determined in other species to be related to the variability in fertility traits of males and females, and genetic markers for components of seminal plasma and semen quality traits proposed as predictors of fertility. Discovery of genetic markers impacting fertility in the stallion could be applied immediately for genotype assisted selection of breeding stallions. One promising example are the genes coding for equine cysteine-rich secretory proteins (CRISPs); previous studies have shown that CRISPs play a role in the fertilizing ability of male animals. CRISP-3 has been proposed as a potential marker for freezability in stallions, and a polymorphism in the CRISP-3 gene has been reported to correlate with fertility in Hanoverian stallions.

### **Sources of variability and limitations**

Despite many advances in semen processing techniques there are still many potential sources of variability that may influence the result when it comes to obtaining a pregnancy, aside from the inherent fertility of the mare or stallion. Although new tools and techniques offer many advantages, they are only as accurate or successful as the technician that is using them. The skill and experience of the clinician processing the semen or breeding the mare can ultimately impact the potential fertility of any breeding. For example, when it comes to semen quality at the time of insemination many factors may have come in to play during the collection, processing and packaging of the breeding dose including:

- stallion collection frequency and the number of mares booked to the stallion that day may influence the number of sperm in the breeding dose.
- The accuracy of preparation of that breeding dose may be influenced by errors in the determination of sperm concentration, measurement of volume or insufficient mixing of the semen prior to dividing up the breeding doses.
- The semen quality may be negatively impacted by inappropriate semen collection and handling techniques, from temperature deviations to poor hygiene practices.
- The semen may not have been packaged appropriately in the shipping container, the shipping container may not hold temperature as it should, or it may have been tampered with or compromised during shipment.

No single advancement in semen handling protocols is going to guarantee fertility when so many potential variables exist to impact the final result. Therefore, a clinician must educate their clients about these sources of variability and encourage them to purchase semen or breedings from reputable breeding farms or processing facilities with demonstrated experience and success. One of the many challenges within the equine breeding industry is that there are very few regulations concerning the collection, processing, distribution and insemination of stallion semen in the US. Any owner who can manage to extract semen from his or her stallion can without any prior training, experience, certification or license, sell semen from that stallion without restriction of any kind. There are also no regulations concerning standards for semen quality, e.g., the number of sperm in a dose, or minimum recommended values for motility or morphology, or for the health status of the stallion.

The World Breeding Federation for Sport Horses proposed semen standards several years ago, and all registries (and thus their members) that are part of the WBFSH are encouraged to adopt these standards. Member laboratories of the Select Breeders Affiliate Laboratory Network have also agreed to follow a set of self-regulated guidelines with respect to quality standards for frozen semen. The American Association of Equine Practitioners (AAEP) has published “Biosecurity guidelines for control of venereally transmitted diseases” that provides recommendations for equine viral arteritis and other potential venereal diseases. Whether controls should be put in place to regulate semen collection and processing facilities in the US is a topic beyond the scope of this review, nonetheless it is mentioned here simply as a possible means to improve reproductive outcomes.

Progress is inevitable and the future looks bright, particularly in the field of genetics and in the burgeoning tools available for analysis of sperm function. It is likely the next decade will produce amazing new technologies that will benefit the equine breeding industry.

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