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Semen Extenders and Sperm Selection Techniques in Stallions

Extensores de sêmen e técnicas de seleção espermática em garanhões

Márcio Menezes Nunes[1*], Maria Luiza Munhoz[2], Gustavo Ferrer Carneiro[3]

[1]German Standard Group, Dubai, UAE
[2]MK Arabians Stud, Ajman, UAE
[3]Universidade Federal Rural de Pernambuco (DMV/UFRPE), Recife, PE, Brasil
*Email: mmnunes@gsgroup.co

Resumo

A preservação do sêmen equino é parte importante da eficiência reprodutiva e é altamente influenciada pela variabilidade individual do garanhão. Embora a motilidade espermática não seja diretamente associada à fertilidade, ela consiste em um indicador da integridade metabólica. Durante o armazenamento, o metabolismo espermático gera espécies reativas de oxigênio (ROS), que podem induzir estresse oxidativo, levando a danos nas membranas celulares e no DNA, redução da motilidade e comprometimento da fertilidade. Mudanças no ambiente da amostra, incluindo flutuações de pH e osmóticas, bem como contaminações, comprometem ainda mais a viabilidade espermática. A qualidade do sêmen pós-coleta é afetada por múltiplos fatores, incluindo a composição do diluente, taxa de diluição, temperatura de armazenamento e o plasma seminal. Devido à variação individual significativa, protocolos padronizados são frequentemente inadequados. Estratégias como testes de ejaculados fracionados, remoção do plasma seminal e técnicas de seleção espermática — incluindo centrifugação em gradientes de densidade e seleção microfluídica — melhoram a qualidade e a longevidade da amostra. Avanços recentes em formulações de diluentes e diversificação nas temperaturas de armazenamento, particularmente com abordagens voltadas para o controle do estresse oxidativo, estenderam a vida útil do sêmen. No geral, protocolos individualizados de manipulação de sêmen são essenciais para otimizar a preservação espermática e os resultados de fertilidade em garanhões.

Palavras-chave: Estresse oxidativo, Seleção espermática, Criopreservação, variabilidade individual, Plasma seminal.

Abstract

Equine semen preservation is a major determinant of reproductive efficiency and is highly influenced by individual stallion variability. Although sperm motility is not directly predictive of fertility, it remains a practical indicator of metabolic integrity. During storage, sperm metabolism generates reactive oxygen species (ROS), which can induce oxidative stress, leading to membrane and DNA damage, reduced motility, and impaired fertility. Environmental changes, including pH and osmotic fluctuations, as well as contamination, further compromise sperm viability. Post-collection sperm quality is affected by multiple factors, including extender composition, dilution rate, storage temperature, and seminal plasma. Due to significant individual variation, standardized protocols are often inadequate. Strategies such as split-sample testing, seminal plasma removal, and sperm selection techniques—including density centrifugation and microfluidics—improve sample quality and longevity. Recent advances in extender formulations and storage approaches, particularly those targeting oxidative stress, have extended semen shelf life. Overall, individualized semen handling protocols are essential to optimize sperm preservation and fertility outcomes in stallions.

Keywords: *Oxidative stress, Sperm selection, Cryopreservation, individual variability, Seminal plasma*

Introduction

The primary objective of equine semen handling is to preserve sample quality during storage or, when possible, enhance it through the application of sperm selection techniques. Despite the low direct correlation between motility and fertility, samples exhibiting high-motility are generally indicative of healthy sperm metabolism (Amann, 1989). This reflects whether quality is being maintained, lost, or improved under a

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given protocol. Furthermore, the impact of morphological abnormalities on fertility, depends on the nature and severity of the defect. Certain abnormalities show strong correlation with reduced fertility (Love, 2011), and their removal from the ejaculate may increase reproductive efficiency (Varner, 2016).

The environment of semen sample is responsible for maintaining sperm viability and fertility potential, either protecting the sperm cells for extended survival or damaging them by reducing motility, viability, or both (Amann, 1989).

Healthy energy production through sperm metabolism physiologically produces substances known as reactive oxygen species (ROS), which can induce cellular self-damage (Gibb et al., 2014). The impact of ROS becomes more significant as storage time increases, a factor that must be considered during protocol selection. These substances, among other effects, lead to the oxidation of several cellular structures, damaging structural membrane lipids and nuclear DNA. This results in a drop in total motility, alterations in sperm kinematics standard, activation of cellular apoptotic death, compromised fertility, or later embryonic mitosis defects that lead to early embryonic loss or abortion (Gibb and Aitken, 2016). The sperm sample environment can be progressively affected by pH oscillations and osmotic changes resulting from natural metabolism or cell death. Such changes can compromise the remaining live cells and challenge their continued survival (Griggers et al., 2001).

Several factors may influence sperm quality once a sample is diluted or stored. While semen handling techniques, such as centrifugation, play a crucial role, other discrete harmful factors can also interfere. These include the type of extender used, the dilution rate, the storage temperature, bacterial load, subclinical urine contamination, and the raw semen quality itself, including the composition of the seminal plasma (Nunes et. al, 2015; Nunes et al., 2019).

The aim of this manuscript is to suggest a different perspective regarding the semen issues faced in equine practice. There is an established mindset that blames the stallion for failures in different semen presentations; however, once the real causes of sperm harm are understood, and healthy sperm is seen as a victim of wrong protocols and mishandling, the benefits of customizing protocols become clear. This stimulates laboratory leaders to search for excellence and not accept less than the best performance possible.

Individual Variation

Due to the considerable variation in individual sperm characteristics among stallions, the use of a single standardize extender composition when aiming to maximize shelf life and fertility rates is often insufficient. Because nutritional demands, membrane reinforcement, and protection requirements vary so widely, the most reliable approach is to conduct a split sample test. This involves comparing various commercial extenders which combine different substrates such as sugars, membrane protectors, antioxidants, and antibiotics using the same sperm concentration and evaluating quality at standardized intervals (Le Frapper et al., 2010).

Storage conditions, including room temperature, must also be compared, as there is a close correlation between this, sperm metabolism, and longevity. Higher temperatures accelerate sperm function, leading to increased ROS production and a shortened lifespan (Gibb et al., 2015). Generally, stallion sperm is stored at a concentration of 25 to 100 × 10^6 sperm/mL at either 5°C or 17°C (Varner et al, 1987; Gibb et al., 2018). These parameters are often adjusted based on the levels of cholesterol and phosphatidylcholine in the membrane, which determine the sperm's resistance to damage when exposed to lower temperatures (Drin-Bennett and White, 1977).

When considering freezing extenders, the freezing process presents an increased challenge to sperm structure and metabolism. Extenders must offer membrane reinforcement, nutritional support, improved antioxidant protection, and cryoprotectants to avoid cellular damage, including injuries related to water crystal formation during the freezing and thawing processes (Hammerstedt et. al, 1990). However, following this same logic and considering broad individual stallion variability, it is not reasonable to expect consistent high sperm quality if all samples are subjected to the same extender or cooling and freezing curves (Amann and Pickett, 1987). To maximize freezing results, it is recommended to perform a freezing test by splitting the sample according to the extenders being tested, as well as different stabilization periods and cooling curves, prior to selecting freezing protocols (Nunes at al., 2024a). This should be done using standardized sperm concentrations and thawing processes for all samples. Preferably, DNA, structural, and metabolic cellular parameters should be checked alongside sperm kinematics to choose the best protocol for each individual (Amann and Hammerstedt, 1993; Mocé and Graham, 2008).

Given the large individual genetic variation leading to variable cellular preferences, stallions are often classified according to their semen preservation capacity. In this context, stallion may be categorized as "good cooler" or "bad cooler", referring to their ability to maintain motility during refrigerated storage

(males maintaining or losing more than 30- 40% of original motility after 24 hours at 5°C, respectively (Brinsko et al., 2000). Similarly, stallion can be described as "good freezer" or "bad freezer" based upon post-thaw sperm quality (males generally above 40% of progressive motility or unable to maintain more than 20% progressive motility after thawing, respectively (Tischner, 1979), is inadequate if starting with a high-quality raw sample. Lower quality after semen handling reveals an inadequate protocol choice, leading to cellular alterations and damage at different levels. Certainly, there are individuals with weaker cellular characteristics for supporting low temperatures; these stallions require extra support to successfully undergo dilution and temperature drops, marking them as more sensitive to cryoinjuries. Due to this intrinsic inability to withstand semen handling challenges despite starting with high-quality raw semen, a classification of "more selective" or "less selective" is more representative. This perspective encourages the continued search for better extenders for more demanding stallions that fail to maintain semen quality after cooling or freezing. At the same time, it acknowledges that some stallions are highly versatile, achieving good results across many different protocols.

Freezing Extenders

During the last several decades, freezing extenders and their associated protocols have evolved considerably. These advancements utilize various combinations of intracellular and extracellular cryoprotectants, with a focus on smaller molecules that cross cell membranes more rapidly (Squires et al., 2004). Single-step protocols and ready-to-use commercial options have also become available, ranging from those requiring frozen storage to more recent lyophilized versions that remain stable at room temperature for months while yielding similar or improved results (Nunes et al., 2024b). This progress has certainly been motivated by the growing demand in the equine industry and the profitability of commercializing frozen semen doses.

However, despite this constant evolution, many protocols ignore the influence of the individual stallion on the final results. This often leads to rigid protocols that pair an extender with a specific freezing curve, whether fast or slow, based primarily on its dehydration capacity and cryoprotectant concentration. Unfortunately, this approach fails to account for unique sperm membrane characteristics. Suboptimal results often occur because freezing curves are not tested independently of the extender's recommended cooling rate (Munhoz et al, 2024). In some cases, post-thaw results are jeopardized by the assumption that slower curves are inherently safer, leading to the misclassification of a stallion's freezability (Nunes et al., 2024a; Munhoz et al., 2024b).

Another frequently ignored influence on freezing is the choice of centrifugation media, which is typically a cooling extender. This choice can dramatically alter motility results after thawing, even if centrifugation occurs shortly after initial collection or after brief cooled storage. Often, no difference in motility is visible between extenders at the time of centrifugation, yet the choice may trigger underlying cellular alterations that only become apparent after the more stressful freezing process (Nunes et al., 2025b).

Cooling Extenders

Over the decades, driven by market demand, cooling extenders have evolved into various combinations of sugars, antibiotics, and cellular membrane supports, such as cholesterol (Moraes et al., 2015). These formulas typically utilize milk or milk derivatives, with a few including egg yolk, and are presented in two forms: powder to be mixed with purified water or liquid ready-to-use. Despite these advancements, available formulas for years failed to consistently keep sperm alive for more than three days under refrigeration. This short shelf-life was often blamed on the stallion's individual inability to handle low temperatures, leading to their classification as "poor coolers." Marketing strategies frequently reinforced this idea by offering products specifically developed for "weak" sperm or certain breeds, rather than highlighting the need for extender trials to address individual variation or critiquing the use of standardized global dilution protocols.

Recently, a new type of extender has entered the market focusing primarily on protecting cells from damage caused by metabolic reactive oxygen species (ROS). By including antioxidants in its formula and maintaining a storage temperature of 17°C instead of the usual 5°C, it avoids the structural damage associated with temperature drops in more fragile samples (Gibb et al., 2018). Initially, for commercial reasons, this generation of extenders was marketed as a solution for "bad cooler" stallions. This was a contradictory mindset; by changing the protocol, individuals previously considered inadequate were suddenly able to survive up to seven days at 17°C. This proved that the limited shelf-life was often related to the extenders ignoring specific sperm demands rather than the stallion itself.

To achieve these results, a better quality, preferably purified, sample was required. While the higher temperature prevents membrane damage, it leads to higher cellular metabolism and oxidative stress, necessitating adjusted antioxidant protection that does not block the processes essential for sperm capacitation and fertility (Medica et al., 2026; Peña and Gibb, 2022). This new scenario also had to address bacterial and fungal growth at warmer temperatures over longer incubation periods by including antimicrobial agents (Price et al., 2008). For longer shelf-life and better standardization, a formula without animal proteins was ideal. However, because standard stallion semen collection often results in the sperm being mixed with seminal plasma, where binder of sperm proteins (BSPs) can compromise the membrane during long exposure, an initial dilution with milk-based extenders remains beneficial (Bergeron and Manjunath, 2012). The casein in these extenders protects the membrane structure during initial exposure. After mitigating the deleterious effects of the seminal plasma, it is removed by centrifugation, and the resulting sperm pellet is resuspended in the new extender, designated as UoN by the authors (Gibb et al., 2018).

Removal of Seminal Plasma

One of the mandatory steps for keeping the sperm cell alive longer is the mitigation of the harmful effects of seminal plasma through proper dilution or, preferably, its removal (Rigby et al., 2001). Seminal plasma is a natural component of the stallion ejaculate produced by the accessory glands in the male reproductive tract. Among many other functions, it is responsible for promoting sperm motility after release from the epididymis (Nunes et al., 2019) and for the immunomodulation of the endometrial inflammatory response immediately after intrauterine ejaculation (Troedsson et al., 2005). Both are ephemeral actions, not naturally selected throughout the species' evolution to last for a long time. The sperm cell was not designed to interact with seminal plasma for extended periods, as occurs in semen doses that are only diluted and this prolonged exposure causes membrane damage by depleting primarily lipids and cholesterol from its composition (Bergeron and Manjunath, 2012).

Seminal plasma removal can be performed via centrifugation, followed by eliminating the supernatant through aspiration. Precautions must be taken to avoid excessive g-force or prolonged processing, as these can generate a compact pellet at the bottom of the centrifuge tube, posing a high risk of mechanical cellular damage. The suggested centrifugation force for semen samples diluted with milk-based extenders is up to 600 g for a maximum of 20 minutes (Loomis, 2006).

To minimize sperm loss while aspirating the seminal plasma, up to 1000 g can be used for 15 minutes to push all cells to the bottom of the conical tube. However, to prevent excessive cellular compaction, a cushioned protocol is highly recommended by adding iodixanol colloid beneath the sample (Ecot et al., 2005).

As an alternative for seminal plasma removal that does not require centrifugation, specialized semen filters are available on the market (Roach et al., 2016). These are often marketed as a more affordable procedure when a centrifuge is unavailable or as an alternative for stallions that do not tolerate centrifugation well. However, their benefits remain controversial; the lengthy processing time may lead to temperature oscillations, bacterial contamination, and cellular damage. Furthermore, exposing the sample to high concentrations for an extended period during processing can result in osmotic shock and concentrated ROS production at room temperature.

Sperm Selection

Colloid Centrifugation

An improved method for removing seminal plasma and initial extenders, which are used immediately after collection to provide basic cellular protection, is density centrifugation. This process involves using a moderate g force to pass the sample through a selective colloid gradient (Morrell, 2009).

This technique allows the most robust sperm, characterized by better progressive motility, fewer morphological defects, and higher density, to cross the density gradient faster and reach the bottom of the conical tube using a swing out rotor centrifuge (Morrell and Nunes, 2018). This method can utilize either a single-phase gradient (single layer centrifugation) or two distinct phases (double layer centrifugation) where liquids of different densities are layered to refine the selection process (Morrell and Rodriguez-Martinez, 2011).

Throughout its daily use in semen laboratories, further applications and advantages of this technique have become more in the spotlight. It results in samples with longer survival rates even when

using standard milk-based extenders. This is achieved not only by removing seminal plasma and its harmful effects but also by filtering out morphologically imperfect and dead sperm cells. Both of these cell types release high levels of reactive oxygen species (ROS), which negatively alter the sample environment and shorten shelf life (Morrell et al., 2013). Among these benefits, since abnormal morphology often indicates poor spermatogenesis, these cells frequently carry DNA issues. Consequently, colloid selection yields a sample with significantly lower overall DNA damage (Al-Kass et al., 2025).

The density sperm selection is based on actively and progressively moving cells. As a result, the bacterial load (Morrell et al., 2014) any urine (Ellerbrock et al., 2018) present in the original sample are significantly reduced, generating a sample with higher motility and lower contamination. This technique can be effectively applied to fresh diluted samples before any targeted use (Nunes et al., 2024b) to create an improved starting sample for processing. It is also helpful for thawed semen straws (Nunes et al., 2016) and epididymal samples prior or after freezing (Santos et al, 2017; Santos et al., 2020) to remove dead cells as well as common blood, bacterial, and cellular debris.

This purification reinforces the idea that the classification of some sires as "bad freezers" or "bad coolers" is often unfair. By starting protocols with a purified sample, many of these stallions are able to produce high quality semen in both chilled and frozen presentations (Nunes et al., 2024). This proves that previous poor results were often a consequence of a highly impure original sample rather than the sire himself, ultimately allowing for the successful shipping and freezing of doses from stallions previously labelled as poor quality individuals.

Microfluidics

The most recent method for sperm separation involves the use of microfluidic membranes. This technique selects sperm based on head dimensions using specific pore sizes and the ability of the cells to actively cross the membrane for subsequent retrieval. This results in a sample with improved kinematics and superior morphology (Huang et al., 2023). Because this method does not require centrifugation or colloid preparations, it is an easy technique that can be effectively managed even by a basic skilled operator.

One device currently available on the market includes models that can be loaded with 800 µl or 3000 µl, with the latter being suitable for both bovine and equine use. This method is applicable to any semen sample, whether raw, chilled, or thawed (Samuel et al., 2018). However, a more active sample will result in better sperm retrieval, making it preferable to use an extender that supports high motility during the processing time at 37°C.

Using stallion semen in a device designed for bovine samples resulted in higher sperm recovery but lower motility, demonstrating that it was less effective at selecting the most motile population. In a trial testing concentration of 60, 120, and 180 million total sperm per ml loaded into a 3000 µl device, sperm recovery increased proportionally with the initial load, suggesting the membrane's maximum capacity had not yet been reached or blocked (Nunes et al., 2025a). For procedures such as ICSI and IVF, which require only a few high-quality sperm, these devices are highly useful as they can retrieve approximately 25% of the total initial load.

Further scientific investigation is required to determine the maximum sperm load for the 3000 µl devices and how this influences the total sperm recovered. Establishing these limits would make the method more viable for field use with raw-diluted semen, chilled doses, and frozen-thawed straws, aiming for the recovery of a minimum artificial insemination dose of selected sperm cells with expected improved fertility. Additionally, a recent unpublished study (Nunes et al., 2025a; Munhoz et al, 2025) indicated that beyond improvements in motility and morphology, this approach also reduces bacterial load and urine contamination, thereby extending the viability of semen doses throughout the storage period. Both studies are scheduled to be presented at the International Congress of Animal Reproduction 2026, to be held in Obihiro, Japan.

Techniques Combinations

A combination of microfluidic selection using thawed semen and a resuspension of the recovered cells with a long shelf-life extender resulted in good embryo recovery using a protocol with fixed time inseminations (Morris et al., 2024). This proves that selecting a high-quality sperm population and removing freezing extenders, dead cells, and harmful substances from the dose environment results in prolonged sperm viability and fertility. The UoN extender trial mentioned earlier (Gibb et al., 2018) was the first to achieve seven-day sperm storage, combining its advanced antioxidant formula with single-layer

centrifugation, the trial successfully purified samples prior to storage, minimizing damage from cell degradation and contaminants.

In another trial, collected diluted semen samples submitted to microfluidic selection demonstrated that selected samples had superior quality and maintained better motility during refrigerated storage when a long shelf-life extender was used compared to a milk-based extender (Munhoz et al., 2025a).

In the Middle East, the first pregnancy obtained with a long shelf-life extender was made possible using stallion semen that could not be maintained for three days at 5°C in a milk-based extender (Munhoz et al., 2025b). By using cushioned centrifugation and resuspending the sample with this new generation extender, the semen was successfully used for insemination six days after collection. This same sample was evaluated for up to 10 days with good motility. Recent publications demonstrate that, with or without density centrifugation sperm selection, available extenders are able to maintain semen doses at 5°C or 17°C for up to 14 days while still achieving good pregnancy rates (Brito et al., 2025).

Final Considerations

Focusing on stallion individualities and protocol customization makes it possible to achieve outstanding results with high quality raw semen for freezing or cooling. It is often unfair to classify these sires as "bad coolers" or "bad freezers" if only one or few protocols were tested. These more selective stallions are able to achieve results as good as any others, but they demand more effort to determine the proper protocols. It is essential to keep in mind that the goal for any semen handling must be to maintain the highest possible initial quality by customizing extenders, cooling curves, and storage temperatures (Freitas and Nunes, 2024).

Low quality samples, and even high-quality ones, can benefit from centrifugation methods and sperm selection protocols. These methods remove seminal plasma and reduce its deleterious effects, while also improving quality, shelf life, and fertility by reducing bacterial load, removing dead sperm, mitigating ROS damage, and clearing urine contamination.

Ultimately, customized protocols combined with advanced semen handling techniques result in improved doses with higher motility, shelf life, and fertility.

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