

Effects of equine seminal plasma on fertility and impacts on reproductive biotechnologies: a comprehensive review

Tayná da Silva, Shara Silva, Júlia Marlière, Bruna Waddington
Veterinary Department, Federal University of Viçosa, Viçosa, MG, Brazil

Abstract

Stallion fertility is crucial for the equine industry, yet selection of stallions focuses on phenotypical, temperamental, and performance traits, with limited attention to reproductive performance. Seminal plasma is composed of fluids from the testes, epididymis, and accessory glands; it has a central role in sperm survival and preservation by providing energy, antioxidants, and proteins. Biochemical composition of seminal plasma, including specific proteins and enzymes, can affect sperm quality and cryopreservation success. Literature presents conflicting views on the effects of maintaining, removing, or adding seminal plasma after cryopreservation. Individual variations in seminal plasma composition can have substantial impact on stallion fertility and predict the success of these biotechnologies.

Keywords: Stallion, sperm, ejaculate, cryopreservation, fertility

Introduction

Stallion fertility is a crucial factor within the equine industry. However, unlike other production species, stallions are primarily selected according to their phenotypical conformation, athletic aptitude, temperament, genetic potential, and financial value, with limited emphasis on reproductive performance.¹ Several factors can affect fertility, stemming from genetics, climate change, behavioral and environmental characteristics, breeding management, exercise regimen and competition, and pathologies, requiring clinical and pathological diagnoses to determine the reproductive prognosis.²⁻⁷

Before a new stallion is introduced into reproductive activities, it should have a thorough examination in which features such as pedigree and behavioral characteristics, overall health and reproductive soundness (both anatomical and functional) should be assessed.⁸ Semen biotechnologies are widely employed in the equine industry, offering greater flexibility for artificial insemination that has grown in popularity, particularly when the primary goal is to accelerate genetic improvement through the production of superior offspring.^{9,10}

Seminal quality is decisive for reproductive success in biotechnology application. Processes such as semen cooling and, in particular, freezing, extend seminal viability but can cause damage to sperm due to irreversible plasma membrane

lesions, reducing fertilization potential.^{11,12} Stallion ejaculate consists of a liquid and a cellular fraction, expelled, though not uniformly, in a variable number of squirts. In general, according to sperm concentration, the ejaculate can be divided into 3 consecutive fractions: preperm, spermrich, and postperm.¹³

Seminal plasma constitutes the liquid portion of the ejaculate and comprises fluids produced and secreted by testes, epididymides, and accessory sexual glands.¹⁴ Its components have a substantial role in sperm survival by providing energy substrate to sperm, as well as amino acids, lipids, proteins, steroid hormones, enzymatic and nonenzymatic antioxidants, and micronutrients like selenium, zinc, copper, and iron, among others.¹⁵⁻²¹ There is disagreement regarding the addition or removal of seminal plasma during cryopreservation. Therefore, the objective of this review is to compile current available data about the seminal plasma profile and its impacts on reproductive biotechnologies and fertility.

Seminal plasma

As in other mammalian species, stallion sperm are not promptly capable of fertilization once they leave testes.²² Posttesticular maturation occurs in 2 steps: maturation and capacitation/acrosome reaction. Maturation occurs during sperm transit in the epididymis and capacitation and

acrosome reactions occur in mare's reproductive tract. Both of these events comprise a series of structural and functional changes to sperm so that they become fertilization-competent.^{19,23} Some of the critical changes include acquisition of progressive motility and ability to recognize and bind to zona pellucida, chromatin condensation, cholesterol depletion with increase in membrane fluidity, increase of cytoplasmic pH, cAMP, and calcium concentrations, calcium influx through the acrosomal membrane, and exocytosis.²⁴⁻²⁶

During ejaculation, the prespermatic fraction is the first to be released and removes impurities from the urethra, being mainly composed of fluids originating from the bulbourethral glands.¹³ Sperm-rich fraction is then released, characterized by its milky appearance due to high sperm concentration (containing up to 80% of the total sperm) and its composition derives from secretions of the epididymis, ampulla, and deferens ducts.²⁷ Although the last fraction does not contain substantial sperm, it is responsible for transporting residual sperm from stallion's urethra through vesicular glands' secretions.¹³ The volume of the ejaculate fractions can be altered by excessive teasing or sexual preparation of the stallion and false mounts (increases the volume of seminal plasma) and consequently, the total volume of semen.²⁸

Biochemical composition of the seminal plasma

Proteins and enzymes

Proteins are the main component of the seminal plasma, and epididymis is the site where ~ 70% of seminal proteins are secreted.²⁹ Although it is the most abundant in the seminal plasma, stallion seminal plasma protein concentration is lower than other mammalian species, with a mean concentration of 10 mg/ml. This is of particular interest when evaluating the role of seminal plasma components due to their participation in crucial processes before fertilization.³⁰ Exact information regarding fertility-associated proteins remains unclear. Although much research has been carried out on the topic, current information about those proteins is still insufficient to establish diagnostic protocols for stallion fertility based on seminal plasma composition.³¹

Some of the major proteins present in the stallion seminal plasma are horse seminal plasma proteins (HSP), a group of 8 recognized low molecular weight proteins.³² With the exception of HSP-4, all HSPs are able to bind to the sperm surface at ejaculation;³⁰ 2 of these proteins, HSP-1 and HSP-2, account for up to 80% of the total seminal protein content. They belong, although not exclusively, to 3 structurally varying seminal protein groups: HSP-1 and HSP-2 belong to the fibronectin type II proteins, HSP-3 belongs to the cysteine-rich secretory proteins (CRISPs), and HSP-7 belongs to the spermadhesins.^{32,33}

HSP-1 and -2 are capable of binding to sperm surface through choline phospholipids present in the gamete membrane, leading to cholesterol efflux, an important event for sperm capacitation.^{26,34} However, the influence of the concentration of these proteins in seminal plasma on fertility was discussed and is questionable. Some reports observed high concentrations of HSP-1 and -2 in the ejaculates of stallions with poor fertility.^{17,35,36} A possible explanation for this finding is the ability of these proteins to increase the permeability of the sperm membrane, compromising its integrity.³⁷

CRISP proteins are divided into 3 main classes, characterized by their structural composition of 16 cysteine residues: CRISP-1, CRISP-2, and CRISP-3 (HSP-3).^{32,33} They are the product of secretions from the epididymis, ampulla and seminal vesicle, and throughout the reproductive tract, CRISP-3 is in higher concentration compared to others.³⁸ Its presence has effects not only on sperm but also on the reproductive tract of the mare after breeding.

In fresh semen, high concentration of CRISP-3/HSP-3 in the seminal plasma was associated with increased sperm motility and seminal quality index.³⁹ It also has an immunomodulatory effect on the mare reproductive tract during the physiological uterine postbreeding inflammatory response, suppressing the binding between sperm and polymorphonuclear neutrophils and increasing fertility.^{40,41}

HSP-4 and HSP-5 are proteins with little or no clear functions documented at this time. HSP-4 is presumed to be related to a calcitonin-like product, impacting sperm motility in men, whereas HSP-5 has not yet been associated with any specific protein.^{32,42} In stallions, the HSP-7 is the only identified member of the sperm adhesins. It is homologous to boar spermadhesin (AWN) protein and its participation during sperm binding to the zona pellucida.^{30,43} HSP-6 and HSP-8 are isoforms of the same protein, belonging to the kallikrein family, but their function is not well established.³²

Immunoglobulins are also present in the seminal plasma. Izumo sperm-egg fusion protein 4 (IZUMO4) was isolated from equine seminal plasma,⁴⁴ a protein belonging to the immunoglobulin group. It was suggested that its function is correlated to prefertilization stages, such as capacitation.⁴⁴ The Izumo multiprotein family is composed of 4 proteins; Izumo 1, Izumo 2, Izumo 3, and Izumo 4 and has already been described as a sperm membrane protein in mice and humans.^{45,46} In these species, the izumo immunoglobulins are expressed on the cellular membrane surface, being essential for gamete fusion during fertilization.

Some enzymes have been isolated and described in equine seminal plasma. Alkaline phosphatases are a group of isoenzymes widely distributed through various horse tissues, such as intestinal mucosa, liver, bones, renal tubule, lungs, and placenta.⁴⁷⁻⁵⁰ Its sources include testicular and epididymal fluids, and for this reason, it can be used as an ejaculation completion marker in normal stallions. Low alkaline phosphatase concentration in the seminal plasma may indicate ejaculatory failure and/or ampullary blockage, being an important clinical tool to differentiate these conditions from azoospermia.⁵¹

Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are gelatinases responsible for degrading extracellular matrix components during tissue restructuring, in physiological and pathological conditions. They are secreted in latent forms and are present in all fractions of the stallion ejaculate. However, there still no evidence to support the use of metalloproteinases expression as a marker for stallion semen quality.⁵²

Enzymatic and nonenzymatic antioxidants

The large group of enzymes identified in the stallion seminal plasma is related to the oxidative balance. As aerobic cells, sperm face the oxidative paradox: oxygen is needed for energy production, but its metabolites can cause significant, and

sometimes irreversible, cellular damage.⁵³ Reactive oxygen species (ROS) act as mediators for the transfer of electrons in several normal biochemical reactions. All aerobic organisms are continually subject to the oxidizing effects of these metabolites, as ROS is the result of aerobic metabolism.⁵⁴

Stallion sperm, unlike human sperm, predominantly use oxidative phosphorylation to produce energy.⁵³ The increased energy production through phosphorylation results in a rapid production of ROS. Under normal conditions, ROS production is balanced by the action of antioxidant systems, both enzymatic and nonenzymatic. Moreover, ROS are necessary mediators for physiological sperm processes, such as tyrosine phosphorylation and capacitation.⁵⁵ However, the accumulation of ROS leads to the oxidation of biomolecules, including peroxidation of lipid membranes, protein damage, carbohydrate oxidation, and destruction of nuclear and mitochondrial DNA and RNA.⁵⁶ Mammalian sperm, due to the high concentration of polyunsaturated fatty acids in the plasma membrane, are particularly sensitive to oxidative damage, especially lipid peroxidation.⁵⁷

Catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase are antioxidant enzymes that act as ROS scavengers and have been identified in the stallion seminal plasma.^{58,59} Superoxide dismutase activity has been associated with a function other than ROS neutralization. In association with lactoferrin, an iron-binding protein identified in stallion epididymal secretion, superoxide dismutase-3 forms a complex that binds itself to nonviable sperm, facilitating their elimination by phagocytosis in mare's reproductive tract.^{18,60} Previously mentioned, HSP-1 and HSP-2 have also been suggested to act as chaperones against oxidative damage and may be able to prevent ROS production.⁶¹

Minerals can contribute, alone or in combination with enzymes, to sperm quality. Calcium, selenium, zinc, copper, and molybdenum are positively associated with sperm morphology, motility, and fertility, whereas higher iron and chrome concentrations were associated with lower fertility in Arabian stallions.^{21,62,63} Copper, zinc, and manganese are important cofactors for superoxide dismutase activity.⁶⁴

Other seminal components

The carbohydrate concentration in the stallion seminal plasma is substantially lower compared to bull and ram ejaculates, and it is suggested that the gel secreted by the seminal vesicles is the main source of carbohydrates. Inositol and glucose are in stallion semen, whereas fructose is only in insignificant concentrations.^{65,66} The lipid content of seminal plasma is low, consisting mainly of phospholipids and cholesterol. The phospholipid-to-cholesterol ratio in seminal plasma is crucial for maintaining membrane integrity and controlling the rate of sperm capacitation through the acrosomal reaction. Infertile stallions with normal spermograms have a phospholipid:cholesterol ratio 2.5 times > fertile stallions (Figure).⁶⁷

A considerable number of hormones can be identified in the seminal plasma. Leptin is directly correlated with the percentage of abnormal sperm in Arabian stallions.⁶⁸ Sperm motility and acrosomal integrity can be positively associated with seminal cortisol, progesterone and insulin concentrations.⁶⁹ Prostaglandin E₂ acts on mare's reproductive tract, increasing

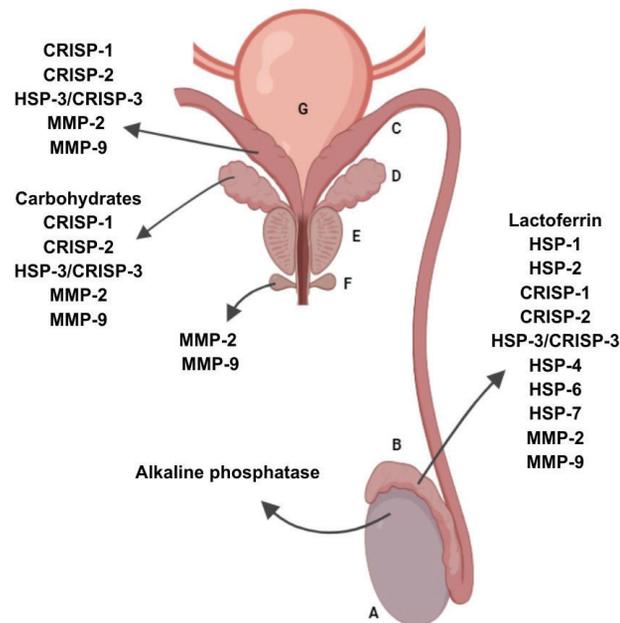


Figure. Stallion seminal plasma components whose production and secretion sites have been identified. A: testicle; B: epididymis; C: ampulla of vas deferens; D: vesicular gland; E: prostate gland; F: bulbourethral gland; G: bladder. CRISP: cysteine-rich secretory protein; HSP: horse seminal plasma protein; MMP: matrix metalloproteinase

the oviduct lumen diameter, thereby facilitating sperm transportation. Seminal prostaglandin F_{2α} concentrations are higher in stallions than in bulls and can affect uterine mechanical clearance during physiological postbreeding endometritis.^{70,71}

Impacts on biotechnologies: cooling and freezing

Reproductive technologies are an essential part of the equine industry and the possibility of selling cooled or frozen semen from genetically valuable stallions is one of its pillars.^{10,72} Use of these biotechnologies enables long-term semen storage and preservation, safe transportation over long distances without compromising quality, and reduced risk of spreading reproductive diseases.⁷³

The equine species is considered challenging for cryopreservation, and stallions can be classified as 'good' or 'poor' freezers based on postthawing seminal evaluations. It is estimated that 20-50% of stallions are poor freezers. Poor freezers have low tolerance to the cryopreservation process, resulting in low progressive sperm motility (< 35%) and a high percentage of dead and immotile sperm.⁷⁴

Sperm quality can be assessed in various ways; common methods use total sperm motility, progressive sperm motility, membrane and acrosomal integrity, and chromatin condensation.⁷⁵ Individual differences between seminal plasma composition and the freezability of stallions is important to consider, as some individuals may not have satisfactory results despite having adequate seminal parameters prefreezing or cooling. For this reason, studies have focused on proteomics and specific biochemical profiling of seminal plasma

with the goal of identifying differences between good and poor freezers.^{69,74,76,77}

Reduced semen fertility after cooling or freezing processes is correlated with drastic temperature changes, osmotic pressure, pH changes, early capacitation, and oxidative stress, all of which affect cellular architecture and biochemical composition.⁷⁸⁻⁸¹ At the final stages of spermatogenesis, sperm become incapable of repairing biomolecular damage, and their metabolism becomes simplified, making the damage caused by these processes irreversible.⁸²

In the literature, there is still no consensus among authors on the maintenance, removal, or postthaw addition of seminal plasma to improve sperm parameters. Some authors report improved postthaw sperm mobility, increased membrane stability and reduced capacitation rates with the addition of seminal plasma.^{72,81,83,84} Others reported no effect on sperm quality.⁸⁵⁻⁸⁸

There was no significant difference in lipid peroxidation after the postthaw addition of seminal plasma.⁸¹

DNA integrity was preserved at 24-48 hours by prior removal of seminal plasma, whereas damage was observed with its addition.⁸⁹ Addition of only autologous seminal plasma after 48 hours of cool semen storage was detrimental to total and progressive sperm motility but there was a reported increase in kinematic parameters with the combination of commercial extenders and 2 concentrations of seminal plasma.⁹⁰

These contradictory results can be attributed to the many differences in methodology present in each study, such as sperm selection, the type of extender used, the use of homologous or heterologous seminal plasma and its concentration when present, the timing of seminal plasma addition (before or postthaw/cooling), and individual variation in seminal plasma composition.

Individual variations in the composition of the seminal plasma are often observed between good and poor freezers. Lower concentrations of vitamin E were in the seminal plasma of good freezers, but the importance of this finding is yet unclear.⁷⁶ In addition, 6 proteins as discriminant variables were used for identifying good freezer stallions; 1 was mitochondrial NADH dehydrogenase (ubiquinone) a component of the mitochondrial respiratory chain that in its reduced form acts as an antioxidant.^{74,91} Differences in phospholipid profiles, evaluated with lipidomics, were also identified between good and poor freezers.⁷⁷ Good freezer stallions had higher concentrations of cortisol, estradiol, insulin, triiodothyronine, and thyroxine in their seminal plasma.⁶⁹

Conclusion

A comprehensive understanding of seminal plasma composition and function in stallions is key to improving the use of reproductive technologies and, consequently, fertility in general. Although substantial progress has been made in identification of seminal plasma proteins, enzymes, and antioxidants of fertile stallions, gaps remain in the practical application of this knowledge, particularly in semen cryopreservation. Individual variations among stallions highlights the need for

personalized approaches to optimize cryopreservation results and ensure sperm fertility. Therefore, additional research is needed to develop techniques that account for individual variabilities, aiming to maximize reproductive potential.

Conflict of interest

None to report.

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