Review Report

Stallion sperm cryopreservation: concepts in cryopreservation-induced sperm damage, processing, analysis, and utilization in assisted reproductive technologies*

Camilo Hernández-Avilés
Equine Fertility Laboratory, Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA

Abstract

Semen cryopreservation remains the only viable method for long-term stallion sperm preservation. Increased demand for frozen/thawed sperm in Assisted Reproductive Technologies (ARTs) continues to result in a considerable body of research determining the effects of cryopreservation on stallion sperm and clinical interpretation of sperm quality in frozen/thawed semen. Some concepts regarding the impacts of cryopreservation on stallion sperm or methods for sperm cryopreservation might limit the widespread implementation of this technique in private practice settings. Clinically relevant data on stallion sperm cryopreservation, various protocols for sperm cryopreservation, concepts on postthaw sperm quality analysis, and utilization of frozen/thawed sperm for ARTs are summarized.

Keywords: Stallion sperm, cryopreservation, cryoprotectants, semen extender, postthaw quality

Introduction

Sperm cryopreservation, to date, is the primary method for long-term sperm preservation in domestic animals, including horses. Since the initial reports by Russian,[1] British,[2] Japanese,[3,4] German,[5] and Polish[6] scientists regarding stallion sperm cryopreservation and the first report of successful insemination of a mare with frozen/thawed (epididymal) sperm,[7] there has been an exponential increase in the 'quest' for suitable and repeatable methods for sperm cryopreservation that may eventually replace fresh or cool-stored semen utilization for artificial insemination (AI) of mares. Multiple benefits of sperm cryopreservation and AI in horses have been described.[8] Because of these, European, Asian (Japan, China), and North American workers have dedicated extensive research programs focused on optimizing AI in mares with frozen/thawed semen, trying to emulate dairy breeding industry programs of 1960's to 1970's.

Studies conducted in 1970's to 1980's[9] were reviewed,[8,10] which reported 30-60% per cycle pregnancy rates in mares inseminated (daily until end of estrus) with frozen/thawed sperm. Recent retrospective studies[11] (> 2,000 mares bred in 2004) reported 46-48% per cycle pregnancy rates in mares inseminated twice in their cycle (24 hours apart). Currently, similar per cycle pregnancy rates can be obtained by 1 insemination performed immediately after induced (with a synchronizing agent) ovulation (determined via transrectal palpation and ultrasonography, every 6-8 hours, starting 12-24 hours after treatment).[12-15] Variations[16] of this protocol 'simplified' ovulation synchronization, frequency of transrectal examinations, and AI after detected ovulation. Overall, it is apparent that insemination of frozen/thawed stallion sperm can yield widely accepted fertility rates (i.e. per cycle pregnancy rates of 40-60%) for commercial settings, provided adequate breeding management, and mare and stallion intrinsic fertility are met. Nevertheless, any of these results are affected by other factors, such as availability of frozen sperm from a given stallion (often limited by stallion owner/agent), semen processing and resulting postthaw sperm quality, and availability and knowledge of the personnel (i.e. veterinarian) involved in insemination.

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CONTACT Camilo Hernández-Avilés **chernandez@cvm.tamu.edu**

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More mares are currently bred with frozen/thawed sperm than before; yet, use of this technology for AI may still be less than fresh or cool-stored semen. Such a trend has not changed since mid-1990's,[17] and may be attributed to multiple factors, including specific breed registries regulations (e.g. only until 2000, the American Quarter Horse Association [world's largest breed registry] allowed foals registration conceived by AI with frozen/thawed sperm), difficulties in standardization of stallion sperm cryopreservation methods under field conditions,[18] lack of consensus regarding 'minimum' criteria for postthaw sperm quality,[18,19] and the relative 'low' relationship between in vitro sperm quality assays and in vivo stallion fertility.[20-23] Although the aim is not to provide extensive review of what has or has not been done regarding sperm cryopreservation in stallions, author prefers to review some relevant concepts on stallion sperm cryopreservation and discuss specific recent data that originated (author and his coworkers) regarding cryopreservation of stallion sperm, analysis of postthaw quality in frozen/thawed stallion semen, and frozen/thawed stallion sperm use for intracytoplasmic sperm injection (ICSI) in mares.

Cellular and molecular changes due to cryopreservation of stallion sperm: what else can we ask them to survive?

Author names this section as a tribute to a seminal review paper[24] by Roy Hammerstedt who in conjunction with James Graham and John Nolan conducted several studies on certain effects of cryopreservation on sperm plasma membranes, particularly in ruminants, using flow cytometry-based assays to study sperm function.[25-27] Certainly, the title ('what we ask them to survive') of the paper[24] attested to challenges researchers and clinicians faced then that we still face when devising methods to maximize survival and fertilizing ability of frozen/thawed stallion sperm. After all, it is surprising that sperm subpopulations are still able to survive and maintain fertilizing competence after sustaining such marked change in their membrane composition and overall function as a result of: 1. temperature-associated metabolic restriction; 2. conformational and functional changes at the organelle level (i.e. plasma membranes, mitochondria) due to exposure to supra (5-0°C) and subzero (< 0°C) temperatures; 3. osmotic changes during cryopreservation and after thawing caused by exposure to high molecular weight penetrating and nonpenetrating cryoprotectants and water freezing; and 4. resulting cellular (i.e. oxidative stress, apoptosis) and molecular (e.g. protein posttranslational modifications) changes that follow osmotic stress (Figure 1). Some of these changes will be discussed.

Sperm plasma membrane, similar to somatic cells, is composed of a phospholipid bilayer with extracellular, intracellular, and embedded transmembrane proteins, fatty acids, and a glycocalyx with potential roles during sperm oviduct/oocyte interactions.[28-30] More comprehensive reviews[31-34] described composition and function of this fascinating organelle, particularly during fertilization. Considering sperm plasma membrane intricacy and composition, it is not surprising that multiple changes at the phospholipid or protein conformation level would result in membrane damage and subsequently, sperm death.

For this review, consider the multiple steps required to freeze stallion sperm (Figure 2). Although initial dilution of the ejaculate with a prewarmed skim milk or casein-based extender (generally isosmotic: 300-350 mOsm/kg) does not result in considerable changes to plasma membrane, temperature reduction (from 37 to ~ 22-20°C) during centrifugation theoretically would cause changes in sperm metabolic rate[35] and semen freezing extender addition would affect plasma membrane organization due to hyperosmolality (semen freezing extenders have an osmolality that ranges 800-1200 mOsm/kg).[36] Additionally, temperature reduction during the initial prefreezing cooling phase (20-5°C) will induce conformational modifications to plasma membrane architecture due to the so-called 'thermotropic phase' change, whereby the plasma membrane phospholipids change from a 'fluid' (liquid-crystalline) to a 'gel' state due to interactions among hydrophobic phospholipid acyl chains (Figure 1).[37-40] These changes, in conjunction with reduced metabolic rate due to temperature restriction, have induced higher membrane permeability, mainly to ions such as potassium and calcium,[41] alterations in membrane potential and permeability, loss of intracellular calcium regulation, and reduced sperm motility and membrane intactness in other mammalian sperm models, such as boars or ruminants (Figure 1).[24,41] To author's knowledge, some of these changes have not yet been studied in stallion sperm, but it is generally assumed that similar alterations would occur during cooling and cryopreservation.

After dilution of sperm with a freezing extender and exposure to lower temperatures (i.e. 5°C), additional conformational changes occur to plasma membrane due to 'lyotropic membrane phase transitions,' wherein water within the plasma membrane begins to freeze due to exposure to temperatures near 0°C.[42] Furthermore, when temperature reaches below 0°C, extracellular ice formation begins due to extracellular water freezing. Such an increase in extracellular milieu osmolality further induces osmotic changes that will cause sperm dehydration (Figure 1). Although sperm dehydration is pivotal for survival after cryopreservation and thawing, excessive cell dehydration would result in plasma membrane rupture and cell death.[43] Studies[44-46] have reported that freshly ejaculated stallion sperm can withstand specific ranges of 'anisosmolality,' mainly when sperm are suspended in a medium at 300 mOsm/kg (isosmolar), rapidly exposed to hyperosmolar conditions (900 mOsm/kg) and then exposed again to isosmolar conditions. Interestingly, a transmission electron microscopy study[47] reported that hypoosmotic conditions (75-100 mOsm/kg) are more harmful to plasma membrane and mitochondrial structure of fresh stallion sperm compared to hyperosmotic conditions (900 mOsm/kg), particularly when sperm are rapidly removed from hyperosmotic to hypoosmotic media. Such data can be interpreted as rapid exposure from hyperosmotic (during freezing) to isoosmotic conditions (after thawing) causes most damage to stallion sperm. Nevertheless, serial dilution of cryopreserved stallion sperm into a skim milk-based extender with decreasing concentrations of cryoprotectant (glycerol [gly]) to avoid sudden changes in sperm osmolality did not yield higher motility or plasma membrane intactness than immediate exposure from hyperosmotic to isosmotic conditions.[48] Hence, the exact mechanism by which exposure to hyperosmotic conditions during cryopreservation (i.e. cryopreservation extender, water freezing, or both) and hypoosmotic conditions during thawing affect stallion sperm quality is not entirely understood. Changes to fresh stallion sperm exposed to osmotic imbalances (also known as 'osmotic excursions') in plasma membrane and mitochondrial structure and function (i.e. intactness and membrane potential) have been extensively reported.[44-47] Additionally, it has been described that exposure of fresh stallion sperm to osmotic excursions, particularly iso-to-hyperosmolality, results in changes in sperm cell volume but not dramatic decreases in

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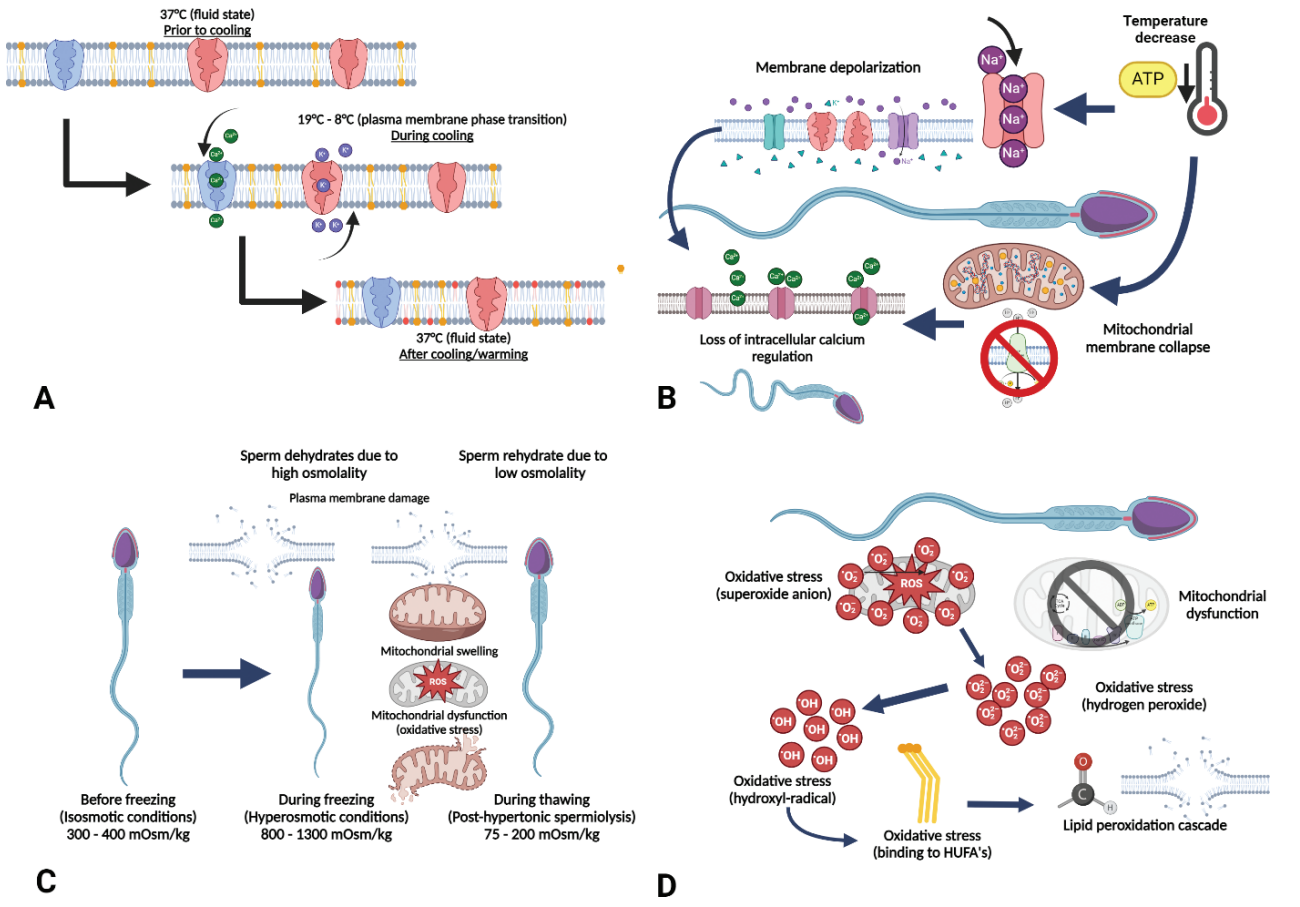

Figure 1. A graphical representation of some of the cellular changes that stallion sperm undergo during cryopreservation, including A. plasma membrane conformation changes due to reduced temperature; B. metabolic rate reduction due to reduced temperatures; C. osmotic-associated stress due to exposure to hyperosmolality during freezing; and D. oxidative stress associated to osmotic damage to the sperm mitochondria. Figure created with BioRender (www.biorender.com).
ROS: Reactive oxygen species; HUFA's: Highly unsaturated fatty acids

the percentage of plasma membrane-intact sperm.[49] In contrast, exposure from hypo-to-isosmolality resulted in mitochondrial membrane potential collapse.[49] Such changes in mitochondrial membrane potential have been associated with decreased mitochondrial function, deregulation of reactive oxygen species (ROS) production, and occurrence of mitochondrial-mediated apoptosis (Figure 1).[50-54]

Relationship between osmotic and oxidative stress (or decreased oxidation-reduction [redox] balance) in fresh stallion sperm was initially demonstrated[55]; stallion sperm exposed to osmotic excursions display an increased production of superoxide anion, a common byproduct of mitochondrial metabolism that in turn was potentially associated with activation of nicotinamide adenine dinucleotide phosphate oxidase.[55] Additionally, multiple studies[50-54] have elucidated various cellular and molecular changes stallion sperm undergo after a freezing/thawing cycle (Figure 1). After freezing and thawing, a subpopulation of plasma membrane-intact sperm had increased concentrations of lipid peroxidation byproduct 4-hydroxynonenal, activated caspases (3 and 7), membrane depolarization, and increased intracellular sodium concentrations and also reduced mitochondrial membrane potential and sperm motility. Readers are referred to more extensive reviews regarding the effects of cryopreservation procedures on stallion sperm, particularly those focused on the occurrence of oxidative insults due to redox imbalance.[56-58] Interestingly, these studies reported the relevance of mitochondrial function in stallion sperm subjected to freezing/thawing that further emphasizes the pivotal role of mitochondria for normal function of stallion sperm under in vitro conditions.[59-62] Hence, devising methodologies to maintain the mitochondrial function of stallion sperm has become a common objective for researchers working on stallion sperm cryopreservation.

Utilization of newer laboratory techniques, such as mass spectrometry-based technologies to study protein (proteomics), lipid (lipidomics), and metabolite (metabolomics) composition of sperm may shed light not only on cryoinjury mechanisms but also on potential targets for improving current protocols for sperm cryopreservation in stallions. In this regard, mass spectrometry-based techniques have been recently employed to determine changes in stallion sperm proteome after a freezing/thawing cycle, whereby a lower abundance of proteins associated with oxidative phosphorylation (the main metabolic pathway utilized by freshly ejaculated stallion sperm to produce ATP), and some proteins associated to sperm-oocyte interactions were observed in frozen/thawed compared to freshly ejaculated sperm.[63] Studies also reported that stallion sperm subjected to a freezing/thawing cycle display a lower abundance of antioxidant

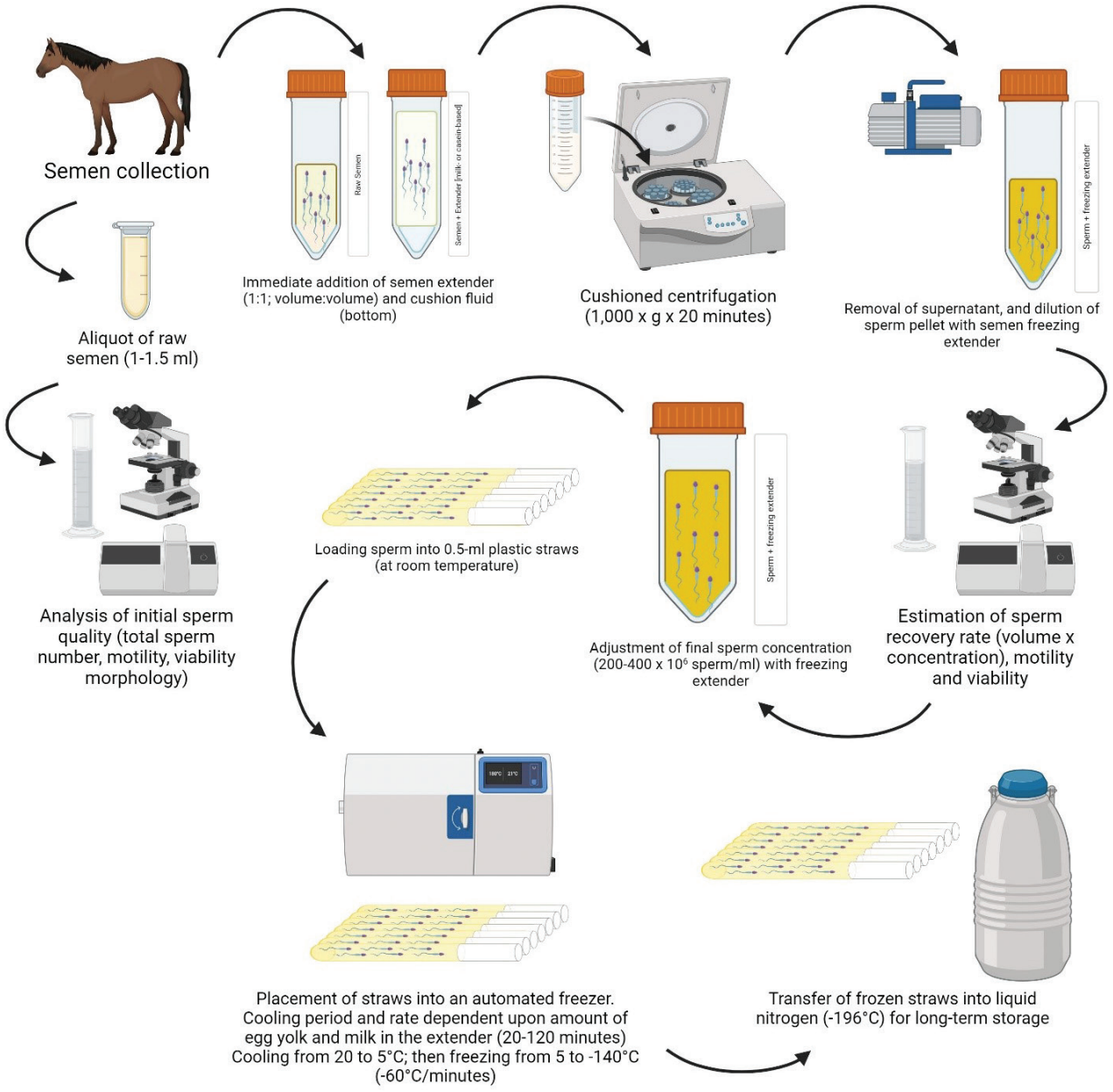

Figure 2. A graphical representation of a typical cryopreservation protocol utilized in the author's laboratory for stallion sperm. Figure created with BioRender (www.biorender.com).

enzyme systems, such as superoxide dismutase that are pivotal for redox balance maintenance.[64] Moreover, studies from this same group of researchers have also attempted to identify proteins of differential abundance in stallions that display higher postthaw motility (i.e. 'good freezers') when compared to 'poor freezer' stallions. These proteins include those associated with mitochondrial function, antioxidant enzyme systems, and seminal plasma-associated proteins.[65,66] In summary, these new molecular biology technologies are promising tools to elucidate further the 'pathophysiology' of cryopreservation-induced damage in stallion sperm, provided a proper validation of some of these 'biomarkers' (i.e. proteins, lipids, and metabolites) is performed. In the years to come, we will observe an exponential increase in the utilization of these techniques in equine reproductive medicine.

Some clinically related concepts regarding stallion sperm cryopreservation

After considering the vast array of cellular and molecular changes that stallion sperm undergo during cryopreservation and thawing, it is evident that specific cryopreservation process steps would be of higher interest while designing methods to maximize sperm survival after thawing. To author, it is noteworthy that many current techniques (semen extenders, cooling, freezing, and thawing methods) for stallion sperm cryopreservation were initially adapted from other species (i.e. cattle) and have not changed dramatically during last 2 decades. Many commercial and 'homemade' preparations of semen extenders employed currently were used and reported in scientific literature; nevertheless, most of these formulations are based on the same components initially devised

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more than 7 decades ago. Surprisingly, such a simple mixture of sugars, phospholipid sources, antibiotics, and penetrating cryoprotectants can prolong fertilizing capacity of sperm subpopulations for an indefinite period!

One of the long-standing concepts of stallion sperm cryopreservation has been the use of slow prefreeze cooling rates to maintain adequate postthaw sperm quality, particularly when utilizing gly as a penetrating cryoprotectant. As mentioned above, changes in the plasma membrane phase (liquid-to-gel phase) are caused by noncontrolled temperature reduction; thus, using fast prefreeze cooling rates (> 1 °C/minute) would result in the so-called 'cold shock.' Sperm plasma membrane composition, particularly cholesterol-to-phospholipid ratio, has been suggested as a critical factor that allows sperm to withstand plasma membrane transition phases discussed above.[29,67] Optimal cooling rate for stallion sperm exposed to temperatures from 20 to 5 °C has been reported to range between –0.1 to –0.01 °C/minute to minimize plasma membrane phase transitions.[68] Such a 'slow' cooling rate will require ∼ 120 minutes before exposure to liquid nitrogen vapors. Slow (0.05-0.1 °C/minute) cooling (from 20 to 5 °C) of stallion sperm diluted in skim milk + 4% egg yolk + gly (INRA-82) semen freezing extender yielded higher postthaw motion characteristics compared to rapid (–60 °C/minute) prefreeze cooling.[69] The study also compared postthaw motility of sperm cryopreserved in INRA-82 versus sperm cryopreserved in a 20% egg yolk + gly + lactose-ethylenediaminetetraacetic acid (EDTA) extender; prefreeze cooling rate did not influence postthaw quality of sperm cryopreserved in lactose-EDTA, higher postthaw motion characteristics were generally obtained with the INRA-82 extender. In contrast, higher postthaw motion characteristics and plasma membrane intactness in stallion sperm cryopreserved in lactose-EDTA (LE®; Animal Reproduction Systems [ARS], Ontario, CA, USA) than in an INRA-82-based extender (MFR5®; ARS), independent of prefreeze cooling rate employed, have been reported.[70,71] Furthermore, stallion sperm processed in LE® extender at –5 (5 minutes cooling), –1 (20 minutes cooling), or –0.1 °C/minute (120 minutes cooling) yielded similar postthaw sperm quality parameters, whereas sperm processed in the MFR5® extender required a 'slow' cooling rate (–0.1 °C/minute) to yield an acceptable postthaw sperm motility and membrane intactness values (30-40% motility, 40-50% plasma membrane-intact sperm).[71]

Under field conditions, it is common to use 'fast' prefreeze cooling rates when utilizing semen cryopreservation extenders that contain low molecular weight penetrating cryoprotectants (i.e. amides, dimethylformamide [DMF], or methyl formamide [MF]). In general, several authors recommend the use of prefreeze cooling periods (20-5 °C) of 20-30 minutes (≥ –1 °C/minute) before exposing sperm to liquid nitrogen vapors.[72-74] The rationale behind this concept is that amides have a higher permeability coefficient due to their low molecular weight and thus would penetrate sperm plasma membrane faster. We recently compared the use of semen extenders with (skim milk + egg yolk + 2% gly + 3% MF [CryoMax MFR5®; ARS; CMMFR5]; 2% egg yolk 3% MF [CryoMax LE®; ARS; CMLE]) or without (skim milk-egg yolk-gly [MFR5®]; egg yolk-gly [LE®]) amides when exposing stallion sperm to 'slow' (–0.1 °C/minute; prefreeze cooling phase of 120 minutes before freezing) or 'moderate' (–1 °C/minute; prefreeze cooling phase of 20 minutes before freezing) cooling rates.[71] Although we observed higher postthaw sperm motion characteristics and plasma membrane intactness in the face of lower lipid peroxidation or apoptosis in stallion sperm cryopreserved in CMLE® or LE® than in MFR5® or CMMFR5® extenders, these results were not associated with precooling rate or penetrating cryoprotectant employed. As such, our studies indicated that prefreeze cooling rate, and thus the period required to cool stallion sperm from 20 to 5 °C before exposure to liquid nitrogen vapors, is dictated by egg yolk quantity in freezing extender and milk proteins (presence or absence), and not by the presence of low molecular weight cryoprotectants such as amides.

An additional concept regarding stallion sperm cryopreservation has been replacement of high molecular weight cryoprotectant gly (92.09 g/mol) by cryoprotectants of lower molecular weight, including ethylene glycol (62.07 g/mol), dimethyl sulfoxide (DMSO; 78.13 g/mol), methyl formamide (MF; 59.06 g/mol), and DMF (73.09 g/mol). Studies indicated that exposure and rapid removal of fresh stallion sperm from a medium that contained 1.0 M gly resulted in the highest proportion of sperm with plasma membrane damage compared to stallion sperm subjected to the same conditions (rapid exposure and removal of cryoprotectant) but with 1.0 M ethylene glycol, DMSO, or propylene glycol.[44] These studies suggested stallion sperm was considerably sensitive to rapid cryoprotectant removal, particularly when exposed to gly. In the early 2000s, various studies in Colorado,[75] Brazil,[73,76-78,] and France[79] indicated that supplementation of semen freezing extenders with DMF yielded similar or higher postthaw sperm quality parameters when compared to gly-containing extenders. Other studies did not report any advantage regarding postthaw sperm motility or plasma membrane intactness when cryopreserving stallion sperm with amides.[80,81] Studies by Brazilian[72] and Colorado/Saskatchewan[82] researchers reported higher fertility rates for stallion sperm cryopreserved with DMF than with gly, whereas French workers did not observe differences in pregnancy rates.[79] Currently, the hypothesis regarding benefits of cryopreserving stallion sperm with amide-containing semen extenders is that due to their lower molecular weight, less osmotic stress is caused to stallion sperm, diminishing plasma membrane and mitochondrial damage. Nevertheless, conflicting results have been published in this regard. A study[83] indicated that stallion sperm cryopreserved in skim milk and egg yolk-based semen freezing extender supplemented with either 4% DMF or a combination of 2.5% DMF and 1.5% gly yielded higher postthaw plasma membrane-intact sperm (4-5% increase) and a lower proportion of sperm with low mitochondrial function (5-10% decrease) than sperm cryopreserved with 4% gly. Conversely, mitochondrial oxygen consumption of stallion sperm cryopreserved in an amide-containing semen freezing extender (BotuCrio®, Botupharma USA, Phoenix, AZ, USA) was higher compared to gly-containing extenders (INRA-96®, MFR5®, LE®) despite similar postthaw motion characteristics, plasma membrane intactness, and ROS production were observed in stallion sperm cryopreserved with LE® than in BotuCrio®.[85] We recently studied whether cryopreservation of stallion sperm with gly-containing extenders (LE® or MFR5®) yielded a higher proportion of sperm with early plasma membrane changes (associated with apoptosis) or higher lipid peroxidation in plasma membrane-intact sperm (associated with mitochondrial dysfunction and redox imbalance) when compared to the same semen extenders containing a combination of gly and MF (CMLE® or CMMFR5®).[72] We did not detect differences in any of these parameters when comparing LE® versus CMLE® or MFR5® vs. CMMFR5®; in contrast, a higher proportion of early plasma membrane changes and lipid peroxidation in plasma membrane-intact sperm were observed in stallion sperm cryopreserved in MFR5® or CMMFR5® versus LE® or CMLE®. We concluded that even when amides have a higher

plasma membrane permeability coefficient than gly,[46] they do not confer higher protection to the sperm plasma membrane nor mitochondria (based on ROS production) than only with gly. An aspect that warrants further research, even though the limitations of performing in vivo fertility trials in horses, is determining whether the fertility of stallions whose sperm has been cryopreserved in amide-containing extenders is higher than the obtained when inseminating mares with sperm cryopreserved in gly-containing extenders.

Finally, another factor that warrants further consideration are the beneficial effects of increasing the cholesterol concentrations at sperm plasma membrane to enhance sperm survival after cooling and cryopreservation. Cholesterol-to-phospholipid molar ratio in plasma membranes of stallion sperm (0.36) has been reported to be lower than that of bull (0.45), rabbit (0.88), or man (0.99) whereas closer to that of rooster (0.30) and boar (0.26) sperm.[29,67] This has been suggested as a main factor that affects the ability of stallion sperm to withstand the cooling and cryopreservation processes.[67,85] Hence, various methods have been devised to incorporate more cholesterol in sperm plasma membrane, with use of cholesterol-loaded cyclodextrins (CLCs) the most popular. These cyclodextrins are cyclic oligosaccharides with a hydrophilic external face and a hydrophobic core that can 'encapsulate' compounds like cholesterol.[85] Several studies have indicated that the addition of CLCs (generally 1.5 mg CLC per 120 x 10^6 sperm) resulted in a 5-10% increase in postthaw motility and plasma membrane intactness compared to nonCLC-supplemented extenders.[86-88] Although some authors initially suggested that increasing the molar cholesterol concentration by adding CLCs to stallion sperm also reduced acrosomal exocytosis and in vivo fertility rates,[87] more recent studies have refuted those findings.[89] Considering the data above, it would be expected then that stallions whose sperm yield higher postthaw sperm quality after cryopreservation also would have a higher cholesterol-to-phospholipids ratio in their sperm plasma membranes, but a preliminary study did not find a linear association between postthaw sperm motility and plasma membrane cholesterol-to-phospholipid ratio of fresh sperm.[90] Certainly, more research regarding the relationship between sperm plasma membrane composition and tolerance to cooling or cryopreservation in stallions is required. Interestingly, recent data indicated that egg yolk replacement in semen cryopreservation extender by CLCs also allowed the reduction in the concentration of penetrating cryoprotectant (gly) required to maximize the postthaw motility and plasma membrane intactness of stallion sperm.[91] Currently, addition of CLCs, in conjunction with purified milk proteins (caseins), to semen cooling extenders is common (i.e. BotuSemen Gold®; BotuPharma USA), and it would be interesting to determine whether egg yolk could be replaced from semen cryopreservation extenders for stallions by adding CLCs. Perhaps this would lead to developing more 'chemically defined' media for long-term storage of stallion sperm.

Postthaw sperm quality analysis in stallions: beyond the concept of progressive motility

One of the long-standing 'dilemmas' in theriogenology, particularly in equine reproductive medicine, is the relationship between sperm quality measures and in vivo fertility. The sperm quality assay most employed by practitioners in the field is the estimation of sperm motility, as it is assumed that motile sperm subpopulations would reach the oviduct after insemination and fertilize the oocyte. Although this may be partially true, many changes in sperm caused by cryopreservation may be unintentionally ignored by just assessing postthaw motility. Many years ago, to create standard values or guidelines for practitioners to 'judge' whether sperm quality in a given sample (frozen/thawed sperm) would be consistent with a positive outcome (pregnancy), the equine breeding industry adopted a minimum of 30% (35% for European countries) 'progressively' motile sperm, to deem that an ejaculate was commercially acceptable.[17,19,92] Whereas tracking down the exact publications that suggested such a cutoff value was difficult for the author, it appears that by the mid 1970's when German,[9] Polish,[93] and (then) Czechoslovakian[94] scientists began developing commercial programs for stallion sperm cryopreservation, the terms 'progressively motile' 'motile' and '30%' were already incorporated, and often used interchangeably, in scientific jargon. None of these studies described why such a cutoff value was used nor what the concept of 'progressivity' entailed. Still, it can be assumed that these values and terminologies were extrapolated from studies conducted in other species. Examining more carefully at other publications at that time regarding stallion sperm analysis and fertility potential, the concept of progressive motility was defined as 'cells that are actively moving forward'[95] or 'cells that are moving across the field of view.'[96] Neither of these definitions specifically dictated how 'forward' or 'across the field' should or should not be interpreted, but basically, they suggested that any sperm that are not moving in circles can be deemed as progressive. Although this argument may seem philosophical rather than clinical, author considers this debate necessary for multiple reasons: 1. definition of progressive motility is not standard per se, even when Computer Assisted Sperm Analysis (CASA) systems are employed; progressive motility is a composite measure (average path velocity – VAP [μm/s]; straightness – STR [%]) that can be altered by modifying the threshold values that a given CASA system uses to detect progressive or nonprogressive sperm, resulting in differences on postthaw progressive motility[97]; 2. stallion sperm tend to display curvilinear rather than linear trajectories, potentially due to the high incidence of abaxial attachment of the sperm midpiece that should not be considered as a morphologic abnormality but rather a distinctive (i.e. normal) stallion sperm feature.[98-101] Thus, unless a spermatozoon is moving in very tight circles in a 'rotational' rather than 'translational' fashion, it is difficult to argue that such spermatozoon is not being 'progressively motile' (Figure 3); 3. using percentual values as an indicator of sperm quality rather than the actual absolute value of sperm displaying a given characteristic (e.g. motile or 'progressively motile') leads to misconceptions regarding the fertility potential of a clinical sample (i.e. batch of cryopreserved semen), as doing it so would ignore that sperm motility, or the lack of thereof, is of a compensable nature. Even when the percentage of motile (or 'progressively motile') sperm is low in a straw of cryopreserved sperm, if more straws are inseminated then the critical number of sperm displaying such characteristic (i.e. motility) required to 'theoretically' achieve maximal fertility can be reached; and 4. the relationship between in vivo fertility and postthaw motility (including progressive motility) is generally low,[20-23] and a combination of assays may provide a better appraisal of fertility potential of a sample, as long as it is understood that this will not be 100% accurate in predicting fertility. It has recently been brought to author's attention that the semen standards established by the World Breeding Federation of Sport Horses (WBFSH)[92] for frozen/thawed stallion sperm are not currently available for consultation and that various European laboratories that process frozen stallion sperm are no longer abiding to the historical threshold value of 35% or more progressive motility to consider an ejaculate

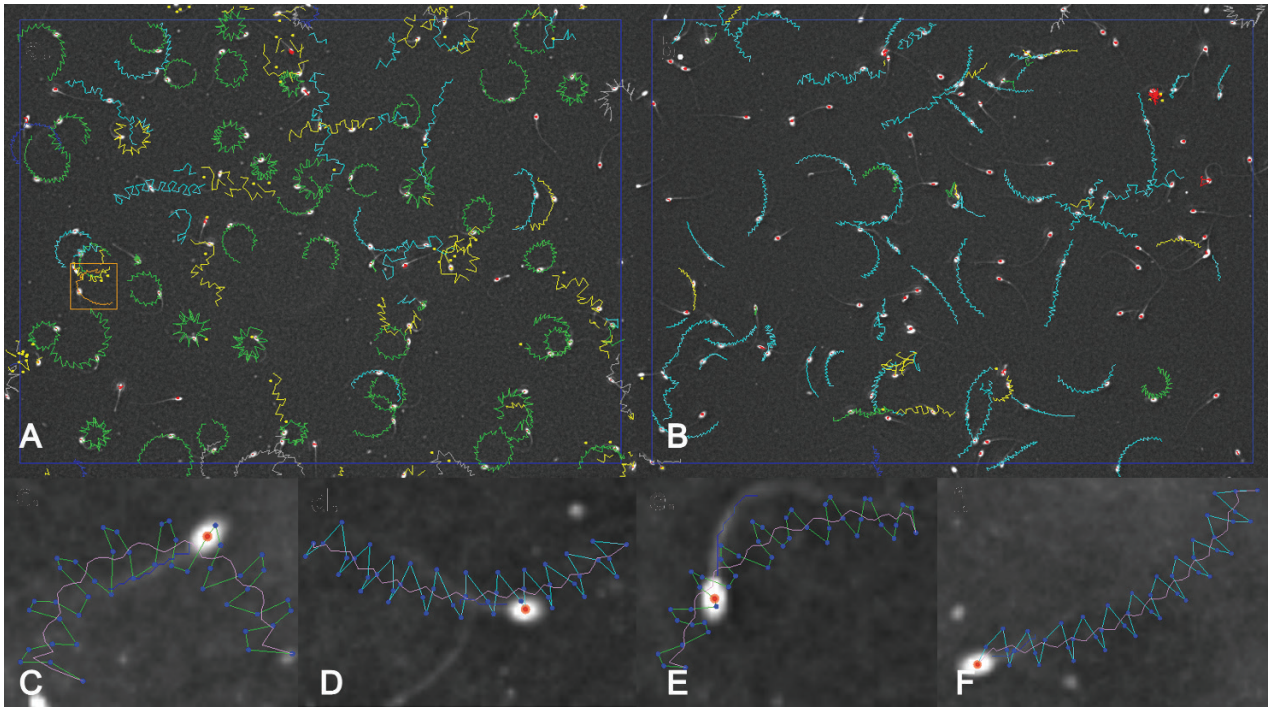

Figure 3. Representative sperm track printouts for sperm motion characteristics analyses in stallion semen using a Computer-Assisted Sperm Motion Analyzer (CASA) system (IVOS II®, Hamilton Thorne, Beverly, MA, USA) before (A) and after cryopreservation (B). Note that the percentage of progressively motile sperm (PMOT), defined by this system as VAP ≥ 30 µm/second and STR ≥ 50% (light blue tracks), was lower in fresh (total motility [TMOT] – 85%; PMOT – 36%; curvilinear velocity [VCL] – 232 µm/second) than in frozen/thawed sperm (TMOT – 69%; PMOT – 53%; VCL – 114 µm/second). Additional images of sperm displaying motile (C and D) or progressively motile (D and F) tracks from other stallion samples are presented. Note the similarities among the trajectories of these sperm despite the system classifying them differently. Definitions for PMOT were VAP ≥ 30 µm/second and STR ≥ 50% for images C and D, whereas VAP ≥ 50 µm/second and STR ≥ 75% for images E and F. Percentages of post-thaw motion characteristics were TMOT – 55% and PMOT 41% for C and D, and TMOT – 54% and PMOT 34% for E and F.

as commercially acceptable. Yet, many other laboratories, publications (scientific and lay), and textbooks still refer to that cutoff value.

Introduction of additional sperm quality assays, primarily based on fluorescent dyes, has allowed us not only to study in depth the changes that sperm undergo during the cryopreservation process but also provided us a better picture of other attributes that are necessary to be considered, besides sperm motility, to judge the fertility potential of frozen/thawed stallion sperm. Indeed, estimating plasma membrane intactness (also termed 'viability') should be considered as important as estimating sperm motility. It is assumed that immotile sperm are 'dead,' although these sperm may still possess an intact plasma membrane and may resume motion if their surrounding conditions (i.e. following insemination) are changed. This has been demonstrated for fresh and cool-stored stallion sperm that has been exposed to high levels of seminal plasma (i.e. 50%, volume:volume),[102] and that still may yield acceptable fertility following 4 days of cooled storage despite displaying < 5% motile sperm (having > 70% viable sperm).[103] Certainly, in these studies, the common factor was that the stallions enrolled presented high intrinsic sperm quality (i.e. sperm morphology), which should be considered when interpreting discrepancies between sperm motility and viability. In frozen/thawed stallion sperm, we observed that differences between values of postthaw sperm motility and viability are lower (i.e. the absolute difference between motility and viability is minimal) when the intrinsic sperm quality (i.e. sperm morphology and DNA quality) is high. At the same time, such discrepancies increased as the intrinsic sperm quality decreased.[104] From a clinical perspective, this indicated that it is highly likely that poor postthaw sperm motility is related to low intrinsic sperm quality from a particular stallion (or ejaculate) and that the analysis of postthaw sperm viability, in conjunction with sperm motility and morphology analyses, would offer additional insight regarding the quality of that batch of frozen/thawed semen.

In general, sperm viability is determined by using exclusion dyes, that is, nonpermeable dyes to the plasma membrane. Under field conditions, eosin/nigrosin (E/N) stain (also known as Hancock's stain) has been extensively reported for such purpose. Although it appears that E/N yields similar results regarding sperm viability in fresh stallion semen,[102,105] it does overestimate this parameter when analyzing cool-stored[103] or frozen/thawed sperm.[105] Considering the increased utilization of fluorescence-based techniques for sperm quality analysis in clinical settings, practitioners have become more familiar with other dyes, such as propidium iodide (PI) or ethidium homodimer-1 (EthD-1) that are nonpermeable to the plasma membrane and thus will identify nonviable ('dead') sperm populations (using PI or EthD-1, sperm will fluoresce red upon excitation with a 488 nm laser). A very popular semiautomated, fluorescence-based cell counter (NucleoCounter SP-100™ [NC], Chemometec, Allerød,

Denmark) utilizes microfluidic cassettes preloaded with PI to determine sperm concentration and viability.[106-108] For stallion sperm, use of NC for estimation of sperm viability has been compared to flow cytometry (FC) when analyzing fresh or cool-stored semen.[107] This study indicated that, overall, both methods (NC and FC) yielded similar results in terms of sperm viability. Thus, NC could be considered a less expensive and more 'user friendly' alternative to FC. We recently compared NC and FC use to estimate sperm viability in frozen/thawed stallion semen.[104] Our results indicated that across a broad spectrum of intrinsic stallion sperm quality (i.e. sperm morphology and DNA quality) 'agreement' (interpreted as the absolute difference between 2 measures or 2 measurement methods) between both methods was consistently high.

While writing this portion of the document, the author wonders if soon the equine breeding industry will move away from utilizing just progressive motility as an indicator of postthaw sperm quality and will adopt both 'more advanced' measures of sperm quality (e.g. viability, DNA quality) in conjunction with more 'traditional' sperm quality assays (e.g. sperm morphology and total motility) that are less deceiving and may have a higher relationship with in vivo fertility of stallions. By any means, the author's intent is not to suggest that sperm motility should not be considered when analyzing postthaw sperm quality, but rather such a parameter should not solely be the measure of postthaw sperm quality utilized to deem whether an ejaculate is 'freezable' or not, nor to determine the fertility potential of a batch of frozen/thawed stallion sperm. Interestingly, a study conducted with fresh and cool-stored stallion sperm had a significant relationship between measures of sperm total motility and normal morphology with the first-cycle pregnancy rates of 88 stallions that was not evident when analyzing progressive motility.[109] Furthermore, in a recent work,[110] combination of a battery of assays (CASA-derived measures of sperm total motility, morphology, viability, and DNA integrity determined by flow cytometry) accounted for up to 95% of in vivo fertility variation from a group of 42 stallions utilized to breed a minimum of 25 mares/stallion with frozen/thawed semen.[110] Although more extensive studies in commercial settings are warranted, it is evident that the concept of progressive motility as a measure of sperm quality should be thoroughly revisited.

Use of frozen/thawed stallion sperm for in vitro production of equine embryos by ICSI: does it only take one?

In vitro production (IVP) of equine embryos by ICSI has become a routine procedure in equine reproductive medicine.[111-113] It would not be surprising that, shortly, more pregnancies will be established by IVP embryos than by conventional embryo transfer (in vivo embryos). Use of frozen/thawed semen for IVP in horses is quite advantageous as straws of frozen semen can either be 'cut' (8-10 small cuts made from a 0.5 ml straw; ICSI cuts) or thawed and refrozen into more dilute straws (1-2 x 10^6 sperm/ml; ICSI doses).[114] Additionally, frozen semen can be utilized at any time of the year without coordinating semen collection and shipment with the timing required for fertilization of in vitro-matured oocytes, and doses of frozen/thawed sperm from old or deceased stallions that were frozen many years ago can still be utilized. It is safe to assume that currently, the primary driving force behind ICSI use in the equine breeding industry is related to the stallion, as many stallion owners or managers have realized the convenience of utilizing ICSI to maximize the offspring available from their genetically valuable stallions.

The first study[115] to report the use of frozen/thawed stallion sperm for ICSI, with resulting pregnancies utilized sperm from a fertile and a subfertile stallion frozen in an egg yolk-gly-based extender and produced 3 pregnancies from the fertile stallion and 1 from the subfertile stallion following ICSI on in vivo-matured oocytes that were transferred into the oviducts of recipient mares within 4 hours after ICSI. First pregnancies and live foals after utilizing frozen/thawed sperm for ICSI on in vitro-matured equine oocytes recovered from abattoir-derived ovaries were reported in the UK,[116] and first comparisons on the efficiency of oocyte activation and initial embryonic development (i.e. 16 cells) of in vitro matured equine oocytes fertilized by ICSI with either fresh or frozen/thawed sperm were reported in the USA.[117] At the 2002 International Symposium on Equine Reproduction (Ft. Collins, CO, USA; https://www.sciencedirect.com/journal/theriogenology/vol/58/issue/2), most of the works related to ICSI in horses reported the use of frozen/thawed stallion sperm for oocyte fertilization.[118-121] Since then, all publications about IVP in horses have employed frozen/thawed stallion sperm, except for a recent preliminary report[122] from a commercial ICSI program that used cool-stored sperm, whereby almost 1 fold increase in embryo production (26 versus 13%) and 20% more pregnancies were observed compared to frozen/thawed sperm. More controlled studies, whereby the same stallions and mares are enrolled, are necessary to determine whether cool-stored sperm would yield higher embryo production than frozen/thawed sperm.

As described above, one of the advantages of utilizing frozen/thawed stallion sperm is sperm that was cryopreserved many years ago can be thawed, rediluted at a much lower sperm concentration (1-2 x 10^6 sperm/ml) and then refrozen. Blastocyst production after ICSI using stallion sperm frozen once (control) or twice in a skim milk-egg yolk-gly-based extender yielded similar blastocyst development rates (27 versus 23%, respectively).[123] However, the cleavage rate (> 8 blastomeres per embryo) was higher in the control group (75 versus 55%).[123] These researchers also indicated that refrozen sperm from a subfertile stallion (whose percentage of sperm DNA damage was > 25% [considered high[124]]) yielded a similar proportion of blastocysts by ICSI compared to sperm that was frozen once (9 versus 9%, respectively). Although the experience with refrozen sperm in the US has been overall satisfactory, a recent retrospective report from a clinical program in Europe indicated that the experience with refrozen sperm has yielded poor cleavage and blastocyst rates (21 and 4%, respectively) after ICSI.[113] During 2023, in our commercial ICSI program at the Equine Fertility Laboratory – Texas A&M University, we utilized refrozen sperm from 4 stallions (reprocessed between 2010 and 2013, when these stallions were already dead) to fertilize in vitro-matured oocytes recovered by transvaginal oocyte aspiration (TVA) from 6 mares (Ramírez-Agámez, Hernández-Avilés: unpublished observations; Table). Overall, the cleavage and blastocyst rates obtained were 52 and 21%, respectively, slightly lower than those obtained in our program during the same period for frozen/thawed sperm that has been cryopreserved once (64 and 29%, respectively) but within values that are currently considered as commercially acceptable.

Our group recently studied whether some factors during cryopreservation of stallion sperm would impact the outcomes (i.e. cleavage and blastocyst rates) after ICSI on in

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Table. Postthaw sperm motility (determined subjectively; phase-contrast microscopy at 200 x magnification), sperm morphology (assessed by differential interference contrast [DIC] microscopy; 1,000 x magnification), number of mares, transvaginal oocyte aspiration (TVA) cycles, number of in vitro-matured oocytes fertilized by intracytoplasmic sperm injection (ICSI), cleavage and blastocyst rates of 4 stallions whose sperm was frozen between 1995-2005, then thawed and refrozen to prepare ICSI doses. All ICSI doses were cryopreserved after 2010 using the MFR5® semen freezing extender (Animal Reproduction Systems, Ontario, CA).

Stallion	Percent motility	Percent normal sperm	Mares (n)	TVA/ICSI cycles	IVM oocytes fertilized	Cleaved embryos/ICSI session	Blastocysts/ ICSI session
A	5%	43%	2	3	16	10 (63%)	3 (19%)
B	10%	55%	1	1	12	3 (25%)	2 (17%)
C	5%	61%	2	2	12	7 (58%)	4 (33%)
D	2%	37%	1	3	8	5 (63%)	1 (13%)

Cleavage rate was determined on day 5 postICSI, and a cleaved embryo (> 8 blastomeres).
Blastocyst rate was determined from day 7 to day 10 postICSI.

vitro-matured equine oocytes.[125] Particularly, we were interested in determining whether the amount of egg yolk (LE® or CMLE® versus MFR5® or CMMFR5®) and the penetrating cryoprotectant combination (LE® or MFR5® versus CMLE® or CMMFR5®) would yield more or less IVP embryos after ICSI. Overall, semen frozen in MFR5® yielded almost 3 times more blastocysts than semen frozen in LE® (26 versus 10%, respectively) and 5 times more blastocysts than semen frozen in CMLE® or CMMFR5® (5 and 5%, respectively). Interestingly, in that study, sperm frozen in MFR5® also yielded the lowest postthaw motility (38%) and higher percentage of lipid peroxidation in viable sperm (35%) compared to other treatments. Two main conclusions were obtained from this experiment: 1. apparently, extenders formulated with amides and glycerol yield lower blastocysts compared to gly-only containing extenders and 2. higher blastocyst rates were obtained in sperm that was cryopreserved only with gly, and in which higher membrane destabilization (lipid peroxidation) resulted after cryopreservation. To date, it is still unknown why extenders formulated with amides yielded considerably lower rates of blastocyst production after ICSI. Still, we hypothesized that a potential toxic effect of these cryoprotectants might have had a role in the ability of in vitro matured oocytes to become embryos. Current studies in our laboratory focus on determining whether cryopreservation of stallion sperm with amide-only containing semen extenders would also yield a lower blastocyst rate after ICSI than gly-only containing semen extenders. Interestingly, in that same study, we thawed ejaculated or epididymal sperm from a fertile stallion that was cryopreserved initially in CMLE® extender and refrozen in MFR5® extender.[125] Then, ICSI doses from each group were utilized for ICSI on in vitro-matured oocytes, and differences in cleavage and blastocyst rates were not observed (57 versus 77% and 17 versus 17%, respectively). Our results not only confirmed those previously reported[123] regarding blastocyst production by ICSI using refrozen stallion sperm but also indicated that sperm that was initially cryopreserved in CMLE® (which yielded 5% blastocyst rate after ICSI) could be 'rescued' by refreezing in MFR5® extender.

Another point worth discussing is the relationship between postthaw sperm quality parameters and ICSI outcomes in horses. In human reproductive medicine, application of ICSI was initially intended to overcome infertility due to poor sperm quality or low sperm numbers.[126-128] Currently, utilization of ICSI has outgrown conventional in vitro fertilization (IVF), and it has become the procedure of choice for IVP of human embryos.[129-131] Studies in human reproductive medicine focused on the relationship between postthaw sperm quality and ICSI outcomes (pregnancy rates and live births) have revealed that analysis of sperm DNA quality may provide the highest predictive value of positive ICSI outcomes.[132-135] In horses, some experimental studies have indicated that sperm of poor postthaw motility can still be utilized to produce IVP embryos and pregnancies.[121,123] To author's knowledge, only 1 study conducted in a commercial ICSI program has determined the relationship between certain postthaw sperm quality parameters (i.e. normal morphology – E/N staining; viability – E/N staining; plasma membrane function – hypoosmotic swelling test [HOST]; DNA damage – sperm chromatin dispersion assay) blastocyst production, and pregnancy rates after ICSI.[136] These researchers estimated sperm postthaw quality after sperm selection by swim-up; they reported that percentage of viable sperm and percentage of sperm with functional plasma membrane (HOST +) were predictors of positive ICSI outcomes (blastocyst development and pregnancy on day 28 after transfer). It is important to mention that these researchers estimated such parameters only after sperm selection before ICSI that would bias the relationship between initial postthaw sperm quality and ICSI outcomes. Further studies in this area are warranted to determine whether there is/are a minimum value/s of postthaw quality that should be considered acceptable, even for ICSI purposes.

Concluding remarks

Although alternative methods for sperm storage in liquid form (i.e. semen extenders for ambient temperature storage)[137] or in the frozen state (i.e. sperm vitrification)[138,139] have been recently reported and might have promising results regarding sperm quality parameters and fertility potential, production and utilization of frozen/thawed sperm is still, and most likely will be, the main method for long-term preservation of stallion sperm. Many studies have been conducted in the last 2 decades regarding cellular and molecular effects of cryopreservation on stallion sperm, that indicated most of the deleterious effects of this procedure are associated with osmotic and oxidative stress and their consequences to various sperm organelles. Remarkably, mitochondria of stallion sperm are a sensitive target to cryopreservation-induced damage; thus, current and future studies will focus on preserving adequate mitochondrial function. Although various advances in sperm quality estimation have been published in the last 2 decades, estimation of postthaw sperm quality still heavily relies on the

analysis of sperm motility. Even though this assay is fundamental, postthaw sperm quality analyses and clinical interpretations should depend not only on this test but also on a combination of assays that can be applied under field conditions. Application of assisted reproductive technologies, and particularly ICSI, in horses has increased the utilization of frozen/thawed stallion sperm. With the recent report regarding a repeatable method for conventional IVF in horses,[140] we will witness an increase in the number of studies that will validate the use of frozen/thawed sperm in IVF. Although IVF may not replace ICSI in a commercial setting, most research efforts will likely be focused on studying the relationship between postthaw sperm quality and in vitro fertility in stallions.

Conflicts of Interest

No conflicts of interest to declare.

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