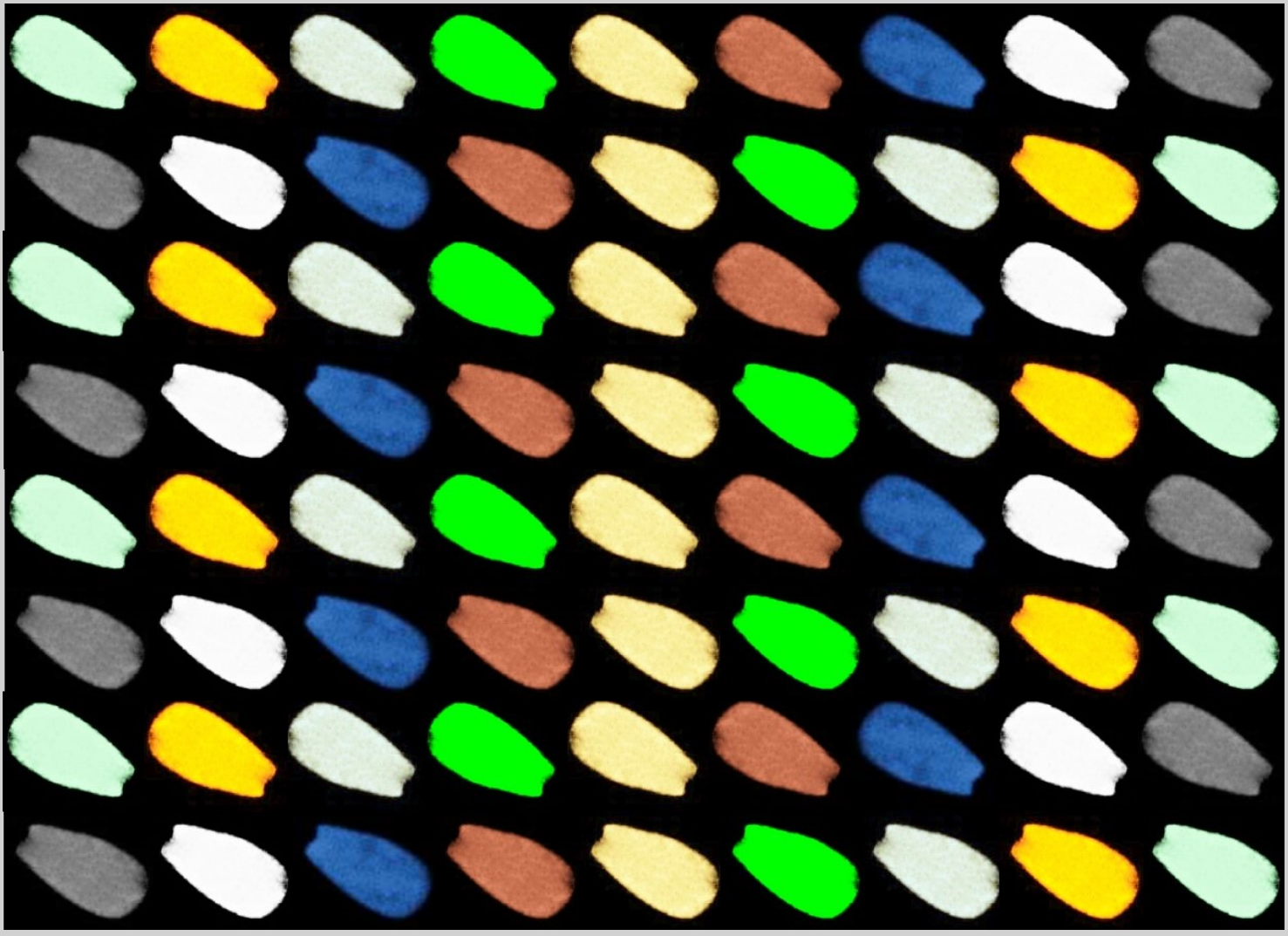
ISSN 2542-3045
Deposito Legal: ppi20162TA4753

VOL 4, NÚMERO 1, ENERO-JUNIO 2019
VOL 4, ISSUE 1, JANUARY-JUNE 2019

JOURNAL OF VETERINARY ANDROLOGY



Editada por/Edited by:
Centro de Estudios Superiores e Investigación en Ciencias del Agro (CESICA)
La Fría-Táchira, Venezuela

Review Article/Artículo de Revisión

CURRENT TRENDS ON STALLION SEMEN EVALUATION: WHAT OTHER METHODS CAN BE USED TO IMPROVE OUR CAPACITY FOR SEMEN QUALITY ASSESSMENT?

TENDENCIAS ACTUALES EN LA EVALUACIÓN DE SEMEN EQUINO: ¿QUÉ OTROS MÉTODOS SE PUEDEN UTILIZAR PARA MEJORAR NUESTRA CAPACIDAD EN LA EVALUACIÓN DE LA CALIDAD DEL SEMEN?

CAMILO HERNÁNDEZ-AVILÉS[1,2], JORGE ZAMBRANO-VARÓN[1], CLAUDIA JIMÉNEZ-ESCOBAR[1]

[1]Reproductive Biotechnology Laboratory, Section of Theriogenology and Herd Health, Department of Animal Health, School of Veterinary Medicine, Universidad Nacional de Colombia, Edificio 561B, Carrera 30 Calle 45-05, Bogotá DC, Colombia.
[2]Current address: Section of Theriogenology, Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA, 77843-4475.
Correspondence should be addressed to (La correspondencia debe dirigirse a): C. Hernández-Avilés chernandez@cvm.tamu.edu

ABSTRACT

Semen evaluation is an important component for the assessment of the stallion breeding potential, as well as for the diagnosis of subfertility/infertility cases. Furthermore, accurate estimation of the damage suffered by the sperm cell after cooling or freezing procedures is necessary for the development of newer procedures to maintain sperm integrity and function. Nevertheless, commonly used methods for sperm quality evaluation (sperm motility or sperm morphology) are not completely associated with the fertilizing potential of the spermatozoa under *in vitro* conditions, and in the best-case scenario are poor- to- moderately associated with *in vivo* fertility. In recent years, the introduction of advanced methods based on the use of fluorochromes for sperm evaluation has improved the clinician's and researcher's capacity to account for the differences on the fertility potential between sires, as well as critically evaluate the effect of several methods to preserve stallion sperm. The aim of this paper was to review some of the current fluorescence-based methods used for the evaluation of equine semen as an alternative for the selection of breeding stallions, the diagnosis of subfertility/infertility, and the estimation of optimal protocols for sperm preservation under laboratory conditions.

Keywords: Stallion, spermatozoa, fluorescent probe, sperm membrane, fertility.
JOURNAL OF VETERINARY ANDROLOGY (2019) 4(1):01-19

RESUMEN

La evaluación del semen es un componente importante en la evaluación del potencial reproductivo de sementales, así como para el diagnóstico de casos de subfertilidad / infertilidad. Además, es necesario realizar una estimación precisa del daño sufrido por la célula espermática después de los procedimientos de refrigeración o criopreservación para el desarrollo de nuevos procedimientos a fin de mantener la integridad y la función del espermatozoide. Sin embargo, los métodos comúnmente utilizados para la evaluación de la calidad espermática (motilidad o morfología espermática) no están completamente asociados con el potencial de fertilización del espermatozoide en condiciones *in vitro*, y en el mejor de los casos están poco a moderadamente asociados con la fertilidad *in vivo*. En los últimos años, la introducción de métodos avanzados basados en el uso de fluorocromos para la evaluación espermática ha mejorado la capacidad del clínico y del investigador para explicar las diferencias en el potencial de fertilidad entre los sementales, así como evaluar críticamente el efecto de varios métodos para preservar el espermatozoide equino. El objetivo de este trabajo fue revisar algunos de los métodos actuales basados en fluorescencia que están siendo utilizados para la evaluación del semen equino como una alternativa para la selección de sementales reproductores, el diagnóstico de subfertilidad / infertilidad y la estimación de protocolos óptimos para la preservación del espermatozoide bajo condiciones de laboratorio.

Palabras clave: Semental, espermatozoide, sonda fluorescente, membrana espermática, fertilidad.
JOURNAL OF VETERINARY ANDROLOGY (2019) 4(1):01-19

Received/Recibido: 13/12/2018; Accepted/Aceptado: 06/03/2019

INTRODUCTION

The equine breeding industry has increased exponentially in the last 30 years in different countries around the world, where the use of different assisted reproductive technologies (ARTs) such as artificial insemination (AI) with cooled or frozen semen, embryo transfer (ET) and more recently intracytoplasmic-sperm injection (ICSI) are now considered as a routine procedure for veterinarians (Hinrichs, 2018; Panarace et al., 2014). The possibility to spread genetics from superior sires worldwide has increased the popularity of these ARTs among breeders, owners and stud farms, which highlights the importance of correctly assessing the reproductive potential of stallions.

As compared to other domestic species such as bulls or boars, wherein the selection or culling criteria are almost based on their reproductive capacity, stallions are selected by their pedigree, conformation and/or athletic performance. Thus, there is a significant percentage of stallions whose semen does not satisfy the "high-quality" criteria used for other species, leading to an important proportion of studs with marginal semen quality with possible impaired fertility (Varner, 2016; Miró-Morán et al., 2013). Moreover, while the use of semen technologies such as sperm cryopreservation or sex-sorting is highly developed in other species, their use in the equine breeding industry is relatively scarce. This is mainly related to several physiological characteristics of the equine spermatozoa, which makes it more susceptible to cellular and molecular damage during cooling, freezing, and sex-sorting. Although the mechanisms related to those effects are beyond the scope of this paper, the main effects of those techniques are related to osmotic and oxidative imbalances, which culminates on several alterations in cellular organelles such as plasma membrane, acrosome, mitochondria and DNA integrity, among others (Peña et al., 2015; Balao da Silva et al., 2016; Martín-Muñoz et al., 2015). It is therefore imperative to use a battery of tests for semen evaluation in order to correctly estimate semen quality and fertility of a given group of stallions. The aim of this paper is to review the current techniques used for analyze stallion sperm quality, either in clinical or laboratory situations.

ARE THE SPERM MOTILITY AND MORPHOLOGY EVALUATION ENOUGH AS ASSAYS TO ESTIMATE STALLION SPERM QUALITY?

Traditionally, assessment of sperm motility and morphology have been considered landmarks of stallion semen evaluation. The Manual for Clinical Assessment of Stallion Fertility published by the Society for Theriogenology (SFT) uses both tests to classify the fertility potential of a stallion. Based on the SFT guidelines, a stud must ejaculate a minimum of 1 billion of progressively motile-morphologically normal sperm to be considered as a satisfactory prospective breeder (Kenney et al., 1983). These two semen quality tests were included in the stallion breeding soundness examination by their easiness to be conducted under field conditions with limited equipment and were adapted from the SFT bull breeding soundness examination guidelines (Ball et al., 1983; Hopkins & Spitzer, 1997).

In general, the estimation of sperm motility is performed using a conventional light microscope or a phase-contrast microscope at 200-400X equipped with a warmed stage to control temperature fluctuations that could reduce sperm motility and generate interpretation errors. As quality endpoints, the clinician or researcher determines subjectively the percentage of sperm displaying any kind of motion (total motility, TMOT), the percentage of sperm displaying a straight-forward motion (progressive motility, PMOT), and in some cases sperm velocity. Historically the percentage of progressively motile sperm has been associated with high sperm quality. A value of 60% PMOT in fresh semen, 30% PMOT in cooled semen (after 24-48 hours of storage), and 30% PMOT in frozen/thawed semen have been considered as threshold values for fertile stallions (Kenney et al., 1983; Hurtgen, 1992; Loomis & Graham, 2008).

Recently, the use of computer-assisted sperm analyzers (CASA) is considered as the most reliable method to evaluate sperm motion characteristics in different species, including the stallion (Amann & Waberski, 2014; Loomis & Graham, 2008; Jasko et al., 1992). These systems not only can estimate the percentages of total and progressive motility but also can determine different sperm subpopulations based on velocity indexes or motion characteristics that are associated with physiological status such as hyperactivation (Amann & Waberski, 2014). Several studies about the relationship between sperm motion analyzed by CASA and stallion fertility have been published (Jasko et al., 1992; Quintero-Moreno et al., 2003; Kuisma et al., 2006; Love, 2011); however, most of those studies consistently demonstrate a low relationship between sperm motion characteristics and pregnancy rates. Jasko et al. (1992) reported that the assessment of sperm total motility by CASA was poorly correlated with per cycle fertility rate of stallions ($r = 0.34$; $P < 0.01$), while Love (2011) observed that the only sperm motion feature (analyzed by CASA) that had a high association with seasonal pregnancy rate, percent pregnant/cycle and percent pregnant/first cycle was total motility ($r = 0.42$, 0.59, and 0.64, respectively; $P < 0.05$). Moreover, the capacity to obtain consistent results of sperm motion characteristics by CASA in a non-biased manner is highly dependent on the type of chamber, dilution factor used before testing, type of sample

analyzed (raw, extended, frozen), and even between brands of sperm analyzers (Yesté et al., 2018; Hoogewijs et al., 2012; Hoogewijs et al., 2011). These factors partially explain why there is no consensus between laboratories, research stations or stud farms about the motion characteristics that could be used for classifying a stallion as fertile or subfertile. In fact, a study conducted by Janson-Whitesell et al. (2014) showed how the same group of stallions could be classified as satisfactory prospective breeders or not, just by using two different CASA settings for sperm motion analysis.

Assessment of sperm morphological features has been commonly conducted using samples prepared with background stains, such as eosin-nigrosin or trypan-blue (using conventional light microscopy), wet mount samples fixed with buffered-formalin solution (using phase-contrast or differential interference contrast-DIC microscopy), or more advanced techniques such as computed assisted sperm morphometry or electron microscopy (Voss et al., 1981; Veeramachaneni et al., 1993; Gravance et al., 1996; Love et al., 2000; Brito et al., 2011). Although various sperm morphology classification systems have been published in the literature, these are based mainly in the estimation of the percentage of normal sperm cells, and the percentages of head, midpiece, and principal piece abnormalities. Based on these types of defects, some studies have reported a low relationship between the percentage of morphologically normal sperm and per cycle fertility ($r = 0.34$, $P < 0.01$; Jasko et al., 1990), percent of pregnant/cycle and percent of pregnant/first cycle ($r = 0.42$ and 0.39, respectively; $P < 0.05$; Love, 2011). Likewise, Love et al., (2000) observed that the sperm morphological defects that had an influence on the odds of pregnancy were the percentages of abnormal heads (OR: 0.970, $P < 0.02$), detached heads (OR: 0.888, $P < 0.05$), abnormal midpieces (OR: 0.883, $P < 0.0001$), coiled tails (OR: 0.839, $P < 0.002$), and premature germ cells (OR: 0.421, $P < 0.0005$).

As mentioned above with sperm motility, the evaluation of sperm morphology is particularly dependent of other factors inherent to the technique, such as processing method, type and quality of the equipment used, training of the personnel conducting the evaluation, environmental conditions in which the test is performed, among others (Brito et al., 2011; Murcia-Robayo et al., 2018). In general, the use of the wet mount technique is superior to the use of background stains such as eosin-nigrosin, due to the potential effect to induce artifactual changes on the sperm shape during processing (particularly coiled and bent tails, or detached heads; Brito et al., 2011); it also limits the capacity to detect subtle but fertility limiting defects, such as abnormalities on the midpiece, head or acrosome (Love, 2018). However, the use of wet-mount samples requires the investment on high-quality phase-contrast or DIC microscopes, which are expensive, and require special training of the personnel involved in sperm evaluation.

FLUORESCENT-BASED METHODS FOR OBJECTIVE EVALUATION OF STALLION SPERMATOZOA

Since the late 80's, several researchers have proposed the use of a wide battery of tests to evaluate different sperm features that could be related with male fertility or could explain why the sperm do not survive well after cooling or cryopreservation (Amann & Hammerstedt, 1993; Colenbrander et al., 2003). In a review published, Varner & Johnson (2007) proposed a list of several processes that the spermatozoa must undergo during its development, maturation, and ejaculation, as well as several attributes that a spermatozoon must possess to fertilize an oocyte (Table 1). These traits include several characteristics of the sperm plasma membrane, acrosome membrane, mitochondria, DNA and molecular mechanisms which cannot be assessed directly or indirectly by the simple determination of sperm motility and morphology.

Therefore, the incorporation of techniques that allow the evaluation of the characteristics mentioned above is necessary for the clinical and research arena. Recent developments on computational based technologies for somatic cell analysis such as flow cytometry have increased also the use of these technologies in andrology. Nowadays, it is common to find laboratories around the world where the sperm quality evaluation in different species, including the stallion, is based on flow cytometry or fluorescence microscopy analysis. Among the fluorescence-based techniques, evaluation of sperm plasma membrane intactness, acrosome membrane intactness and function, mitochondrial functionality, DNA integrity, oxidative stress status and some molecular pathways related with the fertilization process are frequently reported. Part of this manuscript will be focused on the most common probes that could be used for this purpose.

PLASMA MEMBRANE INTACTNESS: THE CONCEPT OF THE LIVE/DEAD SPERM

The sperm plasma membrane is a bilayer composed mainly by phospholipids, cholesterol, transmembrane proteins and glycocalyx (Parks & Lynch, 1992; Gadella, 2008). Its main functions are related to physiological processes such as osmoregulation, ionic interchange, and the preparation of the sperm capacitation and subsequent acrosome reaction (Boerke et al., 2008; Gadella et al., 2008; Drobnis et al., 1993). Given this, an intact and functional plasma membrane is vital for both sperm survival after cooling, freezing/thawing or sex-sorting, as well as when

assessing stallion sperm quality and fertility. The concept of viability has been commonly associated with plasma membrane intactness, and several methods for its assessment are based on the use of fluorochromes or fluorescent dyes (Peña et al., 2018; Love, 2012; Love et al., 2003; Casey et al., 1993).

Table 1. A partial list of attributes that stallion sperm must to possess in order to successfully travel through the mare's reproductive tract, fertilize an oocyte and produce an embryo. Adapted from Varner & Johnson, 2007.

Attribute	Mechanisms and methods associated with its function
Capacity to move from the insemination site, through the uterus, until the oviduct	Fully functional machinery related to energy production-mitochondrial function; assessment of sperm motion characteristics
Highly compacted chromatin as a protective mechanism against environmental injury	DNA integrity assessment through SCSA, COMET, TUNEL or 8OHdG techniques
Alterations in the sperm plasma membrane during formation of spermatozoal reservoir within the oviduct, and acquisition of maturational changes required for zona pellucida binding	Plasma membrane and acrosome intactness; evaluation of sperm capacitation by immunolabeling of protein tyrosine phosphorylation
Penetration through the cumulus-oocyte-complex and zona pellucida	Intact and fully functional acrosome membranes
Oocyte activation and embryo development	Spermatozoon-derived factor-Phospholipase C Zeta
Pronucleus formation in the zygote	DNA integrity assessment through SCSA, COMET, TUNEL or 8OHdG techniques

The most commonly used techniques for assessing sperm plasma membrane intactness include the combination of 2 or even 3 fluorescent probes. The use of membrane impermeable probes such as propidium iodide (PI) or ethidium homodimer-1 (EthD-1) in combination with membrane permeable probes such as SYBR-14 or 6-carboxyfluorescein diacetate (CFDA) is among the most common methods both in clinical and research settings (Garner & Johnson, 1995; Merkies et al., 2000). Others have included to these combinations the use of bisbenzimides or membrane permeable dyes (particularly Hoechst 33342), mostly when flow cytometry is used, for gating out of the analysis all the non-sperm particles that could generate interpretation errors (Martínez-Pastor et al., 2010). Regardless of their chemical classification, all these probes are targeted to bind the DNA and differ fundamentally in their capacity to cross intact or damaged plasma membranes, emitting a fluorescent signal when excited using the appropriate wavelength (Table 2). After excitation with a laser or a fluorescent beam, SYBR-14 and CFDA emit green fluorescence, PI and EthD-1 emit red fluorescence, and Hoechst 33258 and 33342 emit blue fluorescence; thus, after the use of an adequate combination of fluorescent probes the clinician or researcher is able to determine percentages of intact and damaged sperm plasma membrane (Figure 1). All the fluorescent probes mentioned above can be used either with fluorescence microscopy or flow cytometry, being the last one considered as a more reliable, fast and objective method for sperm analysis (Peña et al., 2016; Petrunkina et al., 2007). It can also be combined with other compartmental probes such as acrosomal or mitochondrial probes for the simultaneous analysis of sperm integrity and function (Love et al., 2003; Hernández-Avilés et al., 2018a). Furthermore, there is a commercially available automated-cell counter (NucleoCounter SP-100®, Chemometec, Allerød, Denmark) for evaluation of stallion sperm plasma membrane intactness (Love, 2012; Foster et al., 2011). This device uses disposable cassettes loaded with propidium iodide to determine the total sperm number in the sample, and then the proportion of that sperm with damaged plasma membranes based on the exclusion of PI in less than 2 minutes, being particularly useful in research stations and stud farms.

Table 2. Fluorescent probes commonly used in clinical and research situations for multiparametric assessment of stallion sperm quality using either flow cytometry or fluorescence microscopy.

Organelle or function	Interpretation	Fluorescent probe	Excitation/Emission wavelength	Staining pattern
Plasma membrane intactness	Damaged plasma membrane	Propidium iodide	535/617 nm	Red staining over the sperm head
		Hoechst 33258	352/461 nm	Blue staining over the sperm head
		Ethidium homodimer	528/617 nm	Red staining over the sperm head
	Intact plasma membrane	SYBR-14	485/517 nm	Green staining over the sperm head
		6-Carboxyfluorescein-diacetate	492/517 nm	Green staining over the sperm head
Acrosome membrane intactness	Damaged/reacted acrosome	FITC-PSA or FITC-PNA	490/525 nm	Green staining over the acrosomal region
Mitochondrial membrane intactness and function	Mitochondrial intactness	Rhodamine 123 or MitoTracker Green	490/534 nm	Strong green fluorescence over the midpiece
	High mitochondrial membrane potential	JC-1	535/590 nm	Red-orange staining over the midpiece
	Low mitochondrial membrane potential		485/530 nm	Green-none staining over the midpiece
DNA Integrity	Intact DNA	Sperm Chromatin Structure Assay (SCSA)	500/526 nm	Green staining over the sperm head
	Damaged DNA		460/650 nm	Orange-red staining over the sperm head
ROS production and oxidative stress	Cellular superoxide anion production	Dihydroethidium	518/605 nm	Red staining over the sperm head
	Mitochondrial superoxide anion	MitoSOX Red	510/580 nm	Red staining over the midpiece
	Lipid peroxidation	C11-BODIPY	510/590 nm	Shift from red to green staining over the sperm
Capacitation	Protein tyrosine phosphorylation	FITC-conjugated monoclonal antibody	490/525 nm	Green staining pattern over the sperm principal piece
Oocyte activation after fertilization	Phospholipase C-Zeta	FITC-conjugated monoclonal antibody	490/525 nm	Green staining pattern over the acrosome region and sperm principal piece

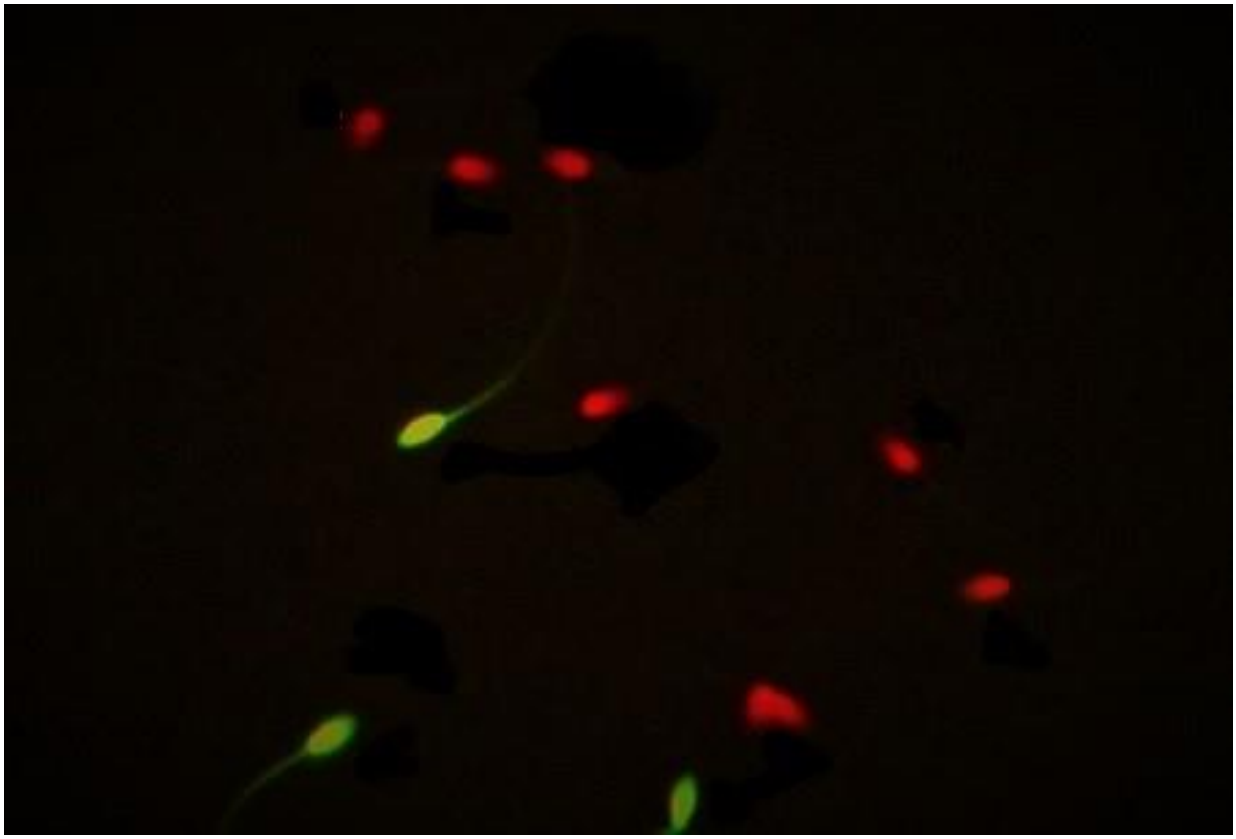


Figure 1. Frozen-thawed stallion spermatozoa stained using the combination of SYBR-14 and propidium iodide, for analysis of sperm plasma membrane intactness. Green spermatozoa are considered as spermatozoa with intact plasma membrane (live sperm), while red spermatozoa are considered as spermatozoa with damaged plasma membrane (dead sperm). 400X magnification.

More recently, other authors have proposed the use of fixable stains for sperm viability assessment such as Fixable Live/Dead Red stain (Thermo Fisher Scientific, Waltham, MA, USA; Teague et al., 2018; Trentin et al., 2018). These probes are based on the binding of the dye to free cellular amines, resulting in an intense fluorescent staining in dead cells. The advantages of these fixable stains are related to the easiness to fix the samples in a remote location for further analysis in a suitable laboratory, without incurring on artifactual changes induced by storage conditions or storage time. Additionally, their unique emission spectra allow to use multicolor flow cytometry for simultaneous assessment of several sperm features (Peña et al., 2018; Peña et al., 2016).

Other authors have proposed the use of dyes that measure the fluidity of the sperm plasma membrane such as Merocyanine-540 or Yo-Pro-1, to determine subtle changes on the plasma membrane related to capacitation or apoptosis (Gallardo-Bolaños, 2014; Rathi et al., 2001). These researchers argue that the use of those fluorescent probes could enhance the detection of early stages of plasma membrane damage; nevertheless, a recent study conducted by Stump et al. (2014) determined that no advantage on the detection of plasma membrane changes was obtained with the combination of Yo-Pro-1/EthD-1 as compared to the use of SYBR-14/PI and flow cytometry in stallion sperm cooled and stored up to 10 days.

ACROSOME MEMBRANE: INTACTNESS, FUNCTION AND THEIR RELATIONSHIP WITH STALLION FERTILITY

The sperm acrosome is a single, modified organelle located in the anterior portion of the sperm head, between the plasma membrane and nuclear envelope. This organelle is enclosed by two membranes, inner and outer acrosomal membrane, which fuses with the sperm plasma membrane during the acrosome reaction and contains several hydrolytic and glycolytic enzymes that are intended for the sperm penetration process through the cumulus-oocyte complex and zona pellucida during fertilization (Eddy & O´Brien, 1994). Due to its relevance during the fertilization process, the assessment of acrosomal status is commonly performed during sperm evaluation in reference laboratories around the world (Love, 2018), particularly for cooled or frozen semen (Bosard et al., 2005; Bedford et al., 2000).

Most frequently used techniques for assessment of acrosome intactness are based on the use of molecules derived from plants, known as lectins, which have a high affinity for carbohydrate residues that are expressed in the inner or outer acrosome membrane. These lectins are conjugated to fluorescent molecules, such as fluorescein isothiocyanate-FITC, which emits a fluorescent green signal when excited with an appropriate wavelength (Table 2). Commercial reagents based on the combination of FITC with the lectins *Pisum sativum* (PSA) or *Arachis hypogaea* (PNA) are the most reported stains for stallion sperm acrosome evaluation (Farlin et al., 1992; Cheng et al., 1996). Both conjugated lectins are commonly combined with viability stains such as propidium iodide or Hoechst 33258 to evaluate simultaneously the plasma membrane and acrosome membrane intactness (Figure 2). This method is particularly useful when flow cytometry is used to evaluate cooled or frozen/thawed semen since the proportion of acrosomal damage is low in stallion fresh sperm unless plasma membrane damage is sustained first (Bedford et al., 2000). With this combination of fluorescent probes, the clinician and researcher can distinguish four different sperm subpopulations: 1) Viable/acrosome-intact sperm, 2) Viable/acrosome damaged sperm, 3) Non-viable/acrosome-intact sperm, 4) Non-viable/acrosome damaged sperm (Figure 3).

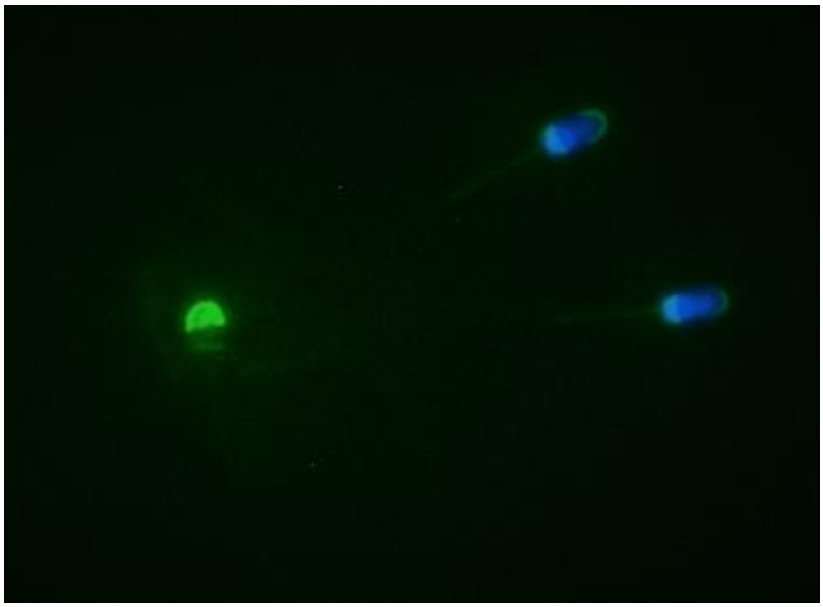


Figure 2. Cooled-stored stallion spermatozoa stained using the combination of Hoechst 33258 and FITC-PNA for simultaneous assessment of sperm plasma membrane and acrosome intactness. Blue spermatozoa are considered as spermatozoa with damaged plasma membrane, while the green staining over the acrosomal region is associated with acrosomal damage or premature acrosome reaction. 400X magnification.

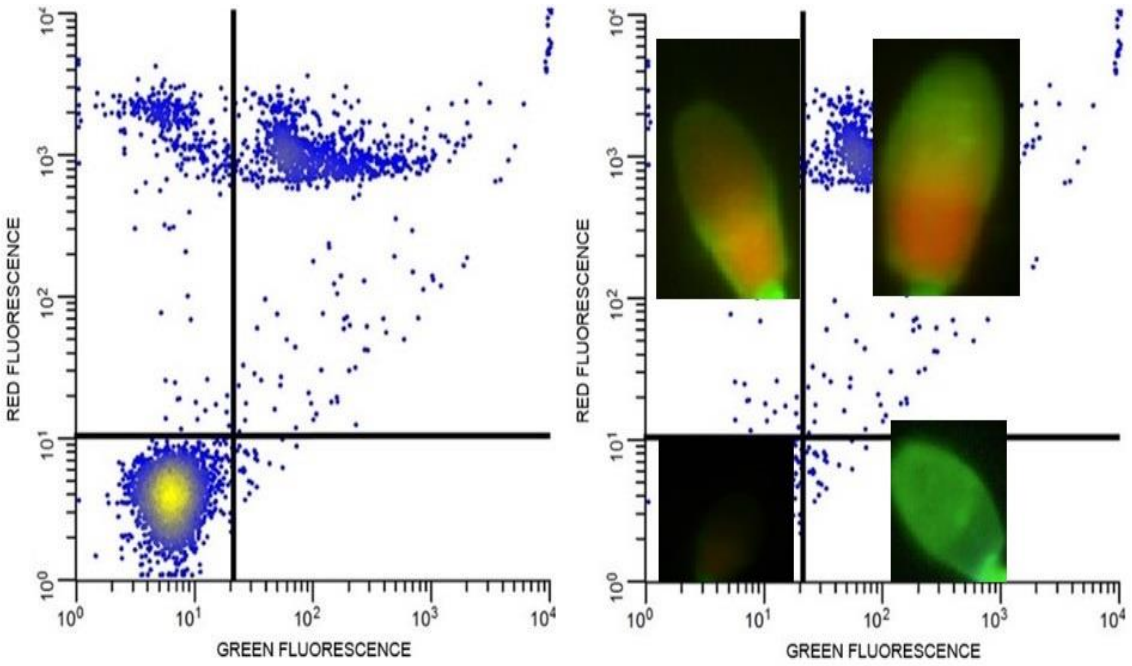


Figure 3. Flow cytometry scattergram representing the simultaneous assessment of sperm plasma membrane and acrosome intactness using the combination of FITC-PSA and propidium iodide in cooled stored stallion sperm, and the corresponding staining patterns after observation under fluorescence microscopy. Left lower quadrant: sperm with intact plasma membrane and acrosome. Right lower quadrant: sperm with intact plasma membrane and disrupted acrosome. Left upper quadrant: sperm with damaged plasma membrane and intact acrosome. Right upper quadrant: sperm with damaged plasma membrane and acrosome.

The assessment of acrosome function is fundamental for the evaluation of stallion fertility, particularly in cases of stallions with good sperm motility, morphology, and even viability but with unexplained subfertility/infertility. Several compounds have been reported to induce acrosome reaction in stallion sperm, as a method to evaluate its functionality, including heparin (Varner et al., 1993), progesterone (Meyers et al., 1995; Rathi et al., 2003), zona-pellucida extracts (Meyers et al., 1996), and calcium ionophores (Varner et al., 2001; 2002). Among these inducers, calcium ionophore is the most commonly used for the clinical assessment of acrosome reaction, mainly because the difficulty to obtain equine oocytes for zona-pellucida binding, or the relatively weak response of the spermatozoa to progesterone stimulation (Meyers et al., 1995). Varner et al. (2001) were the first to report the failure of acrosome reaction after stimulation with calcium ionophore A23187 in a group of five stallions with a history of subfertility or infertility (per cycle fertility rate less than 20%) but with satisfactory semen quality and testicular size based on SFT guidelines. In this study, they evaluated acrosome reaction by transmission electron microscopy, considered as the gold standard. However, the use of electron microscopy is limited by the cost associated with the technique and equipment, as well as the personnel training involved in the sample processing, and the number of spermatozoa that could be evaluated (100-200 sample). Later, this same group of researchers reported the induction of acrosome reaction by calcium ionophore stimulation with the use of flow cytometry and FITC-PSA staining (Figure 4; Bosard et al., 2005), making it easier and faster to conduct. They reported that 36% of the sperm from fertile stallions were able to acrosome react after exposure to A23187, as compared to 11% of the sperm from subfertile stallions. Although a cause of the acrosome reaction failure in stallions has not been clearly established, Brinsko et al. (2007) reported that cholesterol to phospholipids ratio in sperm and seminal plasma from subfertile stallions was increased as compared to fertile stallions, implying that an excess of cholesterol and subsequent reduction of the fusogenic capacity of the plasma and acrosome membranes during the acrosome reaction could explain the altered fertility. Moreover, Raudsepp et al. (2012) reported the association of a genetic trait in the chromosome 13 (FKBP6) with the failure of acrosome reaction of spermatozoa from seven Thoroughbred stallions after exposure to calcium ionophore.

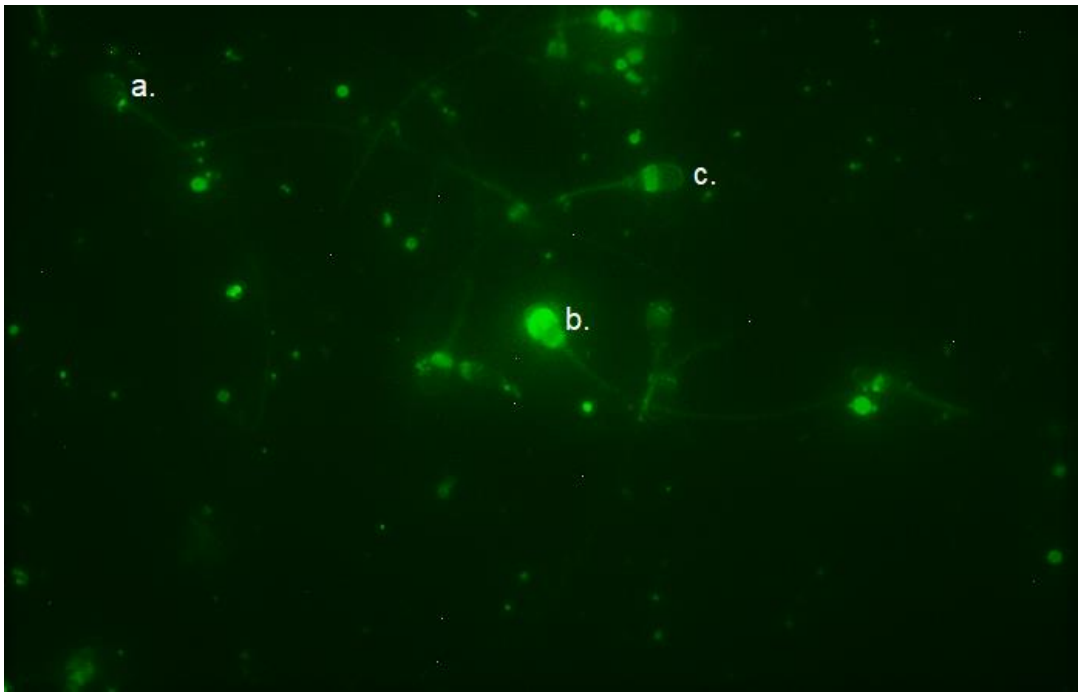


Figure 4. Freshly ejaculated stallion sperm incubated with calcium ionophore A23187 and stained with FITC-PNA for assessment of acrosome function. Some staining patterns can be observed in this image, related to different stages during the acrosome reaction: a. Non-reacted acrosome. b. Partially reacted acrosome-spermatozoa undergoing acrosome reaction. c. Fully reacted acrosome. 400X magnification.

ASSESSMENT OF MITOCHONDRIAL INTEGRITY AND FUNCTION: IS THE MITOCHONDRIA ONLY AN ENERGY FACTORY FOR THE SPERM?

Sperm mitochondria are located in the mid-piece and are organized in a helicoidal pattern around the axoneme. As compared to other species, such as bulls and boars, in which energy production is dependent on the presence of glycolytic enzymes in the flagellum, stallion spermatozoa seem to be mainly dependent on the mitochondrial oxidative phosphorylation pathway to produce ATP required for motility and plasma membrane function (Plaza-Dávila et al., 2016; Gibb et al., 2014). Furthermore, the evidence suggests that sperm mitochondria are severely affected by cooling and cryopreservation, partially explaining the alterations on motility, plasma membrane intactness, and longevity (Hernández-Avilés et al., 2018a; Peña et al., 2015; Macías-García et al., 2012). Given this, the evaluation of sperm mitochondrial function seems to be relevant, particularly when different protocols of stallion semen preservation are tested.

Most methods for mitochondrial evaluation are based on the use of fluorescent probes in combination with flow cytometry or fluorescence microscopy. Rhodamine 123 and MitoTracker, particularly MitoTracker Green, are dyes which are transported by active diffusion into the mitochondrial matrix during respiration. The higher the mitochondrial function, the brighter is the fluorescent green signal over the sperm midpiece that is emitted by these fluorochromes. However, these dyes have not been commonly used for stallion sperm analysis and cannot distinguish between functional states of the mitochondria. Other probe, 5,5´, 6,6´-tetrachloro-1,1´, 3,3´-tetraethyl-benzimidazolyl-carbocyanine iodide, or JC-1 have been extensively used for analysis of stallion sperm mitochondrial function (Ortega-Ferrusola et al., 2009b; Love et al., 2003; Gravance et al., 2000). This dye has the peculiarity of producing a differential staining pattern, depending on whether the mitochondria have a high or low membrane potential. Thus, under high mitochondrial membrane potential (associated with high functionality), the probe emits a red-orange fluorescence when excited, whereas under low mitochondrial membrane potential, the probe emits a green fluorescence. Using fluorescence microscopy, we have observed that the probe is easily vanished from the mid-piece in sperm with non-functional mitochondria, low motility, and plasma membrane damage, which gives an unstained pattern to the mitochondrial helix (Figure 5). Others have also observed inconsistent results when determining the sperm mitochondrial function using JC-1 and flow cytometry, particularly in cooled stallion semen or freshly ejaculated human sperm (Uribe et al., 2017; Stump et al., 2014; Love et al., 2003).

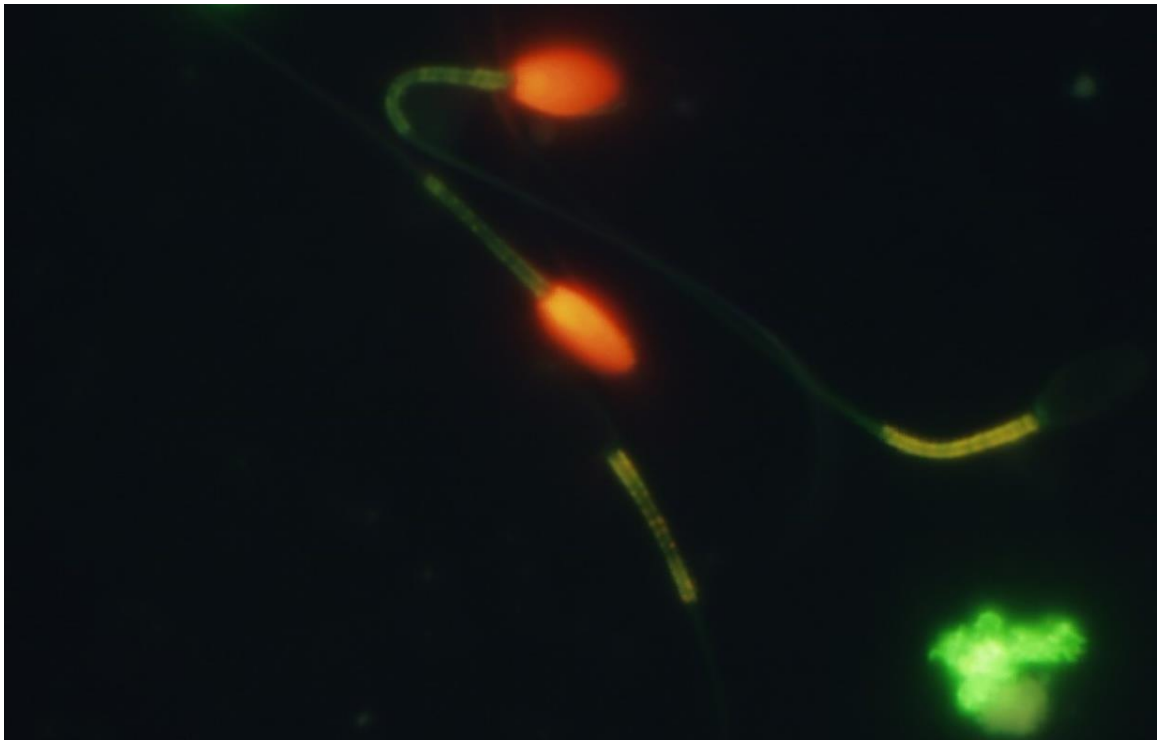


Figure 5. Frozen-thawed stallion spermatozoa stained with the combination of propidium iodide and JC-1 for simultaneous assessment of plasma membrane intactness and mitochondrial function. Spermatozoa with orange midpieces and no staining over the head are considered as sperm with intact plasma membrane and high mitochondrial membrane potential, while spermatozoa with faint green staining over the midpiece and red staining over the head are considered as sperm with damaged plasma membrane and low mitochondrial membrane potential. 1000X magnification.

Other fluorescent probes have also been reported for stallion sperm mitochondrial analysis, including MitoTracker Deep Red, a fluorescent probe with the same ability of JC-1 to discriminate between functional and non-functional mitochondria but with the flexibility to be fixed before staining, allowing the use of this dye in remote locations prior to submission to an appropriate laboratory, or its combination with viability stains for multiparametric flow cytometric analysis (Peña et al., 2018). Recently, a group of researchers has validated an approach to evaluate the function of stallion sperm mitochondria based on the rate of oxygen consumption (Darr et al., 2016a; Darr et al., 2016b). These researchers reported the use of a biosensor plate system, which allows a high-throughput analysis of oxygen consumption in a time-dependent manner, as an approach to quantify the capacity of the mitochondria to undergo cellular respiration and subsequently ATP production. Although this approach allows objectively estimate mitochondrial function, the cost and time required to run this assay make it impractical for a clinical scenario and limited to a few laboratories around the world.

Nevertheless, the evaluation of sperm mitochondrial function has been mainly used to determine the capacity of the stallion spermatozoa to survive during freezing regimens, and more importantly to predict the capacity of a given ejaculate to survive a freezing protocol, when assessment of mitochondrial function is combined with other assays of sperm quality, such as the presence of apoptotic or oxidative stress markers (Yesté et al., 2015; Ortega-Ferrusola et al., 2009b).

DNA INTEGRITY AND STABILITY: EXPLORING THE CAUSES OF EARLY EMBRYONIC DEATH

Due to the high grade of sperm chromatin and associated nucleoproteins compaction that takes place during the spermatogenesis process, it is commonly assumed that the sperm DNA is “non-functional” until the fertilization process occurs. However, this grade of compaction is necessary to protect the sperm DNA during transport through the male and female reproductive tract, and proper fertilization, and embryo development (Evenson et al., 2000; Evenson et al., 1980). Indeed, studies conducted in bovine and primate models have demonstrated that

impaired sperm DNA quality is highly associated with embryo apoptosis and early embryonic death (Burruel et al., 2013; Fatehi et al., 2006). Among the different methods for DNA evaluation in stallion spermatozoa, the most commonly used in clinics and laboratories is the Sperm Chromatin Structure Assay (SCSA), developed initially by Evenson et al. (1980), and validated later for stallion sperm by Kenney et al. (1995). This technique measures the susceptibility of DNA to be denatured after exposure to an acid-detergent solution. The sample is then stained with a metachromatic dye, acridine orange, which binds to the phosphate groups present in single-stranded DNA, generating an orange-red staining pattern over the sperm head; or binds to the double-stranded DNA, generating a green staining pattern over the sperm head. Thus, the sperm exhibiting green staining are considered to have intact DNA, whereas the sperm exhibiting orange-red staining are considered as to have denaturated DNA (Evenson, 2016; Love, 2005). This technique requires the use of a flow cytometer to analyze the proportion of single-stranded or "damaged" DNA in a sample, and several indexes are calculated based on this number (Figure 6). Of particular interest, the percentage of COMP-α_t or Cells Outside the Main Population is the most commonly used parameter to determine the extent of DNA damage (Love, 2005). The SCSA has been successfully used to determine the relationship between DNA damage and subfertility in Thoroughbred stallions (Love & Kenney. 1998), and the effect of cooling regimens on the DNA quality and subsequently stallion fertility (Love et al., 2005; Love et al., 2002).

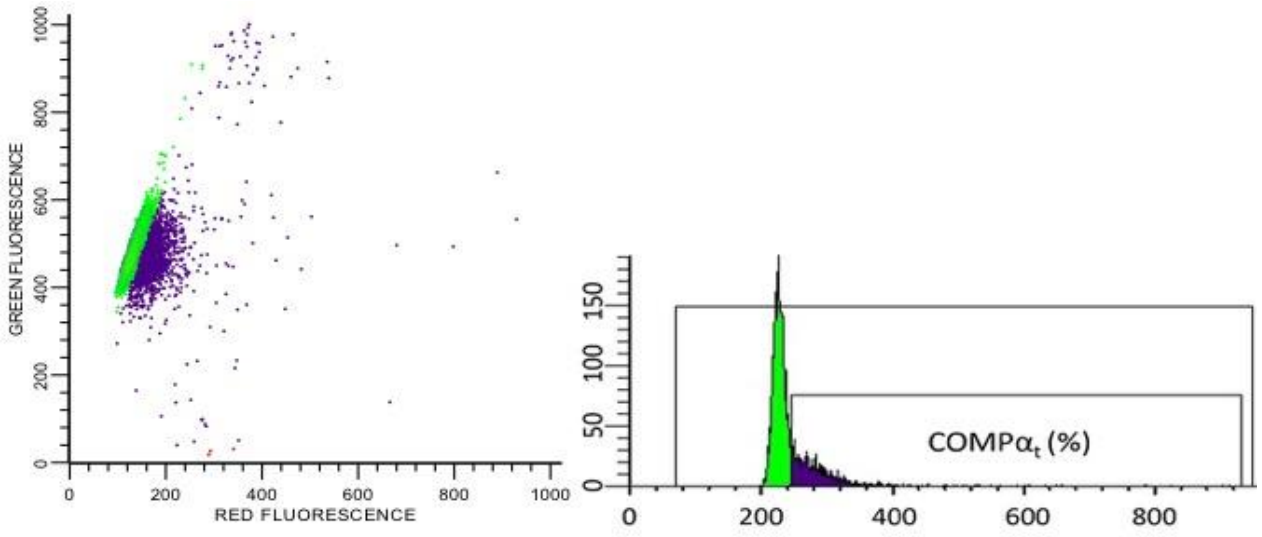


Figure 6. Flow cytometric analysis of DNA integrity in freshly ejaculated stallion sperm using the Sperm Chromatin Structure Assay (SCSA). The image on the left demonstrates an scattergram used for the analysis of the sperm where the green dots represents spermatozoa with intact DNA, while the blue dots represents spermatozoa with susceptible DNA (COMPαt). The image on the right, is a histogram of the analysis to quantify the percentage of COMPαt or sperm with damaged DNA.

Other tests used for sperm DNA evaluation are the COMET and TUNEL (Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling) assays. The COMET assay is a single-cell gel electrophoresis with the formation of a "tail" caused by the migration of a broken DNA strand (Evenson, 2016). This technique has been used to estimate the extent of damage on stallion sperm DNA after cooling, stallion sperm exposed to reactive oxygen species, or in stallions exposed to heat stress (Serafini et al., 2015; Baumber et al., 2003a; Linfor & Meyers, 2002). On the other hand, the TUNEL assay quantifies the incorporation of FITC-labeled-deoxyuridine triphosphate (dUTP) nicks in the DNA strand. These dUTP nicks bind to the single or double DNA strains, depending on the number of spaces available for the nick (Evenson, 2016). Thus, a sperm with compromised DNA will allow the binding of a higher proportion of nicks, leading to a higher proportion of fluorescent light in the sperm head. This technique has been also used for stallion sperm DNA analysis (Serafini et al., 2018); however, comparisons between the COMET and TUNEL assays with the SCSA and their relationship with stallion fertility have not been conducted yet. In any case, the use of these techniques requires the use of high-cost pieces of equipment, such as flow cytometers, as well as highly qualified personnel for conducting the assay and

interpreting the results, making its use less common in a clinical situation, with few laboratories in North America, Europe and Latin America performing these assays for evaluation of stallion fertility.

REACTIVE OXYGEN SPECIES-ROS AND OXIDATIVE STRESS RELATED CHANGES IN SPERM FUNCTION

As mentioned above, the stallion spermatozoa mainly rely on the oxidative phosphorylation pathway to produce energy for the maintenance of motility and viability (Plaza-Dávila et al., 2016). Although energy production through this method is highly efficient, as compared to glycolysis, the formation of toxic-by-products such as reactive oxygen species (ROS) is also constant (about 2% of the oxygen utilized is transformed in ROS; Aitken et al., 2016). This implies that stallion spermatozoa are constantly faced to a relatively high level of oxidative stress, which could be assumed as normal under certain conditions (Aitken, 2017; Gibb et al., 2014). However, when procedures such as centrifugation to remove seminal plasma, cooling, cryopreservation or sex-sorting are performed, the production of ROS is exacerbated leading to self-perpetuating cycle, in which mitochondria, plasma membrane, motility, and DNA integrity are mainly affected (Martín-Muñoz et al., 2018; Balao da Silva et al., 2016; Martín-Muñoz et al., 2015; Baumber et al., 2000). The close relationship between stallion sperm quality and oxidative stress was established approximately 15 years ago, with the pioneering works conducted by Barry Ball and Stuart Meyers at the University of California, Davis. Since then, a whole new set of fluorescence probes and techniques for the assessment ROS production and its effects on the stallion spermatozoa have been published.

As superoxide anion ($O_2^{\bullet}$) is the main ROS produced by stallion sperm mitochondria either in physiological and pathological conditions (Burnaugh et al., 2010; Sabeur & Ball, 2006; Baumber et al., 2003b), the detection of this metabolite is crucial for the study of oxidative stress, particularly when different semen storage protocols are tested (Ertmer et al., 2017; Yesté et al., 2015). Burnaugh et al. (2007) were the first to report the use of the probe dihydroethidium, for assessment of $O_2^{\bullet}$ production in freshly-ejaculated, capacitated, acrosome-reacted and oxidized stallion spermatozoa. This fluorophore is oxidized into ethidium after getting in contact with $O_2^{\bullet}$, generating a red fluorescent light when excited using an appropriate wavelength (Table 2). However, some researchers argue that this probe cannot discriminate between mitochondrial $O_2^{\bullet}$ and cytosolic $O_2^{\bullet}$ production, which has some implications when physiological or pathological ROS production is assessed. Thus, a new generation of probes called MitoSOX red (Molecular Probes, OR, USA) has been used in stallion spermatozoa to discriminate $O_2^{\bullet}$ production not only from mitochondrial or cytosolic origin but also from live and dead spermatozoa when combined with membrane permeable probes (Gibb et al., 2015).

Another approach that has been commonly used for assessment of oxidative stress in stallion sperm is the use of probes for detection of the pathological effects that ROS has on the plasma membrane or DNA. One of the most common causes of sperm death is related to the effect that ROS has on the lipids of the plasma membrane, in a process known as lipid peroxidation (Alvarez & Storey, 1984). For the evaluation of lipid peroxidation, the probe C11-BODIPY has been extensively used with stallion spermatozoa (Ortega-Ferrusola et al., 2009a; Neild et al. 2005; Ball & Vo, 2002). This lipophilic dye binds to the lipids of the plasma membrane, changing its fluorescent light from red to green when the lipids are oxidized. Thus, in sperm suffering lipid peroxidation, the fluorescent signal that is emitted by C11-BODIPY will be green when excited with an appropriate wavelength (Table 2). More recently, lipid peroxidation has been also evaluated in stallion sperm using fluorescent-conjugated antibodies for 4-hydroxynonenal (4-HNE) production (Gibb et al., 2016; Martin-Muñoz et al., 2015). This electrophilic aldehyde is produced during the lipid peroxidation cascade, mainly after degradation of lipids by peroxyl or alkoxyl radicals and has several implications on the perpetuation of plasma membrane degradation and reduction on sperm motility (Aitken et al., 2012).

In the past 5 years, other fluorescent-conjugated antibodies for detection of DNA damage from oxidative origin have been reported (Serafini et al., 2018; Gibb et al., 2015). The 8OHdG technique measures the production of guanine adducts in the DNA chain using a fluorescent-conjugated 8-hydroxy-2´-deoxyguanosine (8OHdG) antibody, either in nuclear and mitochondrial DNA, which is particularly useful for establishing a relationship between oxidative damage due to storage procedures and causes of reduced stallion fertility due to early embryonic death.

MOLECULAR PROCESSES RELATED TO SPERM FERTILITY: CAPACITATION AND OOCYTE ACTIVATION

Nowadays, the advancement on the understanding of sperm physiology in different species such as mice or humans has allowed the discovery of several factors involved in the capacitation process, which can be extrapolated to the stallion sperm. From these, probably the most important molecular event is the protein tyrosine phosphorylation, which is considered a landmark of sperm capacitation (Galantino-Homer et al., 1997; Visconti et al., 1995). Tyrosine phosphorylation has been usually analyzed by western blotting of proteins present in the sperm plasma membrane, including in stallion spermatozoa (McPartlin et al., 2008; González-Fernández et al., 2013); however, this technique is time-consuming and expensive. Therefore, the use of monoclonal antibodies labeled with fluorescent markers is an excellent alternative for assessment of tyrosine phosphorylation and has been successfully reported in stallion sperm being the fastest, the most reliable and easy-to-conduct method used in clinical and research arena (Macías-García et al., 2015; Leemans et al., 2014; González-Fernández et al., 2013).

On the other hand, there is a recent interest in factors affecting fertilization and embryo development, particularly when assisted reproduction techniques are used in humans or horses. From these factors, probably the most evaluated is the presence and function of Phospholipase C-Zeta (PLC-z), a protein from testicular origin which is related with a rise on intracellular calcium concentrations in the oocyte after fertilization, a process necessary for oocyte genome activation and embryonic development (Swann et al., 2006). In infertile men, defects in the expression or abnormal localization of PLC-z have been reported as a cause of male infertility, particularly when intracytoplasmic sperm injection or semen cryopreservation-artificial insemination is used (Nomikos et al., 2011; Heytens et al., 2009). Bedford-Guaus et al., (2012) were the first to report the association between the deficiency on the quantity or localization of PLC-z in sperm from a group of six stallions with normal sperm quality parameters, including sperm motility, morphology, and DNA quality, but with low seasonal pregnancy rates (<20%). Recent studies conducted in Germany and Brazil have also reported the association between the presence of the gene which codifies for PLC-z and stallion per cycle pregnancy rate and seasonal pregnancy rate (Bueno et al., 2018; Schrimpf et al., 2014). Although those studies determined the presence of sperm PLC-z by genetic analysis, preliminary results from Gonzalez-Castro et al., (2018) reported the use of commercial fluorescence-conjugated monoclonal antibodies for evaluation of PLC-z in conjunction with flow cytometry and fluorescence microscopy. These results suggest that this technique can be also used in the same manner as the assessment of protein tyrosine phosphorylation.

HOW RELATED TO STALLION FERTILITY ALL THESE NEW SPERM QUALITY TESTS ARE?

The main question that arouses when reviewing the whole battery of new tests that clinicians and researchers have available for stallion sperm quality assessment is: Can those new methods improve the capacity to discriminate between different levels of fertility, or help us to establish the cause of reduced fertility in a given stallion? As mentioned above, the spermatozoa have to undergo several physiological processes through their journey to reach the oocyte, so a model for stallion fertility prediction must include various tests that could evaluate objectively all these requirements (Table 1). Clinicians and researchers must be aware that a single sperm quality test will not allow to determine stallion fertility or sperm damage after storage.

To our knowledge, there are few studies that meet these criteria. Barrier-Battut et al. (2016, 2017) reported the use of a model for stallion fertility prediction either in fresh, cooled and frozen-thawed semen, which included the assessment of total motility (CASA), plasma membrane intactness (SYBR-14/PI), ROS production (peroxides production), acrosome intactness (FITC-PNA and calcium ionophore A23187), DNA integrity (SCSA) and hypoosmotic resistance. Likewise, Love et al. (2015) reported that embryo recovery rate increased as sperm total motility (subjectively assessed), total sperm number on the insemination dose, sperm plasma membrane intactness (propidium iodide and NucleoCounter), DNA integrity (SCSA), and sperm morphology increased. Remarkably, this last study classified stallions in groups of high (>65%) and average embryo recovery rate capacity (approximately 50%). Suggested threshold values were: Total motility: ≥65%, progressive motility: ≥45%, sperm plasma membrane intactness: ≥71%, morphologically normal sperm: ≥47%, total sperm number in the insemination dose: 1.14 billion sperm, and COMPαt: ≤26.8%.

From all the parameters mentioned above, maybe sperm plasma membrane and DNA intactness could be considered as the most relevant to fertility. Kiser et al. (2014) reported no statistical differences on pregnancy rates when a group of mares was bred with cooled semen stored for 96 hours that had reduced sperm motility (<5% TMOT) but with high viability (74%), or with fresh semen with high sperm motility (75% TMOT) and high viability (80%). Similarly, Hernández-Aviles et al., (2018b) reported that sperm motility but no viability is affected by reducing the amount of glucose in the extender, and more importantly that sperm motility can be easily reestablished in cooled semen stored for 5

days, just by centrifugation and resuspension in a glucose-containing extender. Furthermore, studies conducted by Choi et al. (2011) suggest that freeze-dried sperm, which are immotile, but still viable and with intact DNA can be used for intracytoplasmic sperm injection, with similar pregnancy and live foals rates produced by motile spermatozoa. Taken together, these results suggest that even immotile but viable sperm are still able to resume motility and be competent for fertilization, which reinforces the relevance of sperm plasma membrane intactness and DNA integrity assessment.

CONCLUSIONS

To summarize, there are several methods available for clinicians and researchers for stallion sperm analysis. Most of these methods are based on the use of fluorescent markers in combination with fluorescence microscopy or flow cytometry. The utility of these techniques relies on the assessment between different sperm organelles and functions, which are needed by the spermatozoa during its travel through the male and female reproductive tract, the subsequent oocyte fertilization and early embryo development. Some of these techniques, such as motility, plasma membrane intactness, acrosome function, DNA integrity, or genetic markers have the potential to discriminate different levels of fertility in a group of stallions, as well as to determine the causes of unexplained subfertility/infertility. Other techniques, such as mitochondrial function and ROS production are particularly useful when different protocols for sperm storage are evaluated. In any case, clinicians and researchers must be aware that a battery of tests evaluating as many parameters as possible might be necessary to achieve a more detailed diagnosis. However, the results of these methods cannot be analyzed by themselves if other factors such as management of mare fertility are not considered (Brinkerhoff et al., 2010).

REFERENCES

Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. 2016. Causes and consequences of oxidative stress in spermatozoa. **Reproduction Fertility Development** 28(1-2):1-10.

Aitken RJ, Whiting S, De luliis GN, McClymont S, Mitchell LA, Baker MA. 2012. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species and apoptosis by targeting succinate dehydrogenase. **J Biol Chem** 287:33048-33060.

Aitken RJ. 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. **Molecular Reproduction and Development** 84(10): 1039-1052.

Alvarez JG, Storey BJ. 1984. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. **Biology of Reproduction** 30 (2):833-841.

Amann RP, Hammerstedt RH. 1993. In vitro evaluation of sperm quality: An opinion. **Journal of Andrology** 14:397-406.

Amann RP, Waberski D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential development. **Theriogenology** 81(1):5-17.

Balao da Silva C, Ortega-Ferrusola C, Morrell JM, Rodríguez-Martínez H, Peña FJ. 2016. Flow cytometric chromosomal sex sorting of stallion spermatozoa induces oxidative stress on mitochondria and genomic DNA. **Reproduction in Domestic Animals** 51(1):18-25.

Ball BA, Vo A. 2002. Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C11-BODIPI 581/591. **Journal of Andrology** 23(2):259-269.

Ball L, Ott RS, Mortimer RG, Simons JC. 1983. Manual for breeding soundness examination of bulls. **Journal of the Society for Theriogenology** 12:1-65.

Barrier-Battut I, Kempfer A, Becker J, Lebailly L, Camugli S, Chevrier L. 2016. Development of a new fertility prediction model for stallion semen, including flow cytometry. **Theriogenology** 86:111-1131.

Barrier-Battut I, Kempfer A, Lemasson N, Chevrier L, Camugli S. 2017. Prediction of the fertility of stallion frozen-thawed semen using a combination of computer-assisted motility analysis, microscopical observation and flow cytometry. **Theriogenology** 97:186-200.

Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. **Journal of Andrology** 21(6):895-902.

Baumber J, Ball BA, Linfor JJ, Meyers SA. 2003a. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. **Journal of Andrology** 24(4):621-628.

Baumber J, Sabeur K, Vo A, Ball BA. 2003b. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. **Theriogenology** 60(7):1239-1247.

Bedford SJ, Varner DD, Meyers SA. 2000. Effects of cryopreservation on the acrosomal status of stallion spermatozoa. **Journal of Reproduction and Fertility** Suppl 56:133-140.

Bedford-Guaus SJ, McPartlin LA, Varner DD. 2012. Characterization of equine Phospholipase C Zeta: A review and preliminary results on expression defects in subfertile stallions. **Journal of Equine Veterinary Science** 32:445-450.

Boerke A, Tsai PS, Garcia-Gil N, Brewis IA, Gadella BM. 2008. Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. **Theriogenology** 70:1188-1196.

Bosard T, Love CC, Brinsko SP, Blanchard TL, Thompson J, Varner DD. 2005. Evaluation and diagnosis of acrosome function/dysfunction in the stallion. **Animal Reproduction Science** 89(1-4):215-217.

Brinkerhoff JM, Love CC, Thompson JA, Blodgett G, Teague SR, Varner DD. 2010. Influence of mare age, pre-breeding mare status, breeding methods, and stallion on first cycle pregnancy rates on a large commercial breeding farm. **Animal Reproduction Science** 121:159.

Brinsko SP, Love CC, Bauer JE, Macpherson ML, Varner DD. 2007. Cholesterol-to-phospholipid ratio in whole sperm and seminal plasma from fertile stallions and stallions with unexplained subfertility. **Animal Reproduction Science** 99:65-71.

Brito LF, Greene LM, Kelleman A, Knobbe M, Turner R. 2011. Effect of method and clinician on stallion sperm morphology evaluation. **Theriogenology** 76: 745-750.

Bueno VLC, Bastos HBA, Centeno LAM, Kretzmann NA, Bertolini M, Mattos RC, Rechsteiner FS. 2018. The role of PLCζ and WPB2NL gene expression in semen quality and fertility of stallions. **Journal of Equine Veterinary Science** 66:33-34.

Burnaugh L, Ball BA, Sabeur K, Thomas AD, Meyers SA. 2010. Osmotic stress stimulates generation of superoxide anion by spermatozoa in horses. **Animal Reproduction Science** 117(3-4):249-260.

Burnaugh L, Sabeur K, Ball BA. 2007. Detection of superoxide anion by equine spermatozoa as detected by dihydroethidium. Theriogenology 67: 580 – 589.

Burruel V, Klooster KL, Chitwood J, Ross PJ, Meyers SA. 2013. Oxidative damage to rhesus macaque spermatozoa results in mitotic arrest and transcript abundance changes in early embryos. **Biology of Reproduction** 89(3):72.

Casey PJ, Hillman RB, Robertson KR, Yudin AI, Liu IK, Drobnis EZ. 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. **Journal of Andrology** 14(4):289-297.

Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B. 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. **Journal of Andrology** 17:674-682.

Choi YH, Varner DD, Love CC, Hartman DL, Hinrichs K. 2011. Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. **Reproduction** 142(4):529-538.

Colenbrander B, Gadella BM, Stout TA. 2003. The predictive value of semen analysis in the evaluation of stallion fertility. **Reproduction in Domestic Animals** 38(4):305-311.

Darr CR, Cortopassi GA, Datta S, Varner DD, Meyers SA. 2016a. Mitochondrial oxygen consumption is a unique indicator of stallion spermatozoal health and varies with cryopreservation media. **Theriogenology** 86(5):1382-1392.

Darr CR, Varner DD, Teague S, Cortopassi GA, Datta S, Meyers SA. 2016b. Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility and ROS production. **Biology of Reproduction** 95(2):34.

Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. 1993. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. **Journal of Experimental Zoology** 265(4):432-437.

Eddy EM, O´Brien DA. 1994. The Spermatozoon. In: Knobil E, Neill JD (Eds.), **The Physiology of Reproduction**. 2nd edition. Raven Press, NY. pp: 29-77.

Ertmer F, Oldenhof H, Schütze S, Rohn K, Wolkers WF, Sieme H. 2017. Induced sub-lethal oxidative damage affects osmotic tolerance and cryosurvival of spermatozoa. **Reproduction Fertility Development** 29(9):1739-1750.

Evenson DP, Darzynkiewicz Z, Melamed MR. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. **Science** 210:1131.

Evenson DP, Jost LK, Varner DD. 2000. Relationship between sperm nuclear protamine free-SH status and susceptibility to DNA denaturation. **Journal of Reproduction and Fertility** Suppl 56:401.

Evenson DP. 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. **Animal Reproduction Science** 169:56-75.

Farlin ME, Jasko DJ, Graham JK, Squires EL. 1992. Assessment of Pisum sativum agglutinin in identifying acrosomal damage in stallion spermatozoa. **Molecular Reproduction and Development** 32:23-27.

Fatehi AN, Bevers MM, Schoevers E, Roelen BA, Colenbrander B, Gadella BM. 2006. DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. **Journal of Andrology** 27(2):176-188.

Foster ML, Love CC, Varner DD, Brinsko SP, Hinrichs K, Teague S, Lacaze K, Blanchard TL. 2011. Comparison of methods for assessing integrity of equine sperm membranes. **Theriogenology** 76:334-341.

Gadella BM. 2008. Sperm membrane physiology and relevance for fertilization. **Animal Reproduction Science** 107:229-236.

Galantino-Homer HL, Visconti PE, Kopf GS. 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. **Biology of Reproduction** 58(3):707-719.

Gallardo-Bolaños JM, Miró-Morán A, Balao da Silva CM, Morillo-Rodríguez A, Plaza-Dávila M, Aparicio IM, Tapia JA, Ortega-Ferrusola C, Peña FJ. 2014. Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. **PLoS One** 7: e30688.

Garner DL, Johnson LA. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. **Biology of Reproduction** 53:276-284.

Gibb Z, Lambourne SR, Aitken RJ. 2014. The paradoxical relationship between stallion fertility and oxidative stress. **Biology of Reproduction** 91(3):77.

Gibb Z, Lambourne SR, Quadrelli J, Smith ND, Aitken RJ. 2015. L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. **Biology of Reproduction** 93(4):104.

Gibb Z, Lambourne SR, Curry BJ, Hall SE, Aitken RJ. 2016. Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. **Biology of Reproduction** 94(6):133.

Gonzalez-Castro RA, Graham JK, Carnevale EM. 2018. Localization and quantitative expression of Phospholipase C Zeta in equine sperm using commercial antibodies. **Reproduction Fertility Development** 30(1):228.

González-Fernández L, Macías-García B, Loux SC, Varner DD, Hinrichs K. 2013. Focal adhesion kinases and calcium/calmodulin dependent protein kinases regulate protein tyrosine phosphorylation in stallion sperm. **Biology of Reproduction** 88(6):138.

Gravance CG, Garner DL, Baumber J, Ball BA. 2000. Assessment of equine sperm mitochondrial function using JC-1. **Theriogenology** 53(9):1691-1703.

Gravance CG, Liu IK, Davis RO, Hughes JP, Casey PJ. 1996. Quantification of normal head morphometry of stallion spermatozoa. **Journal of Reproduction and Fertility** 108(1):41-46.

Hernández-Avilés C, Gómez-Romero M, Buitrago-Horta R, Lozano-Márquez H, Jiménez-Escobar C, Zambrano-Varón J. 2018a. Evaluation of post-thaw sperm function and integrity parameters under different freezing regimens in Colombian Paso Fino stallions. **Journal of Equine Veterinary Science** 67:7-14.

Hernández-Avilés C, Love CC, Serafini R, Ghosh S, Teague SR, La Caze KA, Varner DD. 2018b. The effects of extender glucose concentration and storage temperature on stallion semen quality following cooled storage. **Journal of Equine Veterinary Science** 66:46.

Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon SY, Fissore RA, Hamer R, Deane CM, Ruas M, Grasa P, Soleimani R, Cuvelier CA, Gerris J, Dhont M, Deforce D, Leybaert L, De Sutter P. 2009. Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. **Human Reproduction** 24:2417-2428.

Hinrichs K. 2018. Assisted reproductive techniques in mares. **Reproduction in Domestic Animals** 53(Suppl. 2):4-13.

Hoogewijs MK, de Vlieguer SP, Govaere JL, de Schauwer C, de Kruif A, van Soom A. 2012. Influence of counting chamber type on CASA outcomes of equine semen analysis. **Equine Veterinary Journal** 44(5):542-549.

Hopkins FM, Spitzer JC. 1997. The new society for theriogenology breeding soundness evaluation system. **Veterinary Clinics North America: Food Animal Practice** 13:283-293.

Hurtgen JP. 1992. Evaluation of the stallion for Breeding soundness. **Veterinary Clinics North America: Equine Practice** 8(1):149-165.
Janson-Whitesell M, McDonnell SM, Turner RM. 2014. Modernizing the stallion breeding soundness evaluation. **Journal of Equine Veterinary Science** 34:50-51.

Jasko DJ, Lein DH, Foote RH. 1990. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1998). **Journal of the American Veterinary Medical Association** 197(3):389-394.

Jasko DJ, Little TV, Lein DH, Foote RH. 1992. Comparison of spermatozoa movement and semen characteristics with fertility in stallions: 64 cases (1987-1988). **Journal of the American Veterinary Medical Association** 200:979-985.

Kenney RM, Evenson DP, Garcia MC, Love CC. 1995. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. **Biology of Reproduction** 52 (Issue Monograph Series 1): 647-653.

Kenney RM, Hurtgen J, Pierson R, Witherspoon D, Simons J. 1983. Theriogenology and the equine, Part II: The stallion. **Society for Theriogenology Manual for Clinical Fertility Evaluation of the Stallion**. Hastings, NE.

Kiser AM, Brinsko SP, Love CC, Varner DD, Sudderth K, Blanchard TL. 2014. Relationship of sperm quality to fertility after 4 days of cooled storage of equine semen. **Journal of Equine Veterinary Science** 34: 602-605.

Kuisma P, Andersson M, Koskinen E, Katila T. 2006. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. **Acta Veterinaria Scandinavica** 17(48):14.

Leemans B, Gadella BM, Sostaric E, Nelis H, Stout TA, Hoogewijs M, Van Soom A. 2014. Oviduct binding and elevated environmental pH induce protein tyrosine phosphorylation in stallion spermatozoa. **Biology of Reproduction** 91(1):13.

Linfor JJ, Meyers SA. 2002. Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell gel electrophoresis. **Journal of Andrology** 23 (1):107-113.

Loomis PR, Graham JK. 2008. Commercial semen freezing: Individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. **Animal Reproduction Science** 105(1-2):119-128.

Love CC, Noble JK, Standridge SA, Bearden CT, Blanchard TL, Varner DD, Cavinder CA. 2015. The relationship between sperm quality in cool-shipped semen and embryo recovery rate in horses. **Theriogenology** 84:1587-1593.

Love CC, Thompson JA, Brinsko SP, Rigby SL, Blanchard TL, Lowry VK, Varner DD. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. **Theriogenology** 60(6):1127-1138.

Love CC, Thompson JA, Lowry VK, Varner DD. 2002. Effect of storage time and temperature on stallion sperm DNA and fertility. **Theriogenology** 57(3):1135-1142.

Love CC, Varner DD, Thompson JA. 2000. Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. **Journal of Reproduction and Fertility** Suppl 56: 93-100.

Love CC. 2005. The sperm chromatin structure assay: a review of clinical applications. **Animal Reproduction Science** 89(1-4):39-45.

Love CC. 2011. Relationship between sperm motility, morphology and the fertility of stallions. **Theriogenology** 76(3):547-557.

Love CC. 2012. Measurement of concentration and viability in stallion sperm. **Journal of Equine Veterinary Science** 32(8):464-466.

Love CC. 2018. Sperm quality assays: How good are they? The horse perspective. **Animal Reproduction Science** 194:63-70.

Love CC. Brinsko SP, Rigby SL, Thompson JA, Blanchard TL, Varner DD. 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. **Theriogenology** 63:1584-1591.

Macías-García B, González-Fernández L, Loux SC, Rocha AM, Guimaraes T, Peña FJ, Varner DD, Hinrichs K. 2015. Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. **Reproduction** 149: 87-99.

Macías-García B, Miró-Morán A, González-Fernández L, Ortega-Ferrusola C, Morillo-Rodríguez A, Bolaños JM, da Silva CM, Martínez HR, Tapia JA, Peña FJ. 2012. The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. **Journal of Andrology** 33 (1):105-113.

Martínez-Pastor F, Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Anel L, de Paz P. 2010. Probes and techniques for sperm evaluation by flow cytometry. **Reproduction in Domestic Animals** 45(Suppl. 2):67-78.

Martín-Muñoz P, Anel-López L, Ortiz-Rodríguez JM, Álvarez M, de Paz P, Balcao da Silva C, Rodríguez-Martinez R, Gil MC, Anel L, Peña FJ, Ortega-Ferrusola C. 2018. Redox cycling induces spermptosis and necrosis in stallion spermatozoa while the hydroxyl radical (OH●) only induces spermptosis. **Reproduction in Domestic Animals** 53(1):54-67.

Martín-Muñoz P, Ortega-Ferrusola C, Vizuete G, Plaza-Dávila M, Rodríguez-Martínez, Peña FJ. 2015. Depletion of intracellular thiols and increased production of 4-hydroxynonenal that occur during cryopreservation of stallion spermatozoa lead to caspase activation, loss of motility and cell death. **Biology of Reproduction** 93:1-11.

McPartlin LA, Littell J, Mark E, Nelson JL, Travis AJ, Bedford-Guaus SJ. 2008. A defined medium supports changes consistent with capacitation in stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of acrosomal exocytosis. **Theriogenology** 69(5):639-650.

Merkies K, Chenier T, Plante C, Buhr MM. 2000. Assessment of stallion spermatozoa viability by flow cytometry and light microscope analysis. **Theriogenology** 54:1215-1224.

Meyers S, Liu IKM, Overstreet JW, Drobnis EZ. 1996. Sperm-zona pellucida binding and zona-induced acrosome reaction in the horse: comparisons between fertile and subfertile males. **Theriogenology** 46:1277-1288.

Meyers SA, Overstreet JW, Liu IK, Drobnis EZ. 1995. Capacitation in-vitro of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. **Journal of Andrology** 16:47-54.

Miró-Arias M, Gallardo-Bolaños JM, Morillo-Rodríguez A, Balao da Silva C, Aparicio IM, Tapia JA, Valecillo A, Vega-Pla JL, Peña FJ, Ortega-Ferrusola C. 2013. Sperm susceptibility to Poxidative stress in the Retuertas endangered horse. **Journal of Equine Veterinary Science** 33:962-968.

Murcia-Robayo RY, Jouanisson E, Beauchamp G, Diaw M. 2018. Effects of staining method and clinician experience on the evaluation of stallion sperm morphology. **Animal Reproduction Science** 188:165-169.

Neild DM, Brouwers JF, Colenbrander B, Agüero A, Gadella BM. 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. **Molecular Reproduction and Development** 72(2):230-238.

Nomikos M, Elgmati K, Theodoridou M, Calver BL, Cumbes B, Nounesis G, Swann K, Lai FA. 2011. Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and PIP2 hydrolysis activity of sperm PLC zeta. **Biochemical Journal** 434: 211-217.

Ortega-Ferrusola C, González-Fernández L, Morrell JM, Salazar-Sandoval C, Macías-García B, Rodríguez-Martinez H, Tapia JA, Peña FJ. 2009a. Lipid peroxidation, assessed with BODIPI-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. **Reproduction** 138(1):55-63.

Ortega-Ferrusola C, Macías-García B, Gallardo-Bolaños JM, González-Fernández L, Rodríguez-Martínez H, Tapia JA, Peña FJ. 2009b. Apoptotic markers can be used to forecast the freezability of stallion spermatozoa. **Animal Reproduction Science** 114(4):393-403.

Panarace M, Pellegrini RO, Basualdo MO, Bele M, Ursino DA, Cisterna R, Desimone G, Rodríguez E, Medina MJ. 2014. First field results on the use of stallion sex-sorted semen in a large-scale embryo transfer program. **Theriogenology** 81(4):520-525.

Parks JE, Lynch DV. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. **Cryobiology** 29(2):255-266.

Peña FJ, Ball BA, Squires EL. 2018. A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples. **Cytometry Part B: Clinical Cytometry** 94(2):302-311.

Peña FJ, Martín-Muñoz P, Ortega-Ferrusola C. 2016. Flow cytometry probes to evaluate stallion spermatozoa. **Journal of Equine Veterinary Science** 43:S23-S28.

Peña FJ, Plaza Davila M, Ball BA, Squires EL, Martin Muñoz P, Ortega Ferrusola C, Balao da Silva C.2015. The impact of reproductive technologies on stallion mitochondrial function. **Reproduction in Domestic Animals** 40:529-537.

Petrunkina AM, Waberski D, Günzel-Apel AR, Töpfer-Petersen E. 2007. Determinants of sperm quality and fertility in domestic species. **Reproduction** 134:3-17.

Plaza-Davila M, Muñoz PM, Bolaños JM, Stout TA, Gadella BM, Tapia JA, da Silva CB, Ortega-Ferrusola C, Peña FJ. 2016. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. **Reproduction** 152 (6): 683 – 694.

Quintero-Moreno A, Miró J, Teresa-Rigau A, Rodríguez-Gil JE. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. **Theriogenology** 59(9):1973-1990.

Rathi R, Colenbrander B, Bevers MM, Gadella BM. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. **Biology of Reproduction** 65:462-470.

Rathi R, Colenbrander B, Stout TAE, Gadella BM. 2003. Progesterone induces acrosome reaction in stallion spermatozoa via a protein tyrosine kinase dependent pathway. **Molecular Reproduction and Development** 64(1):120-128.

Raudsepp T, McCue ME, Das PJ, Dobson L, Vishnoi M, Fritz KL, Schaefer R, Rendahl AK, Derr JN, Love CC, Varner DD, Chowdhary BP. 2012. Genome-wide association study implicates testis-sperm specific FKBP6 as a susceptibility locus for impaired acrosome reaction in stallions. **PLoS Genet** 8(12):e1003139.

Sabeur K, Ball BA. 2006. Detection of superoxide anion generation by equine spermatozoa. **American Journal of Veterinary Research** 67(4):701-706.

Schrimpf R, Dierks C, Martinsson G, Sieme H, Distl O. 2014. Genome-wide association study identifies Phospholipase C Zeta 1 (PLCz1) as a stallion fertility locus in Hanoverian warmblood horses. **PLoS One** 9(10):e109675.

Serafini R, Varner DD, Bissett W Jr., Blanchard TL, Teague SR, Love CC. 2015. Sperm DNA quality evaluated by comet assay and sperm chromatin structure assay in stallions after unilateral orchiectomy. **Theriogenology** 84(5):833-837.

Serafini R, Varner DD, Blanchard TL, Teague SR, LaCaze K, Love CC. 2018. Effects of seminal plasma and flash-freezing on DNA structure of stallion epididymal sperm exposed to different potentiators of DNA damage. **Theriogenology** 117:34-39.

Stump KE, Teague SR, Love CC, Blanchard TL, Brinsko SP, Long CR, Varner DD. 2014. Assessment of stallion sperm membrane integrity using three different flow cytometric methods. **Journal of Equine Veterinary Science** 34(1):83.

Swann K, Saunders CM, Rogers NT, Lai FA. 2006. PLCζ (zeta): A sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. **Seminars in Cell and Developmental Biology** 17(2):264-73

Teague SR, Ligon EM, Serafini R, Varner DD, Love CC. 2018. Validation of a fixable stain for assessing the viability of stallion sperm. **Journal of Equine Veterinary Science** 66:42.

Trentin JM, González-Castro RA, Rubin MIB, Graham JK, Carnevale EM. 2018. Fixable stain for flow cytometric evaluation of stallion sperm viability. **Journal of Equine Veterinary Science** 66: 70-71.

Uribe P, Villegas JV, Boguen R, Treulen F, Sánchez R, Mallmann P, Isachenko V, Rahimi G, Isachenko E. 2017. Use of the fluorescent dye tetra-methylrhodamine-methyl-ester-perchlorate for mitochondrial membrane potential assessment in human spermatozoa. **Andrologia** 49:e12753.

Varner DD, Bowen JA, Johnson L. 1993. Effect of heparin on capacitation/acrosome reaction of equine sperm. **Archives of Andrology** 31:199-207.

Varner DD, Brinsko SP, Blanchard TL, Love CC, Macpherson ML, Heck RS, Johnson L. 2001. Subfertility in stallions associated with spermatozoal acrosome dysfunction. **Proceedings of the Annual Convention of the AAEP** 47:227-228.

Varner DD, Johnson L. 2007. From a sperm's eye view – revisiting our perception of this intriguing cell. **Proceedings of the Annual Convention of the AAEP** 53:104-177.

Varner DD, Thompson JA, Blanchard TL, Heck R, Love CC, Brinsko SP, Johnson L. 2002. Induction of the acrosome reaction in stallion spermatozoa: effects of incubation temperature, incubation time, and ionophore concentration. **Theriogenology** 58:303-306.

Varner DD. 2016. Approaches to the breeding soundness examination and interpretation or results. **Journal of Equine Veterinary Science** 43(Suppl.):S37-44.

Veeramachaneni DNR, Moeller CL, Pickett BW, Shiner KA, Sawyer HR. 1993. On processing and evaluation of equine seminal samples for cytopathology and fertility assessment: The utility of electron microscopy. **Journal of Equine Veterinary Science** 13:207-215.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. 1995. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. **Development** 121(4):1129-1137.

Voss JL, Pickett BW, Squires EL. 1981. Stallion spermatozoal morphology and motility and their relationship with fertility. **Journal of the American Veterinary Medical Association** 178: 287.

Yesté M, Bonet S, Rodríguez-Gil JE, Rivera del Álamo MM. 2018. Evaluation of sperm motility with CASA-Mot: which factors may influence our measurements? **Reproduction Fertility and Development** 30(6):789-798.

Yesté M, Estrada E, Rocha LG, Marín H, Rodríguez-Gil JE, Miró J. 2015. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. **Andrology** 3(2):395-407.