Review Report

Evaluating sperm fertilizing potential: what can we predict?*

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Abstract

Multiparametric approaches that characterize the functional and metabolic aspects of sperm offer real-time information to discriminate and identify sperm subpopulations with attributes suitable for fertilization. In vivo, sperm requires various abilities to fertilize an oocyte that are less relevant for in vitro fertilization (IVF). Traditional evaluations such as sperm motility, DNA integrity, morphology, and viability as a measure of plasma membrane integrity are less sensitive and specific in detecting sublethal damage that can reduce a sample's fertilizing potential. During in vivo fertilization, the female reproductive tract has a critical role in removing most of the dead sperm and those with sublethal damage that may explain why certain sperm variables have reduced predictive power for IVF success. Assessment of metabolic and physiological status provides new methods to estimate sperm fertilizing potential both in vivo and in vitro by identifying sublethal damage and capacitation-related changes. For IVF and intracytoplasmic sperm injections, sperm factors involved in oocyte activation and embryo development (e.g. phospholipase C zeta 1 [PLCZ1]), are considered the major male factors contributing to the failure of oocyte activation and embryo development. Reduced amounts, abnormal localization, and genetic variability of the PLCZ1 have been identified as factors in male infertility, suggesting potential diagnostic and prognostic value for clinical applications. However, male reproductive performance and fertilization are highly complex processes influenced by several factors. This complexity limits the predictive value of any single sperm assessment for reliably determining fertility outcomes.

Keywords: Fertilization, flow cytometry, stallion sperm, phospholipase C zeta 1

Introduction

Several attributes can be evaluated in vitro to assess sperm quality and estimate the fertilizing competence of sperm samples.[1,2] Sperm motility and morphology are considered as standard, verifiable, rapid, and cost-effective methods for evaluation of semen doses. However, other sperm attributes that include structural and functional aspects of sperm, such as plasma and acrosomal membrane integrity, DNA fragmentation, mitochondrial function, and reactive oxygen species (ROS) production and accumulation, are also associated with male fertility.[1-6] Because several sperm characteristics are required for fertilization, individual sperm evaluations have inherent limitations to estimate potential fertility.[7] Advanced techniques such as flow cytometry and functional assays enable an efficient investigation of multiple parameters in thousands of sperm, thereby providing a higher degree of accuracy, reliability, and repeatability in sperm assessment. Integration of multiple assays for multiparametric evaluation of several sperm attributes can estimate the fertilizing potential of sperm.[8-10]

Various in vitro sperm assessments are associated with sample quality and sperm fertilizing ability in vivo,[5,6] but are less predictive for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).[11-14] This discrepancy exists because many parameters associated with sperm ability to navigate the female reproductive tract to reach the oocyte are not relevant for IVF or ICSI. Female reproductive tract interaction with sperm provides the appropriate number of sperm in a suitable physiological state for fertilizing oocytes after they reach the uterine tube, to ensure successful fertilization.[15] However, in IVF and ICSI, sperm attributes are bypassed by the intrinsic characteristics of these procedures. The best example is ICSI, in which an individual sperm is chosen by the embryologist, based on motility and morphology at low magnification (200-400 x) as the final criteria for individual sperm selection, negating some aspects of the sperm physiological state and biomolecules required for fertilization and embryo development.[12,16,17] Limited research is available on specific attributes and biomarkers that are critical for characterization of sperm fertilizing potential under in vitro conditions.

*Presented at the 2025 Society for Theriogenology Conference; published after peer review.

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Citation: Clinical Theriogenology 2026, 18, 13232, http://dx.doi.org/10.58292/CT.v18.13232

Sperm evaluation is challenging because semen samples are a heterogenous population of sperm formed by many subpopulations with distinctive attributes. Only a reduced fraction of this large heterogenous population retains attributes necessary for fertilization. In addition, sperm analysis requires expertise, laboratory equipment, cost-effectiveness, accuracy, and repeatability to offer consistent results for clinical applications. This review considers some predictive approaches and the latest advances in multiparametric sperm evaluations for fertility in vivo and in vitro, with a focus on the horse.

Predictive value of sperm attributes for fertility in vivo

Numerous sperm attributes are associated with fertility in vivo, but their predictive value varies in sensitivity and specificity. In dismounted semen samples and fresh sperm from stallions used for artificial insemination (AI), sperm parameters such as total and progressive motility, path and progressive velocity, and morphologically normal sperm were associated with increased pregnancy rates per cycle.[18,19] The sperm quality parameters most highly correlated with fertility outcome (pregnancy rates, pregnancy rates per cycle, and pregnancy in the first cycle) were total motility ($r \geq 0.37$) and morphologically normal sperm ($r \geq 0.39$).[18,19] Sperm DNA fragmentation, evaluated by sperm chromatin structure assay,[20] also exposed important negative correlations with pregnancy rates in the first cycle ($r = -0.42$) and per cycle ($r = -0.42$).[18,21] Recently, fresh stallion sperm from dismounted semen samples ($n = 143$) were evaluated for motility and processed in a double-chamber device (Samson™ System; Memphasys, Sydney, NSW, Australia) for sorting and reaction with water-soluble tetrazolium salt 1 (WST1), a membrane-impermeant probe, which in the presence of sperm proportionally becomes reduced to a formazan product that can be quantified by spectrophotometry to assess cell viability and count.[22] Reduction of WST-1 to formazan appears to be mediated by electrons released at the sperm cell surface, apparently by electrons released by the oxidation of NADH via a plasma membrane electron transport system.[23] The NADH activity is involved in the maintenance of intracellular NADH/NAD+ redox balance and regulation of glycolytic flux.[24] Using discriminant analysis for predicting pregnancy rate, the volume of the dismount sample, sperm concentration motility, and kinematics variables reached an accuracy of 65.5%, misclassifying 34.5% of samples. With the addition of the WST1 reduction test, as measure of the metabolic state of the sperm, and motility variables after sperm sorting, the prediction (68.3%) did not significantly change.[22] Due to important variations in reproductive performance among stallions, the discriminant analysis was optimized and individually performed per stallion, predicting the outcome of a given cover with accuracy between 79 and 100%.[22] Overall, elements of a traditional sperm analysis, motion characteristics, and metabolic state are predictive parameters to estimate sperm fertilizing potential and accurately predict the likelihood of establishing a pregnancy. However, individual differences in stallion fertility and reproductive performance prevent the development of a generalized predictive model.

The association between stallion sperm quality in cooled-shipped samples and embryo recovery rates as a measure of fertility has also been investigated.[19,25] Threshold values for total and progressive motility, morphologically normal sperm, and DNA fragmentation between samples that yielded average (41-54%) and high (62-75%) embryo recovery rates were ≥ 65%, ≥ 45%, ≥ 47%, and ≤ 27%, respectively.[19,25]

In attempting to predict fertility in cooled stallion sperm used for AI, a large number of structural and functional attributes were evaluated in sperm doses from 43 stallions with variable fertility. Evaluations were aimed at assessing motility and kinematics, morphology, viability, mitochondrial activity, oxidation level, acrosome and DNA integrity, plasma membrane stability, and hypoosmotic resistance (hypoosmotic swelling test, HOS).[6] Conventional parameters (29 variables) related to motility, morphology, and viability assessed by eosin-nigrosin staining were probed by factorial discriminant analysis to identify optimal combinations of variables to differentiate fertility groups. The analysis of conventional sperm variables allowed an incomplete distinction among fertility groups (> 55% fertile; 55-45% medium; and < 45% subfertile), misclassifying 18% of stallions. However, when a minimum of selected functional sperm variables related to mitochondrial function, plasma and acrosomal membrane response to acrosomal exocytosis induction by ionophore, DNA fragmentation, and HOS response were included, the same analysis precisely classified every stallion into its correct fertility group.[6] According to this data analysis, examining just 6 ejaculates from a stallion might be sufficient to accurately characterize the stallion's fertility.[6] Furthermore, a regression analysis using the best combination of 20 variables (including kinematics, mitochondrial function, oxidative status, plasma and acrosomal membrane response to ionophore, DNA fragmentation, and HOS response) explained 94.2% of the variation in fertility. This model resulted in a high coefficient of determination (adjusted R-squared) of 0.942 between the predicted and observed fertility, confirming a high level of prediction.[6] Although these analytical metrics clearly characterize fresh and cooled sperm and are a valuable tool for assessing fertilizing potential, there are still several practical limitations that hinder the reliable prediction of fertility outcomes in the field. These limitations include the large variability in individual stallion fertility, inconsistencies in breeding management, and the difficulty in obtaining enough cycles per stallion to effectively control for the mare effect.

In the equine industry, large numbers of AI are performed using frozen-thawed sperm that are cryopreserved in various extenders and conditions. A study investigated the association of motility, morphology, response to sperm survival test, and DNA fragmentation of frozen-thawed sperm ($n = 17$), and stallion's age with pregnancy rate.[26] This sperm survival test has the tolerance in function of time (up to 100 hours) of fresh sperm prior to cryopreservation incubated in lactose-chelate-citrate-yolk medium containing 3.5% glycerol diluted 1:3 (volume/volume) at 4 °C. Sperm survival was determined by the time when the sample retains > 5% progressive motility.[26] Sperm morphology did not associate with pregnancy rates. Pregnancy rates had positive correlations with postthaw progressive motility ($r = 0.87$) and sperm survival response ($r = 0.84$) and a negative correlation ($r = -0.94$) with DNA fragmentation measured by a modified sperm chromatin dispersion test.[26] However, this high correlation must be taken with precautions and limited to the experimental condition of this study. Some stallion sperm samples displayed a high value (> 60%) of DNA fragmentation, which is not common in stallion sperm analysis. Stallion's age was positively correlated ($r = 0.51$; $p = 0.04$) to pregnancy rates but with less strength, as the association between stallion age and fertility outcome was not linear.[26] Similar multiparametric and statistical approaches were used for cooled sperm, parameters from frozen-thawed stallion sperm ($n = 33$) and were tested to predict pregnancy rates per cycle.[5] When only motility and kinematics were used, the best-fit model accounted for 74.2% of the variability in fertility as a response. However, when

Citation: Clinical Theriogenology 2026, 18, 13232, http://dx.doi.org/10.58292/CT.v18.13232

a combination of 25 variables including motility, morphology, viability, oxidation level, acrosome integrity, DNA integrity, and hypoosmotic response were used, the model predictive power markedly improved. This comprehensive set of variables explained 94.5% of the fertility variability and resulted in a high coefficient of determination (adjusted R-squared) of 0.98 between the predicted and observed pregnancy rates per cycle.[5] Also, data analysis of this study suggested that the evaluation of 3 straws might be suitable to characterize a cryopreserved ejaculate.[5] Interestingly, the predictive model specifically for frozen-thawed sperm included average path velocity (VAP) and rapid VAP, morphology, and DNA fragmentation.[5] These variables likely are more sensitive and specific variables for fertilization after insemination with a reduced frozen-thawed sperm count. These models demonstrate that although motility and kinematics evaluations are useful, a multiparametric assessment incorporating functional and structural integrity markers is necessary for highly accurate fertility prediction of cryopreserved stallion sperm.

Predictive values of sperm attributes for fertility in vitro

Sperm viability, morphology, plasma membrane function, and DNA integrity are traits frequently assessed for sperm quality that are associated with fertilization in vivo. In equine ICSI, sperm selected are evaluated using methods (eosin–nigrosin staining for viability and morphology, HOS test for membrane function, and sperm chromatin dispersion for DNA fragmentation) appropriate for low sperm numbers.[11] We observed that sperm morphology and DNA fragmentation did not differ in samples that resulted in a positive or negative outcome for cleavage, embryo development, and pregnancy after ICSI. However, the proportion of viable sperm (eosin negative) was higher in sperm in which oocyte injections resulted in cleavage and embryo development. Only sperm samples with higher plasma membrane functionality (HOS+) differed in oocyte injections that resulted in the establishment of pregnancy of ICSI-produced embryos. In univariate regression analyses, cleavage and blastocyst rates increased with higher sperm viability, but pregnancy did not. Cleavage and pregnancy rates increased with higher sperm plasma membrane functionality. Sperm morphology and DNA fragmentation did not have predictive value. Using stepwise multiple regression analysis, sperm viability, plasma membrane functionality, and DNA fragmentation were retained in the analysis for cleavage and blastocyst rates, but only sperm viability exhibited a predictive value for cleavage. For pregnancy, sperm plasma membrane functionality and DNA fragmentation were retained in the stepwise model, but only sperm plasma membrane functionality exhibited a significant predictive value.[11] Most oocytes were injected with sperm from samples with < 21% DNA fragmentation. Although DNA fragmentation is a sensitive variable, it has low specificity for predictive power as shown in these findings,[11] suggesting that DNA fragmentation at low range values is not predictive of embryo development and pregnancy as observed in humans.[27] The absence of predictive value for ICSI success of sperm morphology evaluations can be explained by the fact that the individual sperm for injection is arbitrarily selected by the embryologist based on gross morphology and motility, cancelling out the predictive influence of the morphology evaluation of the sperm sample. Overall, the likelihood of oocytes injected with sperm to develop into an embryo increases when sperm are selected from a population with higher membrane integrity and function.

Failure of oocyte activation is considered an important factor contributing to the low success rate of equine ICSI.[28] The association of sperm plasma integrity and functionality with ICSI success suggested that certain membrane components are required for oocyte activation.[11] After gamete fusion, a sperm-borne oocyte activating factor is released into the ooplasm that triggers calcium (Ca^{2+}) to promote cortical reaction, resumption of meiosis, and pronuclear formation.[29] The major candidate for this role is phospholipase Z zeta 1 (PLCZ1), the smallest phospholipase isoform, was first reported and the molecular structure and function of PLCZ1 as a sperm-borne oocyte activator factor was characterized using a mouse model.[30] In mammals, PLCZ1 is conserved among studied species and predominantly localizes in the acrosomal, equatorial, and postacrosomal regions of the sperm head.[30-39] Molecular characterization and location of PLCZ1 in stallion sperm was subsequently described.[34] As presumed, PLCZ1 is located in the acrosomal and postacrosomal regions in stallion sperm, but it is also notably located in the tail, where it remains catalytically active.[34] Furthermore, PLCZ1 is released into the ooplasm and activates a phosphatidylinositol biphosphate signal pathway to induce oscillatory release of Ca^{2+} from the endoplasmic reticulum and protein kinase activation. The Ca^{2+} oscillations and protein kinase regulate downstream pathways that coordinately promote cortical reaction, resumption of meiosis and pronuclear formation to complete oocyte activation.[40] The characterization of PLCZ1 has been primarily done in men and mice, with less known about the PLCZ1 impact on IVF and ICSI in other species, such as cattle and horses.

In various mammalian species, a relationship among male infertility, ICSI failure and reduction, abnormal localization, and genetic variants of PLCZ1 has been documented (reviewed).[39] In a preliminary study in cattle, we identified bulls with sperm that resulted in a high cleavage rate (≥ 70%) in IVF had significantly greater PLCZ1 abundance than bulls whose sperm yielded a low cleavage rate (< 70%).[13] In horses, the abundance of PLCZ1 exhibits a wide range of values in frozen-thawed sperm from fertile stallions.[41] It is anticipated that this variability could affect the sperm's ability to fertilize and therefore impact fertility outcome. In an earlier report, PLCZ1 abundance in fresh sperm showed an inconsistency of results among stallions with a low (< 30%) or high (> 30%) pregnancy rate for fertility in vivo. Stallions with low pregnancy rates displayed lower or similar PLCZ1 abundance than stallions with high pregnancy rates. Interestingly, subfertile stallions had adequate progressive motile and morphologically normal sperm but showed a lack, reduction, or abnormal localization of PLCZ1.[42,43] In our laboratory, we established immunocytochemistry and flow cytometric protocols to identify and quantify PLCZ1 in bull and stallion sperm after validation of commercial antibodies (Figure 1).[13,17,41] Using heterologous and homologous ICSI, we observed that bovine and equine oocytes injected with frozen-thawed stallion sperm from samples with low PLCZ1 evaluated by flow cytometry consistently revealed lower cleavage rates after sperm injection.[17,41] Frozen-thawed sperm samples displaying high abundance of PLCZ1 also showed a greater proportion of sperm exhibiting positive labeling for PLCZ1 and a specific localization pattern of PLCZ1 in the acrosomal and postacrosomal regions.[17] Under experimental conditions, equine oocytes injected with frozen-thawed sperm from a sample with low PLCZ1 were 77% less likely to cleave.[17] Using retrospective data from our laboratory and logistic regression analysis, we documented that equine oocytes injected with frozen-thawed stallion sperm demonstrated significantly lower cleavage rates (60.9%; 282/463) compared to oocytes

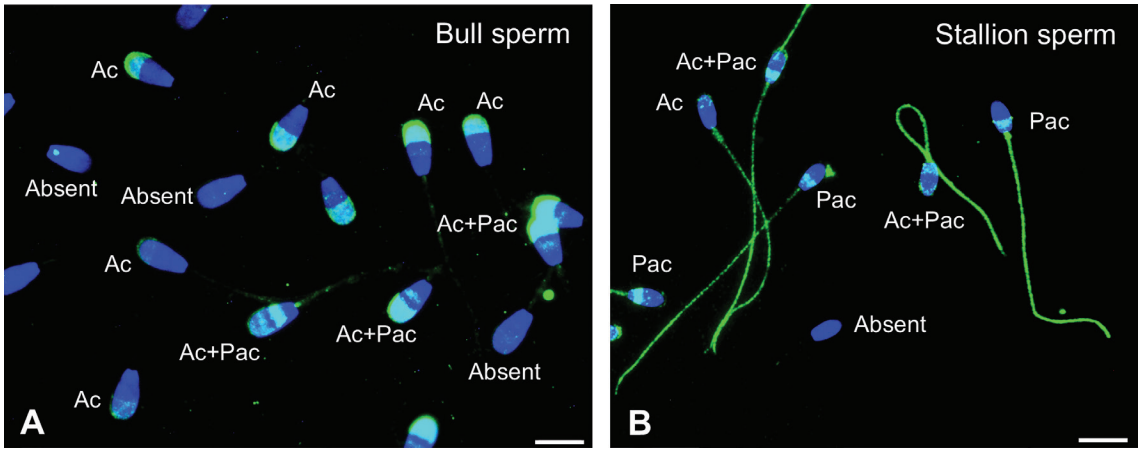


Figure 1. Representative immunofluorescent images of phospholipase C zeta 1 (PLCZ1) localization in frozen-thawed sperm from bulls and stallions using commercial antibodies. Localization of PLCZ1 was assessed using a rabbit antiPLCZ1 antibody of human origin (MyBioSource, San Diego, CA), goat antirabbit IgG-H+L Alexa Fluor™ 488 (Invitrogen, Eugene, OR), and Hoechst 33342. (A) Bull sperm have specific PLCZ1 immunoreactivity in the acrosomal (Ac) and postacrosomal (Pac) regions of the sperm head. (B) Stallion sperm have specific PLCZ1 immunodetection in the acrosomal region (Ac), postacrosomal (Pac) region of the sperm head, and, particularly, in the tail. Both samples also have sperm without immunoreactivity for PLCZ1. Image magnification: 1000 x. Scale bar = 10 µm

injected with cooled sperm (71.1%; 81/114). Specifically, oocytes fertilized with frozen-thawed sperm were 36.6% less likely to cleave (95% confidence interval of 0.35-0.85). This difference strongly suggests that the sperm components required for initiating oocyte activation are impaired by cryopreservation.[17] We compared membrane integrity and PLCZ1 abundance in fresh, frozen, and refrozen stallion sperm. Repeated freezing cycles progressively impaired acrosomal and plasma membranes in the sperm. A significant finding was that freezing caused a loss of PLCZ1 in the sperm subpopulation that retained intact plasma membranes and acrosomes when compared to fresh samples. However, the PLCZ1 abundance was similar between frozen and refrozen sperm. The reduction of sperm survival and loss of PLCZ1 are critical factors that limit the competence of sperm for oocyte activation.[17] Protein abundance and localization of PLCZ1 in sperm have intrinsic variations among stallions that can be exacerbated by cryopreservation, negatively impacting oocyte activation and the success of IVF and ICSI. The male factor has a critical role in oocyte activation, especially when sperm sources come from a heterogeneous population of stallions and from samples cryopreserved under conditions that may compromise the molecular components required for both oocyte activation and embryo development.

Sperm assessments and multiparametric approach

Basic semen analysis involves semen volume and sperm motility, concentration and morphology. These parameters are critical in animal reproduction to determine the total number of sperm available for breeding opportunities and processing insemination doses. Current methods for sperm concentration include the use of hemocytometer, Mackler® chamber, spectrophotometer (densimeter), computer-assisted semen analysis systems (CASA), flow cytometer, and Nucleocounter.[44,45] Hemocytometers and Nucleocounter are considered the gold standard method in animal andrology.[44,45] However, several studies report high correlation between hemocytometer and automated estimations using densitometers, Nucleocounter, and flow cytometers (reviewed[45]). For motility, a direct and visual evaluation involves microscopic observation of a properly diluted semen sample, usually at 200-400 x magnification, using a heated stage set to 37-39 °C. Accurate estimation of total and progressive motility of the sample requires correctly aligned phase contrast or differential interference contrast microscopes. Total motility describes the percentage of sperm exhibiting any type of movement, and progressive motility is the percentage of sperm moving with an arbitrary minimum velocity and a relatively straightforward motion pattern.[46] Computer-assisted semen analysis (CASA) systems allow automated motility evaluation of individual sperm, providing results considered more accurate than visual estimation.[46] These systems utilize specialized hardware and software to digitize and analyze sequential images of sperm motion, generating detailed kinematic data of individual sperm.[46] Sperm motion parameters include velocity (curvilinear straight-line and average path and velocity), velocity ratios (linearity, straightness and wobble) and sperm wobble characteristics (amplitude of lateral head displacement and beat-cross frequency). Some sperm motion parameters may not be comparable across CASA systems, as the algorithms and settings used to process the results differ among equipment and laboratories.[46] However, the precision, accuracy, repeatability, and practicability of sperm concentration and motility estimates are strongly influenced by: operator skills (especially in subjective assessment); limitations inherent to the method used (including sperm sample handling and dilution); adjustments of sperm concentration to assess within the working range; biological and practical species-specific considerations; equipment specifications; and settings in case of automated equipment.[44-46]

Routine microscopic examination of sperm morphology includes the use of brightfield, phase contrast, and differential interference contrast microscopy. In routine clinical applications, the recommended magnification is 1,000 x and the count is 200 sperm per sample.[47] Various stains and preparations can be used for evaluating sperm morphology; eosin-nigrosin is frequently used for practical sperm staining and provides sperm viability (reviewed[47]). The differential interference contrast microscopy of fixed, unstained sperm at 1,000 x is considered the gold standard as it allows for easy depiction

Citation: Clinical Theriogenology 2026, 18, 13232, http://dx.doi.org/10.58292/CT.v18.13232

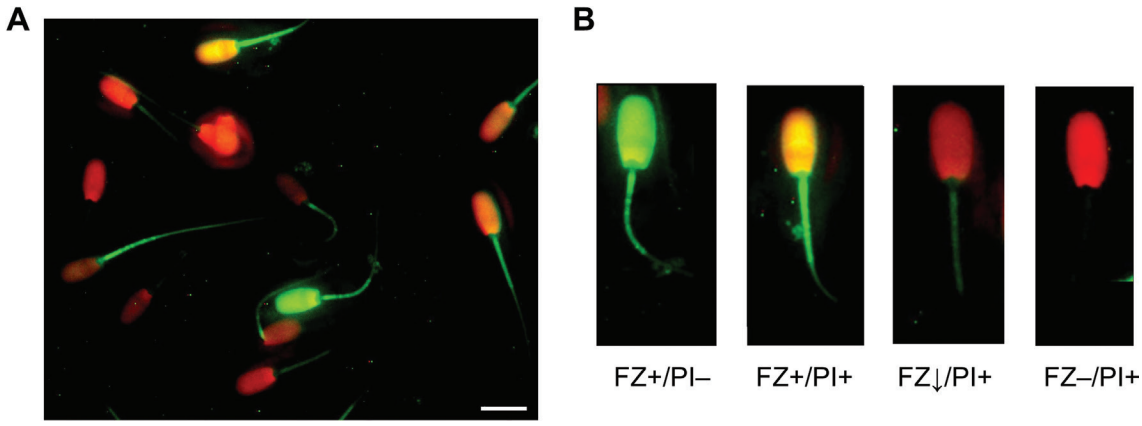
of certain types of sperm abnormalities. However, stained and unstained methods generally yield similar sperm classifications.[47] The accuracy and repeatability of sperm morphology evaluations are markedly influenced by operator skills as a subjective assessment, which demands standardization, continuous education and quality assurance programs for consistent results.

In practice, the decrease in sperm quality after collection mainly occurs by intrinsic cellular events related to prolonged storage or cryopreservation. These events are associated with reductions in motility, plasma membrane integrity, and mitochondrial activity and increases in DNA fragmentation.[48-53] Most of these events, in their first stages, are sublethal to the sperm and difficult to detect by traditional sperm assessment, but they eventually result in sperm death,[54] reducing sperm fertility potential and productive parameters.[48-57] Flow cytometry allows rapid and robust assessment of thousands of sperm. Implementation of multicolor flow cytometry enables simultaneous evaluation of multiple attributes within the same sperm population. A well-established and validated protocol to assess viability in sperm from several species uses a combination of SYBR®14 (SYBR; Invitrogen, Waltham, MA, USA) and propidium iodide (PI).[58-61] Briefly, SYBR is a membrane-permeant nuclear dye that enters sperm with intact plasma membranes and binds their DNA, emitting a bright green fluorescence when excited. Propidium iodide enters sperm from the base of the head only if the plasma membrane is damaged. Both dyes target DNA, thus avoiding the ambiguity of dyes that label separate cellular organelles. Based on the loss of plasma membrane integrity, sperm are classified as 'dead' when they are permeable to PI and 'viable or live' when they exclude PI.[59,61] An important advantage of this staining is that it can be performed with flow cytometry for robust evaluation. However, cell viability distinction is only based on plasma membrane damage, likely related to changes in membrane permeability to allow the incorporation of PI.

Current development of flow cytometers and fluorochromes facilitates simultaneous assessment of multiple sperm functions and compartments within the same sample, enabling rapid analysis of thousands of cells with greater statistical power. Current multicolor flow cytometric panels involve the use of lectin peanut agglutinin (PNA) from *Arachis hypogaea* conjugated with a fluorochrome for assessing acrosome integrity and MitoTracker™ (Invitrogen) probes for mitochondrial activity, among others. Lectin PNA binds to β-galactose moieties in the outer acrosomal membrane only when the plasma membrane and outer acrosomal membrane of fresh sperm are disrupted but does not provide information regarding the molecules involved in fertilization.[58] Mitochondrial activity is crucial for sperm function, providing energy required for motility, participating in calcium signaling, and producing reactive oxygen species (ROS) that drive physiological changes associated with capacitation, hyperactivation, and fertilization.[62-64] We validated the use of a 4-multicolor panel using Hoechst 33342, PNA-Alexa Fluor™ 488 conjugate, PI, and MitoTracker™ Deep Red FM to simultaneously evaluate viability, acrosome integrity, and mitochondrial activity.[65] We compared a 2- and 3-multicolor panel based on SYBR/PI and the proposed 4-multicolor panel in cooled (n = 132) and frozen-thawed (n = 254) boar sperm samples. Sperm exhibiting Hoechst+/PNA–/PI–/MitoTracker+ were classified as live sperm with intact acrosomes and high mitochondrial activity. For validation purposes, comparisons between 2 and 3 multicolor panels with the 4 multicolor panel for the percentage of live, live-acrosome intact, and dead-acrosome reacted sperm were strongly correlated ($r > 0.71$; $p < 0.0001$) and agreement analysis using Bland-Altman plots demonstrated that both assays resulted in similar values for both cooled and frozen-thawed boar sperm samples. Percentages of sperm having high mitochondrial activity between 2- and 4-multicolor panels were highly correlated ($r = 0.98$; $p < 0.0001$), and the agreement assessment confirmed similar outcomes.[65] Similar approach was applied to compare a 5 multicolor panel in boar and stallion sperm for evaluation of mitochondrial activity, plasma membrane integrity and lipid disorder, acrosomal status, and marking DNA using Rhodamine 123, Merocyanine 540, PI, PNA-Alexa Fluor 647 conjugate, and Hoechst 33342, respectively. The 5 multicolor panel had strong correlations with single-color panels for each variable in boar and stallion sperm ($r > 0.92$; $p < 0.01$). Also, there were high concordance correlation coefficients ($r > 0.91$; $p < 0.01$) and agreement for all parameters in both species.[66] The development of multicolor panels to evaluate sperm provides new insights into sperm quality of samples from various species.

In dairy bulls, 20 Holstein-Friesian bull sires with > 900 AI with frozen-thawed sperm as first services per year were designated high (n = 10) or low (n = 10) fertility bulls, based on their annual 56 day nonreturn rate. Only ejaculates (n = 91; 4 or 5 ejaculates/bull) with a volume ≥ 2 ml, sperm concentration ≥ 500 $\times 10^6$ sperm/ml, and motility ≥ 70% were further processed for cryopreservation. A multicolor panel that included calcein violet, PI, PNA-pycoerythrin conjugate, Fluo-4, and cyanine dye DiIC1 to assess esterase activity, plasma and acrosomal membrane integrity, intracellular calcium (Ca^{2+}) concentrations, and mitochondrial membrane potential, respectively, was used to assess sperm quality and fertility outcome.[67] This panel permitted the identification of 18 sperm subpopulations, revealed for 2 or more of a fluorochrome combination. Esterase activity, plasma and acrosomal membrane integrity, low intracellular Ca^{2+} concentrations, and high mitochondrial membrane potential did not differ between high and low fertility bulls. Interestingly, the percentage of sperm with low intracellular Ca^{2+} concentrations within the subpopulation with intact plasma and acrosomal membranes was greater in high than in low fertility bulls. Using random forest analysis, ⅔ of the ejaculates were correctly assigned to their fertility group. The sperm subpopulation exhibiting intact plasma and acrosomal membrane with low intracellular Ca^{2+} was the most important fertility predictor among the 18 distinct sperm subpopulations identified by this multicolor panel.[67] Sperm that concurrently have intact plasma and acrosomal membranes, high mitochondrial function, and low intracellular Ca^{2+} likely have not undergone early capacitation changes that can reduce fertilizing potential at AI. The use of multicolor flow cytometric assays confirms the functional heterogeneity of sperm samples and can contribute to more accurate characterization of sperm, increasing the predictive value for male fertility in vivo.

Recent research has successfully demonstrated the relation between intracellular zinc ion (Zn^{2+}) fluxes and both sperm viability and capacitation state. Intracellular Zn^{2+} in sperm can be measured using FluoZin™-3 (Invitrogen), a highly selective and cell-permeant fluorochrome indicator. This fluorochrome has a strong binding affinity for Zn^{2+} that is not affected by Ca^{2+}, with a > 50-fold increase in fluorescence when exposed to saturating concentrations of Zn^{2+}. This technique was utilized to describe the Zn^{2+} fluxes and associated plasma membrane remodeling that occur in boar sperm during capacitation.[68] A noncapacitated sperm displays high Zn^{2+} in the head, midpiece, and the proximal segment of the principal piece, which



[AQ4] **Figure 2.** Representative epifluorescence microscopic image of boar sperm tested for intracellular zinc and plasma membrane integrity. (A) Boar sperm were stored in liquid state for 7 days at 17°C and stained with FluoZin-3™ (FZ) and propidium iodide (PI). (B) FZ+/PI–: sperm have high FZ green fluorescence in head and midpiece with no PI, corresponding with noncapacitated state; FZ+/PI+: sperm have a reduction in FZ fluorescence intensity and increasing PI fluorescence in the head (increasing red). FZ↓/PI+: sperm have low FZ fluorescence restricted to the midpiece and increasing PI fluorescence; FZ–/PI+: sperm with no FZ fluorescence and high florescence intensity of PI. Image magnification: 1000 x. Scale bar = 10 µm

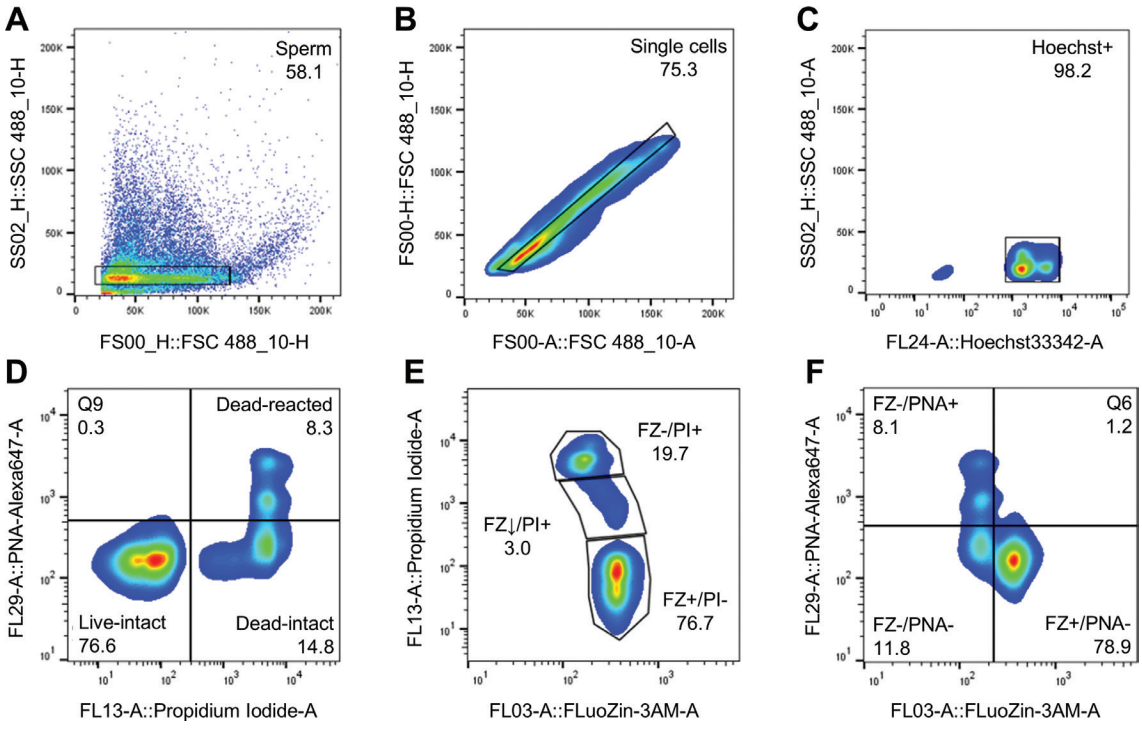


Figure 3. Representative cytograms of flow cytometric evaluation of boar sperm using a 4-multicolor panel for acrosomal and plasma membrane integrity and intracellular zinc content as an indicator of capacitation state. Sperm were stained with Hoechst 33342 (Hoechst) for DNA detection, FluoZin-3™ (FZ) for detection of intracellular zinc content, propidium iodide (PI) for plasma membrane integrity, and PNA-Alexa™647 conjugate for acrosome status. (A) Sperm were acquired, (B) cell aggregates were excluded, and (C) Hoechst-positive events (Hoechst+) were considered as sperm and gated into the following cytograms. (D) Sperm with intact plasma and acrosomal membranes (PI–/PNA–) were identified as live-intact sperm in the left lower quadrant. (E-F) FZ+/PI– and FZ+/PNA647– events were considered sperm exhibiting high intracellular zinc (FZ+) with intact plasma (PI–) and acrosomal (PNA647–) membranes in noncapacitated state

is called Signature 1. As capacitation progresses, Zn^{2+} fluorescence intensity is first reduced and then limited to the midpiece corresponding to Signatures 2 and 3, respectively. Finally, Zn^{2+} fluorescence becomes absent in Signature 4.[68]

In boar sperm, The PI– subpopulation with no acrosomal remodeling (PNA–) had a high Zn^{2+} (Signature 1). As PI is incorporated (PI+) into the sperm, indicating plasma membrane changes, Zn^{2+} is reduced (Signature 2) and then limited

Citation: Clinical Theriogenology 2026, 18, 13232, http://dx.doi.org/10.58292/CT.v18.13232

to the midpiece (Signature 3). Concurrently, an increase in acrosomal remodeling is evident by PNA binding (PNA+) in Signatures 3 and 4. In Signature 4, sperm exhibit high permeability to PI (PI+), acrosomal exocytosis (PNA+) and absence of Zn^{2+}, representing changes associated with a capacitated state and death. In addition, sperm displaying Signature 2 are hyperactivated and quickly transition to Signature 3 (associated with acrosomal remodeling). These findings indicate that PI intensity changes during capacitation, and 2 distinctive PI+ subpopulations are apparent: a PI+ live subpopulation with plasma membrane change and other PI+ cell death.[68] This rapid progression suggests that the final steps of sperm capacitation happen very quickly, and if the sperm fails to fertilize, cell death occurs.[68] The assessment of intracellular Zn^{2+} in sperm samples could serve as an indicator of early changes related to capacitation state that could impair sperm quality and fertilizing ability.

Capacitation state and Zn^{2+} concentrations in semen doses are associated with sperm quality and fertility.[68] After collection, semen of most species is diluted or resuspended in extender, notably reducing the concentration-dependent efficiency of several seminal decapacitating factors such as spermadhesines and Zn^{2+} that are responsible for keeping sperm in a noncapacitated state prior to insemination or storage.[69] In studies of commercial boar semen doses stored in liquid state at 17° or 5°C,[70,71] we identified 4 sperm subpopulations stained with FluoZin™-3 (FZ) and PI for validation purposes: 1. sperm with intense FZ fluorescence in the head and tail and negative for PI; 2. sperm with the same FZ distribution but lower intensity and incorporation of PI; 3. sperm with a marked reduction in FZ fluorescence restricted to the midpiece and increased PI fluorescence; and 4. sperm only exhibiting high PI fluorescence (Figure 2). To use a similar approach to evaluate semen doses, we used conventional flow cytometry and a multicolor panel that included Hoechst 33342, FluoZin™-3, PI, and PNA-Alexa Fluor 647 conjugate to assess intracellular Zn^{2+}, plasma membrane integrity, and acrosome status (Figure 3). In doses stored at 17°C, sperm with high Zn^{2+} with intact plasma and acrosomal membranes considered in noncapacitated state, were reduced in function of time, especially in the first 4 days.[70] The percentages of high Zn^{2+} sperm were significantly correlated ($r = 0.65$) to total motility. Interestingly, there was a subset of samples on day 7 of storage that had a reduced proportion of sperm with high Zn^{2+} (< 50%) that retained high total motility (> 65%),[70] suggesting boar sperm underwent capacitation changes but still retained motility during storage. In paired boar semen doses stored either at 17° or 5°C, we noticed that the reduction in noncapacitated sperm with high Zn^{2+} and intact plasma and acrosomal membranes during storage was not modified by storage temperature but increased by bacterial contamination, more evident at 17°C.[71] In a small trial to examine the relationship between Zn^{2+} concentrations in sperm and fertility, boars ($n = 4$) with variable fertility used for AI with 17°C-stored doses, Zn^{2+} concentrations differed between high- and low-fertility boars. After inducing capacitation, high-fertility boars proportionally exhibited more Signature 3 sperm (Zn^{2+} reduced and limited to midpiece) than low-fertility boars that displayed a minimal proportion,[68] suggesting that high-fertility boars have higher proportion of noncapacitated sperm that were able to capacitate and reach fertilizing ability. Evaluation of intracellular Zn^{2+} in sperm is a powerful tool to add to multiparametric evaluations to characterize the capacitation state and detect early changes that ultimately can affect sperm quality, fertilizing potential, and consequently animal production.

Concluding remarks

Our current understanding of sperm physiology enables us to identify, assess, and quantify specific attributes involved in sperm structure and function. This includes sperm's ability to survive storage and undergo the necessary changes to achieve fertilizing potential under in vivo and in vitro conditions. Advanced techniques and functional assays facilitate efficient multiparametric analysis of several sperm characteristics in many sperm, improving the accuracy and reliability of sperm assessment. The integration of targeted multiparametric methods for sensitivity and specificity to reduce costs in a timely manner can accurately predict sperm fertilizing potential. However, there are limitations, as male reproductive performance and fertilization are complex, sophisticated, and multifaceted events by nature.

Male fertility, particularly in the context of IVF and ICSI, is markedly influenced by the sperm contribution to oocyte activation. Males inherently differ in the abundance and location of PLCZ1 within sperm. The genetic variants, combined with variability in sperm quality and the detrimental effects of cryopreservation, can alter PLCZ1, impacting sperm's ability to activate the oocyte and the overall success of IVF and ICSI procedures. However, further research is required to determine factors that limit male fertility. It is also necessary to determine if assessment methods and predictive models are universally applicable across species or if they must be adapted by species-specific differences.

Conflict of interest

Author has no conflict of interest.

Acknowledgement

Author acknowledges Cecil and Irene Hylton Foundation and Membrane Protective Technologies for funding, and Vista Equine Colorado LLC and Saw Ranch for sample donation.

References

1. Peña FJ, Ortega Ferrusola C, Martín Muñoz P: New flow cytometry approaches in equine andrology. Theriogenology 2016;86:366-372. doi: 10.1016/j.theriogenology.2016.04.050
2. Peña FJ, Martin-Muñoz P, Ortega-Ferrusola C: Advances in flow cytometry in basic and applied equine andrology. Anim Reprod 2017;14:136-142. doi: 10.21451/1984-3143-AR892
3. Gonzalez-Castro RA, Peña FJ, Herickhoff LA: Validation of a new multiparametric protocol to assess viability, acrosome integrity and mitochondrial activity in cooled and frozen thawed boar spermatozoa. Cytometry B Clin Cytom 2022;102:400-408. doi: 10.1002/cyto.b.22058
4. Shepherd MJ, Gonzalez-Castro RA, Herickhoff LA: Application of antioxidants in extender on bull sperm cryopreservation to reduce the male effect in dairy fertility. J Dairy Sci 2024;107:10027-10040. doi: 10.3168/jds.2024-24872
5. Barrier Battut I, Kempfer A, Lemasson N, et al: Prediction of the fertility of stallion frozen-thawed semen using a combination of computer-assisted motility analysis, microscopical observation and flow cytometry. Theriogenology 2017;97:186-200. doi: 10.1016/j.theriogenology.2017.04.036

6. Barrier Battut I, Kempfer A, Becker J, et al: Development of a new fertility prediction model for stallion semen, including flow cytometry. Theriogenology 2016;86:1111-1131. doi: 10.1016/j.theriogenology.2016.04.001
7. Graham JK: Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 2001;68:239-247. doi: 10.1016/S0378-4320(01)00160-9
8. Jung M, Rüdiger K, Schulze M: In vitro measures for assessing boar semen fertility. Reprod Domest Anim 2015;50:20-24. doi: 10.1111/rda.12533
9. Kuhlgatz DA, Kuhlgatz C, Aepli M, et al: Development of predictive models for boar semen quality. Theriogenology 2019;134:129-140. doi: 10.1016/j.theriogenology.2019.05.024
10. Torres MA, Díaz R, Boguen R, et al: Novel flow cytometry analyses of boar sperm viability: can the addition of whole sperm-rich fraction seminal plasma to frozen-thawed boar sperm affect it? PLoS One 2016;11:1-20. doi: 10.1371/journal.pone.0160988
11. Gonzalez-Castro RA, Carnevale EM: Association of equine sperm population parameters with outcome of intracytoplasmic sperm injections. Theriogenology 2018;119:114-120. doi: 10.1016/j.theriogenology.2018.06.027
12. Gonzalez-Castro RA, Carnevale EM: Use of microfluidics to sort stallion sperm for intracytoplasmic sperm injection. Anim Reprod Sci 2019;202:1-9. doi: 10.1016/j.anireprosci.2018.12.012
13. Gonzalez-Castro R, Porflidt C, Bartfield J, et al: Cleavage rates after bovine IVF are affected by relative abundance of sperm phospholipase C zeta 1. Reprod Fertil Dev 2025;37:RDv37n1Ab133. doi: 10.1071/RDv37n1Ab133
14. Fernández-Montoro A, Araftpoor E, De Coster T, et al: Decoding bull fertility in vitro: a proteomics exploration from sperm to blastocyst. Reproduction 2025 Mar 19;169(4):e240296. doi: 10.1530/REP-24-0296
15. Suarez SS: Formation of a reservoir of sperm in the oviduct. Reprod Dom Anim 2002;37:140-143. doi: 10.1046/j.1439-0531.2002.00346.x
16. Sessions-Bresnahan DR, Graham JK, Carnevale EM: Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro fertilization, perivitelline, and intracytoplasmic sperm injections. Theriogenology 2014;82:274-282. doi: 10.1016/j.theriogenology.2014.04.002
17. Gonzalez-Castro RA, Whitcomb LA, Pinsinski EC, et al: Cryopreservation of equine spermatozoa reduces plasma membrane integrity and phospholipase C zeta 1 content as associated with oocyte activation. Andrology 2024;12:918-931. doi: 10.1111/andr.13517.
18. Love CC: Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76:547-557. doi: 10.1016/j.theriogenology.2011.03.007
19. Love CC: Modern techniques for semen evaluation. Vet Clin North Am Equine Pract 2016;32:531-546. doi: 10.1016/j.cveq.2016.07.006
20. Evenson DP: The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim Reprod Sci 2016;169:56-75. doi: 10.1016/j.anireprosci.2016.01.017
21. Kenney RM, Evenson DP, Garcia MC, et al: Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. Biol Reprod 1995;52(1):647-653. doi: 10.1093/biolreprod/52.monograph_series1.647
22. Aitken RJ, Lambourne S, Medica AJ: Predicting the outcome of Thoroughbred stallion matings on the basis of dismount semen sample analyses. Reproduction 2023;165:281-288. doi: 10.1530/REP-22-0309
23. Berridge MV, Tan AS: High-capacity redox control at the plasma membrane of mammalian cells: trans-membrane, cell surface, and serum NADH-oxidases. Antioxid Redox Signal 2000;2:231-242. doi: 10.1089/ars.2000.2.2-231
24. Gray JP, Eisen T, Cline GW, et al: Plasma membrane electron transport in pancreatic β-cells is mediated in part by NQO1. Am J Physiol Endocrinol Metab 2011 Jul;301(1):E113-21. doi: 10.1152/ajpendo.00673.2010
25. Love CC, Noble JK, Standridge SA, et al: The relationship between sperm quality in cool-shipped semen and embryo recovery rate in horses. Theriogenology 2015;84:1587.e4-1593.e4. doi: 10.1016/j.theriogenology.2015.08.008
26. Atroshchenko MM, Arkhangelskaya E, Isaev DA, et al: Reproductive characteristics of thawed stallion sperm. Animals 2019;9(12):1099. doi: 10.3390/ani9121099
27. Cissen M, van Wely M, Scholten I, et al: Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: a systematic review and meta-analysis. PLoS One 2016;11:e0165125. doi: 10.1371/journal.pone.0165125
28. Ruggeri E, Deluca KF, Galli C, et al: Cytoskeletal alterations associated with donor age and culture interval for equine oocytes and potential zygotes that failed to cleave after intracytoplasmic sperm injection. Reprod Fertil Dev 2015;27:944-956. doi: 10.1071/RD14468
29. Dozortsev D, Qian C, Ermilov A, et al: Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm-oocyte interaction. Hum Reprod 1997;12:2792-2796. doi: 10.1093/humrep/12.12.2792
30. Saunders CM, Larman MG, Parrington J, et al: PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development 2002;129:3533-3544. doi: 10.1242/dev.129.15.3533
31. Escoffier J, Yassine S, Lee HC, et al: Subcellular localization of phospholipase Cζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. Mol Hum Reprod 2014;21:157-168. doi: 10.1093/molehr/gau098
32. Heytens E, Schmitt-John T, Moser JM, et al: Reduced fertilization after ICSI and abnormal phospholipase C zeta presence in spermatozoa from the wobbler mouse. Reprod Biomed Online 2010;21:742-749. doi: 10.1016/j.rbmo.2010.07.006
33. Fujimoto S, Yoshida N, Fukui T, et al: Mammalian phospholipase C zeta induces oocyte activation from the sperm perinuclear matrix. Dev Biol 2004;274:370-383. doi: 10.1016/j.ydbio.2004.07.025

34. Bedford-Guaus SJ, McPartlin LA, Xie J, et al: Molecular cloning and characterization of pospholipase C Zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein. Biol Reprod 2011;85:78-88. doi: 10.1095/biolreprod.110.089466
35. Sato K, Wakai T, Seita Y, et al: Molecular characteristics of horse phospholipase C zeta (PLCζ). Anim Sci J 2013;84:359-368. doi: 10.1111/asj.12044
36. Cooney MA, Malcuit C, Cheon B, et al: Species-specific differences in the activity and nuclear localization of murine and bovine phospholipase C zeta 1. Biol Reprod 2010;83:92-101. doi: 10.1095/biolreprod.109.079814
37. Arroyo-Salvo C, Cogollo Villarreal MY, Clérico G, et al: The ability of donkey sperm to induce oocyte activation and mule embryo development after ICSI. Theriogenology 2024;218:200-207. doi: 10.1016/j.theriogenology.2024.02.002
38. Yoon SY, Fissore RA: Release of phospholipase C zeta and [Ca2+]i oscillation-inducing activity during mammalian fertilization. Reproduction 2007;134:695-704. doi: 10.1530/REP-07-0259
39. Gonzalez-Castro RA, Carnevale EM: Phospholipase C Zeta 1 (PLCZ1): the function and potential for fertility assessment and in vitro embryo production in cattle and horses. Vet Sci 2023;10(12):698. doi: 10.3390/vetsci10120698
40. Nomikos M, Kashir J, Swann K, et al: Sperm PLCζ: from structure to Ca2+ oscillations, egg activation and therapeutic potential. FEBS Lett 2013;587:3609-3616. doi: 10.1016/j.febslet.2013.10.008
41. Gonzalez-Castro RA, Amoroso-Sanches F, Stokes JE, et al: Localisation of phospholipase Cζ1 (PLCZ1) and postacrosomal WW-binding protein (WBP2 N-terminal like) on equine spermatozoa and flow cytometry quantification of PLCZ1 and association with cleavage *in vitro*. Reprod Fertil Dev 2019;31:1778-1792. doi: 10.1071/RD19217
42. Bedford-Guaus SJ, McPartlin LA, Varner DD: Characterization of equine phospholipase C Zeta: a review and preliminary results on expression defects in subfertile stallions. J Equine Vet Sci 2012;32:445-450. doi: 10.1016/j.jevs.2012.06.002
43. Gradil C, Yoon S, Brown J, et al: PLC zeta: a marker of fertility for stallions? Anim Reprod Sci 2006;94:23-25. doi: 10.1016/j.anireprosci.2006.03.095
44. Brito LFC: Quality assurance applied to semen analysis. Anim Reprod Sci 2024;270:107615. doi: 10.1016/j.anireprosci.2024.107615
45. Brito LFC, Althouse GC, Aurich C, et al: Andrology laboratory review: evaluation of sperm concentration. Theriogenology 2016;85(9):1507-1527. doi: 10.1016/j.theriogenology.2016.01.002
46. Peter A, Brito L, Althouse G, et al: Andrology laboratory review: evaluation of sperm motility. Clinical Theriogenology 2021;13:24-36. doi: 10.58292/ct.v13.9359
47. Chenoweth P, Brito L, Peter A, et al: Andrology laboratory review: evaluation of sperm morphology. Clinical Theriogenology 2024;16. doi: 10.58292/ct.v16.10600
48. Jäkel H, Henning H, Luther AM, et al: Assessment of chilling injury in hypothermic stored boar spermatozoa by multicolor flow cytometry. Cytometry Part A 2021;99:1033-1041. doi: 10.1002/cyto.a.24301
49. Henning H, Petrunkina AM, Harrison RAP, et al: Bivalent response to long-term storage in liquid-preserved boar semen: a flow cytometric analysis. Cytometry A 2012;81A:576-587. doi: 10.1002/cyto.a.22058
50. Rodriguez AL, van Soom A, Arsenakis I, et al: Boar management and semen handling factors affect the quality of boar extended semen. Porc Health Manag 2017;3:1-12. doi: 10.1186/s40813-017-0062-5
51. Hammerstedt H, Graham K, Nolan P: Cryopreservation what of mammalian sperm: we ask them to survive other retrace. J Androl 1990;11:73-88. doi: 10.1002/j.1939-4640.1990.tb01583.x
52. Amann RP, Pickett BW: Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7:145-173. doi: 10.1016/S0737-0806(87)80025-4
53. Sieme H, Oldenhof H, Wolkers WF: Sperm membrane behaviour during cooling and cryopreservation. Reprod Dom Anim 2015;50:20-26. doi: 10.1111/rda.12594
54. Suarez SS: Interactions of gametes with the female reproductive tract. Cell Tissue Res 2016;363:185-194. doi: 10.1007/s00441-015-2244-2
55. Knox RV: Artificial insemination in pigs today. Theriogenology 2016;85:83-93. doi: 10.1016/j.theriogenology.2015.07.009
56. Waberski D, Luther AM, Grünther B, et al: Sperm function in vitro and fertility after antibiotic-free, hypothermic storage of liquid preserved boar semen. Sci Rep 2019;9:1-10. doi: 10.1038/s41598-019-51319-1
57. Jäkel H, Scheinpflug K, Mühldorfer K, et al: In vitro performance and in vivo fertility of antibiotic-free preserved boar semen stored at 5 °C. J Anim Sci Biotechnol 2021;12:1-12. doi: 10.1186/s40104-020-00530-6
58. Boe-Hansen GB, Satake N: An update on boar semen assessments by flow cytometry and CASA. Theriogenology 2019;137:93-103. doi: 10.1016/j.theriogenology.2019.05.043.
59. Garner D, Johnson L: Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995;53:276-284. doi: 10.1095/biolreprod53.2.276
60. Satake N, Boe-Hansen G: Flow cytometry in the assessment of sperm function parameters: viability, acrosomal integrity, membrane stability, and mitochondrial status. In: Álvarez-Rodríguez M: editor. Spermatology: Methods and Protocols. New York, NY; Springer US: 2025. p. 289-303.
61. Garner DL, Johnson LA, Yue ST, et al: Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 1994;15:620-629. doi: 10.1002/j.1939-4640.1994.tb00510.x
62. Meyers S, Bulkeley E, Foutouhi A: Sperm mitochondrial regulation in motility and fertility in horses. Reprod Dom Anim 2019;54:22-28. doi: 10.1111/rda.13461
63. Vertika S, Singh KK, Rajender S: Mitochondria, spermatogenesis, and male infertility – An update. Mitochondrion 2020;54:26-40. doi: 10.1016/j.mito.2020.06.003
64. Aitken RJ: Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol Reprod Dev 2017;84:1039-1052. doi: 10.1002/mrd.22871
65. Gonzalez-Castro RA, Peña FJ, Herickhoff LA: Validation of a new multiparametric protocol to assess viability, acrosome integrity and mitochondrial activity in cooled and frozen thawed boar spermatozoa. Cytometry B Clin Cytom 2022;102(5):400-408. doi: 10.1002/cyto.b.22058

66. Quirino M, Jakop U, Mellagi APG, et al: A 5-color flow cytometry panel to assess plasma membrane integrity, acrosomal status, membrane lipid organization and mitochondrial activity of boar and stallion spermatozoa following liquid semen storage. Anim Reprod Sci 2022;247:107076. doi: 10.1016/j.anireprosci.2022.107076
67. Bucher K, Malama E, Siuda M, et al: Multicolor flow cytometric analysis of cryopreserved bovine sperm: a tool for the evaluation of bull fertility. J Dairy Sci 2019;102:11652-11669. doi: 10.3168/jds.2019-16572
68. Kerns K, Zigo M, Drobnis EZ, et al: Zinc ion flux during mammalian sperm capacitation. Nat Commun 2018;9(1):2061. doi: 10.1038/s41467-018-04523-y
69. Sutovsky P, Kerns K, Zigo M, et al: Boar semen improvement through sperm capacitation management, with emphasis on zinc ion homeostasis. Theriogenology 2019;137:50-55. doi: 10.1016/j.theriogenology.2019.05.037
70. Gonzalez-Castro RA, Herickhoff LA: Effect of holding period on viability, Acrosome status and intracellular zinc of boar sperm during liquid storage. J Anim Sci 2021;99:476. doi: 10.1093/jas/skab235.381
71. Gonzalez-Castro RA, Peña FJ, Herickhoff LA: Spermatozoa cooled to 5°C one day after collection from porcine commercial semen doses retain sperm functionality with reduced bacterial load. Andrology 2024;12:186-197. doi: 10.1111/andr.13441