Animal 17 (2023) 100720

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Animal board invited review: An update on the methods for semen quality evaluation in swine – from farm to the lab

Carolina Maside [a,b,1], Sandra Recuero [a,b,1], Albert Salas-Huetos [a,b,c,d], Jordi Ribas-Maynou [a,b,e], Marc Yeste [a,b,f,*]

[a] Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain
[b] Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona, ES-17003 Girona, Spain
[c] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA 02115, United States
[d] Consorcio CIBER, M.P., Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), ES-28029 Madrid, Spain
[e] Institute for Biogenesis Research, Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96822, United States
[f] Catalan Institution for Research and Advanced Studies (ICREA), ES-08010 Barcelona, Spain

ARTICLE INFO

Article history:
Received 8 March 2022
Revised 15 January 2023
Accepted 17 January 2023
Available online 25 January 2023

Keywords:
DNA integrity
Flow cytometry
Morphology
Motility
Sperm viability

ABSTRACT

Pig breeding is mainly conducted through artificial insemination with liquid-stored semen. It is, therefore, crucial to ensure that sperm quality is over the standard thresholds, as reduced sperm motility, morphology or plasma membrane integrity are associated with reduced farrowing rates and litter sizes. This work aims to summarise the methods utilised in farms and research laboratories to evaluate sperm quality in pigs. The conventional spermiogram consists in the assessment of sperm concentration, motility and morphology, which are the most estimated variables in farms. Yet, while the determination of these sperm parameters is enough for farms to prepare seminal doses, other tests, usually carried out in specialised laboratories, may be required when boar studs exhibit a decreased reproductive performance. These methods include the evaluation of functional sperm parameters, such as plasma membrane integrity and fluidity, intracellular levels of calcium and reactive oxygen species, mitochondrial activity, and acrosome integrity, using fluorescent probes and flow cytometry. Furthermore, sperm chromatin condensation and DNA integrity, despite not being routinely assessed, may also help determine the causes of reduced fertilising capacity. Sperm DNA integrity can be evaluated through direct (Comet, transferase deoxynucleotide nick end labelling (**TUNEL**) and its in situ nick variant) or indirect tests (Sperm Chromatin Structure Assay, Sperm Chromatin Dispersion Test), whereas chromatin condensation can be determined with Chromomycin A3. Considering the high degree of chromatin packaging in pig sperm, which only have protamine 1, growing evidence suggests that complete decondensation of that chromatin is needed before DNA fragmentation through TUNEL or Comet can be examined.

Implications

Evaluating semen quality is crucial to ensure proper reproductive performance in pig breeding. This review updates the methods used to assess semen quality and discusses the source of variations and limitations of different techniques. It summarises from the simplest to the most advanced tools to determine conventional sperm parameters, usually conducted in farms. More sophisticated techniques carried out in specialised laboratories are also covered, including the assessment of sperm physiological status and chromatin integrity. The take-home message is the need to standardise the methods for semen analyses in order to reduce variability and robustly predict boar fertilising capacity.

Introduction

In most of the pork-producing countries, pig breeding is mainly conducted through artificial insemination with liquid-stored semen at 17 °C (Knox, 2016). Although sperm cryopreservation is the most efficient method for long-term storage, the use of frozen-thawed pig sperm for artificial insemination remains marginal (<1%) due to its significantly lower reproductive efficiency compared to liquid-stored semen at 17 °C (Waberski et al.,

* Corresponding author at: Unit of Cell Biology, Department of Biology, Faculty of Sciences, University of Girona, ES-17003 Girona, Spain.
E-mail address: marc.yeste@udg.edu (M. Yeste).
[1] Joint first authors.

https://doi.org/10.1016/j.animal.2023.100720
1751-7311/

2019a; 2019b). In addition, semen production is performed on stud farms that collect, evaluate, dilute, package and distribute seminal doses among artificial insemination centres (Schulze et al., 2019). The evaluation of sperm quality is, in this scenario, very relevant, as it determines the number of seminal doses produced by a given ejaculate (Bonet et al., 2012). While sperm quality in farms is mainly evaluated on the basis of sperm concentration, motility and, in some cases, morphology, additional functional tests have been set over the last years.

The main aim of this review is to describe the methods used to determine pig sperm quality and functionality parameters in farms and research laboratories, which are summarised in Fig. 1 and Table 1. The article starts by describing the assessment of sperm motility and morphology, then covers the determination of functional parameters, such as plasma membrane integrity and levels of reactive oxygen species (**ROS**), evaluated with specific fluorochromes, and finally refers to the estimation of chromatin condensation and DNA integrity. As mentioned later, most of these tests were first established for human sperm and were subsequently transferred to livestock sperm. Moreover, the methods referred to herein are used to evaluate both liquid-stored and cryopreserved pig sperm, despite the former being far more used than the latter for routine breeding in this species.

Evaluation of conventional spermiogram parameters

Evaluation of sperm concentration, motility and morphology are among the most frequently performed analyses to determine sperm quality in animal breeding centres and specialised laboratories (Sancho et al., 2004). Yet, differences between studies indicate that these methods would much benefit from further standardisation, especially regarding the analysis of sperm morphology and motility, and that of the functional parameters described in the next section.

Sperm concentration

A widely used method to determine sperm concentration is manually counting using counting chambers (haemocytometer), including Neubauer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), Bürker (BT, Brand, Wertheim, Germany), Makler (Sefi Medical Instruments, Haifa, Israel) and Thoma (Hecht-Assistant, Sondheim, Germany). Although it is a low-cost and simple option to evaluate sperm concentration, it is important to highlight that this approach entails high subjectivity and variability between observers (Christensen et al., 2005).

Spectrophotometers are also utilised for assessing sperm concentration by measuring light transmission through the diluted semen sample. This approach involves the establishment of a calibration curve as spectrophotometers do not directly quantify sperm (Brito et al., 2016); to avoid high variability between measurements, calibration of these curves needs to be carefully conducted (Camus et al., 2011). On the other hand, Nucleocounter® SP-100 (ChemoMetec, Denmark) is a fast and reliable device for determining sperm concentration and viability through its integrated fluorescence microscope; the main disadvantage, however, is the elevated cost of the instrument (Grossfeld et al., 2022). Similarly, flow cytometry is another rapid and automated strategy to assess sperm concentration using beads and viability staining (Hansen et al., 2002). Although the acquisition costs of the equipment are elevated, flow cytometry allows the analysis of several sperm function parameters, as will be explained in more detail in Section 3.

While modern computer-assisted semen analysis (**CASA**) systems are usually employed for the examination of sperm motility

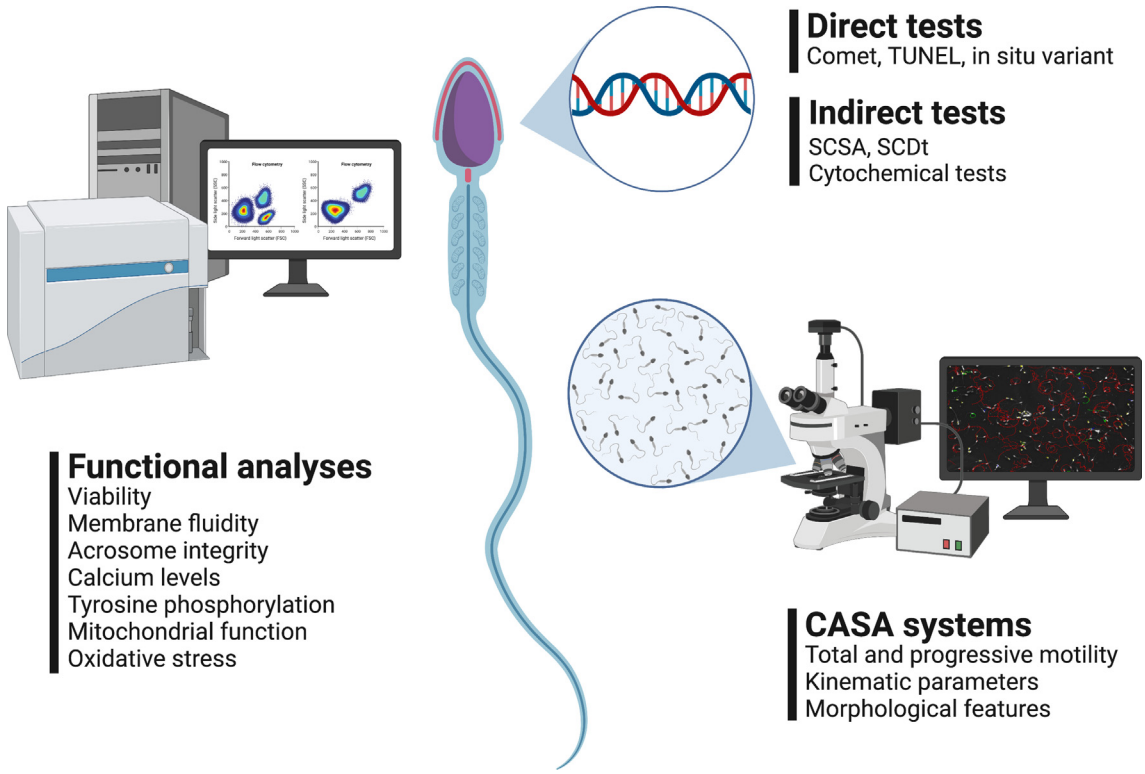

Fig. 1. Main methods used to determine pig sperm quality and functionality parameters in farms and research laboratories (created with BioRender.com). Abbreviations: CASA, Computer-Assisted Semen Analysis; SCSA, Sperm Chromatin Structure Assay; SCDt, Sperm Chromatin Dispersion Test; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end.

Table 1
Main techniques used for the evaluation of pig sperm quality, and their advantages and disadvantages.

Technique	Advantages	Disadvantages
Computer-assisted analyses (CASA system)	– Evaluation of sperm motility, kinematic parameters, morphology, and concentration – Specific software for porcine species is available – Fast and objective analysis – Requires a high number of cells (1 000 sperm cells/replicate)	– High acquisition costs – Trained staff
Conventional microscopy	– Analysis of sperm motility, morphology, and concentration – Low cost	– Time-consuming process – Subjective analysis
Flow cytometry	– Fast and objective analysis of multiple cell parameters – Quantification of fluorescence intensity – Requires a high number of cells (10 000 sperm cells/replicate)	– High acquisition costs – Trained staff
Fluorescence Microscopy	– Emphasis on subcellular localisation	– Time-consuming process
SCSA	– Fast and robust test – Provides DNA fragmentation index (DFI) and DNA stainability – Requires a high number of cells (10 000 sperm cells/replicate)	– Expensive equipment – Trained staff
SCDt	– Simple and fast – Low cost – Highly reproducible	– High variation between observers
TUNEL	– High sensitivity and specificity – Does not need a great volume/amount of sample	– Lacks from standardisation – May underestimate DNA fragmentation in pig sperm
Comet assay	– Sensitive and versatile – Does not need a great volume/amount of sample	– Time-consuming process – Trained staff – High variation between observers – Limited data available for pig species

Abbreviations: SCSA, Sperm chromatin structure assay; SCDt, Sperm chromatin dispersion test; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end.

and kinematics (see Section 2.2), its use for determining sperm concentration is increasing. This system consists of a phase-contrast microscope and a video camera connected to a computer with a commercial software. It provides an objective and detailed measurement of several aspects of a semen sample or an individual spermatozoon, including sperm concentration, motility and morphology (Boe-Hansen and Satake, 2019).

Sperm motility

Total and progressive sperm motility are evaluated through a phase-contrast microscope (100–200× magnification), either subjectively by eye or using automated CASA devices, which are more robust and reliable (Broekhuijse et al., 2011). When a subjective evaluation is made, sperm vigour is, besides total and progressive motility, commonly assessed. The score values for sperm vigour comprise a scale from 0 to 5, in which 0 represents total immobility and 5 the fast and most vigorous movement (Ax et al., 2016).

As pig sperm are often stored at 15–17 °C, preincubation of samples at 38 °C is needed to activate sperm motility (Passarelli et al., 2020). It is also worth mentioning that although a specific concentration for assessing sperm motility has not been established, elevated sperm count makes the differentiation of individual motility patterns difficult and misestimates the calculation of kinematic parameters by CASA. Furthermore, excessive dilution can decrease sperm motility. For this reason, a range between 2×10^6 and 50×10^6 sperm/mL is recommended for the determination of sperm motility, particularly when a CASA system is employed (Yeste et al., 2018). Additionally, and in contrast to human species, there are no standard guidelines for the evaluation of pig semen, nor are quality thresholds available. In spite of this, different studies have established a value of ≥70–80% total motile sperm (Broekhuijse et al., 2012a and 2012b; López Rodríguez et al., 2013) which, in general, is a widely accepted range.

The development of CASA technology in the early 1980s provided more accurate results and reduced the subjectivity regarding motion sperm traits (Amann and Katz, 2004). Not only does evaluation of sperm motility through CASA allow the determination of total and progressive motility but also that of distinct kinematic parameters (Table 2), which are described with more detail in previous reviews (Yeste et al., 2018; Boe-Hansen and Satake, 2019). Based on multivariate analyses of these individual kinematic parameters, one may identify the different sperm subpopulations in an ejaculate (Estrada et al., 2017). While some farms evaluate sperm motility subjectively, there are many that utilise a CASA, as most research laboratories do. Yet, no comprehensive study about the proportions of animal breeding centres utilising CASA devices has been published. On the other hand, different commercial CASA systems are currently available for mammalian species, some of which have specific modules/settings for pig sperm, including AndroVision (Minitüb, Tiefenbach, Germany), Hamilton Thorne Sperm Analyser (IVOS/CEROS; Hamilton Thorne Biosciences, Beverly, MA, USA), Sperm Class Analyzer (**SCA**; Microptic, Barcelona, Spain), and Integrated Semen Analysis System (**ISAS**; Proiser, Valencia, Spain) (Boe-Hansen and Satake, 2019). Because of the high cost of commercial CASA systems, there are laboratories

Table 2
Main kinematic parameters provided by the CASA system in the evaluation of pig sperm motility.

Abbreviation	Parameter	Description	Units
VSL	Straight line velocity	Average velocity of the sperm in a straight line from the beginning to the end of the path	μm/s
VAP	Average path velocity	Average velocity of the smoothed path of the sperm	μm/s
VCL	Curvilinear velocity	Average velocity along the sperm trajectory	μm/s
STR	Percentage of straightness	VSL/VAP ratio	%
LIN	Percentage of linearity	VSL/VCL ratio	%
WOB	Wobble coefficient	VAP/VCL ratio	%
BCF	Beat cross-frequency		Hz
ALH	Amplitude of lateral head displacement		μm

Abbreviations: CASA, Computer-Assisted Semen Analysis.

that envisage the use of an open CASA. Some open-source CASA systems, which are based on the ImageJ software, have been developed to evaluate horse (Giaretta et al., 2017) and sheep sperm (Alquézar-Baeta et al., 2019). To the best of the authors' knowledge, nevertheless, no specific open-source CASA has been developed for pig sperm, nor has this invention been tested in this species.

In the context of semen preservation and artificial insemination, evaluation of sperm motility through CASA enables the discrimination between good and bad quality ejaculates (Broekhuijse et al., 2012b). In fact, several attempts to investigate whether sperm motility and kinematic parameters can predict swine fertility have been made, with a wide variety of results (Hirai et al., 2001; Broekhuijse et al., 2012a and 2012b; Schulze et al., 2013 and 2014; Tremoen et al., 2018; Winters et al., 2018; Barquero et al., 2021a; 2021b). For example, Broekhuijse et al. (2012a) found that the total number of piglets born was positively correlated with average path velocity and the amplitude of lateral head displacement, but negatively correlated with straight line velocity. They also observed that farrowing rates were negatively correlated with beat cross-frequency (Broekhuijse et al., 2012a). Winters et al. (2018) reported a positive association of beat cross-frequency and curvilinear velocity with pregnancy rates, and Barquero et al. (2021a) demonstrated that beat cross-frequency and curvilinear velocity, together with average path velocity and lateral head displacement, had a significant, albeit limited, capacity to predict the litter size (Barquero et al., 2021a). Hirai et al. (2001) did not observe any association between sperm motility and litter size, but found that a higher proportion of slow sperm was associated with greater non-returning rates (>86%) (Hirai et al., 2001).

It is also worth mentioning that differences in motility parameters between pig breeds have been reported (Tremoen et al., 2018). Hence, taking into consideration other factors and variables when using sperm motility to predict reproductive performance is important, especially because it is more difficult to predict high than low fertility. Besides differences between breeds, males or even ejaculates, care must be taken when comparing motility outcomes, as diverse laboratory conditions may also have an effect. Indeed, several factors and conditions affect motility parameters, such as the distinct counting chambers used (e.g., Makler or Leja; Quintero-Moreno et al., 2004; Basioura et al., 2019), the incubation time (Vyt et al., 2008), the length of video recording (Valverde et al., 2019), the algorithms used by the software, or even the different settings and thresholds established to calculate kinematic parameters. For this reason, it is important that authors carefully describe the conditions and settings used for motility analysis in their studies.

Sperm morphology and agglutination

Sperm malformations in pigs include immature cells presenting cytoplasmic droplets (at proximal or distal position), aberrant sperm with tail defects (including coiled tails, and tails folded at the connection piece, mid-piece or Jensen's ring) and alterations in the size and shape of the head (Briz and Fàbrega, 2013). Amongst these, the most common abnormalities observed in pig sperm are cytoplasmic droplets and bent tails (Schulze et al., 2014). In non-pathological conditions, a proportion ≥80% morphologically normal sperm is expected in a pig ejaculate, with <20% morphologically abnormal sperm and <15% sperm with cytoplasmic droplets (López Rodríguez et al., 2013).

Sperm morphology is evaluated at 200–400× magnification under a phase-contrast or bright-field microscope. The aforementioned CASA systems (AndroVision, IVOS, CEROS, SCA, and ISAS) allow the evaluation of sperm morphology, in addition to motility. This assessment requires sperm cell staining (using conventional bright-field microscopy or fluorescent probes) or wet mounting (using a phase-contrast microscopy) (Boe-Hansen and Satake, 2019). In a similar fashion to motility, sperm morphology can also be evaluated through a semi-automatic method using the open-access software ImageJ; this approach needs sperm cells to be previously stained with dyes (bright-field microscopy) or fluorescent probes (Yániz et al., 2016).

As previously mentioned, there is a lack of standard guidelines for the evaluation of pig sperm. Sperm staining with eosin plus nigrosine or eosin plus gentian are widely used not only for the evaluation of plasma membrane integrity but also for that of pig sperm morphology (Banaszewska et al., 2011). It is important to highlight that the staining technique affects sperm head morphometry and, thus, establishing general head dimensions in unstained sperm and determining the most optimal staining protocol is essential (Czubaszek et al., 2019). In this context, SpermBlue staining has been proposed to be the best method for sperm morphology assessment in pigs (Czubaszek et al., 2019).

Apart from the variation derived from protocols and technical procedures, and as reported for sperm motility, morphological differences have also been observed between pig breeds and individuals (Saravia et al., 2007), and separate ejaculate portions may exhibit different head morphometric features (Peña et al., 2005a). Besides, sperm morphology has been correlated to other variables, such as DNA integrity and fertility, including non-return rates to oestrus, farrowing rates and litter sizes (Yeste et al., 2010; McPherson et al., 2014). In effect, proximal cytoplasmic droplets are associated with DNA fragmentation, and reduced farrowing rates and litter sizes (Boe-Hansen et al., 2008; Schulze et al., 2013). The ability of sperm to bind the oviductal epithelium is also negatively correlated with the proportions of both aberrant sperm and sperm with cytoplasmic droplets (Petrunkina et al., 2001). Moreover, Barquero et al. (2021b) reported the existence of separate sperm morphology subpopulations, which differ in head size. Interestingly, different parameters measuring the size and shape of the sperm head, such as length, ellipticity, elongation, and regularity are predictive for litter size (Barquero et al., 2021b), and high non-return rates to oestrus are associated with a smaller, less elongated sperm head (Hirai et al., 2001).

The origin of sperm morphological abnormalities is, in many cases, presumed to be congenital. Zhao et al. (2020) identified several genes potentially linked to the main morphological alterations, including proximal (*NSF, WNT3, WNT9B, LYZL6*) and distal (*ARSA, SYCE3, MOV10L1, CBR1*) droplets, bent (*FGF1*) and coiled tails (*ARSA, SYCE3*), and distal mid-piece reflex (*OMA1, PFN1, PELP1*). A Gene Ontology (**GO**) enrichment analysis revealed that these genes may play a role in spermatogenesis, testis function and plasma membrane architecture (Zhao et al., 2020). This finding is of special interest for genetic selection, because, as mentioned previously, fertility parameters (i.e., farrowing rates, litter sizes) are impaired by morphological alterations.

Finally, the evaluation of sperm morphology may be run in parallel to that of agglutination. Sperm agglutination is usually caused by bacterial contamination or immunological alterations, interferes with sperm movement, and negatively affects fertilising ability. It is determined under a phase-contrast microscope at 200–400×, and samples are classified according to the degree of agglutination as follows: 0 or absent, 1 or low (<10% agglutinated cells), 2 or moderate (10–15% agglutinated cells), and 3 or severe (>15% agglutinated cells; Pinart et al., 2017).

Evaluation of sperm function parameters

In addition to conventional sperm variables, there are other function parameters that may also help predict fertilising ability (Table 3). These parameters reflect the physiological status of

Table 3
Main functional pig sperm parameters and fluorochromes used for flow cytometry and fluorescence microscopy assessment.

Functional cell parameters		Fluorochromes	Functional assessment
Sperm Viability		SYBR-14/PI	Plasma membrane integrity
		YO-PRO-1, CFDA, Ethidium homodimer-1, Hoechst 33258, Annexin-V	Alterations associated with apoptotic-like changes
Capacitation status	Membrane fluidity	M-540/YO-PRO-1	Membrane lipid disorder
	Calcium levels	Fluo-3 AM	Intracellular calcium levels
	Acrosome reaction	PNA-FITC, PSA-FITC	Integrity of acrosomal membrane
	Tyrosine phosphorylation	Anti-phospho-tyrosine antibody-FITC	Tyrosine phosphorylation levels
Mitochondrial activity		JC-1	Mitochondrial membrane potential
		MitoTracker	Active mitochondria staining
		DiOC6(3)	Mitochondrial membrane potential
		Tetramethylrhodamine methyl	Mitochondrial membrane potential
Oxidative stress		H_2DCFDA	Intracellular levels of reactive oxygen species
		DHE	Intracellular superoxide levels
		MitoSOX	Mitochondrial superoxide levels
		BODIPY$^{581/591}$	Membrane lipid peroxidation
		DAF-2 DA	Reactive nitrogen species
		DHR 123	Reactive nitrogen species
		Fluorescein-boronate	Reactive nitrogen species

Abbreviations: PI, Propidium iodide; CFDA, 6-carboxymethylfluorescein diacetate; M-540, Merocyanine 540; PNA-FITC, *Arachis hypogaea* agglutinin conjugated to fluorescein-labelled; PSA, *Pisum sativum* agglutinin; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; H_2DCFDA, 2′7′-dichlorodihydrofluorescein diacetate; DHE, Dihydroethidium; DAF-2 DA, 4,5-Diaminofluorescein diacetate; DHR 123, Dihydrorhodamine 123.

sperm cells and can be evaluated through microscopy (fluorescence or confocal) and flow cytometry. When a flow cytometer is not available or when emphasis on the localisation rather than on the intensity of fluorescence is made, an optical microscope with a fluorescence module can be used.

Despite being initially utilised for cell biology and other research applications, flow cytometry is increasingly employed for routine semen assessment in many andrology laboratories. This expanded use is due to the possibility of running rapid, accurate analyses with a high number of cells. In a flow cytometer, the sperm population is gated on the basis of two optical detectors, forward scatter and side scatter, which identify cells for size and granularity, respectively. The representation of these two variables in a histogram leads to a characteristic L-shaped distribution that corresponds to the sperm population. Because pig semen may contain other events apart from sperm and some debris have forward scatter and side scatter features similar to sperm, the usage of DNA probes is needed to detect and subtract those particles from the sperm population. The most common fluorochromes employed for this purpose are Hoechst 33342, Syto59 and SYBR-14 (Castro-González et al., 2010; Torres et al., 2021). In the following subsections, the most applied protocols to evaluate sperm functional parameters (plasma membrane status, acrosome integrity, mitochondrial function, oxidative stress…) are described (Table 3).

Plasma membrane integrity

Plasma membrane of pig sperm is rich in polyunsaturated fatty acids and has low cholesterol content, which makes it more susceptible to damage compared to other species. The integrity of sperm plasma membrane is known to be essential for an optimal sperm function and has good predictive value for fertility (Yeste et al., 2010). Moreover, liquid-storage and, particularly, cryopreservation can induce changes in the organisation of membrane lipids and proteins, which drives alterations in membrane permeability (Johnson et al., 2000). It is therefore crucial to make an accurate assessment of plasma membrane integrity.

The previous attempts to determine plasma membrane integrity were conducted under a bright-field microscope and involved eosin-nigrosine staining (Pintado et al., 2000). In the 1980s, the first study assessing plasma membrane integrity of pig sperm with fluorochromes was published. This report used 6-carboxymethylfluorescein diacetate (**CFDA**), a membrane-permeable stain retained in the cytoplasm, and propidium iodide (**PI**), an impermeable nuclear fluorochrome; samples were evaluated using a flow cytometer (Garner et al., 1986). Nowadays, the most used stain for pig sperm viability is SYBR-14, a membrane-permeable DNA fluorochrome, and PI that, as aforementioned, penetrates membrane-damaged sperm (Garner et al., 1996). Both probes are excited with a 488-nm laser, which most cytometers include. SYBR-14 emits green fluorescence for viable cells at 518 nm and PI stains damaged sperm in red fluorescence (636 nm), displacing SYBR-14. Specifically, there is a commercial kit combining SYBR-14 and PI (Live-Dead Sperm Viability Kit L-7011; Molecular Probes Inc., Eugene, OR, USA), which allows identifying debris particles in addition to viable and non-viable sperm.

The market also offers other fluorochromes, which makes viability analysis versatile and able to be combined with other tests. For instance, fluorochromes like Syto® are used to evaluate plasma membrane integrity and other sperm functional variables simultaneously (Murphy et al., 2017; Li et al., 2018). There are nucleic acid-binding stains that are only able to enter cells with a compromised plasma membrane. Other changes in sperm membrane characteristics, such as alterations associated with apoptotic-like changes, are detected with probes like YO-PRO-1, CFDA, ethidium homodimer-1, Hoechst 33258 and Annexin-V (Garner et al., 1986; Li et al., 2018; Peña et al., 2005b). Moreover, amine-reactive dyes, which interact with free amines in the cytoplasm, are fixable viability dyes for flow cytometry. These dyes have several advantages as they may detect non-viable/damaged sperm in fixed samples (Perfetto et al., 2010). When membranes are damaged, amine-reactive dyes interact with intracellular amines increasing the brightness of labelling. In horse sperm, for example, the fixable viability dye Zombie® is used in combination with MitoTracker Deep, and this allows the simultaneous assessment of sperm viability and mitochondrial membrane potential (Peña et al., 2018).

Acrosome integrity

Acrosome integrity can be assessed with lectins conjugated to fluorescein-labelled (**FITC**) *Pisum sativum* agglutinin (**PSA**) or *Arachis hypogaea* agglutinin (**PNA**), combined with a viability probe like PI (Waterhouse et al., 2004; Martín-Hidalgo et al., 2011). Lectins interact with glycosidic residues of damaged/exocytosed acrosomal membrane, and how results have to be interpreted depends

on whether the sperm membrane has previously been permeabilised. Currently, PNA is also available conjugated with Alexa Flour 488 (Alvarez-Rodriguez et al., 2018) or Alexa Fluor 647 (Murphy et al., 2017).

Sperm capacitation

Sperm capacitation plays a crucial role during fertilisation and involves, among other changes, an increase in membrane fluidity, calcium levels and mitochondrial membrane potential. The fluidity of the sperm plasma membrane is commonly evaluated as membrane lipid disorder with Merocyanine 540 (M-540) in combination with YO-PRO-1 (Martín-Hidalgo et al., 2011; Yeste et al., 2015). This combination enables the detection of viable and non-viable sperm with high or low plasma membrane lipid disorder. Flow cytometry and fluorescence microscopy have also been used to measure calcium levels in pig sperm before and after in vitro capacitation with Fluo-3 AM (Yeste et al., 2015; Schulze et al., 2017). This probe is a green fluorochrome that is excited with a conventional 488-nm laser. The fluorescence intensity of Fluo-3 is proportional to the levels of intracellular calcium (Caballero et al., 2009). Finally, an anti-tyrosine phosphorylation antibody conjugated to FITC can be employed in combination with fluorescence microscopy or flow cytometry to identify subpopulations of non-capacitated and capacitated sperm (Torres et al., 2016).

Mitochondrial integrity and activity

Mitochondria play a vital role in the regulation of sperm function (i.e., apoptotic-like changes, calcium homeostasis, production of ROS, and so on; Moraes and Meyers, 2018). There are different fluorescent probes to evaluate mitochondrial function in sperm, including 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (**JC-1**) and MitoTracker®, which identify sperm with active and non-active mitochondria (Martín-Hidalgo et al., 2011; Guo et al., 2017). Particularly, JC-1 is widely employed to assess mitochondrial membrane potential. This probe is excited with a 488-nm laser and exhibits dual fluorescence: green fluorescence (monomers) under low mitochondrial membrane potential and orange fluorescence (aggregates) under high mitochondrial membrane potential (Garner and Thomas, 1999). This double fluorescence may represent, in some flow cytometers, an inconvenient when a viability dye that involves the same laser and/or detector has to be used. On the other hand, Mitotracker® probes label active mitochondria and have some advantages, as they are highly specific, have a wide range of excitation and emission wavelengths, and some are even fixable. A lesser-known probe is DiOC6(3), a cell-permeable fluorochrome that targets functional mitochondria in viable cells (Kadirvel et al., 2009). While JC-1 has been reported to be more specific than DiOC6(3) in cattle (Marchetti et al., 2004), whether DiOC6(3) is better than JC-1 for the assessment of mitochondrial membrane potential in pig sperm is yet to be determined. In the last years, another alternative probe to JC-1 known as tetramethylrhodamine methyl ester perchlorate has been utilised to examine changes in the mitochondrial membrane potential of human (Uribe et al., 2017), cattle (Mizrahi and Breitbart, 2014) and pig sperm (Pezo et al., 2020), with an efficiency comparable to that of the commonly used JC-1. The tetramethylrhodamine methyl ester perchlorate is cell-permeable and emits red/orange fluorescence proportional to mitochondrial membrane potential.

Oxidative stress

Oxidative stress occurs when there is an imbalance between ROS and the antioxidant defence machinery. At normal physiological levels, ROS regulate essential mechanisms such as sperm maturation, capacitation, acrosome reaction and fertilisation. High concentrations of ROS, however, are associated with reduced sperm motility, lipid peroxidation of sperm membrane and DNA damage (Brouwers et al., 2005). For this reason, it is another variable that needs to be determined.

Different fluorescent probes to estimate the intracellular levels of ROS are available. The 2′7′-dichlorodihydrofluorescein diacetate (**H_2DCFDA**) is extensively used to detect total ROS in pig sperm (Li et al., 2018; Delgado-Bermúdez et al., 2019). The H_2DCFDA is a non-fluorescent cell-permeable probe that, in the presence of ROS, is oxidised into dichlorofluorescein (**DCF**), which emits green fluorescence (504 nm) (Guthrie and Welch, 2006). Dihydroethidium (**DHE**) is a probe that, in the presence of superoxides, is oxidised into ethidium. Ethidium binds to DNA and emits red fluorescence at 610 nm (Guthrie and Welch, 2006); so the higher the intensity of the ethidium, the higher the levels of superoxides. Moreover, MitoSOX Red, which is a derivative of DHE, detects mitochondrial ROS levels (Guo et al., 2017). H_2DCFDA and DHE can be combined with viability dyes, such as PI or YO-PRO-1, to detect ROS levels in viable and non-viable sperm (Delgado-Bermúdez et al., 2019).

Reactive oxygen species are known to induce lipid peroxidation of the sperm plasma membrane. A dual fluorescence probe, BODIPY$^{581/591}$, has been reported to detect lipid peroxidation; this fluorochrome can be combined with viability stains, thus providing the degree of lipid peroxidation in viable and non-viable sperm (Brouwers et al., 2005; Guthrie and Welch, 2006). This probe emits different wavelengths according to the oxidation status (non-oxidised membrane in red and peroxidised membrane in green).

The assessment of reactive nitrogen species could also be a valuable sperm quality and fertility biomarker. The reactive nitrogen species are considered a subgroup of ROS and include nitric oxide, peroxynitrite and nitroxyl ion, amongst others. Excessive amounts of nitric oxide or peroxynitrite have been associated with detrimental effects on the function and fertilising ability of pig sperm (Moran et al., 2008; Serrano et al., 2020). Nitric oxide can be determined through the cell-permeable fluorescence probe 4,5-Diaminofluorescein diacetate (**DAF-2 DA**) in different cell types including sperm (Moran et al., 2008). When it reacts with nitric oxide, DAF-2 DA becomes highly fluorescent. Peroxynitrite levels in pig sperm are measured with dihydrorhodamine 123 (**DHR 123**), as this probe is oxidised by peroxynitrite to rhodamine 123, and the fluorescence of the latter is proportional to the levels of the former (Serrano et al., 2020). Another highly sensitive fluorochrome for the evaluation of peroxynitrite is fluorescein-boronate, which has been validated in human but not yet in pig sperm (Uribe et al., 2020).

Multiple fluorochrome staining for the evaluation of sperm function

Nowadays, the development of flow cytometers with many lasers and optical channels allows multiwavelength analyses measuring more than one parameter simultaneously. The application of multiparametric assays improves the robustness of sperm evaluation and provides better prediction of fertilising ability, as a single assay gives information about more than one sperm functional parameter at the same time; this allows looking into the relationship between functional parameters. While there are some multiparametric studies using a large number of colours, not much research in pigs has been conducted. In fact, the simultaneous use of different fluorochromes to evaluate sperm function was first validated with fluorescence microscopy (de Andrade et al., 2007; Celeghini et al., 2007) and then adapted to flow cytometry. For example, a five-colour staining including fixable live dead violet dye®, Cell Event® green, Mitotracker® deep red, DiSBAC 2 and an

antiCD-44 APCFire antibody was set in horses (Ortega-Ferrusola et al., 2017). In cattle, Bucher et al. (2019) developed a protocol with five fluorochromes that simultaneously assessed esterase activity, plasma membrane integrity, acrosomal status, intracellular calcium levels and mitochondrial membrane potential. Recently, sperm viability, acrosome integrity and mitochondrial activity have been reported to be evaluated together using a four-colour protocol in pigs (Gonzalez-Castro et al., 2022). In the near future, it is very likely that the use of multiple fluorochrome staining will be extended.

Sperm nuclear integrity

General concepts

The sperm head consists of two main components: the nucleus and the acrosome. The nucleus contains the chromatin, which is formed by DNA and nucleoproteins (Steger and Balhorn, 2018). While, in somatic cells, chromatin is composed of DNA and histones, which regulate DNA condensation, organise chromatin into the classical nucleosome/solenoid structure and modulate gene expression, most of these histones (85–95%) are replaced by protamines in mature sperm (Balhorn, 1982). Protamines are small, arginine- and cysteine-rich proteins that replace histones during spermiogenesis (nucleohistone-to-nucleoprotamine transition; Bao and Bedford, 2016). Over this process, the nucleosomal structure is disassembled and topoisomereases allow the supercoiling of DNA, which results in its packaging into a very small space (Wang et al., 2019).

The replacement of most histones by protamines supports sperm chromatin to be packaged into highly condensed toroidal structures, each toroid packages about 50 kb of DNA (Torres-Flores and Hernández-Hernández, 2020). Retained histones maintain the organisation in nucleosomes and form the toroid linker regions, which are nuclease-sensitive segments of DNA that are associated with nuclear proteinaceous matrix filaments (matrix attached regions; Gold et al., 2018). In addition, toroid linker and histone-bound DNA regions are sensitive to damage by external agents. The presence of arginine allows for strong binding to DNA, and the degree of condensation is increased by sixfold through cross-linking via the formation of disulphide bonds (cysteines), which occurs during epididymal maturation (Ward, 2018). It is worth mentioning that the organisation of sperm chromatin throughout the nucleus is thought to be non-random, and some authors have proposed that histone-rich areas are distributed over the periphery, whereas the most abundant, protamine-rich chromatin occupies the centre of the nucleus (Zini and Libman, 2006). Moreover, whereas telomeres of each chromosome are positioned towards the nuclear periphery, centromeres distribute in internal areas (Zalensky and Zalenskaya, 2007). Upon formation of the male pronucleus after fertilisation, sperm chromatin undergoes severe changes in their conformation, involving the disassembly of the toroidal structures and the condensation of DNA in somatic nucleosomal configuration (Barrachina et al., 2018). The structure of sperm chromatin in toroids and toroid linker regions may also bring epigenetic information for the initiation of DNA replication of the male pronucleus (Shaman et al., 2007).

Aetiology and causes of chromatin and DNA damage

Chromatin compaction and DNA integrity may be affected by both extrinsic and intrinsic factors. Extrinsic factors include storage in both liquid and cryopreserved states (iatrogenic damage), gamete handling, infections, post-testicular oxidative stress and gonadotoxic effects. Particularly, liquid-storage of pig semen at 18 °C for four days decreases total thiols and disulphide bonds, and increases protamine deficiency (Khezri et al., 2019). Intrinsic factors underlying DNA damage are abortive apoptosis, errors in meiotic recombination and oxidative stress (Ribas-Maynou and Yeste, 2020; Ribas-Maynou et al., 2020a). Furthermore, if not enough protamines replace histones (deprotamination/histone retention), chromatin is considered to be immature and wrongly packaged. This turns sperm DNA more susceptible to damage by extrinsic factors. In vitro fertilisation experiments conducted in pigs support that alterations in sperm DNA compromise embryo development rather than the ability of sperm to fertilise the oocyte (Mateo-Otero et al., 2022).

Assessment of sperm DNA integrity

The integrity of DNA/chromatin is indispensable for sperm to be capable of fertilising an oocyte and is important for subsequent embryo development (Virro et al., 2004). While sperm DNA integrity is not routinely evaluated in farm animals and all tests were originally established in humans, the integrity of sperm nucleus may need to be considered when the reproductive performance of a given boar stud is reduced.

In the 1960/1970s, sperm DNA damage was determined for the first time in humans using acridine orange staining (Gledhill et al., 1966). In the 1980s, Prof. Evenson developed the sperm chromatin structure assay (**SCSA**), a flow cytometry test currently employed to estimate DNA integrity in pigs (Evenson, 2016). In the 1990s, the Comet test, based on single-cell electrophoresis, the terminal deoxynucleotidyl transferase deoxynucleotide (**dUTP**) nick end labelling (**TUNEL**; Cissen et al., 2016) and the sperm chromatin dispersion test were established (**SCDt**; Schlegel and Paduch, 2005).

Sperm DNA integrity can be assessed through direct (Comet, TUNEL and its in situ nick variant) and indirect tests (SCSA and SCDt; Table 4). Chromatin packaging can be evaluated with aniline blue, toluidine blue and Chromomycin A3 staining, and through the disruption of disulphide bonds between the thiol groups of cysteines, which is determined with 2,2′-dipyrydil disulphide and spectrophotometry, or with dibromobimane followed by dodecyl sulphate-treated polyacrylamide gel electrophoresis (SDS–PAGE) (Yeste et al., 2014a, 2014b).

Sperm chromatin structure assay

The SCSA was developed during the 1980s and is based on the removal of nucleoproteins through acid denaturation (Evenson, 2016). Because damaged DNA is more susceptible to acid denaturation, this step leads to the formation of single-strand DNA when DNA breaks are present (Angelopoulos et al., 1998). Following this, sperm cells are stained with acridine orange, a cationic fluorochrome that binds double-stranded DNA and remains as a monomer when the DNA is intact, emitting green fluorescence. When the DNA is fragmented and thus single-strands are formed after acid denaturation, acridine orange aggregates and emits red fluorescence. Samples are then analysed with a flow cytometer, which provides the proportion of sperm with higher susceptibility to DNA breaks (Love, 2005). Two main parameters in this test, DNA fragmentation index (**DFI**) and sperm DNA stainability, are determined. The DNA fragmentation index, which corresponds to the ratio between red fluorescence (denaturated DNA) and green plus red fluorescence, refers to sperm whose denatured DNA is single-stranded. At present, the DFI threshold for infertility in pig sperm is not clear, but a high DFI is known to be related to reduced fertility (Boe-Hansen et al., 2008). In humans, a DFI of $\geq$25% is linked to fertilisation failure and risk of pregnancy failure (Simon et al., 2017), despite existing controversy regarding the threshold values across studies (Ribas-Maynou et al., 2013). Apart from DFI, SCSA also

Table 4
Main techniques to evaluate pig sperm DNA fragmentation.

Test	Principle	Damage Detected	Range
SCSA	Acid denaturation and staining with acridine orange. Fragmented DNA is more susceptible to denaturing conditions and thus emits red fluorescence.	Single-stranded breaks Double-stranded breaks	0–3%
SCDt	Acid denaturation, lysis of sperm membranes and extraction of protamines. Fragmented DNA is more dispersed than non-fragmented DNA. In most species, especially those containing both protamine 1 and protamine 2, a halo is not observed in fragmented sperm because DNA is highly dispersed. In species with only one protamine 1, such as the pig, damaged sperm present a halo whereas non-fragmented sperm do not.	Single-stranded breaks Double-stranded breaks	0–22%
TUNEL	Labelling of 3′-breaks with fluorescent UDP and TdT transferase	Single-stranded breaks Double-stranded breaks	5–20%
Alkaline Comet	Lysis of sperm membranes and extraction of protamines, alkaline denaturation and electrophoresis at alkaline pH	Single-stranded breaks Double-stranded breaks	0–50%
Neutral Comet	Lysis of sperm membranes and extraction of protamines and electrophoresis at neutral pH	Single-stranded breaks	0–5%

Abbreviations: SCSA, Sperm Chromatin Structure Assay; SCDt, Sperm Chromatin Dispersion Test; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end.

allows calculating the proportion of sperm DNA stainability, which refers to histone retention in immature sperm. Evidence concerning the relationship between the proportion of sperm DNA stainability and reproductive performance in pig sperm is sparse, but in humans, there is controversy about the usefulness of this biomarker to predict fertility outcomes (Bungum et al., 2008).

As mentioned previously, not many reports have evaluated DNA fragmentation in pigs, but some that used SCSA to do so reported a positive relationship of DNA integrity with fertilising capacity, farrowing rates and litter sizes (Myromslien et al., 2019). The DFIs reported for pigs are, in general, low for liquid-stored and frozen-thawed sperm. For example, Schäfer et al. (2017) found that DFI of frozen-thawed sperm ranged between 1.0 and 1.6%. These low values were also observed by Bucci et al. (2018). In addition, Myromslien et al. (2019) evaluated sperm DFI with SCSA in Landrace and Duroc Norwegian boars and found that males with high DFI (>3%) gave smaller litters. Moreover, Schulze et al. (2020) investigated the potentially harmful effects of bisphenol A diglycidyl-ether, a component of plastic bags, on sperm quality, and saw that, after 72 h of storage at 17 °C, sperm DFI was very low (2%). Furthermore, Deori et al. (2020) interrogated on how single-layer centrifugation affects sperm quality, and detected no differences in DFI apart from the figures being very low (<3%). Nerozzi et al. (2020) also utilised SCSA and observed that Roundup or glyphosate did not have any detrimental impact on sperm DNA integrity; again DFI figures were low (<5%). Another study investigating the effect of low-temperature preservation on the DNA integrity of pig sperm also reported very low DFI (<2%; Waberski et al., 2019a; 2019b). All these observations suggest that, in pigs, DNA fragmentation is low; yet, as discussed below, it could also be that insufficient chromatin decondensation was one of the reasons explaining such a low degree of sperm DFI.

Sperm chromatin dispersion test

The Sperm Chromatin Dispersion test (**SCDt**) is also known as the halo test, because, after being exposed to acid denaturation and after the removal of nucleoproteins, pig sperm with fragmented DNA show a halo, whereas those with an intact DNA do not (Pérez-Llano et al., 2006). This assay, which requires sperm to be embedded in an agarose matrix, can be combined with bright-field (using dyes such as eosin or azure B) or fluorescence microscopy (employing fluorochromes like PI or DAPI). While the SCDt is a simple, fast, cheap and highly reproducible test that does not need expensive devices, the variation between observers may be high (Fernández et al., 2005).

Data available thus far also indicate that, in general, the proportions of sperm with fragmented DNA evaluated through the SCDt are low. In the case of liquid-stored pig semen, the proportion of sperm with fragmented DNA is lower than 5% (Valencia et al., 2021). Storage of sperm at 17 °C in a medium without antibiotics may increase the proportion of sperm with fragmented DNA up to >20% after 72 h. This increase is likely to be caused by bacterial contamination, as the presence of antibiotics, such as gentamicin, aminoglycoside, cephalosporin, lincomycin or spectinomycin, partly counteracts that DNA fragmentation increase (Tvrdá et al, 2021).

The terminal deoxynucleotidyl transferase-mediated deoxynucleotide nick end labelling assay

The TUNEL assay has been less used than SCSA or SCDt to determine DNA fragmentation in pig sperm. The TUNEL assay is based on the binding of dUTP to the 3′-hydroxyl break-ends (nicks) of single- and double-stranded fragmented DNA (Sharma et al., 2021). The attachment of terminal dUTP to the break-ends is catalysed by deoxynucleotidyl transferase, a template-independent DNA polymerase. Because dUTPs are covalently linked to a fluorescent probe, like fluorescein isothiocyanate, detection of fragmented DNA must be performed with a fluorescence microscope or a flow cytometer. The intensity of the fluorescent signal is proportional to the number of strand breaks, so that the larger the fluorescence intensity, the higher the proportion of sperm with fragmented DNA (Matás et al., 2011). This probe is usually combined with DNA-staining fluorochromes that, like DAPI or Hoechst, label the DNA of all cells and can thus counterstain the nuclei.

The TUNEL assay has high sensitivity and specificity but, despite different modifications to the protocol having been made to increase its robustness, it lacks from standardisation even in humans (Sharma et al., 2021). The threshold for the TUNEL assay has not been set but, in humans, it has been suggested that men showing a proportion of sperm with fragmented DNA $\geq$ 17% are infertile (Ribas-Maynou et al., 2013; Dutta et al., 2021). In pigs, the proportion of sperm with fragmented DNA is also low, but differs between studies. In effect, whereas Bryła and Trzcińska (2015) found the proportions of sperm with fragmented DNA to range between 1.8 and 2.3%, Peña et al. (2019) reported a range between 2 and 22% in boars exposed to tropical conditions. Interestingly, when the diet of these boars was supplemented with an antioxidant supplement containing distinct ingredients (Vitamin E, Vitamin C, Folic acid, β-carotene, Zinc, Selenium, Garlic powder), the proportion of sperm with fragmented DNA decreased to less than 10%.

On the other hand, it is worth mentioning that because of large chromatin condensation in pig sperm, the TUNEL assay may underestimate the proportion of sperm with fragmented DNA. This high

degree of packaging, probably ought to the fact that pig sperm chromatin only contains P1 (and not P2), could impede TdT to access DNA breaks (Ribas-Maynou et al., 2021a). Remarkably, chromatin decondensation through DTT increases the ability of TUNEL to detect sperm DNA fragmentation in this species (from 1.89% ± 1.63% to 8.74% ± 6.05%; Ribas-Maynou et al., 2021a).

The Comet assay

The Comet assay is based on single-cell gel electrophoresis and allows determining single- and double-stranded DNA breaks in a given sperm cell (Hughes et al., 1996). Because, when submitted to an electric field, DNA fragments can be separated on the basis of their size and charge, broken strands - which are charged negatively - migrate to the positive anode. Since the presence of breaks originates separate sperm DNA fragments, each of these molecules migrate in a specific manner depending on the size, so that after applying an electric field and after staining with a fluorescent probe, they resemble a comet (Ribas-Maynou and Benet, 2019). The fluorescence intensity and the length of the Comet tail are proportional to the extent of DNA damage in an individual sperm cell. The two main parameters evaluated are olive tail moment and proportion of tail DNA (Mateo-Otero et al., 2022).

As mentioned previously, the Comet assay can evaluate single- and double-stranded breaks, which may have a different origin and a distinct impact on sperm fertilising ability and embryo development (Ribas-Maynou et al., 2021b; Ribas-Maynou et al., 2020b). The determination of single- or double-stranded DNA breaks depends on the application of an alkaline pH solution that denaturates double-stranded DNA after chromatin is fully decondensed. When the DNA is not denatured and electrophoresis is conducted at neutral pH, the extent of damage reflects double-stranded breaks (Neutral Comet). When an alkaline buffer is used to denature DNA, both single- and double-stranded breaks (Alkaline Comet) can be determined (Mateo-Otero et al., 2022).

While the Comet assay has been reported to be the most specific and sensitive method in humans (Ribas-Maynou et al., 2013), and presents good correlation with TUNEL, SCD or SCSA assays, not many data from pigs are available. Reports in humans show that a value of DNA fragmentation above 40% is related to a 9.5 times higher risk of fertilisation failure (Simon et al., 2017). In pigs, the proportions of DNA fragmentation in frozen-thawed sperm given by the neutral Comet are similar to those provided by SCDt and lower than 5% (Fraser et al., 2010). Other studies evaluating liquid-stored semen (Li et al., 2022) and cryopreserved sperm from the Okinawan native Agu pig (Tatemoto et al., 2022) also reported low proportion of sperm DNA fragmentation. Recent evidence, nonetheless, suggests that most previous studies could have underestimated DNA fragmentation because they did not apply lysis treatments to completely decondense pig sperm chromatin (Ribas-Maynou et al., 2021d). Indeed, when chromatin is completely decondensed, the DNA damage detected in pig sperm may be higher than that previously thought, especially in the case of single-stranded DNA breaks (Ribas-Maynou et al., 2021c).

Assessment of chromatin condensation

There are other tests, known as cytochemical (aniline blue, toluidine blue and chromomycin A3), that, despite not being as used as the previous ones, may provide information on chromatin condensation (Dutta et al., 2021). On the one hand, toluidine blue is a cationic dye that binds negatively charged phosphate groups, so that it allows for the detection of sperm chromatin anomalies. As sperm with poor DNA integrity expose phosphate residues, they are stained in purple (Erenpreiss et al., 2001). The more intense the staining, the lower the degree of intact chromatin. In contrast, sperm with a tightly compacted DNA are slightly stained because they do not have free phosphate residues. Although this test is not much utilised at present, due to the better performance of the aforementioned ones, results obtained from acridine orange and toluidine blue are correlated (Ajina et al., 2017).

Chromomycin A3 is a fluorescent probe that measures the degree of protamine deficiency through binding to the same sites as protamines. The intensity of chromomycin A3 can be evaluated under a fluorescence microscope or through a flow cytometer. The higher the intensity of the staining, the larger the extent of protamine deficiency and aberrant chromatin packaging (Llavanera et al., 2021; Ribas-Maynou et al., 2021a; 2021c). Ribas-Maynou et al. (2021c) compared the performance of direct and indirect methods for the evaluation of DNA fragmentation in frozen-thawed pig sperm (TUNEL, TUNEL with previous chromatin decondensation, SCSA, alkaline and neutral SCDt, alkaline and neutral Comet assays) with chromomycin A3 outcomes. While the degree of chromatin condensation and the proportion of sperm with an intact DNA were found to be significantly correlated in direct methods (TUNEL, TUNEL with previous chromatin decondensation, and alkaline and neutral Comet) and chromomycin A3, this was not the case of the indirect techniques (SCD and SCSA). This differs from what was reported in humans, where TUNEL, SCSA, alkaline SCD, and alkaline Comet are correlated. Yet, the absence of correlation between some of the tests could be explained by the different aspects of chromatin structure and packaging that each method evaluates. In that case, one could conclude that both sperm DNA fragmentation and chromatin protamination should be determined to understand how sperm nuclear integrity ultimately affects fertilising ability.

Nuclear (de)condensation and DNA fragmentation

While, in humans, a high proportion of sperm DNA fragmentation is associated with men with idiopathic infertility (Ribas-Maynou et al., 2020a), the low incidence of DNA fragmentation in pigs leads one to interrogate to which extent this damage is related to a reduced reproductive performance. Indeed, and as extensively discussed in the previous sections, the proportion of sperm with fragmented DNA in pigs is purported to be low. The degree of chromatin condensation in pig sperm, however, could explain why most literature reports those low proportions of sperm DNA fragmentation.

Mounting evidence supports that full chromatin decondensation is needed in order for sperm DNA fragmentation to be properly evaluated. Not only does this requirement apply for the Comet assay but also for the other tests, including SCDt and TUNEL. In fact, insufficient decondensation could be the reason for the low proportion of DNA fragmentation found in fresh and frozen-thawed pig sperm using different methods. As shown by Ribas-Maynou et al. (2021d and 2021e), incubation with proteinase K for at least 180 min is required to completely decondense pig sperm chromatin. This additional step to decondense sperm chromatin appears to be needed for those species that have their chromatin condensed with protamine 1, such as bovine, but not for those that have protamine 1 and 2, like human or equine (Ribas-Maynou et al., 2021d). This additional decondensation step thus emerges as a modification of the protocol that should be envisaged by future studies conducted in the field.

Conclusions

Artificial insemination with liquid-stored semen is widely used for pig breeding. In this context, having suitable techniques to evaluate sperm quality is required. At present, most farms producing

semen doses assess sperm concentration, motility and morphology. There are, notwithstanding, other techniques that, despite not being routinely utilised, can shed some light on the problems underlying reduced sperm quality and/or fertilising ability. These techniques involve the usage of fluorescent probes in combination with fluorescence microscopy or flow cytometry (membrane integrity, intracellular calcium, ROS levels). In addition, different techniques are available to determine DNA fragmentation and chromatin condensation. These methods, nevertheless, should take into account that pig sperm chromatin is hard to decondense, so that one should not discount that the low levels of DNA fragmentation reported in the literature for this species are related to an incomplete chromatin decondensation prior to running the tests.

Ethics approval

Not applicable.

Data and model availability statement

Not applicable. As it is a review article, there was no production of new data.

Author ORCIDs

Carolina Maside: https://orcid.org/0000-0003-0872-9122.
Sandra Recuero: https://orcid.org/0000-0001-9341-1164.
Albert Salas-Huetos: https://orcid.org/0000-0001-5914-6862.
Jordi Ribas-Maynou: https://orcid.org/0000-0002-9101-2044.
Marc Yeste: https://orcid.org/0000-0002-2209-340X.

Authors' contributions

SR wrote Section 2, **CM** wrote Section 3, and **MY** wrote the other sections. **ASH** and **JRM** made a critical revision of the Manuscript. **MY** also revised and edited the entire Manuscript. All authors contributed to the finalised Manuscript, read and approved the final version.

Declaration of interest

The authors declare that this review article was written in the absence of any commercial or financial relationships that could be defined as a potential conflict of interest.

Acknowledgements

The authors acknowledge the support from other members of TechnoSperm lab, University of Girona, for suggesting the inclusion of flow cytometry variables.

Financial support statement

The authors acknowledge the support from the European Union's Horizon 2020 Research and Innovation programme under the Marie Skłodowska-Curie grant agreement No. 801342 (Tecniospring INDUSTRY; TECSPR-19-1-0003); the Ministry of Science and Innovation, Spain (IJC2019-039615-I), the Catalan Agency for Management of University and Research Grants (2017-SGR-1229), the Catalan Institution for Research and Advanced Studies (ICREA), and the University of Girona (IFUdG and POSTDOC schemes).

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