Chapter 9
The Boar Ejaculate: Sperm Function and Seminal Plasma Analyses

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Abstract A complete study of the boar ejaculate includes both routine sperm quality analysis and complementary tests of sperm function. Rigorous monitoring of both steps permits the fertilising ability of a given ejaculate to be predicted as accurately as possible. With regard to quality analysis and after successfully carrying out a macroscopic inspection of semen (volume, temperature, pH and osmolality), the technician proceeds with a microscope analysis that includes the study of the following relevant semen parameters: concentration, motility, morphology, plasma membrane integrity and osmotic resistance of spermatozoa. This chapter also reviews those tests that assess additional features of the sperm cell, such as its capacitation status, its nuclear integrity and that of its membranes, and also provides information on in vitro fertility assays as estimators of in vivo sperm fertility. Finally, the chapter outlines the main components of seminal plasma and the most important methods in their study.

9.1 Introduction

In the pig sector, the accurate prediction of the fertilising ability of an ejaculate is of great importance. To achieve this objective, we make use of the basic techniques of sperm quality analysis that are routinely applied in the centres of pig production and artificial insemination (AI). However, results from these techniques do not always positively correlate with fertility in vivo (Rodríguez-Martínez 2003; Yeste et al. 2010). That is why, thanks to new technologies that have been implemented in recent years, several complementary assays have been proposed and developed to facilitate reliable estimation of sperm fertilising ability.

Although many of them are still difficult to apply in the sector due to their high cost, turning some tedious practices into simple and standardised protocols are the

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key to the development of state-of-the-art techniques and their implementation in the industry. In this way, the prediction of the fertilising ability of an ejaculate will result in reduced production costs that will significantly offset the expenses incurred.

The total set of techniques is intended to provide a reliable approach to the structure and function of sperm and its surrounding secretions. Specifically, the study of the cell membrane appears to be a good way to evaluate the general viability of the male gamete. Many techniques nowadays resort to fluorochromes to understand processes and structures of sperm such as the acrosome reaction and capacitation, mitochondrial activity, membrane permeability, structure and stability of the nucleus, chromatin integrity, and so on. (Harrison and Vickers 1990; Garner and Johnson 1995; Silva and Gadella 2006).

A thorough approach to the physiology of the sperm cell is essential to establish corrective measures for deviations in fertility; metabolic and enzymatic studies of different chemical components present in the ejaculate help to understand the relationship established between seminal plasma and sperm fertility (Strzezek and Skaweta 1984; Gadea et al. 1998). Studies carried out on the membrane lipid composition of sperm, which broaden our knowledge of the sensitivity of boar spermatozoon to temperature changes (Shadan et al. 2004), and the impairment of nuclear integrity that could be related to fertility problems (Evenson et al. 2002), definitely contributes to the success of fertilisation. Additionally, the study of regulation systems governing sperm cell capacitation and acrosome reaction provide clues for handling ejaculates better.

Undoubtedly, the best in vitro method to predict the fertility of a given ejaculate is to prove its ability for penetrating oocytes (Brown et al. 1990). This method, mostly used in the form of heterologous penetration of zona-free hamster oocytes, has a good rate of correlation with in vivo pregnancy indexes, but it is very expensive to practice and does not provide enough information about the recognition process between gametes or, more concretely, about the molecular interactions occurred when binding oviductal membranes or penetrating the zona pellucida. Other methods, such as the homologous penetration test, provide more information about these processes; however, their reliability to estimate the fertilising ability of a boar ejaculate is still limited (Zhao et al. 2002; Foxcroft et al. 2008).

The information provided by all these techniques will also help to improve sperm quality results achieved for freshly diluted semen (see Chap. 10) and for cryopreserved sperm (see Chap. 11), the two semen storage systems that are necessary to maintain the complex machinery of the swine industry.

9.2 The Boar Ejaculate

In pigs, first ejaculations occur at the beginning of puberty, between 5 and 6 months of age. Boars are considered to be post-pubertal from 8 to 12 months, and mature from 1 year onward (Martín 1982; Hugues and Varley 1984; Sancho 2002; Cordova-Izquierdo et al. 2004). A boar ejaculation has a volume averaging

200–300 mL and contains between 10×10^9 and 100×10^9 sperm cells (Foote 2002; Casas et al. 2010) (see Sect. 9.2.3) immersed in the seminal plasma that mostly consists of secretions from the accessory sex glands and less from the epididymis and testis (see Chap. 3).

9.2.1 Parts of an Ejaculate: Sperm and the Seminal Plasma

Full discharge of semen occurs during the ejaculation process. The whole ejaculate appears as a viscous, creamy, slightly yellowish or greyish fluid. In boars, the ejaculation process takes approximately 10–30 min within which there are three phases (see Sect. 9.2.2). The boar ejaculate consists of two main parts or fractions: the sperm fraction containing the sperm cells, representing 10–30 % of the final volume, and the liquid fraction containing the seminal plasma and representing the remaining 90–70 % of the total ejaculate. In the last one, only up to 5 % of fluid comes from the secretions of the seminiferous tubules in the testes and from the epididymides; the remaining liquid comes from the secretions of the accessory glands (Table 9.1).

The seminal plasma, the fluid fraction of semen after removal of sperm by centrifugation or filtration, is the liquid nutrient medium that allows the maintenance of cell viability; that is, it is responsible for ejaculate survival along the first passages of the female tract after insemination, and helps/participates in the metabolism of spermatozoa. Seminal plasma mainly consists of organic and inorganic compounds, namely carbohydrates, lipids, amino acids and proteins of high and poor molecular weight (Pursel et al. 1973; Mann and Lutwak-Mann 1982). Their proportions vary among species and depend on the interval between ejaculations and on the health of the animal (Caballero et al. 2004; Strzezek et al. 2005; Maxwell et al. 2007).

In this sense, several studies show that seminal plasma contains specific protein factors that influence the function and fertilising capacity of sperm in several mammalian species including the pig. Furthermore, these factors appear to interact with the environment of the female genital tract during sperm transport to the site of fertilisation (Barrios et al. 2000; Centurion et al. 2003; Moura et al. 2006) (Table 9.2).

Boar seminal plasma contains fructose, glucose and sorbitol as its main sugar components. All these molecules are important energy sources, essential for sperm movement and metabolism. Additionally, the main sperm receptor proteins are glucose and fructose transporters (GLUT-3 and GLUT-5, respectively) belonging to a gene family encoding seven proteins with identical transmembrane domains

Table 9.1 Accessory sex gland contribution to seminal plasma (Martín 1982; Buxadé 1984; Sancho 2002)

Accessory sex glands	Contribution (%)
Testis and epididymis	2–5
Seminal vesicles	15–20
Bulbourethral glands	10–15
Prostate	45–60

Table 9.2 Seminal plasma content (Hugues and Varley 1984; Sancho 2002)

Seminal plasma compounds	Normal range values (units in mM)
Na^+	125–252
K^+	17–46
Ca^{2+}	1.5–4.6
Mg^+	2.5–2.4
Cl^-	85–105
Phosphate	0.4
Fructose	0.5
Glucose	0.06–0.3
Sorbitol	0.4
Inositol	28
Lactid acid	2.2
Citric acid	2.6–10.4
Glutamic acid	2
Glycerophosphocholine	4
Glycerophosphoinositol	0.26
Arginine	0.01
Creatinine	0.03
Ergothioneine	0.7
Proteins (mg/mL)	30

(Sancho et al. 2007; Casas et al. 2009). Other studies show the existence of a heterodimer protein consisting of the low molecular weight monomers PSP-I and PSP-II which, added to the dilution medium when concentration of the ejaculate is low, enhance in vitro membrane integrity, motility and mitochondrial activity of spermatozoa (Centurión et al. 2003; Garcia-Hernandez 2007).

9.2.2 Ejaculated Semen Fractions

In boars, three fractions can be obtained from sequence in the whole ejaculate as a result of testicular and epididymal activity, as well as different secretions coming from the accessory sexual glands (Fournier-Delpech and Thibault 1993; Garner and Hafez 1996; Sancho 2002; Cordova-Izquierdo et al. 2004; Peña et al. 2006).

Pre-spermatic fraction: This is formed by the secretions produced by prostate, seminal vesicles and Cowper or bulbourethral glands. The volume of this fraction is about 10–15 ml. It does not contain spermatozoa and normally presents a transparent or clear appearance.

Spermatic rich fraction: The volume of this fraction ranges approximately 70–100 ml and has a milky-white appearance. The testicular activity produces high sperm concentration, varying between 0.5×10^9 and 10^9 spermatozoa per ml. This fraction also contains secretions produced by both the prostate and seminal vesicles. This fraction is the only one collected to prepare seminal doses.

Post-spermatic fraction: The volume of this fraction is about 150–200 ml. It is pale white in appearance and contains few spermatozoa (the concentration is lower than 10^6 spermatozoa per ml). In addition, secretions of gelatinous consistency coming from the prostate and Cowper glands are found in it. This fraction contains a large amount of seminal plasma that acts in such a way as to stimulate the spermatozoa. As basal spermatic metabolism must be achieved for best storage, collection of the post-spermatic fraction when preparing seminal doses is not recommended.

9.2.3 Physiochemical Parameters of a Standard Ejaculate: Volume, Density, Viscosity, Temperature, pH and Osmolality

In terms of volume, the boar ejaculate varies between 150 and 300 ml and exceptionally it can reach 500 ml (Martín 1982; Garner and Hafez 1996; Rothschild 1990; Pinart et al. 1999; Sancho 2002).

The volume is subjected to considerable variations as a result of individual boar characteristics, such as breed, age, reproductive physiology and environmental conditions (Setchell 1991). In comparative terms, it is much higher than in other species of domestic mammals. In goats, for example, it is between 0.2 and 2.5 ml (Setchell 1991); in bulls it is between 5 and 30 ml (Garner and Hafez 1996; Rasbech 1975; Setchell 1991); in horses, it ranges from 30 to 150 ml (Rasbech 1975). In humans, standard values vary between 2 and 6 ml (Ludwig and Frick 1990; Andolz and Bielsa 1995; WHO 2000).

As stated before, the density (in terms of total number of cells) of a boar ejaculate usually varies between 10×10^9 and 100×10^9 sperm cells, which are high values when compared to other species of mammals (Crabo 1997; Sutkeviciene et al. 2005). The viscosity depends on the secretion of the accessory sex glands. The temperature of the ejaculated semen is 37 ± 1 °C and the pH is usually between 6.85 and 7.9. Variations in semen osmolality depend on the seminal composition that is in turn related to the rate of testosterone secretion resulting from testis activity (Martín 1982).

9.3 Assessment of Sperm Quality and General Viability

The first evaluation of semen, immediately after extraction, corresponds to a macroscopic control to confirm normal volume, colour, smell, viscosity and density. The normal ejaculate, consisting of the sperm-rich fraction, must be of considerable volume (70–100 ml), the intensity depending on the concentration, and should have a milky white colour. This colour can be somewhat yellowish but it normally has a similar appearance to that of skimmed milk. Occasionally, small amounts of

blood, usually originating from the urethra, may be present in the ejaculate, which gives the semen a pinkish hue. This does not reduce the fertility or the viability of the ejaculate, but when a darker red colour is associated with a pungent odour due to possible infections, the ejaculate is excluded from the commercial circuit. Hence, microbiological analyses are also required to ensure the hygienic status of semen (see Chap. 10).

Sperm quality analysis of semen can include both cell and biochemical parameters (Briz 1994; Knobil and Neil 1994; Sancho 2002). After measuring the volume, a careful microscopic examination of semen, including parameters of quality (concentration, motility, morphology, plasma membrane integrity and osmotic tolerance), is performed in order to assure the reproductive performance of a given ejaculate. Conventional semen evaluation is mainly focused on measuring volume, sperm concentration and, the percentage of spermatozoa that is progressively motile and morphologically normal (Amann and Hammerstedt 1993; Gadea 2005; Foxcroft et al. 2008). Even though microscopic evaluations are the standard for accepting or rejecting ejaculates or sperm doses, it is important not to forget obvious visual and olfactory characteristics of semen.

Although the parameters mentioned above are traditionally used for the evaluation of a given ejaculate, several authors affirm that these are not always accurately correlated with sperm fertility (Gillan et al. 2005; Holt 2005). Therefore, other parameters measuring the structural, physiological and metabolic status of the sperm cell are necessary to better predict fertilising ability; these are the following: plasma membrane integrity, osmotic tolerance, capacitation status, membrane fluidity, nuclear integrity, status of the mitochondrial sheath (MS), percentage of apoptotic spermatozoa, levels of reactive oxygen species (ROS), acrosome integrity and the activity of different metabolites and enzymes. All these assessments must be carried out using a large number of replicates because of the high level of variability within and between individual samples and the subjectivity of some assays (Woelders 1991). These will be dealt with in greater depth in the following corresponding sections.

9.3.1 Sperm Concentration

One step in the evaluation of boar semen is the determination of sperm concentration that, apart from contributing to the fertility diagnosis, establishes the number of doses that can be obtained from a single ejaculate. In this way, the concentration has a direct economic implication in porcine AI centres as well as in AI efficiency, depending on the number of spermatozoa used at each insemination (Camus et al. 2011).

Therefore, accurate assessment of sperm concentration is essential for AI organisation in boars, although there is no agreed method to use as a standard.

Various techniques are available to evaluate sperm concentration of mammalian species; in most laboratories it is routinely assessed by the use of a counting

chamber such as: Bürker (BT, Brand, Wertheim, Germany), Thoma (TH-50 and TH-100; Hecht-Assistant, Sondheim, Germany) and Makler (Sefi Medical Instruments, Haifa, Israel) (Prathalingam et al. 2006; Mrkun et al. 2007). This method consists of a microscope slide which can hold a specific volume of semen sample and a cover glass with a grid marked on its surface that makes it possible to calculate the sperm count (Fig. 9.1). This technique is widely used because of its simplicity, low cost and reproducibility (Mrkun et al. 2007); however, this method has some handicaps like time consumption, and high variability in results due to subjectivity (Knuth et al. 1989) and the use of different types of chambers (Christensen et al. 2005).

Spectrophotometers and colorimeters are widely used for determining sperm concentration not only in boars in many AI centres, but also in other mammalian species (Woelders 1991). Both instruments measure the sample opacity as the percentage of light transmittance through an aliquot of the diluted ejaculate. In a machine specially calibrated for seminal doses, this percentage is directly plotted against a standard curve performed by the manufacturer. These automated systems allow a quicker estimate of the concentration, but great variability exists in transmittance between different ejaculates because of the different compound profile and concentrations. So, to overcome this difficulty, calibration curves need to be precisely evaluated before use (Maes et al. 2010; Camus et al. 2011).

Recently, several novel techniques have been proposed which involve computer assistance to determine sperm concentration, namely Computer-Assisted Semen Analysers (CASA): Sperm Class Analyser (SCA, Microptics S.L., Barcelona, Spain), Integrated Semen Analysis System (ISAS, Proisers, Valencia, Spain), Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, United Kingdom) or Sperm Vision® (Minitüb, Tiefenbach, Germany). Several commercial companies offer integrated facilities, equipped with a phase contrast microscope, a digital

Fig. 9.1 Makler chamber counting (Sefi Medical Instruments, Haifa, Israel) where a 5 μl sample is loaded before the cover class is put in position to assess sperm concentration (TechnoSperm)

video camera and a specific program for image analysis. These devices improve the accuracy and repeatability of data collection, avoiding errors due to individual subjective evaluation and time saving in the analytical procedure (Maes et al. 2010). As a result, these systems are being used increasingly in AI centres, progressively replacing the counting chamber method.

Another instrument called Nucleocounter SP-100 (Chemomethec, Denmark) can be used for quick analysis of the sperm concentration and the sperm viability. It analyses the image from its integrated fluorescent microscope displaying accurate values. Nevertheless, this instrument is not fully implemented routinely in laboratories (Hansen et al. 2002; Anzar et al. 2009).

Finally, it is important to know that flow cytometry is a tool that also determines mammalian sperm concentration. This device can measure the number of spermatozoa in a sample utilising a variety of fluorochrome staining methods and it can be used as a reference procedure due to its velocity, sensitivity and objectivity in its measurements. However, controversy exists as to whether cytometry can accurately assess sperm concentration; it has been reported in studies using human sperm samples that this parameter could be determined correctly compared to other techniques, but there are investigations that conclude that flow cytometry overestimates sperm concentration (Lu et al. 2007).

9.3.2 Sperm Motility

It is well-known that motility together with concentration are two very important indicators of boar sperm quality for AI centres (Flowers 1997; Rigau et al. 2001; Sancho 2002; Vyt et al. 2008; Tejerina et al. 2008). Specifically, the progressive motility of spermatozoa indirectly indicates unimpaired metabolism as well as undamaged membranes (Johnson et al. 2000) and is the most frequent viability parameter assessed in andrology laboratories.

Visual estimation of motility by phase contrast microscopy is cheap and quick, but its reliability depends on the accuracy of the technician (Woelders 1991; Tejerina et al. 2008). The experience and training of the operator explain the large intra- and inter-assay variation documented in the literature (Dunphy et al. 1989; Yeung et al. 1997; Brazil et al. 2004). Moreover, the correlation between sperm motility and sperm fertilising ability, measured as the fertility rate and the non-returning rate to oestrus within 60 days (NRR_{60d}) is a controversial subject (Berger et al. 1996; Pérez-Llano et al. 2001; Yeste et al. 2010). Nevertheless, its assessment is expected to provide clues to the potential fertility of the spermatozoa.

Other tools to avoid subjectivity have been developed for the assessment of this parameter, such as turbidimetry, laser-Doppler spectroscopy and photometric methods. However, these systems do not take into account the evaluation of each individual spermatozoon and they only allow for a rough estimation of the whole population (Verstegen et al. 2002). There are other automated devices, belonging

to CASA systems, available for motility assessment of porcine spermatozoa. For example, the Hamilton-Thorne Ceros sperm analyser (HTR Ceros 12.1; Hamilton-Thorne Research, Beverly, CA, USA); this assesses the motion of individual spermatozoa by processing digital images of the trajectories over a certain time interval. The signal is digitalised and the information processed by a computer that reconstructs the trajectory of each sperm cell from the position of the head in subsequent frames (Rijsselaere et al. 2002; Vyt et al. 2008). Another homologous system is the sperm quality analyser (SQA). The first version, named SQA-IIC (SQA-IIC, Medical Electronic Systems Ltd, Tirad Carmel, Israel), was consistent and suitable for the estimation of boar semen quality (Vyt et al. 2008) but recently a device specifically designed for boars (SQA-Vp, A-Tech, Los Angeles, California, USA) has been introduced. This device analyses the signals of a mass of motile sperm traversing a light through a capillary tube, and the percentage of motile sperm is obtained by mathematical algorithms. However, it does not provide as many sperm motility and velocity parameters as the HTR system (Vyt et al. 2004; López Rodríguez et al. 2011).

In the last decade, these CASA systems have become commercially available for objective and accurate assessment of sperm motility in pigs and other species. The information provided by these devices is especially interesting with regard to the quality of sperm movement, which is classified into several sperm quality parameters of motility. It is interesting to mention the following: total motility (in percentage), circular trips, curvilinear velocity (VCL, determined by the average velocity measured over the actual point-to-point track followed by the spermatozoon in micrometers per second), straight-linear velocity (VSL, which corresponds to the average velocity measured in a straight line from the beginning to the end of one track in micrometers per second), average velocity (VAP, average velocity of the smoothed cell's pathway in micrometers per second), linearity index (LIN = VSL/VCL in percentage which estimates the proximity of the cell's track to a straight line), straightness index (STR = VSL/VAP, determined as the straightness of the average path), oscillation index (WOB = VAP/VCL, oscillation of the real trajectory with respect to the mean trajectory), amplitude of lateral head displacement (ALH, defined by the amplitude of lateral head displacement along its average trajectory in micrometers per second), beating frequency (BCF, which is the frequency at which the head of the spermatozoon crosses the sperm cell's average path trajectory in Hertz) and progressive motility (sperm showing a given percentage of STR) (Verstegen et al. 2002; Martínez-Pastor et al. 2011) (Table 9.3).

The CASA examination is considered faster than a visual assessment and it can provide much more data with such a degree of accuracy that it can reveal subtle differences in motility not perceivable to the human eye (Tejerina et al. 2008; Casas et al. 2010). Since subjectivity is avoided, there has been increasing interest in including this methodology in the routine analysis of sperm motility (Tejerina et al. 2008), but visual estimation is still the main practice in those AI centres that cannot sustain the expenses of such automated systems. Detailed CASA motility data in pigs can be related to fertility results to a certain extent, as explained in a study undertaken by Holt and collaborators (Holt et al. 1997).

Table 9.3 Semen characteristics and sperm quality parameters (Hugues and Varley 1984; Sancho 2002)

Parameters	Normal range values
Temperature (°C)	37 ± 1
pH	6.8–7.9
Volume (mL)	150–300
Density (number of cells)	10,000–100,000
Osmolality	Variable
Sperm concentration ($\times 10^6$/mL)	200–300
Sperm motility (%)	
Total	>80
Progressive	>60
Sperm viability	
Viables(%)	>75
Sperm morphology (%)	
Normal	>80

An ejaculate assessed by CASA with a minimum of 80 % of total sperm motility and 60 % of progressive motile spermatozoa (established as sperm cells showing more than 45 % of their STR) matches the values for good sperm quality in accordance with investigations carried out in our group. In a given ejaculate, this percentage is subjected to variations due to handling. Although this problem can be solved by incubating sperm samples at 37 °C between 15 and 20 min, which induces motility (Briz 1994), inter-replicate variability is very high (Yeste et al. 2010). Different reports have studied ejaculate structure by analysing subpopulations of spermatozoa in an attempt to explain this variability. In these studies, cluster analysis of sperm motility parameters plays a basic role and manifests the existence of spermatozoa groups with differential behaviour inside the ejaculate (Abaigar et al. 1999; Quintero-Montero et al. 2004; Satake et al. 2006; Rodriguez-Martinez et al. 2009). The relative percentages of these groups in each ejaculate sample could be a reason for the large data deviation observed.

In boars, as well as in other mammalian species, it has been noted that a structure of separate motile subpopulations exists in an ejaculate. This finding allows a better predictive ability of boar semen quality analyses because the ejaculate is not treated as a normal, uniform, distribution model of motility (Quintero-Moreno et al. 2004), permitting better assessment of the ejaculate and its fertility potential (Martínez-Pastor et al. 2011). Although total motility does not seem to be of great importance in vivo fertility, it has been observed that specific motile subpopulation structures can be strongly related to the fertilising ability of a sample (Quintero-Moreno et al. 2004).

Besides, it has been noted that great differences exist in the proportion of motile subpopulations among individuals (Quintero-Moreno et al. 2004), and no standard method exists to assess these different subpopulations. In addition, an improvement in the statistical methods used to disclose different motility patterns is needed, in order to enhance the results and facilitate comparison among studies (Abaigar et al. 1999; Quintero-Moreno et al. 2004; Martínez-Pastor et al. 2011).

9.3.3 Sperm Morphology

The assessment of sperm morphology is an effective tool to estimate the function of the seminiferous epithelium and the epididymal maturation in porcine and other mammalian species, and serves as an additional measure of ejaculate quality and fertility potential (Bonde et al. 1998; Gadea 2005).

Usually, a morphological examination of the boar ejaculate comprises a qualitative and also a quantitative classification of normal and abnormal sperm morphologies. This classification used to be assessed by optical microscopy and a simple staining techniques, such as Eosin nigrosin, Trypan Blue, Giemsa, Papanicolaou or Diff-Quick (Foxcroft et al. 2008). This manual approach has always been problematic because of large variations between technicians and laboratories, extensively reported in the literature (Comhaire et al. 1994; Root Kustritz et al. 1998; Eustache and Auger 2003).

Currently, a contrast phase microscope can provide better resolution without requiring staining the samples and, as in motility assessment; it can be coupled to a CASA system. In this case, the device identifies individual spermatozoa and allows easy classification, which is usually composed of the following main categories: normal or mature spermatozoa, immature spermatozoa with proximal cytoplasmic droplets and aberrant spermatozoa with head or tail abnormalities. In this last category, a more specific subclassification is also provided. Computerised systems help analysis by reducing the time of assessment, but require standardisation of the parameters that allow images of sperm cells and seminal debris and/or superimposed cells to be distinguished. This should be done to minimize variability among analyses performed with these systems in different laboratories (Fig. 9.2).

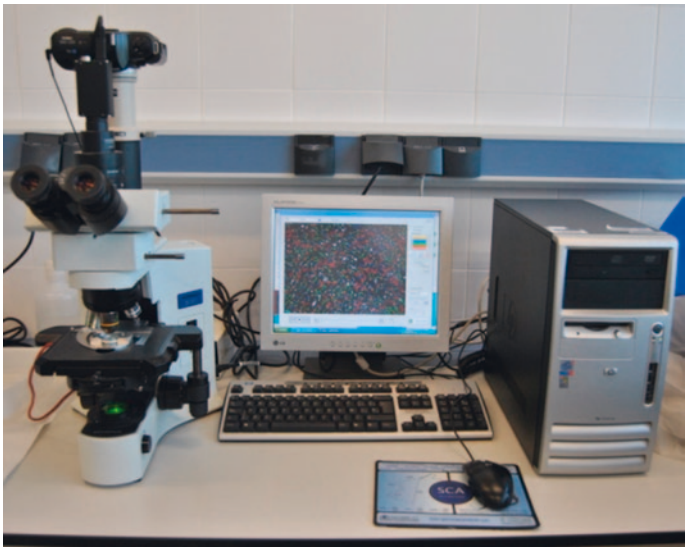

Fig. 9.2 Computer-Assisted Semen Analysis (CASA) system. In the screen a detail from morphology analysis is displayed (TechnoSperm)

A threshold of 75–80 % of mature spermatozoa has been set as the standard value (Rozeboom et al. 2000; Sancho, 2002). When the percentage of immature spermatozoa is higher than 30 %, an epididymal dysfunction is assumed, often caused by a high rhythm of collections (Thibault et al. 1993; Pruneda et al. 2005). Concerning the aberrant spermatozoa, teratozoospermia is considered when the percentage of abnormal cells in an ejaculate is higher than 50 % (Knobil and Neill 1994). Aberrant morphologies can affect the head and/or the tail of the spermatozoon. Depending on the origin, malformations can be primary, when produced in the testicle during spermatogenesis, or secondary, when occurring in the epididymis (Bonet el al. 1995). Head malformations can be presented as aberrations in number (two or more heads), shape (triangular, ovoid, pear formed, etc.) or size (micro or macro cephalic). Tail malformations can be presented as aberrations in number (two or three tails) and length or trajectory (folded or coiled tails) (Bonet et al. 1995; Sancho 2002).

9.3.3.1 Sperm Agglutination

Sperm agglutination is observed when a spermatozoon binds to another spermatozoon by head-to-head or tail-to-tail contact. Under normal conditions, agglutination does not occur and an immunological cause may be suggested when it is often present. It is well-known that bivalent and trivalent cations in boar seminal plasma, as well as long storage, induce sperm agglutination (Sánchez et al. 1991; Yeste et al. 2008b).

9.3.4 Sperm Plasma Membrane Integrity

Although sperm membrane integrity is often confused with terms like “viability”, “live” and “dead”, this nomenclature is not scientifically correct. Even though it is certain that the rupture of the membrane leads to the death of the spermatozoon, almost all dyes and fluorochromes that strictly provide information on the status of the membrane are wrongly believed to be the only indicators of sperm viability. Viability should be understood as a whole cell physiological status, and thus it can be approached with any quality parameter and not only by using membrane integrity; however, this is currently the best estimator.

In the assessment of sperm membrane integrity, porcine ejaculates should contain 85 % of spermatozoa with intact membranes to be considered of good quality (Martin 1982; Buxadé 1984; Briz 1994; Bonet et al. 1995; Briz et al. 1995; Pinart et al. 1999; Sancho 2002).

9.3.4.1 The Use of Stains as a Field Approach

The percentage of membrane integrity can be assessed by conventional optical microscopy with specific dyes including the eosin blue aniline (Shaffer and Almquist 1948) and eosin nigrosin (Dott and Foster 1972; Garcia-Artiga et al. 1994;

WHO 2000; Kvist and Björrndahl 2002; Sancho 2002). These stains are based on the fact that intact membranes are impermeable to the dye, whereas impaired ones are permeable to it. In the eosin-nigrosin test, sperm that is stained in pink red is classified as "intact", whereas sperm that has not been stained is labelled as "damaged" (Dott and Foster 1972; Kvist and Björndahl 2002; Sancho 2002). This approach is still utilised in on-field assessments as it is a cheap procedure that does not require the use of expensive microscopes. The use of stains, however, is not recommended when certain media (like freezing extenders) are used as they could mutually interfere, producing misleading results (Casas et al. 2010).

9.3.4.2 Fluorochrome Probes for Fluorescence Microscopy or Flow Cytometry

There are other methods to assess membrane integrity of boar spermatozoa more accurately than with conventional non-fluorescent dyes. These take advantage of fluorochromes by directly binding to the organelle to be tested either by covalent unions or in other molecules such as lectins (Serrano et al. 2001). Fluorochromes are not only efficient in binding the nucleus but also the acrosome, the MS and other membrane proteins (Gravance et al. 2000). For example, acridine orange is a nuclear probe that has been used in a wide range of mammalian species such as the porcine (Bonet et al. 1995; Pinart et al. 1999). It is based on the nucleic acid selectivity of the fluorochrome, which emits green fluorescence when it binds to non-altered (double stranded) DNA and orange fluorescence when it binds to RNA or denatured (single stranded) DNA. The degree of emission in the orange spectrum has been shown to correlate with male infertility in the sperm chromatin structure assay (Silva and Gadella 2006).

Sperm cells labelled with fluorochromes can be either assessed under fluorescence microscopy or by flow cytometry. Fluorescence microscopy is more subjective than flow cytometry, but the sharpness of fluorescence labelling reduces the incidence of mistakes coming from the observer. Although a flow cytometer is expensive and voluminous it processes samples quickly, automatically and objectively and therefore it is frequently found in clinical laboratories for routine assays. On the other hand, this apparatus requires expensive maintenance and cautious selection of the samples that are to be processed, as particles exceeding a certain size can result in obstruction of filters and pipes.

Double Fluorochrome Labelling

Since the beginning of the twenty-first century, the use of double labelling has been the most used quantitative method for assessing sperm membrane integrity. It is based on using two specific fluorochromes; one labels viable cells by binding enzymes, active only when the cell is alive, or by emitting fluorescence when ionic pumps are working (Shapiro 1998), and the other fluorochrome is specific for non-viable cells as it only enters cells whose plasmalemma is ruptured.

The most commonly used fluorochromes to label boar sperm cells with intact membranes are SYBR-14 (Garner et al. 1994; Gravance et al. 2000; Huo et al. 2002; Love et al. 2003; Puigmulé et al. 2011), Hoechst 33342 (Cai et al. 2005; Hallap et al. 2006) and Hoechst 33258 (Bussalleu et al. 2005; González-Urdiales et al. 2006; Pinart et el. 2006). The last two dyes have been developed by a German company from the molecule bisbenzimide, a membrane-permeable fluorescent dye that intercalates between Adenine–Thymine base pairs in DNA of both viable and non-viable cells. When excited at 346 nm it emits blue fluorescence (488 nm) if bound to DNA. The fluorochromes often used to stain damaged membrane of boar sperm cells are ethidium homodimer (EthD-1) (Yeste et al. 2009) and propidium iodide (PI) (Bussalleu et al. 2005; Puigmulé et al. 2011). The latter is a DNA-intercalating agent widely used as vital dye in cell biology (Garner et al. 2004). When intercalated with the nucleic acid helix and irradiated at 488 nm, it emits fluorescence in the red spectrum (615 nm) (Love et al. 2003; Nagy et al. 2003; Rowland et al. 2003).

A frequently reported combination of fluorescent stains is SYBR-14/PI (Garner and Johnson, 1995). In a flow cytometrical analysis the labelled sperm is excited by an argon ion laser emitting at 488 nm to measure both green SYBR-14 and red PI fluorescence. The green wavelength fluorescence is collected through different band pass filters and data are plotted on a bidimensional graph, each spermatozoon corresponding to a dot. For measuring SYBR-14 fluorescence, a 525 nm band pass filter is used (FL-1), while PI fluorescence is detected through a 635 nm band pass filter (FL-3). Depending on the percentage and quality of the fluorescence each cell is plotted up and down or right and left to the axes and counted in the population of sperm with intact membrane or damaged membrane, or classified as debris. Sperm cells depicting double fluorescence can be considered in the damaged membrane cluster (Nagy et al. 2003; Garner et al. 2004).

Multiple Fluorochrome Labelling

New tests have been developed to assess the integrity not only of the plasmalemma but also of other organelles and vesicles in sperm cells (Nagy et al. 2003; Bussalleu et al. 2005). A simple method of multiple staining with fluorochromes allows the status of the nucleus, the acrosome, and the MS of boar spermatozoa to be assessed objectively and accurately (Bussalleu et al. 2005; Pinart et al. 2006).

First, bisbenzimide and PI are added to the sample and penetrate the sperm membranes to bind DNA. Bisbenzimide penetrates all sperm membranes and produces blue fluorescence of 488 nm when bound to DNA, whereas PI penetrates only damaged membranes and emits red fluorescence of 617 nm (Love et al. 2003), which hides the bisbenzimide emission. Bisbenzimide is monitored through flow cytometry with a sensor using a 500 ± 30 nm band pass filter and PI labelled spermatozoa with a sensor using a 630 ± 20 nm band pass filter.

Second, two more fluorochromes are added to the sperm suspension, these fluorochromes being Mitotracker® Green FM (Molecular Probes Inc., Eugene,

OR) and Alexa Fluor® 488 Conjugate-SBTI (Molecular Probes Inc., Eugene, OR). Mitotracker® Green FM is excited at 490 nm and emits green fluorescence at the 516 nm wavelength. It passively diffuses across the plasma membrane and binds to membrane lipids of functional mitochondria (Fraser et al. 2001; Keij et al. 2000); in contrast, this dye is not retained in those mitochondria with altered membrane potential. Mitotracker® Green FM fluorescence is monitored through flow cytometry with a sensor using a 530 ± 300 nm band pass filter. Alexa Fluor 488 Conjugate-SBTI is formed by the Trypsin inhibitor from soybean (SBTI), which is an inhibitor of the catalytic activity of serine proteases that bind to and inhibit acrosin (Tollner et al. 2000; Fukami et al. 2003) and the fluorochrome Alexa Fluor 488. The fluorochrome is excited at 490 nm and emits green fluorescence of 519 nm. Alexa Fluor 488 Conjugate fluorescence can be monitored through flow cytometry with a sensor using a 530 ± 30 nm band pass filter. Both fluorochromes can also be checked under fluorescence microscopy.

From the results obtained by this technique, spermatozoa can be considered to have either intact membranes (blue-stained nuclei) or damaged membranes (red-stained nuclei). Spermatozoa with intact membranes are classified into three additional categories according to both acrosomal and mitochondrial staining: (1) spermatozoa with intact acrosome and mitochondria that display intense green fluorescence over the acrosomal cap and the MS; (2) spermatozoa with reacted acrosome and intact mitochondria that show intense green fluorescence of the MS. The acrosome displays a patchy disrupted green fluorescence on the equatorial segment, or it is not stained at all; (3) spermatozoa with intact acrosome and altered MS that exhibit intense green fluorescence over the acrosome and a partial or total lack of fluorescence in the MS. Spermatozoa with both the acrosome and mitochondria altered (lack of fluorescence on the acrosome and the MS) are classified into category 4 (Fig. 9.3) (Bussalleu et al. 2005).

9.3.5 Osmotic Tolerance of Sperm

Spermatozoa have different mechanisms of adaptation and resistance to sudden changes in the osmolality of the medium they are in. These mechanisms involve complex metabolic pathways, such as ion channels related to ATPase-dependent Na^+/K^+ type or exchanger of Na^+/K^+, all of them requiring precise regulation.

Boar spermatozoa are sensitive to osmotic changes in the environment, both hypotonic and hypertonic and the ability to respond to osmotic stress is related to cell functionality and can be a good indicator of its physiological state (Yeste et al. 2010). It has been reported that osmotic shock affects the diffusion of phospholipids in the bilayer membrane of spermatozoa (Christova et al. 2002). When sperm cells are exposed to an environment of high osmotic pressure, their motility decreases and the acrosome is damaged (Curry and Watson 1994; Revell and Mrode 1994; Liu and Foote 1998, Rossato et al. 2002; Chantler and Abraham-Peskir 2004; Yeste et al. 2010).

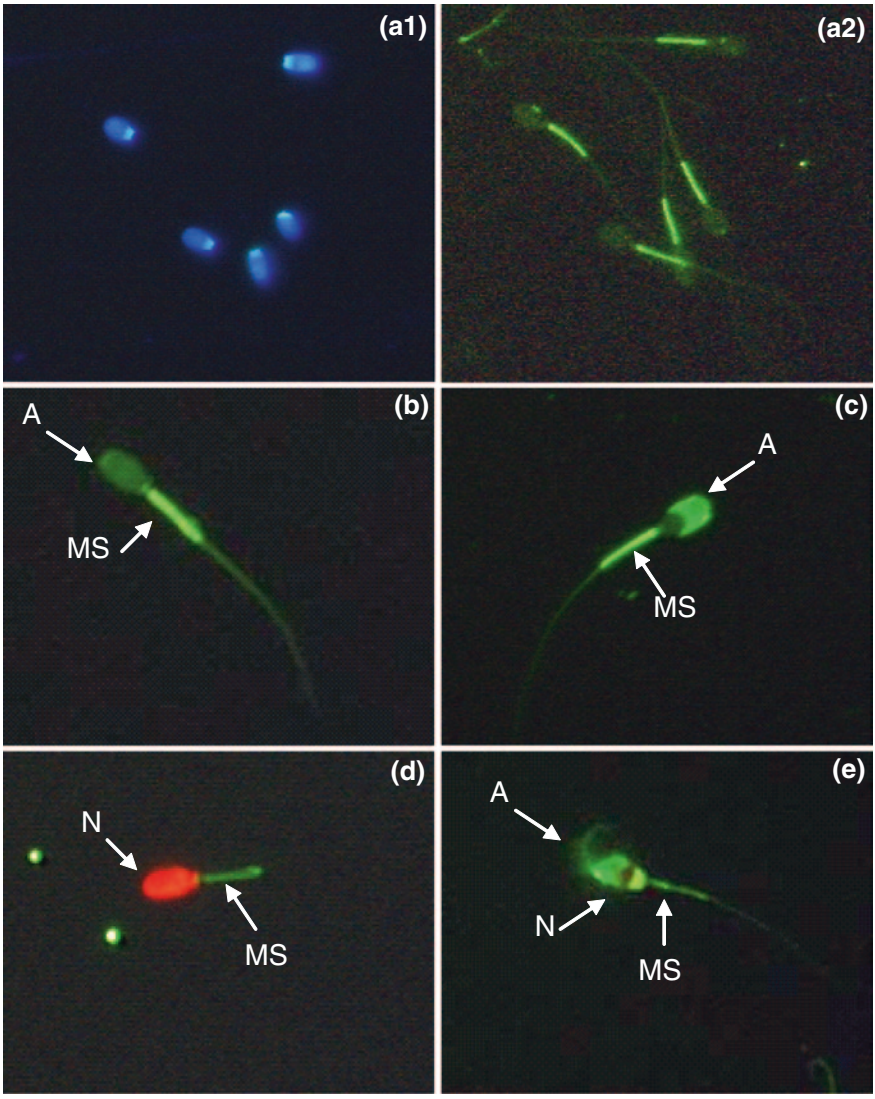

Fig. 9.3 Images of spermatozoa obtained from multiple staining methods in the membrane integrity assay. **a1**–**a2** Viable spermatozoa with intact acrosome and intact MS. The nuclei of viable spermatozoa emit intense blue fluorescence (**a1**), whereas intact acrosomes and intact MSs emit weak and moderate green fluorescence, respectively (**a2**). **b** Viable spermatozoon with reacting acrosome (*A*) and intact mitochondrial sheath (*MS*). Note that the reacting acrosome exhibits moderate green fluorescence. **c** Spermatozoon with altered acrosome (*A*) and intact mitochondrial sheath (*MS*). **d** Non-viable spermatozoon with intact acrosome and intact mitochondrial sheath (*MS*). The intense red fluorescence of the nucleus (*N*) masks the weak green fluorescence of the intact acrosome. **e** Non-viable spermatozoon with reacted acrosome (*A*) and altered mitochondrial sheath (*MS*). Note a weak red fluorescence of the nucleus (*N*) (×40) (Bussalleu et al. 2005)

In boars, an isotonic solution is established between 300 and at 400 mOsm Kg^{-1} (Petrunkina and Topfer-Petersen 2000; Petrunkina et al. 2000; Fraser et al. 2001).

Several tests have been developed to measure the response of boar sperm to osmotic changes as a parameter indicator of semen quality. These methods are the hypo-osmotic swelling test (HOST), the hyperosmotic resistance/swelling test (HRT) and the osmotic resistance test (ORT). All of them are focused on the response of the sperm membrane to sudden changes of osmolality depending on the media in which they are enclosed.

9.3.5.1 Hypo-Osmotic Swelling Test

This technique, which was first described in humans (Jeyendran et al. 1984), measures and evaluates the response of sperm to a hypo-osmotic solution (below 300 $mOsm \cdot Kg^{-1}$) based on the swelling capacity of these cells when trying to balance the ionic content of the external environment. This swelling is manifested in a characteristic curl of the tail that can vary depending on different factors such as individual tolerance or composition of the media (Gonzalez-Urdiales et al. 2006; Bonet et al. 2006) and represents a quick, easy technique to estimate the quality of a given ejaculate from several mammals including the boar.

9.3.5.2 Hyperosmotic Resistance/Swelling Test

This technique is also based on the resistance that spermatozoa exhibit to sudden changes of osmolality. In essence, this test consists of challenging sperm to a solution that has higher osmolality than seminal plasma (hyperosmotic or hypertonic medium) and to another at a similar osmolality (isoosmotic or isotonic medium). The result is the quotient obtained by dividing the percentage of osmotically tolerant sperm (sperm with intact membranes) in isoosmotic medium and the percentage of osmotically tolerant sperm in hyperosmotic medium. Sperm cells can be contrasted through the eosin-nigrosin staining technique (Rodriguez-Gil et al. 1994).

9.3.5.3 Osmotic Resistance Test

The ORT consists of checking the capacity of sperm to resist a hypoosmotic shock by challenging sperm to a hypoosmotic medium ($\leq$150 $mOsm \cdot Kg^{-1}$) and to a medium at a similar osmolality to seminal plasma (~300 $mOsm \cdot Kg^{-1}$). It tests the capacity of spermatozoa to resist changes in osmotic pressure like the ones they will encounter in the female tract. The result is the quotient obtained by dividing the percentage of osmotically tolerant sperm (sperm with intact membranes) in the isoosmotic medium and the percentage of osmotically tolerant

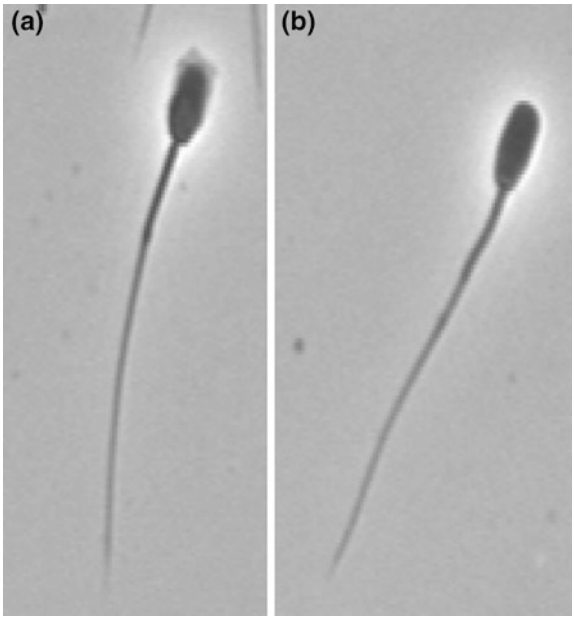

Fig. 9.4 Osmotic tolerance of sperm. **a** spermatozoa with altered acrosomal membrane. **b** Non-reacted spermatozoa (TechnoSperm)

sperm in the hypoosmotic medium. Osmotically, tolerant sperm must resist changes in osmolality and must show a low percentage of altered acrosomal membranes (Bonet et al. 1995; Briz et al. 1995; Sancho et al. 2004; Medrano et al. 2006) (Fig. 9.4).

9.3.6 Sperm Capacitation Status

Capacitation involves several physiological changes that the spermatozoon undergoes at the oviduct and that are essential for achieving its fertility ability. These changes alter its membrane and include reorganisation of proteins, metabolism of phospholipids and reduction in cholesterol levels. They also provoke hyperactivation in terms of sperm motility.

There are different techniques to assess the capacitation status of spermatozoa that analyse part of the physiological changes occurred in the sperm membrane during the capacitating process.

9.3.6.1 CTC Staining

One of the most used techniques to test capacitation status in boar sperm is chlortetracycline (CTC) co-staining with ethidium homodimer (EthD-1). This technique is based on the procedure described by Wang et al. (1995) and Mattioli et al. (1996)

and adapted in boars by Fazeli et al. (1999) and Yeste et al. (2008a, 2009). It consists of the assessment of calcium fluctuations by using the fluorescent antibiotic CTC, which traverses the cell membrane of spermatozoa and enters intracellular compartments containing free calcium (Tsien 1989). After its entrance, CTC becomes negatively charged and binds calcium, becoming more fluorescent; the CTC-calcium complex preferentially binds to hydrophobic regions, such as the cell membrane, resulting in characteristic staining patterns (Gillan et al. 2005). Labelled cells are observed under fluorescence light microscopy using blue-violet illumination. Viable spermatozoa (ethidium homodimer-1 negative) are counted, differentiating three fluorescence patterns: NC/F (uniform fluorescence over the whole head; uncapacitated spermatozoa), C/B (fluorescence-free band in the post-acrosomal region, capacitated spermatozoa) and AR (no fluorescence over the head or a thin fluorescent band in the equatorial segment, acrosome reacted).

A variant of this technique is combined Hoechst 33258/CTC labelling. This dual staining method is based on that described by Perez et al. (1996) and Hewit and England (1998). Spermatozoa are classified as dead (nuclei show bright blue fluorescence over the sperm head), live/non-capacitated (bright green fluorescence distributed uniformly over the entire sperm head with or without a stronger fluorescent line at the equatorial segment), live/capacitated (green fluorescence over the acrosomal region and unlabelled postacrosome), or live/acrosome reacted (sperm showing mottled green fluorescence over the head or no fluorescence at all and green fluorescence only in the post-acrosomal region) (Maxwell and Johnson 1999).

9.3.6.2 M540 and Plasma Membrane Fluidity

Detection of plasma membrane fluidity of boar sperm can be performed using the fluorescent dyes Merocyanine-540 (M540), based on the technique introduced for boar sperm by Harrison et al. (1996). The results analysed using flow cytometry are expressed as the percentage of membrane intact sperm (Yo-Pro-1 negative). In this category, two populations of Merocyanine-540 positive cells are typically observed; low fluorescence/low fluidity and highly ordered, and high fluorescence/high fluidity and greater disorder, which are associated with capacitating and/or acrosome-reacted sperm (Januskauskas et al. 2005; Purdy 2008; Puigmulé et al. 2011).

9.3.6.3 Levels of Intracellular Calcium as an Indicator of Sperm Capacitation (Fluo-3 AM)

Changes in membrane properties and enzyme pathways occurred during capacitation are facilitated by the activation of cell signalling cascades (Salicioni et al. 2007). One result of capacitation in mammalian sperm is an increase in membrane permeability to calcium, which allows rapid movement of calcium into the spermatozoa that is required for the fusion of the plasma membrane and the outer

acrosomal membrane to occur, initiating sperm acrosome reaction (Yanagimachi and Usui 1974; Triana et al. 1980; Landim-Alvarenga et al. 2004). This property could be used to evaluate the capacitating status by the assessment of intracellular levels of calcium (Okazaki et al. 2011) using the fluorescent calcium indicator Fluo-3 AM (Harrison et al. 1993; Kardivel et al. 2009).

9.3.6.4 Assessment of Proacrosin-Acrosin System

Another technique has been developed recently that allows a large number of samples and most of the parameters involved in the capacitating process to be examined.

The capacitated status of spermatozoa can be checked by measuring sperm viability, membrane fluidity and Ca^{2+} influx using flow cytometry (Puigmulé et al. 2011). This set of sensitive assays comprises a reliable method to study the molecular changes occurring in vitro capacitated spermatozoa, using SYBR-14 and PI for the assay of sperm plasma membrane integrity (see Sect. 9.3.4), Merocyanine 540 (M540) and Yo-Pro-1 for the assay of sperm membrane fluidity, and Fluo-3 AM and PI for the estimation of intracellular calcium levels (Harrison et al. 1993; Kardivel et al. 2009; Puigmulé et al. 2011).

9.3.7 Nuclear Integrity of Sperm: DNA Fragmentation

It has been observed in humans that the existence of abnormal semen parameters is associated with the presence of DNA strand breaks (Sakkas et al. 1999). The structure of sperm chromatin in mammals is unique compared with that of somatic cells. In spermiogenesis, chromatin condensation begins when the main histone complexes of round spermatids in DNA are replaced first by transition proteins and finally by small basic protamines. During the passage of sperm through the epididymis, the chromatin condenses fully by forming disulfide bonds between cysteine residues of protamines, which gives a very rigid structure to the nucleus. Therefore, to study the state of the DNA of mature sperm, that structure must be broken to release the chain of nucleotides (Garcia-Macias et al. 2006). Results obtained by Flores et al. (2011) suggested that the boar-sperm nuclear structure is heterogeneous and it is possible to differentiate a zonated pattern with different DNA density and compactness of the precise nucleoprotein structures.

This heterogeneity in chromatin structure, which is associated with disturbances of spermatogenesis, could be related to the variation observed in the susceptibility of DNA to denaturation leading to reduced fertility (Evenson et al. 1980; Januskauskas et al. 2000).

The status of sperm DNA in mammals is analysed by fluorochrome-based techniques, which have specific and complex interactions with chromatin or DNA: single cell gel electrophoresis (COMET) assay (Abu-Hassan et al. 2006; Enciso et al. 2011),

terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay (Lopes et al. 1998; Sun et al. 1997), in situ nick end translation assay (ISNT) (Gorczyca et al. 1993) or in situ ligation (ISL) assay (Hornsby and Didenko 2011), sperm chromatin structure assay (SCSA) (Evenson and Lorna 2000; Evenson and Jost 2001) and, less used due to its inaccuracy is the acridine orange test (AOT) (Evenson et al.1980). All of them are based on the detection of possible breaks in the DNA chain of the mature sperm nucleus using enzymes or induced lysis by heat or acid and subsequent staining of the treated sperm. The need for the use of flow cytometry limits the routine application of these techniques (Garcia-Macias et al. 2006; Gadea et al. 2008; Pérez-Llano et al. 2010; Matás et al. 2011).

Although some studies in humans have shown that the percentage of sperm containing fragmented DNA may be an independent parameter of semen quality and fertilising capacity (Evenson et al. 2002), other studies in boars using the SCSA technique suggest a relation with fertility (Garcia-Macias et al. 2006; Didion et al. 2009). However, it must be said that controversy exists about the extent of DNA fragmentation in boars (Fraser and Strzezek 2007; Flores et al. 2008, 2011) and some studies sustain this phenomenon is rarely observed in this species owing to the tight packaging mediated by protamines although alterations in the overall protamine-DNA structure have not been ruled out (Flores et al. 2008).

Use of these techniques is of great interest in boar semen analysis, although they are not adapted to routine monitoring due to complex matters.

To overcome such inconveniences, a quick, easy technique has been developed in recent years based on the detection of sperm nuclei presenting fragmented DNA, which can be performed in any conventional analysis laboratory. This technique is the sperm chromatin dispersion test (SCDt) and was originally developed for human semen (Fernández et al. 2003) and later adapted to boar semen (Enciso et al. 2006). It presents two possible variations depending on whether staining for bright field microscopy or labelling for fluorescence microscopy are used (Enciso et al. 2006; Flores et al. 2008). This technique involves immersing intact spermatozoa in an agarose matrix, smearing it on slides, dropping an acid solution to denature the DNA and lysing membranes and proteins to freed nucleotides, forming a peripheral halo of dispersion in those spermatozoa containing fragmented DNA. By staining, halos can be visualised by light or fluorescence microscopy and can be counted manually or by software image processing to find the percentage of sperm with fragmented DNA, called the DNA fragmentation index (DFI). In boar, the extremely tough of the sperm in relation to DNA fragmentation makes this assessment less useful than in other species (Gosalvez et al. 2011).

9.3.8 Detection of Apoptotic Spermatozoa

The spermatozoon is a highly specialised cell whose main function is to transport the male haploid genome into the female genital tract and to deliver it during fertilisation of the oocyte. During their genesis in the testis, spermatozoa lose most

of the organelles, except the acrosome (derived from the Golgi vesicle) and a certain subset of mitochondria, which are allocated in the midpiece of the sperm tail and play critical roles in sperm function prior to fertilisation (Peña et al. 2009). However, the spermatozoon should not be interpreted as a simplified version of a somatic cell because its whole physiology is complex like any other. Conventional quality parameters reflect dysfunctions in sperm, but the precise identification of their origin is only achieved after dissection of the mechanisms that hold spermatic activity.

Apoptosis is a complex phenomenon that consists of a fine regulation of cellular proliferation and programmed cell death as a normal component of development. At the earlier stages of apoptosis, plasma membrane becomes slightly permeable and loses asymmetry. When the cell membrane is disturbed phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane and this is identified as a signal for phagocytes (Desagher and Martinou 2000; Peña et al. 2003). The process of apoptosis can be divided into three phases: induction, execution, and degradation (Peña et al. 2009).

When induced apoptosis mitochondrial pores are opened, leading to a decrease in the mitochondrial membrane potential (Peña et al. 2009). During the execution phase, the mitochondrial pores open and trigger the release of pro-apoptotic factors into the cytoplasm, where they are activated leading to the degradation phase (Peña et al. 2009). During this latter phase an increase in the permeability of sperm plasma membrane and the externalisation of the constituent phospholipid PS are produced, which triggers a non-inflammatory recognition of the apoptotic cell by phagocytes (Peña et al. 2009).

According to Peña et al. (2009), when permeability of the inner mitochondrial membrane increases for solutes smaller than 1.5 kDa, the permeability transition pore related to that membrane is opened (Grimm and Brdiczka 2007) and when this occurs, the mitochondrial membrane potential, which relies on the impermeability of the inner mitochondrial membrane for protons, breaks down together with the ability of the cell to synthesise ATP. The blocking of the respiratory chain generates ROS intermediates (Grimm and Brdiczka 2007) that increase the concentration of solutes in the mitochondrial matrix. The increase in osmotic pressure provokes the expansion of the inner mitochondrial membrane, which could even affect the outer membrane, as well as the release of pro-apoptotic factors that leads to cell death (Peña et al. 2009).

One of the techniques used to identify apoptotic cells is the calcium-dependent binding of Annexin-V, which detects the externalisation of PS in plasma membrane (Martin et al. 1995). The Annexin-V binding assay is more sensitive to detecting changes in sperm plasma membrane than other membrane integrity assays since, during the process of apoptosis, the translocation of PS precedes the loss of membrane integrity by several hours; this early sign of apoptosis can be monitored by using Annexin-V conjugated with fluorescein isothiocynate (FITC) and PI in a cytometer equipped with standard optics (Peña et al. 2003).

Another recent, accurate technique is the use of YO-PRO-1 dye and PI together, which provides a sensitive indicator of apoptosis (Trzcinska et al. 2011).

In this, the green fluorescent YO-PRO-1 dye can enter apoptotic cells which exhibit a slight permeability of its membrane, whereas other dyes such as the red fluorescent PI cannot (Trzcinska et al. 2011).

9.3.9 Levels of Reactive Oxygen Species

Seminal plasma is a powerful source of antioxidants (Saleh and Agarwal 2002) and evidence suggests that as long as spermatozoa are suspended in it they are protected from oxidative damage. The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and affect sperm function, both in vivo and in vitro (Aitken 1995). In order to counteract the toxic effect of these ROS, seminal plasma contains the enzymes superoxide dismutase (SOD) and peroxidases, such as catalase (CAT). The SOD converts the O_2^- to H_2O_2, and the CAT converts the H_2O_2 to H_2O and O_2, eliminating potential ROS toxicity (Aitken 1995, Aitken et al. 2010).

One of the most useful techniques for estimating ROS level in boar spermatozoa is the use of the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dye (Gadea 2005; Lampiao et al. 2006; Guthrie and Welch 2008; Awda et al. 2009). This dye is a fluorogenic probe commonly used to detect cellular ROS production. Non-ionised H_2DCFDA is membrane permeable and therefore is able to diffuse readily into cells. Within the cell, the acetate groups are hydrolysed by the intracellular esterase activity forming 2′,7′-dichlorodihydrofluorescein (DCFH), which is polar, and thus stays trapped in the cell. The 2′,7′-Dichlorodihydrofluorescein fluoresces when it is oxidised by H_2O_2 (or lipid peroxides) to yield 2′,7′-dichlorofluorescein (DCF). Measurements by flow cytometry are expressed as mean green intensity fluorescence units as an estimator of ROS generation (Guthrie and Welch 2008; Matás et al. 2010).

Other techniques to assess the production of ROS levels of sperm cell are chemiluminescence methods using luminol (amino-2,3-dihidro-1,4-phthalazinedione; A-8511) and lucigenin (bis-N-methylacridinium nitrate; M-8010) described by Aitken et al. (1992). Levels of ROS are assessed by measuring the luminol- and lucigenin-dependent chemiluminescence with a luminometer.

The ROS levels can also be evaluated by Mitotracker Red/proxylfluorescamine double staining (Wenzel et al. 2005; Flores et al. 2010).

9.3.10 Acrosomal Integrity

Several methods can be used to evaluate acrosome integrity; among them, the most common is the use of plant lectins labelled with a fluorescent probe (Vázquez et al. 2005; see Sect. 9.3.4.2). Lectins are glycoproteins, mainly of non-immune origin and without enzymatic activity, which specifically detect sequences of oligosaccharides

present in the membrane of cells and, in the case of sperm, are involved in primary recognition at the time of oocyte fertilisation. Lectins have two binding sites: specific sugars and glycosylated molecules (Hernández et al. 2006). The first authors to study the cell membrane using lectins were Kashiwabara et al. (1965). To view the location of the lectins these can be conjugated to Alexa Fluor® 488 Conjugate-SBTI (Molecular probes Inc., Eugene, OR) (Bussalleu et al. 2005), fluorochromes such as TRITC and FITC (Nicolson et al. 1977), to peroxidase or to ferritin (Nicolson et al. 1977), to hemocyanin (Kinsey and Koeheler 1976) and to colloidal gold using ultrastructural techniques (Sinowatz and Friess 1983).

Techniques using lectins allow us to locate changes in the distribution of sugars in the sperm membrane. Jimenez et al. (2002) suggested that the membrane glycoconjugates play an important role in the recognition of gametes during fertilisation. Others, such as Schwarz and Koehler (1979), observed changes at the site of attachment of lectins during sperm capacitation.

9.3.11 The Status of the Mitochondrial Sheath

The role of mitochondria in sperm physiology involves two main aspects: the first is related to motility acquisition by the spermatozoon to be able to swim along the female genital tract until reaching and fertilising the oocyte (Peña et al. 2009), and the second is the regulation of sperm death by being a major source of ROS (Lesnefsky and Hoppel 2006; Ott et al. 2007; Erkkila et al. 2006).

Spermatozoa are able to generate energy (in the form of adenosine triphosphate, ATP) by either aerobic or anaerobic metabolic pathways. Monosaccharides are often the main available substrate for spermatozoa both in vivo and or during in vitro handling; by transforming to glucose-6 phosphate (G6-P), they enter the glycolytic pathway to generate pyruvate. Later, pyruvate can either produce extracellular lactate or enter the mitochondrial Krebs cycle. The equilibrium between glycolysis and glycolysis-oxidative phosphorylation depends on factors such as O_2 pressure, the intracellular levels of ATP and the number of intracellular factors, such as nitric oxide (Peña et al. 2009). Moreover, there are possible species-specific differences among mammals, with boars having the highest glycolytic activity (Marin et al. 2003). Mitochondria are found only in the midpiece; thus, oxidative phosphorylation occurs exclusively at this level. However, flagellar kinases and dynein-ATPases need large amounts of ATP to maintain sperm motility (Cao et al. 2006). It has therefore been suggested that the amount of ATP produced in the mitochondria is not enough to diffuse all along the flagellum to provide energy to support motility (Turner 2003) and that other energy sources may exist. Several studies have identified glycolytic enzymes in the principal piece of mammalian spermatozoa, including hexokinase, lactate dehydrogenase and glyceraldehydes 3-phosphate dehydrogenase (Nagdas et al. 2006; Perl et al. 2006).

The second major function of sperm mitochondria is the regulation of cell death (Lesnefsky and Hoppel 2006; Ott et al. 2007; Erkkila et al. 2006).

Mitochondria are a major source of ROS, mainly generated at complexes I and III of the respiratory chain (Krahenbuhl et al. 1991; Gonzalvez and Gottlieb 2007). The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The concentration of superoxide anion in the mitochondria is about 5 to 10-fold higher than in the cytosol or nucleus (Cadenas and Davies 2000), and thus mitochondria might also be a primary target of the damage generated by ROS. Mitochondrial-generated ROS play an important role in the release of Cytochrome-C and other pro-apoptotic proteins, which can trigger caspase activation and apoptosis (Mishra and Shaha 2005; Chan 2006; Tsujimoto and Shimizu 2007; Peña et al. 2009). Mitochondria also perform other diverse cellular functions essential for sperm viability such as modulation of redox status and osmosis, and regulation of Ca^{2+} homeostasis, this cation being involved in metabolic pathways that allow epididymal sperm maturation and fertilisation of the oocyte (Peña et al. 2009).

Several fluorochromes are available to assess the status of the MS in pigs, among them the rhodamine-123 (Fraser et al. 2001) and the iodide of 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbicianine (JC-1) (Huo et al. 2002; Ramió-Lluch et al. 2011). Since fluorochromes that stain the MS are cationic and lipophilic, they are able to passively diffuse across the plasma membrane and accumulate in the negatively charged mitochondrial matrix. Thus, depending on the membrane potential of mitochondria, fluorochromes are accumulated in a greater or lesser degree (Gilmore and Wilson 1999; Fraser et al. 2001).

Another interesting fluorochrome mentioned before (see Sect. 9.3.4.2) is the Mitotracker® Green FM, which passively diffuses across the plasma membrane and binds to membrane lipids of functional mitochondria (Keij et al. 2000; Fraser et al. 2001); it is included in multiple staining methods for assessing sperm cell integrity (Kavac et al. 2003; Bussalleu et al. 2005) and for the identification and localisation of mitochondria (Keij et al. 2000; Fraser et al. 2001). Another fluorochrome that can be used is the Mitotracker Red (Flores et al. 2010).

9.3.12 Metabolic Activity in Boar Spermatozoa: Methods Involving Signal Pathway Transduction and Kinase Activity

In mammals, sperm function is critically controlled through the phosphorylation of specific proteins (Aparicio et al. 2007). Specifically, sperm capacitation involves tyrosine phosphorylation of several proteins (Galantino-Homer et al. 1997; Visconti and Kopf 1998; Baldi et al. 2002) and this phenomenon is also observed in boar spermatozoa (Kalab et al. 1998; Flesch et al. 1999; Bravo et al. 2002).

Various specific signalling pathways mediating phosphorylations during sperm capacitation include intermediate metabolites like protein kinase (PK) A and C (Thundathil et al. 2002), cAMP/PKA (of fibrous sheath protein) and protein tyrosine kinase (PTK) (Visconti et al. 1995). On the other hand, sperm motility appears to depend on the level of phosphorylation of certain specific proteins that is mediated by protein kinases, especially the PKA, and inhibited by protein phosphatases (PP), especially of the PP1 type (Si and Okuno 1999).

Modulation of phosphorylation by PKA is achieved by alterations in the intracellular concentration of the major sperm second messenger- molecule, cyclic adenosine monophosphate (cAMP), brought about by changes in the adenylyl cyclase activity (Holt and Harrison 2002). Two important effectors of motility in natural environment of sperm, bicarbonate and calcium, have been identified as direct activators of adenylyl cyclase (Morton et al. 1974; Okamura et al. 1985; Holt and Harrison 2002). Moreover, numerous studies have shown that pharmacological agents that promote a rise in intracellular levels of cAMP (e.g. inhibitors of cyclic nucleotide phosphodiesterase, such as caffeine or pentoxifylline) can stimulate motile sperm samples poorly. It is therefore supposed that motility shortcomings may often be due to the inability of the spermatozoa to produce and/or maintain sufficient levels of cAMP to stimulate PKA (Magnus et al. 1993; Holt and Harrison 2002). Moreover, studies carried out by Rivlin et al. (2004) show that high concentrations of H_2O_2 inhibit tyrosine phosphorylation, thus corroborating that high levels of ROS are implicated in damaging sperm in the bull (Lopes et al. 1998) and also in the boar (Awda et al. 2009). Because capacitation is regulated by kinases, an indirect way to assess kinase activity is the assessment of the capacitating status of sperm (see Sect. 9.3.6).

9.4 In Vitro Methods for Predicting the Sperm Fertilising Ability

The methods for assessing sperm quality are inaccurate for predicting the fertilising capacity of a given ejaculate even if it displays acceptable sperm quality parameters (Rodríguez-Martínez 2003). Since it is not always possible to carry out in vivo trials, useful in vitro techniques are available that let us know an approximation of the real sperm fertilising ability. These tests are focused on the binding and penetration of the zona pellucida that spermatozoa must overcome to fertilize the oocyte. Therefore, the study of gamete interaction allows sperm fertility to be predicted more accurately.

A number of in vitro assays are available to estimate the capacity of boar sperm to fertilize the oocyte. Some of them are focused on the ability of sperm to develop the specific functions involved in the fertilising process and others are centred on assaying the successful recognition between gametes and the penetration of the oocyte. The most useful tests for predicting boar sperm fertilising capacity are explained in the next sections.

9.4.1 Sperm Binding Assays

Gamete recognition, binding and fusion are highly regulated processes that involve a number of biochemical mechanisms in which a great number of specialised molecules act, even though their particular function is not always known. Sperm binding assays are focused on the study of the molecules that participate in the interaction between gametes prior to penetration and fertilisation. The co-culture of spermatozoa with oocyte substrates or oviductal extracts allows the evaluation of sperm indicators that validate the success of the process. These tests are often contrasted by the same techniques used in the evaluation of the status of spermatozoa (see Sect. 9.3.6).

9.4.1.1 Sperm-Zona Pellucida Binding Test

The zona pellucida (ZP) of the oocyte is the major selective barrier that spermatozoon encounters after its release from the oviductal reservoir (Yanagimachi 1994). The initial contact between spermatozoa and the ZP induces the acrosome reaction, allowing secondary ligands and receptors to act (Rath et al. 2005). The ZP-binding test evaluates the ability for biochemical recognition between the sperm membrane and ZP-receptors, testing the co-culture of spermatozoa with isolated ZP from oocytes and performing an ulterior count of acrosome-reacted spermatozoa (Yanagimachi 1994). Specific recognition, binding and fusion are necessary prior to the penetration of the oocyte and this in vitro test acts as an indicator of the fertilising capacity of the sperm cell, as variations in the number of sperm cells that bind to the ZP between fertile and subfertile boars have been described (Ivanova and Mollova 1993; Lynham and Harrison 1998; Waberski et al. 2005; Collins et al. 2008).

9.4.1.2 Sperm-Oviduct Binding Test

This in vitro test challenges the capacity of the sperm to bind to the oviductal epithelium (Green et al. 2001; Fazeli et al. 2003; Holt et al. 2006) (see also Chap. 6). The sperm population in the oviductal reservoir depends on the initial sperm quality of each ejaculate from each boar, on the site of sperm deposition, and on the number of inseminated sperm (Foxcroft et al. 2008). This assay is interesting since it allows better understanding of sperm-oviductal interactions and can also be proposed for fertility prediction as a complement to other tests designed for the same purpose (Petrunkina et al. 2000; Waberski et al. 2005).

9.4.2 Sperm Penetration Assays

These assays are designed to evaluate in vitro the potential of spermatozoa to overcome the different barriers that participate in the fertilising process.

9.4.2.1 Cervical Mucus Penetration Test

The ability of spermatozoa to penetrate the cervical mucus has been considered essential for fertilisation for a long time (Yanagimachi 1972; Lorton and First 1977; Cupps 1991; Zhao et al. 2002). Since cervical mucus exerts a qualitative selection of sperm entering the cervix (Suarez and Pacey 2006; Martínez-Rodríguez et al. 2012), the cervical mucus penetration test (CMPT) has been proposed as an in vitro laboratory assay for analyzing the fertility of a given ejaculate in many mammalian species. Generally, it consists of a visual assessment of the linear distance covered by the foremost sperm cell in a capillary tube with natural mucus, using the number of spermatozoa accumulated in different segments of the capillary tube as the parameter of analysis (Cox et al. 2002; Martínez-Rodríguez et al. 2012).

Furthermore, Tas et al. (2007) have developed another variant of this technique by using transparent plastic straws instead of capillary tubes. In this test, the total number of penetrating spermatozoa that predetermined distances in cervical mucus is measured on slides. The major problem with this method is the difficulty to standardise the quality of cervical mucus, that is, to obtain large volumes of natural homogeneous cervical mucus. This has been mainly solved for in vitro sperm penetration tests in humans, bulls and rams by formulating synthetic media such as acrylamide, methylcellulose and, hyaluronic acid as substitutes of the natural cervical mucus (Ivic et al. 2002; Tas et al. 2007; O'Hara et al. 2010), but it has not been tried yet in boars.

9.4.2.2 Oocyte Penetration Test (Sperm Penetration Assay–SPA)

This in vitro technique includes two variants: the homologous and the heterologous penetration tests, both based on the assay of successful sperm penetration of oocytes from the same or different species. In boars, the heterologous penetration in vitro test was first described by Imai et al. (1977) by using hamster oocytes; subsequent studies showed that this test correlates positively with semen quality (Clarke and Johnson 1987; Berger and Horton 1988). Nevertheless, this technique has not received widespread attention in porcine species, because it is time consuming and involves high economical costs. Conversely, the heterologous penetration in vitro test has an extra value in human as it avoids using human oocytes which entails ethical implications (Rogers 1985). The inconvenience of these techniques is that they obviate the recognition and attachment of sperm to the oocyte membrane as well as the process of penetration through the ZP.

SPA Using Zona-Free Hamster/Bovine Oocyte Test

A variant of this technique, the zona-free hamster/bovine oocyte test—sperm penetration assay (SPA) has been described as an in vitro heterologous penetration test, which is useful for predicting sperm fertilising capacity in several mammals

including the boar (Yanagimachi et al. 1976). This test requires in vitro preparation of sperm and oocytes in different steps including sperm capacitation, acrosomal reaction, superovulation of females, removal of the zona pellucida from the recovered oocytes, incubation of gametes and finally evaluation of sperm penetration. Nonetheless, Brown et al. (1990) suggested that it might provide a better prediction of fertility than homologous in vitro fertilisation using oocytes with intact zona pellucida. The use of homospecific spermatozoa and oocytes with its zona pellucida intact in in vitro techniques allows all phases of the fertilisation process to be analysed in great detail, including those related with the acquisition of hyperactivity required for in vivo fertilisation. However, it is not always possible to harvest oocytes from the corresponding species and the induction of sperm capacitation (Nagai et al. 1994; Martínez et al. 1996) which, together with the optimal oocyte stage (Mattioli et al. 1990; Martínez et al. 1993), are crucial events when developing this technique. Moreover, if the standardisation and simplification of in vitro fertilisation protocols, including the preparation of cell gametes and the reduction of the economical cost of the whole process were achieved, this would encourage routine use of this technique.

The heterospecific in vitro sperm penetration into zona-intact oocytes is difficult regardless of the phylogenetic distance between species (Yanagimachi 1972; Hanada and Chang 1978). However, if oocytes are freed from the zona pellucida, heterospecific sperm penetration into oocytes is facilitated between some species, including rodents and porcine (Imai et al. 1977). Thus, zona-free hamster oocytes have been widely used for evaluating the fertilising ability of spermatozoa from different species (Shibahara et al. 1998; Brahmkshtri et al. 1999).

SPA Using Zona-Free Hamster/Bovine/Pig Oocyte Test

This assay is the most classical sperm penetration test. It uses zona-free hamster/bovine oocytes for an in vitro assessment of sperm competence required to fuse with the oocyte membrane and to undergo decondensation after penetration (Foxcroft et al. 2008). High positive correlation between the penetration rate and male fertility has been observed in several studies (Berger and Parker 1989; Ivannova and Mollova 1993; Martinez et al.1993) performed a similar assay using zona-free pig oocytes (a homologous in vitro penetration assay) to study the relationship between penetration and boar fertility and they were able to discriminate fertile and sub-fertile boars. However, it must also be stated that other authors have reported that the ability to identify differences in relative fertility among fertile boars is limited (Foxcroft et al. 2008).

9.4.3 In Vitro Fertilisation Assay

The in vitro fertilisation assay (IVF) allows us to evaluate the potential of spermatozoa to complete several biochemical and biophysical changes, such as capacitation, acrosome reaction, sperm-oocyte binding and penetration, and

sperm decondensation, which are all required for successful fertilisation. This test assesses the ability of sperm to fertilize a homologous zona-intact oocyte, generally using immature oocytes collected from fresh ovaries of prepubertal gilts and matured in vitro, a process termed oocyte in vitro maturation (IVM). Immature frozen oocytes (germinal vesicle stage) have been used to reduce time and costs involved in the oocyte maturation process, and also to minimize variation in oocyte quality between replicates of IVF within a same experiment (Martínez et al. 1993; Foxcroft et al. 2008). After 48 h of IVM, the oocytes are freed from cumulus cells by gentle pipetting and are then washed with equilibrating medium and transferred to four-well plates containing fertilisation medium. Sperm diluted to an appropriate concentration of 1×10^5 spermatozoa $\times$ mL^{-1} in IVF medium is added to each fertilisation-well containing the oocytes. Gametes are co-incubated at 39 °C in a humidified atmosphere of 5 % CO_2 for 16–18 h and the success of the IVF assay is tested by evaluating stained oocytes with acetic acid and lacmoid under phase contrast microscopy (Gil et al. 2007), or either by Hoechst staining under fluorescence microscopy (Coy et al. 2005). The parameters evaluated are the number of sperm per oocyte and the following rates: penetration, polyspermy, monospermy, male pronuclear formation (MPN) and potential embryo production (Xu et al. 1998; Rath et al. 1999; Gil et al. 2007).

9.5 Assessment of Seminal Plasma Composition

9.5.1 Common Elements in Seminal plasma and Methods for their Detection

Seminal plasma is a complex mixture of secretions originated in the testes, epididymis and in the male accessory reproductive glands (Yanagimachi 1994). Seminal plasma is not only a vehicle for spermatozoa; there is growing evidence demonstrating that this fluid plays other roles including modulation of sperm function and interaction with the epithelium and secretions of the female genital tract (Rozeboom et al. 2000; Rodríguez-Martinez et al. 2011).

Seminal plasma composition mainly consists of inorganic and organic compounds, such as carbohydrates, lipids, amino acids and proteins (Kordan et al. 1999; Strzezek et al. 2002). Among proteins, spermadhesins represent more than 90 % of their bulk (Calvete et al. 1995; Calvete et al. 1996; Petrunkina et al. 2000; Assreuy et al. 2003). X-ray crystallography has been used to determine the structure of this important group of boar sperm proteins (Varela et al. 1997; Nimtz et al. 1999). The technique consists of the study of the angles and intensities of the diffracted beams produced when an X-ray beam strikes the crystal structure of a protein. Thus, information such as the arrangement of the atoms, their chemical bonds or their disorder can be determined.

Moreover, in these proteomic investigations, affinity chromatography is a frequent resource used to separate heparin-binding proteins from the rest, because these bind to the surface of spermatozoa during ejaculation and are implied in sperm modulation as well as in interaction with the female reproductive tract; in the non-heparin-binding fraction some spermadhesins can be isolated as well (Holody and Strzezek 1999; Centurion et al. 2003; Strzezek et al. 2005). This technique consists of coupling heparin to a water insoluble copolymer in a column using carbodiimide reaction; the boar seminal plasma is loaded into the column with a buffer. Afterwards, non-adsorbed proteins are washed out with a diluent, such as PBS, and heparin-binding proteins attached to the copolymer are eluted with NaCl (Manásková et al. 2003). Chromatography columns can also be used to study protein aggregates by carrying out size exclusion chromatography, which involves size separation and it is usually applied to large protein complexes. Two solutions of proteins are mixed to study their tendency to form aggregates. Afterwards they are eluted through a Sephadex® column or similar and the protein complexes obtained in the fractions are analysed (Manáskova et al. 2003).

If the aim of the study is to analyse the whole proteome, the most used strategy is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). This type of electrophoresis is based on the separation of the pool of proteins by using two features of the peptides: their Isoelectric point (pI) and their size. Hence, there is a first separation or a first dimension called isoelectric focusing (IEF) in which the sample is dropped onto a pH gradient gel. The charge of a protein is affected by its surrounding pH and when an electric potential is applied, making one end of the gel positive and the other negative, proteins in the sample migrate to one or another pole depending on their electrical charge. They stop when they reach their own pI, i.e. the pH point at which their overall charge is 0. The second separation or dimension is made 90° from the first. It consists of a conventional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that allows mass-dependent separation. Since proteins are denatured and bound to sodium dodecyl sulphate (SDS), which is negatively charged, they move to the positive pole at different velocities depending on their size. The 2D-PAGE permits an effective separation of the proteome since it is difficult, if not impossible to find two proteins sharing the same pI and mass; furthermore, this method presents a high resolution so it is not difficult to understand why it has become the base of proteomic analyses (Strzezek et al. 2005). Often, the 2D-PAGE is coupled to mass spectrometry (MS), specifically designed to characterize the protein spots obtained. There are different MS methods, one of the most popular being the matrix-assisted laser desorption/ionisation source with a time-of-flight mass analyser (MALDI-TOFF). In brief, the sample is adsorbed in a solid matrix and is irradiated with a UV laser beam. Therefore, ions and neutral molecules are expelled from the matrix, which form a cloud on the sample and finally protein is ionised by the collisions with these little cations. The system, using an electric field, accelerates the ions and measures the time they take to arrive to the detector and, in this way, it can identify peptide sequences.

A wide range of immunological techniques to study seminal plasma proteins are also available. An example, the enzyme-linked immunosorbent assay (ELISA), is frequently carried out to evaluate the presence of a protein in a sample as well as to determine its concentration. The sample is loaded in the wells of the assay plate and is incubated with a coating buffer; the wells are deactivated to prevent unspecific unions and the sample is incubated with an antiserum against the antigen studied, which is in turn coupled to a fluorescent or colorimetric substrate (Manásková et al. 2003; Nishita et al. 2011).

Regarding the other compounds of seminal plasma, biochemical analyses, such as enzymatic methods to determine the concentrations of lactate, citrate or phosphorus, have been the most used in the last few years (Kamp and Lauterwein 1995). More recently, however, automated equipment has substituted these time-consuming techniques, given their high accuracy and speed. For instance, the DRI-CHEM® analyser (Fuji Film, Tokyo, Japan) fits a wide range of colorimetric and electrolyte tests for measuring molecules like sodium, potassium, chloride and calcium as well as proteins such as albumin (Murase et al. 2007).

Studies using multinuclear magnetic resonance (NMR) spectroscopy have also been performed for boar seminal plasma (Kamp and Lauterwein1995; Kalic et al. 1997). These approaches, apart from allowing an identification of seminal plasma molecules, permit the concentration of these different compounds to be determined (Kamp and Lauterwein 1995).

NMR unveils physical and chemical parameters of molecules like proteins, carbohydrates and nucleic acids that have nuclei with magnetic properties. Concretely, it studies the atomic nucleus of these molecules when they are aligned in a constant magnetic field while they are perturbed with another magnetic field with an octagonal orientation.

Finally, gas chromatography is applied, which consists of vaporising the seminal plasma sample and injecting it in a chromatographic column containing a liquid stationary phase. Then, the sample is transported through this column by an inert, gaseous mobile phase such as helium or an unreactive gas. Thus, the identification of the different compounds is based on the comparison of the retention time of the seminal plasma sample with standards analysed under same conditions.

This type of chromatography is widely applied in order to study fatty acid and carbohydrate content in boar seminal plasma samples (Johnson et al. 1972; Pizzi et al. 2005; Am-in et al. 2011).

9.6 Conclusion

The study of ejaculate quality is obviously a key factor for the successful development and applicability of AI techniques in pigs. The results obtained from the different macroscopic and microscopic analyses of sperm quality, routinely performed in AI centres, sustain the economical yields of reproductive strategies. Conventional techniques can be complemented with more complex methods that

provide detailed information about sperm physiology and a more precise approach to the fertilisation aptitude of a given ejaculate. These latter techniques require specialised equipment and experienced technicians and are therefore still relegated to research purposes or specifically requested when ejaculates coming from high-value genetic boars are required for specific trials.

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