Polish Journal of Veterinary Sciences Vol. 16, No. 4 (2013), 823–833

DOI 10.2478/pjvs-2013-0117

Review

Semen quality assessments and their significance in reproductive technology

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Abstract

Semen quality assessment methods are very important in predicting the fertilizing ability of persevered spermatozoa and to improve animal reproductive technology. This review discusses some of the current laboratory methods used for semen quality assessments, with references to their relevance in the evaluation of male fertility and semen preservation technologies. Semen quality assessment methods include sperm motility evaluations, analyzed with the computer-assisted semen analysis (CASA) system, and plasma membrane integrity evaluations using fluorescent stains, such as Hoechst 33258 (H33258), SYBR-14, propidium iodide (PI), ethidium homodimer (EthD) and 6-carboxyfluorescein diacetate (CFDA), and biochemical tests, such as the measurement of malondialdehyde (MDA) level. This review addresses the significance of specific fluorochromes and ATP measurements for the evaluation of the sperm mitochondrial status. Laboratory methods used for the evaluation of chromatin status, DNA integrity, and apoptotic changes in spermatozoa have been discussed. Special emphasis has been focused on the application of proteomic techniques, such as two-dimensional (2-D) gel electrophoresis and liquid chromatography mass spectrometry (LC-MS/MS), for the identification of the properties and functions of seminal plasma proteins in order to define their role in the fertilization-related processes.

Key words: spermatozoa, seminal plasma, semen quality assessments, reproductive proteomics

Introduction

In recent years, increasing interest in assisted reproduction techniques (ARTs) has led to a significant improvement in animal reproduction. One of the most commonly used tools in ARTs is artificial insemination (AI), which allows maximum use of the sires' genetic potential following liquid storage or cryopreservation of semen (Johnson et al. 2000). However, the assessment of semen quality following preservation requires the application of reliable laboratory methods.

There is ongoing research in developing new laboratory methods to compliment conventional semen analysis, such as sperm concentration, motility and morphology, which are routinely used to assess sperm quality characteristics. It has been suggested that evaluation of the different sperm attributes consider-

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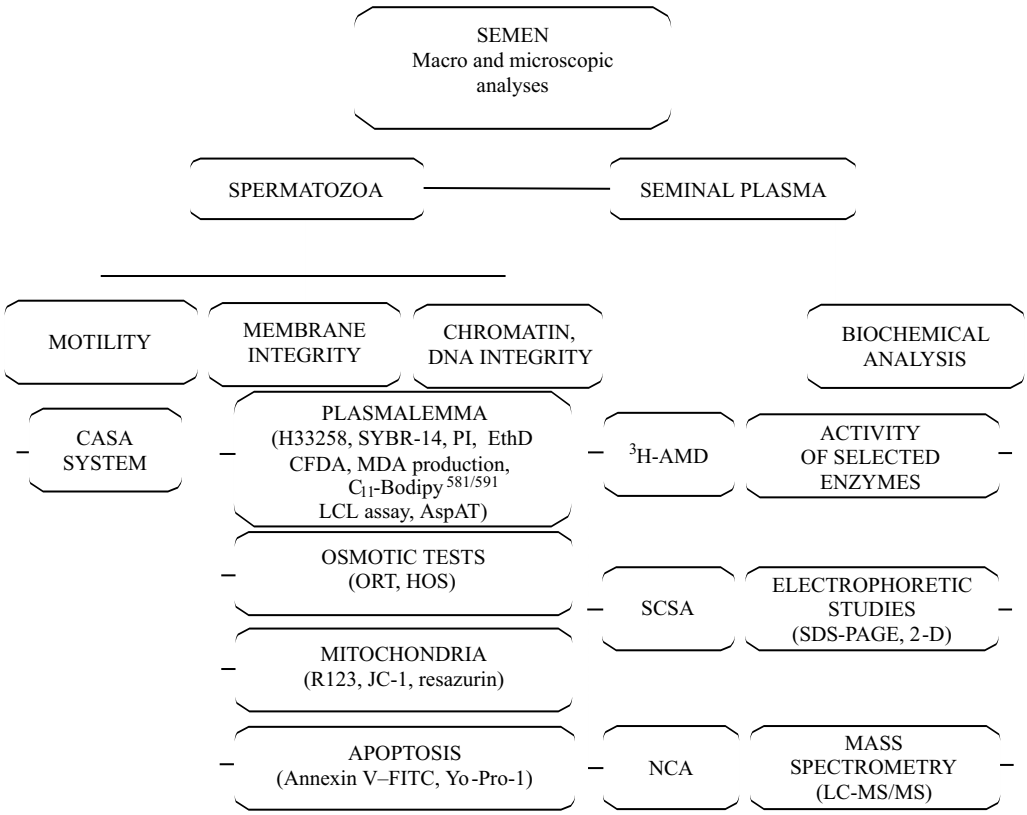

Fig. 1. Methods of semen quality assessment: CASA – computer-assisted semen analysis; H33258 – Hoechst 33258; PI – propidium iodide; EthD – ethidium homodimer; CFDA – 6-carboxyfluorescein diacetate; MDA – malondialdehyde; LCL – luminol chemiluminescence; AspAT – aspartate aminotransferase; ORT – osmotic resistance test; HOS – hypo-osmotic swelling; R123 – rhodamine 123; Annexin V- FITC – Annexin conjugated to fluorescein isothiocyanate; ^{3}H-AMD – tritium-labelled ^{3}H-actinomycin D; SCSA – sperm chromatin structure assay; NCA – neutral comet assay; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2-D – two-dimensional electrophoresis; LC-MS/MS – liquid chromatography mass spectrometry.

ed prerequisite for fertilization gives better estimates of the fertilizing ability of an individual (Rodríguez--Martínez 2003). Therefore, the use of multiple laboratory assays for the assessment of sperm function and seminal plasma quality gives better prediction of the male fertilizing ability.

Sperm motility evaluations have been improved by the use of a computer-assisted semen analysis (CASA) system, which provides detailed information about the mitochondrial status and functional axonemes of spermatozoa (Mortimer 1997). Structural changes in the sperm plasma membrane can be evaluated using specific fluorochrome probes, such as the bisbenzimide stain, Hoechst 33258 (H33258), SYBR-14, propidium iodide (PI), ethidium homodimer (EthD), 6-carboxyfluorescein diacetate (CFDA) and Yo-Pro-1 (Garner et al. 1986, Garner and Johnson 1995, Johnson et al. 2000, Peña et al. 2005, Silva and Gadella 2006, Fraser et al. 2011b, Partyka et al. 2012). Moreover, the osmotic resistance test (ORT) and hypoosmotic swelling (HOS) test have been used to assess the functional integrity of the sperm plasma membrane (Jeyendran et al. 1984, Schilling and Vengust 1987), whereas the fluorochromes, rhodamine 123 (R123) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), have been used to monitor the sperm mitochondrial status (Garner et al. 1997, Silva and Gadella 2006, Fraser et al. 2007, Dziekońska et al. 2009).

It must be emphasized that sperm function is affected by the presence of components of the seminal plasma, and several biochemical tests have been used to analyze its composition. Numerous studies have shown that seminal plasma components, particularly protein substances, have a protective effect on sperm function during semen cryopreservation (Cremades et al. 2005, Fraser et al. 2007, 2010, Fraser and Strzeżek 2007a, Rodríguez-Martínez et al. 2011). Various enzymatic tests and proteomic analyses, using the two-dimensional (2-D) gel electrophoresis, and liquid chromatography and mass spectrometry (LC-MS/MS), have been applied to assess seminal plasma proteome (Torska and Strzeżek 1985,

Ciereszko et al. 1992, 1994, Kordan et al. 2008, 2009b). This review covers a selection of the current laboratory methods used for semen quality assessments, and discusses their relevance in the assessment of male fertility and improvement of semen preservation technologies. Some of the laboratory tests used for the assessment of semen quality are shown in Fig. 1.

Sperm quality assessment methods

Motility

Subjective motility evaluation is commonly used to assess the quality of liquid-stored and cryopreserved semen. Moreover, application of the CASA system for assessment of sperm motility characteristics facilitates the comparison of results with other laboratories, and provides detailed information about the percentage of total motile, locally motile and non-motile spermatozoa (Mortimer 1997, Broekhuijse et al. 2011, Partyka et al. 2012). The CASA system provides numerous sperm motility parameters: straight line velocity (VSL); curvilinear velocity (VCL); average path velocity (VAP); linearity (LIN) expressed as the VSL/VCL×100; straightness (STR) expressed as the VSL/VAP×100; the amplitude of lateral head displacement (ALH); and beat cross frequency (BCF) (Mortimer 1997, Antończyk et al. 2010). The repeatability of CASA results is enhanced when there are optimal sample preparations, standardization of the chamber depth and optimal training of technicians working with the CASA system (Broekhuijse et al. 2011). Among the sperm motility parameters, it was shown that VCL, VSL and ALH were highly correlated with fertility results, and that BCF and ALH might be indicators of the sperm ability to penetrate the zona pellucida surrounding the ovum (Holt et al. 1997, Antończyk et al. 2010). According to Hirano et al. (2001), CASA-derived motility estimates provide reliable prediction of the sperm fertilizing ability, as indicated by the significant correlations between fertilization rates and ALH, VCL and VSL and STR. In another study the CASA system detected different motile sperm populations and subpopulations with various values for VSL, VCL, VAP, ALH and BCF, indicating the heterogeneity of sperm movement patterns in cryopreserved boar semen (Cremades et al. 2005). It has been demonstrated that the CASA system detected significantly higher values for sperm motility and LIN in cryopreserved boar and canine semen supplemented with the synthetic platelet-activating factor, PAF (Kordan and Strzeżek 2002, 2006, Kordan et al. 2009a, 2010). All these studies reaffirm the significance of the CASA system in the analysis of sperm motility patterns during semen preservation.

Status of structural and functional sperm membranes

The integrity of the sperm plasma membrane has been one of the most analyzed parameters due to its major role in acting as a cell boundary and in cell to cell interaction, both in terms of structural integrity and functional intactness (Silva and Gadella 2006). The composition of the sperm plasma membrane, in terms of the protein, lipid and cholesterol contents, is different among various animal species (Parks and Lynch 1992). It has been demonstrated that the sperm plasma membrane with low cholesterol content is more susceptible to cold-shock injury, resulting in increased membrane permeability and the leaking of intracellular enzymes (Parks and Lynch 1992).

In recent years, several fluorochromes have been used for the assessment of sperm cell membrane integrity, and can be used alone or in combination with other fluorochromes for assessing the different functional properties of spermatozoa (Garner and Johnson 1995, Silva and Gadella 2006). Fluorochromes H33258 (excitation, ex and emission, em at 358/488 nm wavelength), PI and EthD (both with ex/em 488/568 nm wavelength) are non-permanent DNA-specific stains, whereas SYBR-14 (ex/em 488/515 nm wavelength) is a membrane permeable DNA stain, and CFDA (ex/em 488/525 nm wavelength) is a non-specific esterase substrate (Garner et al. 1986, Garner and Johnson 1995, Garner et al. 1997, Johnson et al. 2000, Fraser et al. 2001, Silva and Gadella 2006). The H33258 fluorochrome penetrates damaged sperm plasma membrane, binds to DNA and emits blue fluorescence at UV (Silva and Gadella 2006), and has been shown to detect a high percentage of membrane-damaged spermatozoa under aniosmotic conditions (Fraser et al. 2001). On the other hand, SYBR-14 penetrates undamaged sperm plasma membrane, deacylates and binds to DNA, emitting green fluorescence (Garner and Johnson 1995). When CFDA is diffused through undamaged cell membranes it is converted by non-specific esterases to fluorescein, emitting green fluorescence (Garner et al. 1986). Fluorescent staining with SYBR-14 or CFDA is commonly performed with a dead-cell discriminatory fluorochrome, like PI or EthD, which allows the differentiation of viable and non-viable spermatozoa (Garner and Johnson 1995, Fraser et al. 2001, Silva and Gadella 2006).

Fluorescent staining with both CFDA/PI and H33258 assays detected similar populations of membrane-intact spermatozoa, which were less susceptible to aniosmotic conditions (Fraser et al. 2001). Kordan et al. (2010) reported that, even though there were improved post-thaw sperm motility parameters, neither the SYBR-14/PI assay nor H33258 assay detected any beneficial effect of PAF on the plasma membrane integrity of cryopreserved canine spermatozoa. In a recent study, we have shown that CFDA/PI and SYBR-14/PI assays identified similar proportions of frozen-thawed boar spermatozoa with intact membrane, regardless of the sperm source (Fraser et al. 2011b). Nevertheless, the findings of these studies confirmed that semen preservation compromised sperm plasma membrane integrity, further stressing the importance of fluorescent assays to assess semen quality.

The functional membrane integrity of the acrosome and tail of spermatozoa, when exposed to hypoosmotic conditions, can be assessed by ORT and HOS tests, respectively (Schilling and Vengust 1987, Strzeżek et al. 2000, Pinto and Koznik 2008, Gączarzewicz et al. 2010). Moreover, both ORT and HOS tests have been reported to be successfully associated with *in vivo* fertility (Schilling and Vengust 1987, Gadea 2005). According to Yeste et al. (2008), ORT values were significantly reduced following different treatments of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) of liquid-stored boar semen. Pinto and Koznik (2008) found that the percentages of hypoosmotic-reactive spermatozoa were correlated with the proportions of viable spermatozoa following cryopreservation of canine semen. Also, studies have shown that the HOS test was positively correlated with the SYBR-14/PI assay following liquid storage and cryopreservation of boar semen (Gączarzewicz et al. 2010, Fraser et al. 2011b). The results of these studies and those of a previous study (Gadea 2005) have confirmed that both ORT and HOS tests provide predictions of fertility that are similar to those with other sperm membrane assays.

The analysis of lipid peroxidation (LPO) potential, measured as the production of malondialdehyde (MDA) level, is a biochemical laboratory method used for the assessment of the sperm membrane integrity (Strzeżek et al. 2000). Besides the measurements of MDA production, other alternative methods which have been used to monitor the LPO status include the use of the fluorescent probe C_{11}-BODIPY$^{581/591}$ (Ball and Vo 2002) and the luminol chemiluminescence (LCL) assay (Gogol et al. 2007). Quantification of LPO has been monitored with the fluorescent probe C_{11}-BODIPY$^{581/591}$, which emits green fluorescence when there is oxidative damage or red fluorescence if there is no oxidative damage (Ball and Vo 2002). It has been demonstrated that the degree of LPO, detected with BODIPY, was significantly associated with the production of reactive oxygen species (ROS) by the spermatozoa (Aitken et al. 2007). The LCL assay, based on ferrous ion-induced luminescence, has been successfully used to monitor the LPO status and oxidative damage of cryopreserved goat spermatozoa (Gogol et al. 2007).

The male reproductive cells are particularly susceptible to LPO because of a high content of polyunsaturated fatty acids (PUFA) in the sperm membranes and the relatively low activity of antioxidant enzymes (Parks and Lynch 1992, Strzeżek et al. 1999, Koziorowska-Gilun et al. 2010). Lipid peroxidation has been considered to be the main mechanism responsible for sperm damage caused by ROS, leading to impaired sperm function (Sikka 2004). Protection of the sperm function against ROS-induced damage is provided by low molecular weight substances, such as L-glutathione, L-ergothioneine, L-ascorbic acid and tocopherols, and antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Strzeżek et al. 1999, 2005, Kowalówka et al. 2008, Koziorowska-Gilun et al. 2010). In the boar, SOD is the major antioxidant enzyme in spermatozoa and reproductive tract fluids, and a positive relationship between SOD activity and the MDA level was detected in spermatozoa (Strzeżek et al. 2004, Kowalówka et al. 2008, Koziorowska-Gilun et al. 2010). In the stallion, CAT is the major antioxidant enzyme (Ball et al. 2000), whereas SOD activity was highest in canine semen (Strzeżek et al. 2009). It should be emphasized that assessment of the antioxidant defence system in the spermatozoa and seminal plasma provides valuable information on the level of protection provided by various antioxidants against ROS-induced damage.

Measurements of aspartate aminotransferase (AspAT) activity are commonly used to monitor the sperm membrane integrity. It has been reported that AspAT belongs to the group of enzymes of various origin, and is permanently bound to the sperm midpiece, particularly to the mitochondrial membrane (Ciereszko et al. 1992, 1994). Even though the physiological function of AspAT remains unclear, it has been suggested that this enzyme participates in the sperm metabolic processes (Ciereszko et al. 1992). Determination of AspAT activity in sperm extracts subjected to cold shock treatment has been used as a marker for evaluating the integrity of the sperm membrane overlying the midpiece region (Ciereszko et al. 1992). Moreover, it has been shown that in-

creased susceptibility of boar spermatozoa to cold-shock treatment was associated with high levels of MDA production, suggesting impairment in sperm function (Strzeżek et al. 2000). Research studies have demonstrated that high collection frequency of ejaculates and semen preservation caused membrane damage in the midpiece, resulting in a gradual leakage of AspAT (Strzeżek et al. 1995, Gączarzewicz et al. 2010).

Mitochondrial functional efficiency

Sperm mitochondria are localized in the mid-piece area overlying the principal part of the flagellum and produce adenosine triphosphate (ATP) by oxidative phosphorylation (Silva and Gadella 2006). Maintenance of functionally efficient mitochondria is required to protect the sperm function associated with motility. Specific fluorochromes which have been used to assess the sperm mitochondrial status include R123 and JC-1 (Garner et al. 1997, Fraser et al. 2001, Silva and Gadella 2006, Dziekońska et al. 2009).

The R123 fluorochrome (ex/em 488/515-575 nm wavelength) is a strong cationic stain, which accumulates in the sperm mitochondria and emits only green fluorescence when the mitochondria are functionally active (Garner et al. 1997). The fluorochrome JC-1 has been used for the evaluation of changes in the mitochondrial transmembrane potential ($\Delta\Psi m$). This fluorochrome is accumulated in the sperm mitochondria and at low $\Delta\Psi m$ occurs mainly in the form of monomers, which emit green fluorescence (ex/em 488/510-520 nm wavelength), whereas high $\Delta\Psi m$ is associated with the formation of aggregates, which emit red-orange fluorescence (ex/em 488/515-590 nm wavelength). According to Garner et al. (1997), the ability of JC-1 to discriminate between sperm mitochondria exhibiting high membrane potential from those having low to medium membrane potential provided a more rigorous estimate of the sperm metabolic function than the R123 fluorochrome. Furthermore, both JC-1/PI and R-123/PI measurements of the mitochondrial function were highly correlated with each other, with the SYBR-14/PI assay, and with subjective motility estimates of frozen-thawed bull spermatozoa (Garner et al. 1997). Additionally, the fluorescent staining assays, JC-1/PI and R123/PI, have provided valuable information on the mitochondrial status of liquid-stored and cryopreserved boar spermatozoa (Fraser and Strzeżek 2007a,b, Dziekońska et al. 2009).

Measurement of the ATP content in spermatozoa is an additional test, which allows the evaluation of the functional and energy status of the mitochondria. The bioluminescence method, based on the oxidation of luciferin by the enzyme luciferase, is primarily used to determine the ATP content (Dziekońska et al. 2009). Studies have shown that there were positive relationships between ATP measurements and the percentage of spermatozoa with functional mitochondria following preservation of boar and canine semen (Fraser et al. 2007, Dziekońska et al. 2009, Kordan et al. 2010). These methods can be complemented with the spectrophotometric application of the resazurin reduction test (RRT) to evaluate sperm quality (Zrimšek et al. 2004). The RRT is based on the ability of metabolically active sperm cells to reduce the resazurin redox dye to resorufin, and was correlated with sperm characteristics, such as motility and viability (Dart et al. 1994, Zrimšek et al. 2004).

Chromatin and DNA integrity

There is increasing evidence indicating that abnormalities in the male genome may be indicative of male subfertility, regardless of the routine semen parameters (Evenson et al. 1994, 2002, Fraser 2004). A substantial part of the head of a mature spermatozoon is occupied by the nucleus with tightly packed chromatin, and high arginine content in sperm nuclear proteins contributes to a high degree of sperm chromatin stability (Balhorn 1982).

The structural state of sperm chromatin can be assessed, using the radioisotope assay, tritium-labelled actinomycin D (^{3}H-AMD incorporation assay), which has specific affinity for the base pair guanine-cytosine in the DNA structure (Glogowski et al. 1994, Strzeżek et al. 1995, 2000). According to Strzeżek and Kordan (2003), reducing agents, such as glutathione (reduced), heparin, dithiothreitol, 2-mercaptoethanol and sodium lauryl sulfate, had a very strong decondensing action on boar sperm chromatin structure, as indicated by increased ^{3}H-AMD labeling. In contrast, substances, such as bovine serum albumin (BSA) and ethylene diaminetetraacetic acid (EDTA) caused a decrease in ^{3}H-AMD uptake, resulting in hyperstabilization of the chromatin. Using the ^{3}H-AMD incorporation assay, it was demonstrated that cryopreservation markedly affected the chromatin structure of bull and boar spermatozoa (Glogowski et al. 1994, Fraser and Strzeżek 2007b).

Another test that has been used to assess the chromatin integrity is the sperm chromatin structure assay (SCSA), which measures the susceptibility of the sperm chromatin to acid-induced denaturation in-situ (Evenson et al. 1994). The fluorochrome, acridine orange (AO), gives rise to green fluorescence

when bound to native double-strand DNA (dsDNA) and red fluorescence when bound to single-stranded DNA (ssDNA) (Evenson et al. 1994). The proportion of spermatozoa with altered chromatin is determined by flow cytometry analysis (FCM) and is expressed as the DNA fragmentation index (DFI). The SCSA predicts infertility when the DFI exceeds 30% in natural conception (Evenson et al. 2002, Evenson and Wixon 2008), justifying its clinical application. The SCSA has identified compromised chromatin integrity of cryopreserved spermatozoa from different farm animals, such as the boar (Evenson et al. 1994), bull (Janaskauska et al. 2001) and ram (Peris et al. 2004).

The Neutral Comet Assay (NCA) is a very sensitive test for the detection of nuclear DNA damage in individual sperm cells, and can be performed under neutral or alkaline conditions (Fraser 2004). Furthermore, the NCA quantifies dsDNS and ssDNA breaks under electrophoresis and allows the assessment of DNA integrity following semen preservation (Fraser 2004). Using the NCA, it has been shown that prolonged liquid semen storage (Fraser and Strzeżek 2004) and cryopreservation induced DNA damage to boar spermatozoa (Fraser and Strzeżek 2007a, Fraser et al. 2010, 2011a). In our laboratory, we have confirmed that spermatozoa with compromised chromatin integrity, induced by the cryopreservation process, were more susceptible to DNA damage, assessed by the NCA (Fraser et al. 2007b). Besides boar spermatozoa, the NCA has been used to detect DNA damage to cryopreserved spermatozoa from the stallion (Linfor and Meyers 2002), ram (López-Fernández et al. 2008) and bull (Mukhopadhyay et al. 2011).

Apoptotic-like changes

Apoptosis of spermatogenic cells is one of the main mechanisms requires to maintain proper spermatogenesis. Since the presence of apoptotic spermatozoa in fresh semen might be associated with reduced fertility (Anzar et al. 2002), different fluorescent stains have been developed to monitor apoptotic-like changes. Fluorescent assays, such as Yo-Pro-1 (with ex/em 488/515 nm wavelength) and Annexin V-FITC, are commonly used to assess apoptotic-like changes in spermatozoa (Idziorek et al. 1995, Anzar et al. 2002, Peña et al. 2003, 2005).

The fluorochrome Yo-Pro-1 is a semi-permeable DNA-binding probe, which penetrates the cellular membranes after destabilization, under conditions where either PI or EthD does not (Peña et al. 2005). The Yo-Pro-1/EthD fluorescent assay has been used to determine the freezability of boar semen and identified three sperm subpopulations: live spermatozoa with negative fluorescence (Yo-Pro-1$^-$/EthD$^-$); apoptotic spermatozoa (Yo-Pro-1$^+$/EthD$^-$), emitting green fluorescence; and necrotic spermatozoa (Yo-Pro-1$^+$/EthD$^+$), emitting green and red fluorescence (Peña et al. 2005).

Annexin-V (A) is a calcium-dependent phospholipid binding protein that has been used to identify the externalization of phosphatidylserine (PS) occurring during apoptosis (Peña et al. 2003). During apoptosis, PS is translocated from the inner side of the lipid bilayer of the sperm plasma membrane to the outer layer, by which PS becomes exposed to the external sperm surface (Peña et al. 2003, Silva and Gadella 2006). Moreover, Peña et al. (2003) used A-FITC/PI assay (Annexin conjugated to fluorescein isothiocyanate, FITC) to monitor early changes in the sperm plasma membrane of cryopreserved boar spermatozoa and identified four sperm subpopulations: live, non-apoptotic (A-FITC$^-$/PI$^-$); early apoptotic (A-FITC$^+$/PI$^-$); early necrotic (A-FITC$^+$/PI$^+$); and necrotic cells (A-FITC$^-$/PI$^+$). In another study, Trzcińska et al. (2010) used the A-FITC/PI assay to evaluate the semen of transgenic boars, which expressed recombinant α1,2-fucosylotransferase. The authors did not find any significant differences in the percentage of apoptotic, necrotic or live spermatozoa between the semen of transgenic and non-transgenic boars. However, significant differences were found in the percentage of early necrotic and necrotic spermatozoa between the semen of transgenic and non-transgenic boars.

Biochemical assessment of the seminal plasma

Activity of selected enzymes

Accumulating evidence has shown that the seminal plasma proteins are implicated in the protection of spermatozoa against proteolysis, maintenance of sperm function in the female reproductive tract, capacitation and acrosome reactions, which are necessary for the sperm-egg fertilization processes (Strzeżek et al. 2005, Rodríguez-Martínez et al. 2011).

Acrosin, a trypsin-like serine proteinase enzyme occurring in the sperm acrosome, hydrolyzes the zona pellucida (ZP) of the ovum, and plays an important role in the fertilization-related processes (Torska and Strzeżek 1985). In undamaged mammalian spermatozoa, 95-97% of acrosin occurs in an inactive precursor, known as proacrosin, and its premature activation compromises the permeability of the sperm acrosome membranes, resulting in impaired sperm function (de Jonge et al. 1993). Hence, determination of the level

of acrosin activity in the seminal plasma has been proposed as a good indicator of sperm quality.

The activity of low and high molecular weight proteinase inhibitors of the seminal plasma comprises the extracellular defence system, which protects proteins and the reproductive tract tissues against the proteolytic activity of acrosin, when prematurely released from damaged or dead spermatozoa (Strzeżek et al. 2000). It has been suggested that analysis of the antitrypsin activity of proteinase inhibitors is therefore a good indicator of the antiproteolytic properties of the seminal plasma (Strzeżek et al. 2000, Strzeżek and Torska 2001). The authors postulated that antitrypsin activity was positively correlated with protein content and antiperoxidant properties of the seminal plasma as well as with sperm function.

Acid and alkaline phosphatases are good indicators of the secretory functions of the epididymis and accessory sex glands (Strzeżek et al. 1995, 2000). Determination of the activity of these enzymes has been used for the identification of reproductive organ dysfunction. For example, increased activity of acid phosphatases was associated with testicular neoplasms in men (Yousef et al. 2001). Studies in our laboratory have identified four molecular forms of acid phosphatase of boar seminal plasma, with one form of the vesicular glands and epididymis showing affinity for phosphotyrosyl protein residues (Wysocki and Strzeżek 2003, 2006). The findings of these studies suggested that acid phosphatase participates in the regulatory mechanisms for phosphorylation-dephosphorylation of sperm proteins. It has been suggested that tyrosine phosphorylation in freshly ejaculated spermatozoa may facilitate semen freezability, and that there is individual variability among boars, with regard to the level of phosphorylation of tyrosine residues in sperm proteins following semen cryopreservation (Wysocki et al. 2009). Since acid phosphatases, particularly those originating in the vesicle gland secretion, may act as the major decapacitation factors in boar seminal plasma, it has been proposed that the phosphorylation level of tyrosine residues can be used as an indicator of cryocapacitation-induced changes in boar spermatozoa (Wysocki et al. 2009).

Electrophoretic analysis (SDS-PAGE, 2-D) and mass spectrometry (LC-MS/MS)

Even though there are several markers for semen quality, the search for new ones is still required. Taking into consideration the importance of protein systems in animal reproductive processes, many andrological laboratories are looking for new methods and techniques that will be useful for the analysis of sperm and seminal plasma proteins in order to define their function in the male reproductive processes.

Proteomics is a comprehensive analysis of the translation activity and protein profile of a body cell at different life stages (Panisko et al. 2002), and has been developing rapidly in recent years. The proteomic approach comprises protein profiling, spatial structures of proteins and their post-translation modifications and mutual interactions (du Plessis 2011). The research tools used mainly in proteomic studies include 2-D gel electrophoresis and LC-MS/MS.

The classical 2-D gel electrophoresis, in combination with isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is a high-resolution technique which allows the identification of proteins on the basis of their isoelectric point and molecular weight (Yanagida 2002, du Plessis 2011). Although 2-D gel electrophoresis is one of the main analytical methods used in proteomics, it has some methodological limitations; that is, it fails to separate proteins with hydrophobic properties, and extremely acidic and basic, as well as high and low molecular weight proteins (Yanagida 2002).

Mass spectrometry has emerged as a powerful technique which allows a wide range of analytical applications. The LC-MS/MS technique uses liquid chromatography (LC), coupled with tandem mass spectrometry (MS/MS). Furthermore, the mass spectrometry technique uses two sample ionization methods, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray mass spectrometry (ES-MS). These ionization methods allow the determination of proteins with molecular weights of about 200 kDa, with an accuracy of 1 Da, having picomole or femtomole quantities of proteins and peptides. Using a mass spectrometry-based approach, the primary protein structure can also be determined with the use of fast atom bombardment (FAB) or cesium ions (Yanagida 2002).

Reproductive proteomics has been focused on the characterization of the sperm and seminal plasma proteome to better understand and clarify the mechanism involved in the sperm-egg fertilization processes (Strzeżek et al. 2005). The proteomic approach also enables the identification and characterization of seminal plasma proteins, which could affect semen freezability.

It is noteworthy that analytical techniques used in reproductive proteomics enable the identification of the properties and functions of proteins involved in the mechanism regulating the activity of the reproductive tract, and can be used to improve male fertility and clinical diagnosis of reproductive-related dis-

orders (Strzeżek et al. 2005, du Plessis 2011, Rodríguez-Martínez et al. 2011). Kordan et al. (2008) applied the 2-D gel electrophoresis for analysis of polymorphism in boar seminal plasma protein. The authors demonstrated that quantitative and qualitative changes in the composition of the polypeptide maps of boar seminal plasma proteins were dependent on the animal age and the season. Furthermore, it was found that there were marked differences in the electrophoretic profiles between normospermic and oligoasthenoteratozoospermic ejaculates. Such findings suggest that electrophoretic profiling of seminal plasma proteins can be used as a marker for the dysfunction of the reproductive organs and for the identification of high-quality fertile semen for reproductive technology. In another study four conserved polypeptides, with identical molecular weights, were detected in the composition of the polypeptide maps of boar seminal plasma, using the 2-D gel electrophoresis and LC-MS/MS (Kordan et al. 2009b). Mass spectrum analysis of the four conserved polypeptides showed similarity to the family of spermadhesins, particularly epididymal spermadhesin AWN-1, which are implicated in several sperm functions, such as capacitation and the acrosome reaction (Strzeżek et al. 2005, Kordan et al. 2009b).

Killian et al. (1993) identified two seminal plasma proteins (55 kDa, pI 4.5; 26 kDa, pI 6.2) with high fertility in bulls and two proteins (16 kDa, pI 4.1; 16 kDa, pI 6.7) which were associated with low fertility. The 26-kDa fertility-associated protein (FAP) was 75% homologous to prostaglandin D synthase, which plays an important role, both in the development and maturation of spermatozoa (Gerena et al. 1998). Furthermore, three low molecular weight acidic proteins, BSP I, BSP II and BSP III, with an amino acid sequence identical to BSP-A3, BSP-A2 and BSP-A1 proteins, respectively, were identified in bovine seminal plasma (Esch et al. 1983, Manjunath and Sairam 1987). Moreover, the BSP II/BSP III complex, together called PDC-109 (Esch et al. 1983), showed homology to the major protein (MP) synthesized in the bovine vesicular glands (Kemme et al. 1986). It should be noted that the BSP-A1, BSP-A2 and BSP-A3 proteins bind heparin, while the PDC-109 complex binds to the sperm midpiece and prevents Ca^{2+} uptake, thus inhibiting the sperm acrosome reaction (Vijayaraghavan et al. 1989). In the stallion, only one protein, SP-1 (72 kDa, pI 5.6), was positively correlated with fertility, whereas three proteins, SP-2, SP-3 and SP-4 (75 kDa, pI 6.0; 18 kDa, pI 4.3; 16 kDa, pI 6.5 respectively), were highly abundant in the seminal plasma of low fertility males (Brandon et al. 1999).

It can be concluded that recent advancement in the development of subjective and objective assessment methods of semen quality are promising approaches that will help to improve the prediction of fertility of semen following preservation. These methods will contribute significantly to an improvement in animal reproductive techniques, and will aid in the selection of high-quality fertile semen. Furthermore, a greater understanding of the sperm and seminal plasma proteome technology would lead to the identification of proteins which can be used as potential markers for semen quality and fertility.

Acknowledgements

This study was supported by funds from the University of Warmia and Mazury in Olsztyn (No. 0103.0803).

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