Application of semen evaluation techniques

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SUMMARY

Laboratory methods of semen evaluation are used to select males for artificial insemination. The current review describes several techniques that have been recently used for sperm analysis. Conventional microscopic methods in combination with the objective computer-assisted sperm motility and morphology analyzers and flow cytometry, allows to obtain more precise information about the membrane and functional status of spermatozoa. By using several methods to detect motility, viability, acrosomal and capacitation status besides DNA integrity sperm biology and some of the mechanism involved in sperm cry injury can be better understood. The number of possible targets related to sperm quality is increasing, and possible that some of them could enable sperm analysis for predicting freezability and fertility to be improved.

Keywords: *apoptosis, fertility, flow cytometry, plasma membrane integrity, spermatozoa*

ÖSSZEFOGLALÁS

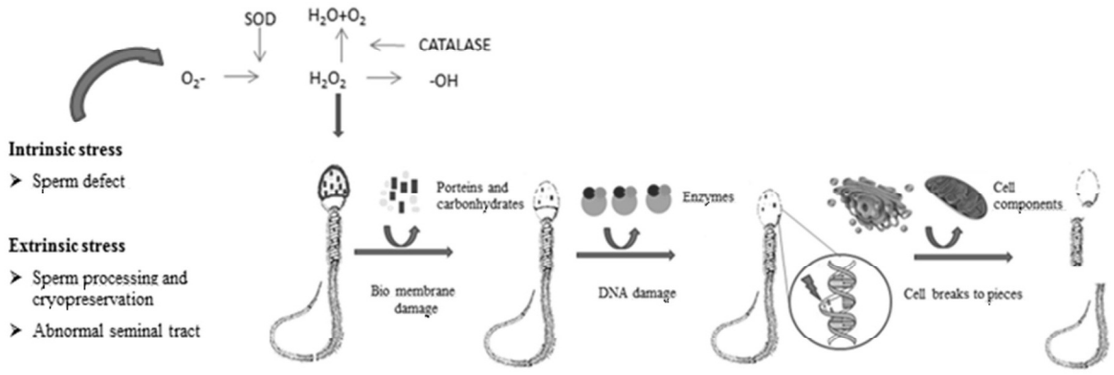
A spermavizsgálati technikák használatával megállapítható, hogy alkalmas-e a hímivarú állat ondója a mesterséges termékenyítésre. Cikkünkben, a gyakorlatban használt spermavizsgálati módszerek kerülnek bemutatásra. Jelenleg a mikroszkópos spermavizsgálat mellett a számítógépes spermaanalízis és a flow citometria használata is a gyakorlat része. Az előbbi módszerek segítségével a sperma motilitását, membránintegritását, akroszóma állapotát, kapacitációs státuszát, DNS integritását lehet meghatározni. Az új technikák alkalmazásával jobban megismerhetőek a spermasejtek életjelenségei, különös tekintettel a spermakonzerválás során fellépő hidegsokk hatására bekövetkezett változásokra. A spermavizsgálati technikák fejlődése továbbá lehetőséget biztosít újabb spermaminőséget befolyásoló tulajdonságok felismerésére, melyek vizsgálatával előre jelezhető az ondó mélyhűtésre való alkalmassága, fertilitása.

Kulcsszavak: *apoptózis, fertilitás, flow citometria, plazma membrán integritás, spermatozoa*

INTRODUCTION

Assisted reproductive technology (ART) methods such as artificial insemination (AI), embryo transfer (ET), in vitro fertilization (IVF) and semen cryopreservation are important tools for genetic improvement, farm animal genetic resource management and conservation. Through AI one ejaculate from a genetically superior male can be used to fertilize several females to maximize the distribution of the favorable genes (Bailey et al., 2000). Another important aspect that should be addressed to the technique is that it improves biosecurity and limits the risk of sexually transmissible diseases. ET also offers the same genetic advantages on the female side and furthermore by using the technique genetic interval can be shortened considering the slow maturity of ruminant species (Foote, 2002). Sperm cryopreservation offers a benefit for restoration of endangered species furthermore successfully frozen and thawed semen can be subsequently used to produce more offspring in farm animals (Evans et al., 2004). Successful freezing of semen from all livestock is a high priority due to the advantages comprising biosecurity and global commercialization of superior genotypes. Nowadays the use of cooled liquid semen for AI dominates among the farm animal species while the use of frozen semen is mostly used in dairy cattle (Rodríguez-Martínez and Peña Vega, 2013). Over the decades of sperm cryopreservation work many specific protocols have been established as standards, nevertheless post-thawed viability and fertility levels of cryopreserved semen in other species still remains low (Madeiros et al., 2002). During the semen cryopreservation process cold shock susceptibility, cooling rate, diluent composition, osmotic stress are some of the main factors affecting the proportion of live sperm cell and the functionality (e.g. membrane stability, membrane receptor ability, nuclear structure) of the cells (Watson, 2000). Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen (e.g. oxygen ions and peroxides). During the time of environmental stress and semen preservation process the level of ROS can increase dramatically and it may result in the damage of the sperm membrane structure and functionality (Salamon and Maxwell, 2000). The above mentioned process is known as oxidative stress. ROS production in the ejaculate consumes antioxidant equivalents from seminal plasma hence lowering the level of protection that can be given to the viable cells in the ejaculate. Most likely infiltrating leucocytes are responsible for lowering the antioxidant capacity in seminal plasma (Aitken and Baker, 1995), nevertheless Cohen-Bacrie et al. (2009) haven't found any correlation between the presence of leukocytes and DNA damage. To prevent sperm cells from the effect of oxidative stress, antioxidants (e.g. ascorbic acid, tocopherol, glutathione peroxidase, resveratrol, curcumin) can be added to the diluent to reduce ROS damage (Salamon and Maxwell, 2000; Sarlós et al., 2002; Buçak et al., 2012). *Figure 1* presents the major factors could indicate oxidative stress.

Figure 1: **The impact of ROS on spermatozoa**



Sperm motility assessment

Sperm motility gives the percentage of all moving sperm cells in the ejaculate and the percentage of sperm with productive flagellar motion such as rapid, linear and forward progression. Sperm cells that are not able to have a proper movement will not reach the egg in order to fertilize it, therefore motility is considered as an important semen quality parameter related to pregnancy rate. Regarding motility sperm cells can be classified into three groups as non-motile, progressively motile or non-progressively motile. A progressively motile sperm cell displays motility along a linear track with rapid, slow, or sluggish forward progression. The new guidelines for human semen processing have classified motility under three categories only, like progressive motility (PR) and non-progressive motility (NP) and immotility (IM), in a contrast in the different animal species the parameter of total motility (TM) is also described (WHO Laboratory Manual, 2010). Exposure to heat or cold the change of pH, osmolality in the diluent, urine in the semen sample or sexual inactivity can have a detrimental effect on sperm motility (O'Hara et al., 2010). There are several methods to asses, semen motility depending on the desire for precision, repeatability and availability of the necessary equipment.

Manual motility assessment

The motility of a sperm cell can be evaluated manually by using a Makler chamber or Neubauer improved chamber or automatically using CASA system (Mortimer and Mortimer, 2013a). Palacín et al. (2013) concluded that the type of the evaluation chamber (slide-coverslip, Makler and ISAS chambers) had a significant effect on the motility results, but precision and accuracy, whereas the drop volume (5 and 10 µl) did not had an effect on the motility results. Taking into account that these manual methods are not so easily calibrated as a spectrophotometer or CASA system the repeatability of the method is not enough good. However, in laboratories with standardized, systematic training and functional quality control, this procedure has been performed routinely for many years (Mortimer, 1994). To describe sperm progressive motility (5) and the rate of progressive movement a 0–5 scale can be used during manual motility evaluation, but motility must be assessed immediately in order to get an accurate measurement. In the scale 0 represents „no movement", 5 „extremely fast forward movement" (Pécsi, 2007). The main disadvantage of the method is that standard semen analysis is a rather subjective technique in the way of term that it is associated with large inter-laboratory variation, even though Yeung et al. (1997) reported that if there is agreement among the laboratories in the assessment of sperm motility evaluation, than CASA system is only necessary to provide the expected percentages of grades. By using both of the techniques there will be also differences between the sperm velocity assessed with the two different methods.

Computer-aided motility analysis

Nowadays AI centers and laboratories mostly use CASA systems for motility assessment in order to avoid subjectivity and to minimize differences in assessing videotaped samples. For the evaluation 40x phase contrast objective is used and at least 200 spermatozoa should be classified in duplicate – at least 400 sperm cells in total – and at least five fields should be assessed in each count. The basic principle behind the microscopy-based CASA system is that a series of successive images of motile spermatozoa within a static field of view is acquired. Most systems use standard video image acquisition rates (frames s^{-1}; Hz), typically 25, 30, 50 or 60 Hz (Holt et al., 2007). CASA systems have different types such as SQA-V, ISAS, IVOS or CASMA-M for the different needs. Future validation of methods for the accurate identification of sperm cells might allow the extension of CASA to the automated analysis of semen in all species. To reach this estimation additional imaging capabilities such as fluorescence will be required to distinguish sperm from high level of debris or other cell types with that has the same size as sperm cells (Mortimer and Mortimer, 2013b). In human semen motility evaluation guidelines classify two categories only as progressive motility (PR) and non-progressive motility (NP), but in the latest additions one more movement is described that includes both slow progressive and sluggish movement. By subjective motility assessment it is hard to distinguish categories, however by the use of ISAS system the spermatozoa

can be divided into four motility classes (1: straight-line progressive, 2: straight slow progressive, 3: non-straight progressive motility, 4: non progressive) by using straight-linear velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) values (Elia et al., 2010). In conclusion, the distinction between sperm motility classes (rapid and slow progressive motility, sluggish and non- progressive motility) is one of the essential parameters in the evaluation of fertilizing ability; to ignore a part of this crucial information. By using Computer-assisted sperm morphometry analysis system (CASMA) not only sperm viability but morphology can be assessed as well. It provides a rapid and accurate analysis of objective morphology parameters e.g. length, width, area, perimeter, ellipticity, form factor, elongation and regularity that have facilitated the standardization of sperm morphometric evaluation. De Paz et al. (2011) compared the above mentioned parameters with male fertility. For the morphometric analysis CASMA is furthermore an image analysis software (NIS –Elements) in combination with an optical microscope (MO-NIS) and SEM-NIS is an image analysis software with a scanning electron microscope was used. Based on the results, only the subpopulations obtained with the MO-NIS procedure showed a significant correlation with male fertility. Vicente-Fiel et al. (2013) developed the automatic method of sperm nuclear morphometry evaluation (CASMA-F) which is combining fluorescent staining (Hoechst 33342) method and image analysis in order to study the sperm nuclear morphometry of different species like cattle, sheep, goat and boar. They concluded that in small ruminant species 3 sperm nucleus size classes can be distinguished, such as: large, average and small size and besides this the relationship between the four species for the sperm nuclear dimensions were the following: bull>ram>boar>goat.

Assessment of sperm viability

Sperm viability is a key measure of semen quality therefore the assessment of sperm viability requires the development of precise methods and evaluation. Sperm viability is a reflection of the proportion of live spermatozoa determined by the evaluation of cellular and/or membrane integrity. The integrity and functional activity of a sperm membrane has an essential importance on the fertilizing potential of the spermatozoa. Most of the viability assays are based on the permeability of the sperm cell membrane to different type of fluorescent or non-fluorescent dyes. All the methods are based on the principle that sperm cells with membrane damage are able to take up the fluorescent dye while membrane intact cells are impermeable for the colorants. Different techniques can be used such as vital stains and fluorescent live-dead assays (Ramalho Santos et al. 2007). Vital stains like eosin, eosin-nigrosin, trypan blue are sufficient for microscopy, nevertheless the repeatability of the methods is questionable (Björndahl et al., 2004). Sperm viability can also be assessed by the sperm cells osmoregulation capacity under hypo-osmotic conditions (150mOsm/L). The hypo-osmotic swelling test (HOS) predicts membrane integrity by determing the ability of the sperm tail membrane to maintain equilibrium between the sperm cells and its environment. Influx of the fluid due to hypo-osmotic stress causes the sperm tail to coil and balloon or „swell" (Ramu and Jeyendran, 2013). Gambardella et al. (2012) compared two sperm viability tests in 100 semen samples with the usage of eosin-nigrosin and hypo-osmotic swelling test (HOS) to validate the functionality of the methods. Regarding the results 15% concordances in the result the other 85% has shown inconsistent results. By using HOS the vitality results were lower than in the reference values given by WHO, defines more than 60% of live cells in fresh human semen. Serafini et al. (2013) conducted a study to test the reliability of trypan blue/Giemsa staining in order to evaluate sperm viability, acrosome status and morphology in stallion semen. The relationship of live sperm with intact acrosome (LSIA) acrosome reacted sperm (ARS) and the number proximal droplets (PD) was compared with fertility results of inseminated mares. Mares inseminated with a higher percentage of LSIA samples had higher percentages of pregnancy rate ($p<0.05$), therefore the authors concluded that trypan blue/Giemsa staining is a useful tool can be used in field, to evaluate sperm membrane integrity and to distinguish the poor and good quality ejaculates in stallion semen. Nagy et al. (1999) also used trypan blue/Giemsa staining to justify the hypothesis that sperm cells with an intact head membrane, but stained and presumably membrane damaged tail are not motile, therefore these cells should be included in the dead category rather than alive in the usual live-dead studies with vital stains. Desalegn (2012) accomplished a study to compare three different staining methods with 0.1% Congo red, 0.16% Chicago sky blue and 0.2% trypan blue with different concentrations (0 control, 0.1% and 0.3%) of formalin fixative in the PBS used for diluting frozen-thawed bull semen samples just before staining. The highest viability results were achieved in the samples stained with 0.16% Chicago sky blue with 0.3% formalin in a contrast with 0.1% Congo red staining with 0.3% formalin, had the lowest viability. The above mentioned techniques are considered easy and rapid, because they are one-step staining methods, only requiring immersion oil and simple light microscopy for observation. The fluorochromes like propidium-iodide, Hoechst 33342, ethidium homodimer-1, SYBR-14, Yo-Pro-1 can be used for fluorescent microscopic and flow cytomertic evaluation as well to determine sperm cell viability (Moskovstev and Librach, 2013). The membrane impermeable stains such as propidium-iodide (PI) and ethidium-homodimer (EH) are the most widely used fluorescent stains for viability, because they are easy to use, rapid and can be excited with the 488 nm laser that, most cytometers include. These dyes enter into cells with broken membrane emitting a red fluorescence when binding to nucleic acids (PI: 636 nm; EH: 617 nm) (Gillian et al., 2005). Yániz et al. (2013) carried out a study to compare the effectiveness and usability of four permeant fluorochromes (CFDA, SYBR-14, Hoechst-33342 and acridine orange) combined with propidium-iodide to assess sperm membrane integrity. They concluded that the most efficient and rapid combination to assess ram sperm membrane integrity

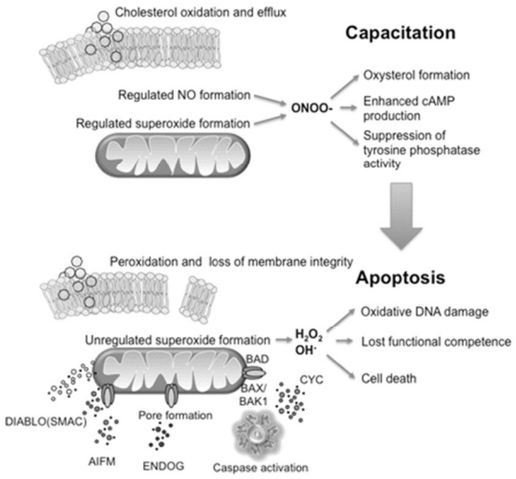
were acridine orange/propidium-iodide and SYBR-14/ propidium-iodide, because by using these combinations there is no need of incubating samples, while in Hoechst groups and CFDA a minimum of 4 and 6 minutes incubation is required to have a sufficient fluorescent intensity. Martínez-Pastor et al. (2010) summarized the recognition of different fluorescent staining methods and came to the conclusion that only using one single test to evaluate the fertilizing potential of sperm cells is incomprehensible, however using several techniques in combination such as motility, acrosomal integrity and fluorescent assessment of membrane integrity can give a significant result about the sperm cells fecundate potential.

Evaluation of acrosomal and capacitation status

During spermatogenesis spermatozoon undergoes a continuous modification, maturation in epididymis and capacitation in the female reproductive tract, but only the capacitated spermatozoa are able of binding the zona-intact egg and undergoing acrosome reaction (Tulsiani et al., 1998). Normally acrosome reaction takes place in the ampulla of the Fallopian tube. Oxidative stress (OS) triggered by the effect of refrigeration/ freezing cause imbalance between the level of reactive oxygen species (ROS) and antioxidants which plays an important role in sperm physiological processes such as capacitation, acrosome reaction, and signaling processes to ensure fertilization (Bansal and Bilsapuri, 2011). Acrosomal status can be investigated in live cell or with fixed sperm staining as well, however if live cell staining is carried out, microscopy or flow cytometry is immediately required for the detection. There are various staining methods which can stain the acrosomal region without also staining the post-acrosomal region. Giemsa and periodic acid-Schiff reagent, triple stain (Bismark brown, rose Bengal and the supravital stain, trypan blue) are suitable for many species (Talbot and Chacon, 1981). Staining sperm with Giemsa stain – by using dual (Kovacs and Foote, 1992) or triple staining technique – four different patterns of sperm can be observed such as (LAR: unstained acrosomal region and unstained post acrosomal region), live spermatozoa with intact acrosome (LAI: pink acrosome and unstained postacrosomal region), dead spermatozoa with acrosome reacted (DAR: unstained acrosomal region and dark postacrosomal region) and dead spermatozoa with acrosome intact (DAI: stained acrosome and dark postacrosomal region. Live (pink) and dead (dark) tail subdomains are also differentiated. The above described methods can be evaluated by bright field microscope, but the Giemsa-stain may reveal the presence or absence of acrosomal contents and therefore indicate only completed acrosome reactions. Nagy et al. (2003) developed a triple fluorescent staining method to simultaneously evaluate viability and acrosome integrity by flow cytometry. By using the method discrimination can be made of living from plasma membrane-damaged sperm cells (using SYBR-14 in combination with PI), acrosome-intact from acrosome-reacting cells (by using PE-PNA), and sperm-specific particles from non-sperm particles (are labeled neither by SYBR-14 nor by PI).

Capacitation is a functional maturation of the spermatozoon in order to be able to fertilize the egg. In vivo it occurs in the uterine tube, in vitro capacitation can occur after semen dilution and during the refrigeration/ freezing process. Capacitation includes multiple biochemical changes, e.g. an efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity and permeability to bicarbonate and calcium ions, changes in protein phosphorylation and protein kinase activity and an increase in Ca^{++} and cyclic adenosine monophosphate (cAMP) levels, described in *Figure 2*. Capacitation has two different signaling events: fast and slow. Slow appears in the female reproductive tract while fast encloses the activation of the vigorous and asymmetric movement of the flagella and these reactions begin to start as soon as the sperm cells leave the epididymis (Ickowicz et al., 2012). Capacitation can be detected by using direct or indirect method. In the indirect method sperm cells which will capacitate will also show acrosome reaction and it can be justified by Giemsa-staining. Muratori et al. (2004) used double staining (Annexin V and mercocyanine 540 and YOPRO-1) and a direct comparison between the staining methods to evaluate capacitation status in human spermatozoa, because the signaling pathways that characterize the process of capacitation of human spermatozoa are still largely unknown. Swim-up and non-selected samples were incubated with capacitation medium for 3 hours. They concluded that the percentage of Ann V^+/PI^- as well as Ann V^+/PI^+ sperm was much greater in unselected than in corresponding swim-up selected sperm. In the unselected samples there was a strong negative correlation between Ann V binding in live sperm, and both sperm morphology and motility. When double staining method was used they have found that fraction of swim-up selected live sperm (Ann V^+/PI^-) binds M540 and it is an apparent contrast with experiments performed in swim-up selected sperm using YOPRO-1 as a tool to discriminate viable sperm. Based on these results the authors concluded that neither probe is able to detect capacitation related membrane modifications. Rathi et al. (2001) compared CTC/Etdh-1 and mercocyanine 540/YOPRO-1 and FITC-PNA assay in stallion spermatozoa. CTC samples were evaluated by fluorescent microscope while mercocyanine 540 and FITC-PNA stained samples were evaluated by using flow cytometry. It was concluded that mercocyanine 540 and FITC-PNA assays were preferable to CTC because the evaluation by flow cytometry is easier to perform, quicker, more objective and the washing and fixation steps that may interfere with apparent cell viability and integrity are not required. Another disadvantage of CTC staining is that the biomarkers and their relationship to the occurence of capacitation and acrosome reaction are remaining unsolved.

Figure 2: **The capacitation-apoptosis highway**



Source: Aitken (2011)

Assessment of DNA damage

Sperm cell DNA damage can be induced by different factors like: abortive apoptosis initiated post meiotically when the ability to drive this process to completion is in decline or oxidative stress which is one of the major contributors to DNA damage in the male germ line. Spermatozoa with DNA damage are able to efficiently fertilize an egg, nevertheless after ferilization it is most likely that poorer fertilization, embryo quality and pregnancy rate can be achieved. This type decreased fertility appears when the paternal genome introduces DNA damage that has not been repaired by the oocyte after fertilization or by the embryo at the maturation period (Fatehi et al., 2006). In the past ten years evaluation of sperm nuclear chromatin integrity has been included in human and other mammalian semen quality assessment techniques. To assess DNA damage in sperm cell several methods can be used like deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, comet assay, sperm chromatin structure assay (SCSA) to detect spermatozoa with fragmented or damaged DNA. The disadvantage of these methods is that – except TUNEL – flow cytometer is required for analysis otherwise low sperm count samples and small testicular biopsies cannot be measured by flow cytometer either (Dugum et al., 2011). Waterhouse et al. (2006) proved that despite the high number of spermatozoa per AI dose from high-quality bulls, both CASA and TUNEL assay were valuable measures for evaluating sperm quality in relation to fertility after AI. Sperm chromatin dispersion (SCD) is a recently developed (Fernandez et al., 2003) method used specifically in sperm cells. The principle of method is built on the precept that sperm with fragment DNA fail to produce characteristic halo of dispersed DNA loops that is observed in sperm with non-fragmented DNA, following acid denaturation and removal of nuclear proteins. Mitchell et al. (2011) highlighted in their study that the conventional TUNEL method was shown to be insensitive and unresponsive to the DNA fragmentation induced in human and mouse spermatozoa, therefore to have more reliable results the chromatin was exposed to 2mM dithiothreitol (DTT) for 45 min prior to fixation. Based on the results the modified version of the assay significantly enhanced the TUNEL signals. Also, DBD-FISH allows in situ detection and quantification of DNA breaks and reveals structural features in sperm chromatin (Fernandez et al., 2000). Ribas-Maynou et al. (2013) compared Tunel assay, SCSA (Sperm Chromatin Structure Assay), SCD (Sperm Chromatin Dispersion) test and alkaline and neutral COMET assay to analyze differences between the methodologies and to establish their cut-off values, sensitivity and specificity in predicting male fertility. The percentage of DNA fragmentation in the sperm cells differed between fertile and infertile men when TUNEL, SCSA, SCD and alkaline COMET assay was used, but with neutral COMET assay no differences were observed between the results. The threshold values for infertility were 20.05% for TUNEL assay, 18.90% for SCSA, 22.75% for SCD test, 45.37% for alkaline COMET and 34.37% for neutral COMET. As a main conclusion they stated that except neutral COMET assay all the above mentioned methods are able to distinguish fertile and infertile man by the intensity of DNA fragmentation. Fraser et al. (2010) conducted a study to compare neutral Comet assay (NCA) and the Sperm-Sus-Halomax (SSH) test to measure post-thaw DNA fragmentation of boar spermatozoa. Both NCA and SSH detected variations among individual boars in terms of post-thaw sperm DNA fragmentation, but by scatter-plot differences they also proved that there can be an agreement between the measurements was detected by NCA and SSH assays.

CONCLUSION

Nowadays it is a requirement that the semen of males serve at AI station should be free from pathogens, infectious diseases and genetic disorders (e.g. chromosome and gene disorders. Beside these the potential fertility of a sperm sample depends on the number of motile, viable and membrane and DNA intact cells that can reach the oviductus to fertilize the oocytes. Nowadays the structural and functional sperm parameters can be acquired in a short time by using computerized systems for sperm motility and fluorescent technologies to access membrane integrity. With the help of new probes like SCSA, Tunel-test evaluated with flow cytometry also offers the opportunity to measure several sperm attributes on thousands of spermatozoa per sperm sample, therefore it is a really useful tool to improve semen quality control extremely and to shed light on the underlying reasons behind male infertility.

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