Chapter 6

Molecular Markers in Sperm Analysis

Rita Payan-Carreira, Paulo Borges,
Fernando Mir and Alain Fontbonne

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52231

1. Introduction

In mammals, the success of fertilization largely depends on gamete fertility potential and consequently on what concerns sperm and oocyte quality they are both equally important.

Sperm contribution to fertilization is usually estimated through evaluation of semen parameters. A loss of fertility potential associated to manipulation and preservation techniques is usually calculated based on the semen characteristics at collection and on the knowledge of the damages associated with the technique to be implemented.

Assessment of sperm quality conventionally relies on microscopic evaluation of sperm parameters including total sperm count, sperm concentration, percentage of motile sperm and percentage of normal sperm morphology. Some of these parameters are correlated with fertility though it does not truthfully predict male fertility [1-3]. Concentration and morphology are considered to be important to evaluate the fertilizing ability of sperm cells, as well as motility and the acrosome status, which are critical elements regarding fertilization. These parameters are currently analysed under light microscopy. Computer-assisted semen analysis (CASA) increases the reliability and the accuracy of the analysis with the increase of cell counting [4,5]. Results of the functional testing (such as the *zona pellucida* binding assay, the hemi-zona essay or the hypoosmotic swelling test) are better correlated with the AI outcome than the results of conventional semen evaluation [1,2].

Nevertheless, these methods have limited prognostic value for the reproductive success of the donor male [6,7]. Discrete and unclear sperm abnormalities impairing the reproductive success of sperm and egg interaction often remain undiagnosed. This is the major limitation for the most conservational *in vitro* methodologies of sperm evaluation, either in humans or animals. Inability of the *in vitro* assessment methods to accurately predict spermatozoa fertility may be attributed to the complexity and multifactorial nature of male fertility.

In the past decades, attempts to escape these limits led to the introduction, in the laboratorial panel, of some sophisticated analyses. Those included the use of fluorescent markers to assess the acrosomal status, the use of vital staining for mitochondrial activity, the use of particular fluorochromes to detect altered sperm chromatin or DNA integrity along with several molecular regulators of thermal and oxidative stress. Proteomic, biochemical, and immunocytochemical approaches are now starting to highlight some key events that may determine the success of the sperm function. Existing functional tests were also retained, such as the hypoosmotic swelling test and the hemi-zone assay, to assess membrane functional integrity and sperm ability to interplay with the oocyte.

Understanding the main determinants of sperm fertility and knowing how fertility changes or is influenced by sperm manipulation (such as cryopreservation and sperm-sorting) would allow to enhance the knowledge on extender design, to accurately estimate sperm fertility and to predict sperm survival after processing. The knowledge to adequately extend the lifespan of cryopreserved sperm would also be improved, in particular on what concerns the programs for genetic biodiversity preservation. Nowadays, the lack of reliable methods allowing the accurate *in vitro* assessment of semen quality, limits our capacity to properly monitor semen freezing-thawing damages and to predict its performance at insemination [8].

Though extensively used in domestic species (such as bovine, pigs and dogs), it is well known and accepted that cryopreservation damages the sperm, with a large number of cells losing their fertility potential after freezing/thawing. Further, it is also common knowledge that individual variations exist on sperm resistance to cell damage during these procedures, justifying why some males are "better freezers" than others, even if no differences are found in fresh semen quality assessment [9, 10].

Determination of additional markers for semen quality is now being explored either as a complementary assessment of sperm quality or as an additional way to study in more detail the side effects of extenders or molecules associated to infertility. Seminal markers reveal molecular pathways that could be suppressed or stimulated by *in vitro* sperm manipulation. Moreover, it may be of utmost importance when considering the development of protocols for sperm cryopreservation of wild and endangered species. Up to now, the extender selection in those species is mainly based on phylogenetic or physiological resemblances and on the trial-and-error approach.

Another issue strengthening the need for additional tests in laboratory assessment of sperm quality relates to the fact that standard seminal parameters (motility, concentration and morphology) currently used for all the species are insufficient to predict fertility and to detect sub-fertile males. In addition, sperm samples are very heterogeneous and although spermatozoa may look the same on traditional semen analysis, more sophisticated methods allow identifying different spermatozoa subpopulations with distinct biochemical and physiological characteristics. It is the combination of sperm cells of different functional competences that largely determines the fertility potential of a specific male.

http://dx.doi.org/10.5772/52231

The search for effective predictors of spermatozoa fertility is now on the table, and the identification of suitable molecules would greatly benefit the semen industry and would strengthen the proposal of new therapies for infertility, in both man and animal. Furthermore, it would allow a better understanding of the side effects of technology (such as freezing/thawing or sex-sorting procedures) upon the sperm integrity and functionality, as well as to evaluate the reasons of some undesirable responses of exotic or endangered species' sperm to preservation.

A large number of factors and molecules have been proposed to be of interest or tested as putative predictors for sperm fertility. Before playing their role in fertilization, spermatozoa are required to survive in the female genitalia, accomplish to reach the place for fertilization and to acquire competence to fertilize the oocyte (Figure 1). This is true, for both the natural mating and the artificial insemination. These are important actions, which reflect a multitude of complex and specialised functions that, in brief, result in sperm survival and fertility. Yet, all these functions would hardly be evaluated together through a sole molecule.

In this review it is the intent to present and discuss the use of new methods for sperm assessment and estimation of spermatozoa fertility.

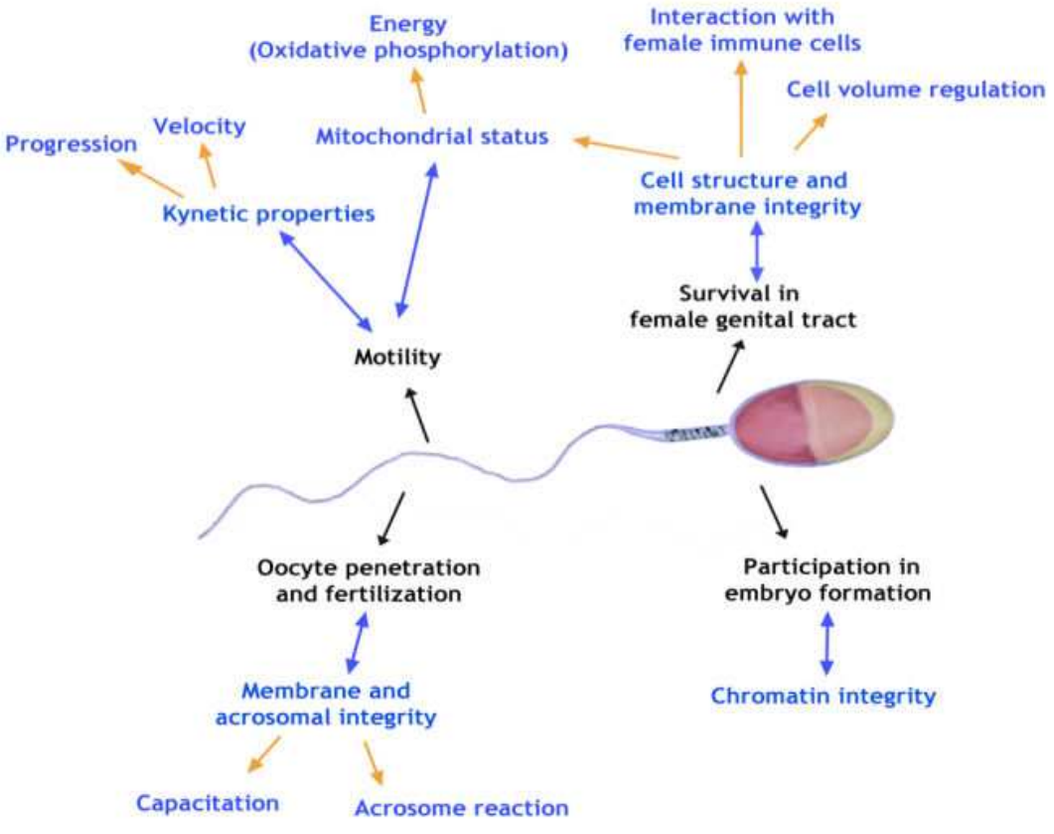

Figure 1. Major cellular mechanisms associated with main roles of the spermatozoon.

2. Proposed side effects for sperm cryopreservation

Sperm cryopreservation is unavoidably linked to a reduction in sperm quality, which has been related to cold shock and freezing damages. The importance of cold shock injuries varies with the species, the composition of the extender, the cryoprotectant selected and the male, among other factors [10,11]. Seldom more than 50% of the sperm population survives cryopreservation [9].

Deleterious effects of freezing/thawing procedures originate a reduction on the sperm life span due to alterations in the structure and functions of spermatozoa. Side effects include altered motility, changes in the plasma membrane and acrosomal integrity and increased DNA fragmentation. All these alterations induce a reduction of the sperm ability to survive in the female reproductive tract and to interact with the oocyte at fertilization [8,12]. In an attempt to compensate these side effects, seminal doses are usually prepared with excessive numbers of spermatozoa in order to improve AI fertility [5,8].

Available cryopreservation techniques have a number of potentially detrimental problems, such as physical and chemical injuries that prone the spermatozoa to cell death and dysfunction (Figure 2). These include [9,13-15]:

- Capacitation-like changes – after freezing/thawing, sperm behaves as if capacitated, which decreases its ability to survive within the female genital tract and to fuse with the oocyte;
- Motility impairment – a decrease in the motility is observed in post-thawed spermatozoa, which tend to exhibit a variable degree of motility weakening, with subsequent hampering of sperm progression till the oviducts and a decrease on the fertility potential;
- Oxidative damages – which may trigger apoptosis and DNA damage when reaching a given threshold. Apoptosis compromises the mitochondrial function, motility and predispose to DNA fragmentation.
- Compromise of the membrane and acrosome integrity - loss of membrane integrity lead to altered ionic transport to the cell, in particular the calcium and water balance, with subsequent loss of the sperm ability for volume regulation and osmoadaptation. Also, it will compromise protein location and/or exposition on the cell's surface, which negatively affects sperm survival, sperm binding to oviductal epithelium and interaction between male and female gametes. In addition, restrain of the acrosome integrity may compromise sperm competence to penetrate the oocyte layers at fertilization;
- DNA and chromatin changes, which may not be directly related to fertilization but are often reported to impair sustainable post-syngamy embryonic development and pregnancy.

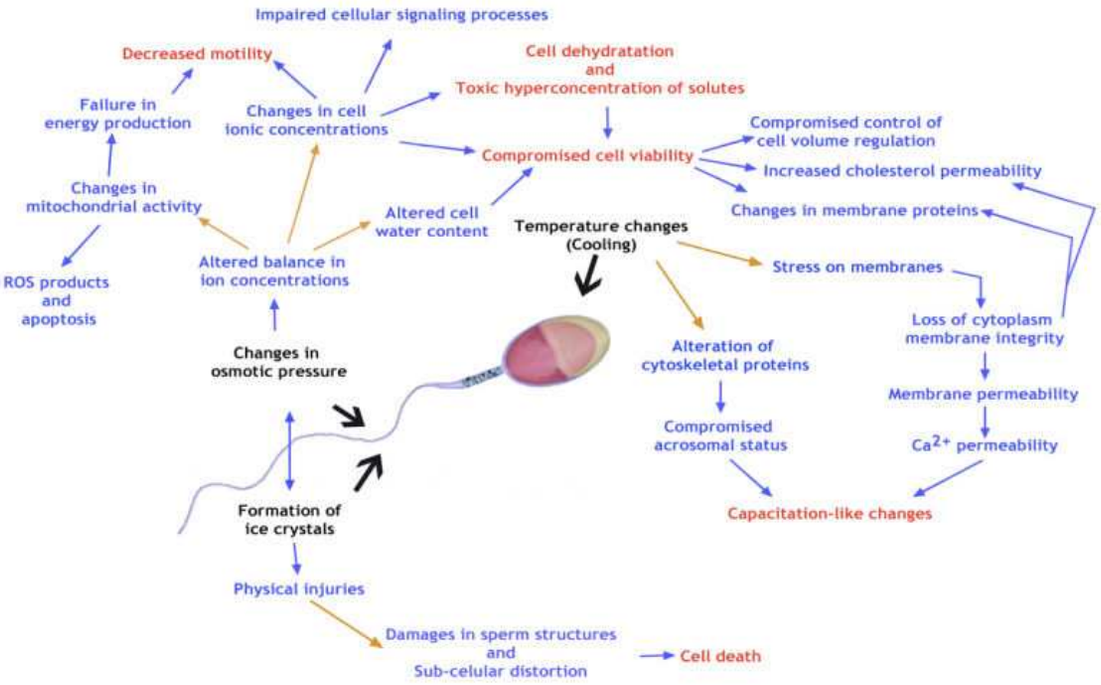

Figure 2. Proposed deleterious effects in sperm cryopreservation [Ca^{2+} - calcium].

3. Biological markers of sperm function

The most frequently used methods of sperm analysis have been pleasantly reviewed in a recent InTech publication [11], driving the main topic of this review into new adjunctive methods available to test sperm quality (Figure 3). These tests can be performed as well in freshly ejaculated sperm or in preserved samples. In the former, it would allow to increase the ability to predict sperm quality, the selection of donor/sperm for cryopreservation and to assess infertility causes. In the later it could be of utmost interest to study the sperm response to preservation trials, such as the design of a new extender. Further, it could also be of importance when studying the sperm response to preservation in new species, where it would allow the identification of the most suitable molecular and functionally-friendly extender or procedure.

3.1. Assessment of events associated with sperm capacitation

For long, it has been accepted that freezing/thawing procedures induce a capacitation-like status that originate losses on the fertilizing potential of spermatozoa. Non-capacitated live sperm cells survive longer in the female genital tract than capacitated sperm [16]. Dysfunction of intracellular pathways associated with calcium (Ca^{2+}) predisposes to acrosome instability and exocytosis of its content. Regulation of protein function by Ca^{2+} signalling pathways is central for most sperm functions and infertility is often found when those signalling pathways are disturbed [17].

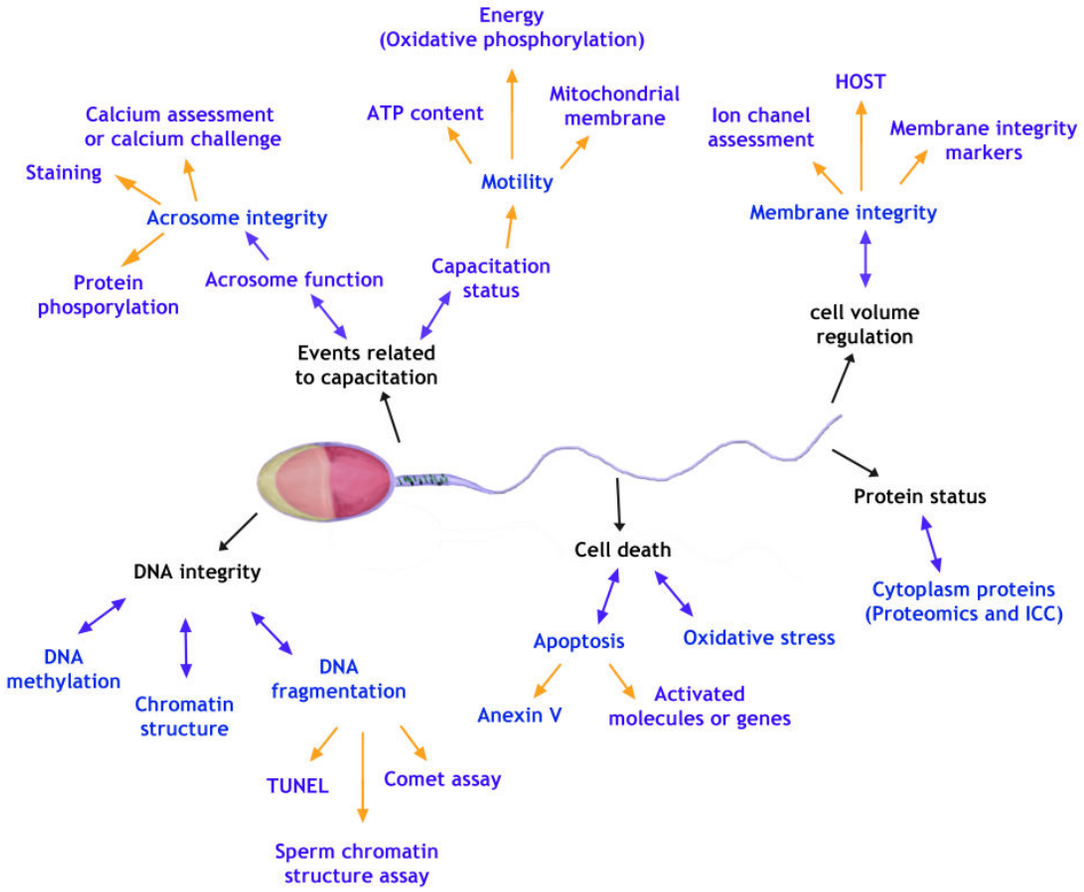

Figure 3. Main objectives for advanced sperm screening are directly related to the assessment of the spermatozoa functions [ICC - immunocytochemistry; HOST - hypoosmotic swelling test; TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling].

As it was mentioned, calcium is an important regulator of intracellular activity. Calcium mobilization has been associated with major sperm functions, such as capacitation, acrosome reaction and hypermotility. Ca^{2+} stores in the sperm are located in the acrosome, neck and mitochondria [17]. Release of Ca^{2+} from its stores triggers the above-mentioned reactions, although it is now suspected that different patterns of calcium release are responsible for different functions. For example, hypermotility is associated with an oscillatory, wave-like pattern of Ca^{2+} release, while capacitation, acrosome reaction and exocytosis of the content are associated with a burst of intracellular Ca^{2+} into the cytoplasm [6,17]. Also, the increase in free intracellular Ca^{2+} is often associated with the stimulation of different, pH-sensitive ion-channels that have been associated with hypermotility and acrosome reaction. Sperm neck Ca^{2+} stores seem to be related with the flagella movement, during hyperactivation [17].

Acrosome membrane integrity is commonly assessed with fluorescent conjugated lectins (PNA- Peanut agglutinin- and PSA- Pisum sativum agglutinin). Absence of fluorescence in the living sperm indicates an intact acrosome, whilst fluorescence is indicative of acrosome disrupted or acrosome-reacted sperm [5,11]. Fluorescent conjugated lectins can be used ei-

ther in flow cytometry [18] or in cell imaging microscopy, and when combined with other vital staining, such as Hoechst 33258 or 33342 and carboxy-SNARF/PI (carboxy-seminaphthorhodafluor/ propidium iodide), or with the hypoosmotic swelling test they also allow to distinguish between non-viable and reacted spermatozoa.

There are other fluorescent tests to evaluate the acrosome, like the chlortetracyclin (CTC) staining, in which fluorescence is activated when there is bounding to free calcium ions. When combined with another fluorescent dye, such as Hoechst 33258, the combined fluorescent staining allows to differentiate from three different sperm populations: the uncapacitated and acrosome intact (F-pattern), the capacitated and acrosome intact (B-pattern) and the capacitated and acrosome reacted (AR-pattern) [5]. Today, CTC staining is a routine test to assess the occurrence of the capacitation and the acrosome reaction; it has also been adapted to flow cytometry analysis.

Additional tests can be performed for assessment of the occurrence of capacitation-like events, using biological markers, molecules known to trigger or participate in the capacitation reaction. Determining the cholesterol efflux, the protein phosphorylation and changes in intracellular calcium are some of the available methodologies. Nevertheless, for some indicators, it is still unclear how they correlate with sperm quality.

Furthermore, the acrosome status may be tested indirectly through calcium assessment or by studying the response of sperm stimulation with calcium ionophores, progesterone or egg vestments [14,15,17,19]. Acrosome defective sperm show poorer responses to calcium testing than do the sperm with intact acrosome [6].

Changes in free Ca^{2+} concentrations in sperm may be studied by flow cytometry or indirectly by an ionophore challenge test, the later generating intracellular calcium signals that trigger the acrosome reaction [14,15,20]. The percentage of reacted spermatozoa is usually determined using a fluorescent dye. Samples with 10 to 30% of reacted spermatozoa have higher fertility potential than samples with less than 10% (this value being considered a threshold) [20].

Recently, it was demonstrated that sperm exposition to progesterone induced similar but more rapid Ca^{2+} signalling pathway, which seems to be independent of a known second messenger system [19]. This behaviour allows the use of this molecule to challenge the sperm acrosome function, as do the ionophore test. For a large number of species, granulosa cells expelled with the oocyte from the ovulatory follicle have the capacity to produce progesterone, which can affect the spermatozoa that approaches the egg for fertilization.

Protein phosphorylation can be studied using different approaches. Detection of phosphotyrosine residues in the spermatozoa can be performed by immunocytochemistry (ICC) in a cytology specimen (over silane- or poly-L-lysine-coated slides), using specific antibodies. The reaction is amplified by the use of secondary antibodies and the reaction may be visualized either with a fluorescent or a non-fluorescent dye. Further, this technique also allows the assessment of sub-cellular changes in the molecule localisation, besides the evaluation of changes in the intensity of immunolabelling [7]. ICC may also extend to other proteins targeting acrosome-related functions.

While ICC locates the molecule inside the cell, the Western blotting technique (also named immunoblotting) may be used for quantification of the protein. After a gel electrophoresis, the extracted proteins are transposed into a membrane and incubated with a primary antibody for the target molecule (the same as for ICC). The reaction is revealed in an X-ray film or a digital image [21]. The use of a cell or a molecular standard, like for the genomic assays, will allow the relative quantification of the protein content in the sample. However, this technique presents a weakness: the possible degradation of the target protein during sample preparation may cause the visualization of multiple bands of different molecular weight.

Mass spectrometry and liquid chromatography, enhancing the separation and the identification of a large number of proteins, are often used for proteomics analysis. However, until now, this method gives a catalogue of hundreds or thousands of proteins that are not easily associated with sperm biological functions [22,23] and consequently there is not a practical interest for the immediate sperm quality assessment. Yet, using specific regions of spermatozoa or particular cell organelles to focus the analysis could turn this approach to be helpful in the assessment of sperm function or specific sperm events.

3.2. Assessment of energy metabolism and sperm motility

Energy metabolism is a key-factor in sperm function. It is supported by ATP pathway, which is found in the background of the most important sperm events, such as hyperactivation, capacitation and protein phosphorylation of the acrosome reaction. It has been shown that high intracellular ATP values correlate with higher survival and vitality post-freezing/thawing [24], while mitochondrial membrane potential mirrors the sperm quality and a better motility pattern. A primary function associated with mitochondria is the ATP synthesis by oxidative phosphorylation, although, energy might also be obtained by glycogenolysis in the sperm tail, a necessary complement to sustain energy in the tail and to maintain an effective movement. In humans, a decrease in mitochondrial activity has been found in patients with history of infertility even when normozoospermic [25]. Also, cumulative evidences suggest that mitochondrial activity is positively correlated with sperm quality and fertility, possibly associated with the fact that healthy mitochondria have a higher membrane potential [25].

The intracellular ATP content may be determined by an enzymatic assay (ATP/NADH-linked enzyme coupling assay) in association with spectrophotometry. On this reaction, the regeneration of hydrolysed ATP is linked to NADH oxidation. The assay measures the differences in NADH, which are proportional to the rate of ATP hydrolysis.

Sperm metabolic function may also be evaluated by assessment of the mitochondrial activity. Integrity of the mitochondrial functioning can be assessed using specific dyes for these organelles [7,43]. Earlier, rhodamine 123 (R123) was frequently used to selectively stain functional mitochondria. It is a potentiometric membrane dye that fluoresces only when the proton gradient over the inner mitochondrial membrane (IMM) is built up. When the proton gradient collapses, the aerobic production of ATP fails, and mitochondria remain unstained [13]. More recently, other dyes have been developed, which selectively bind to respirating mitochondria and become fluorescent after oxidation. These can be used to test mitochon-

drial functionality, which has been correlated with the mitochondrial potential [25]. They are named MitoTracker® and are available in red, green and orange colours. Live sperm cells are suspended to incubate into a solution with the selected probe. Cells may be analysed by flow cytometry, by microplate-based analysis or by epifluorescence microscopy, using cytological preparations. The probes diffuse across the plasma membrane and accumulate in active mitochondria. To determine the percentage of MitoTracker positive sperm, 200 spermatozoa are usually counted per sample, in at least four fields, in a fluorescence microscope [7], varying the spectral wavelength with the probe used. The MitoTracker® can be combined with a vital dye, such as the Hoechst 33342, allowing the separation of different sperm sub-populations: the dead spermatozoa, the live mitochondrial non-competent sperm and the live mitochondrial competent spermatozoa. Also belonging to the MitoTracker dyes, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolylcarbocyanine iodide) is a dual-emission, potential-sensitive probe, which emits different fluorescent colours according to the membrane potential (IMM): after incubation JC-1 is captured by functional mitochondria where it stains in green if the IMM is polarized or in orange or red if the IMM is depolarized. Depolarization of IMM leads to the aggregation of the dye. The ratio of orange-to-green JC-1 fluorescence depends only on the membrane potential, since it is independent of the mitochondrial size, shape or density. Using this staining, a sperm sample can be composed of different combinations of fluorescent cells according to their mitochondrial inner membrane potential [13]. The different labelling patterns may be correlated with parameters such as sperm motility [7].

A different approach to assess the mitochondrial integrity is to assess the presence of sperm mitochondrial proteins through the use of ICC [7,13]. This approach allows the detection and location of target molecules within the cell and the study of the modifications to the expected pattern of immunolabelling. One of the proteins available to study mitochondrial function is the Cytochrome C oxidase or complex IV, which catalyzes the final step in the mitochondrial electron transfer chain. This molecule is regarded as one of the major regulatory molecules for oxidative phosphorylation. As in other ICC, cytological preparations are set to incubate with the specific primary antibody, followed by incubation with the appropriated secondary antibody. The revelation can be obtained with DAB (3,3-Diaminobenzidine) or using DAPI (4',6-diamidino-2-phenylindole) as fluorochrome, under light or epifluorescence microscopy, respectively. The percentage of stained cells is determined over 200 spermatozoa in a minimum of 4 microscope fields.

Heat Shock Proteins (HSP), which are divided in families, are chaperon proteins involved in the protection of intracellular macromolecules against unfolding and aggregation during thermal and osmotic stress. HSP70 and HSP90, which have been found in the sperm, have important functions in the cellular trafficking of proteins other than the refolding and transport of client proteins. The role of HSP's on cell signalling in mature sperm is not clearly understood. It is known that an active cell metabolism, such as ATP production, is required for the expression of heat sock response [26,27]. HSP 70 and HSP 90 have separated ATPase and client protein-binding sites [27] and also distinct roles in sperm function. It has been shown that HSP70 and 90 are targets for protein phosphorylation, which is activated during capaci-

tation and capacitation-like response to sperm manipulation, in a reaction that might be associated with the nitric oxide synthesis during oxidative stress [28]. Sperm is transcriptionally inactive. Thus, HSP content in the spermatozoa is defined at ejaculation and those proteins must be present in the cytosol to help protecting the sperm from injury [29]. Therefore it is expectable to find a reduction of its amount or intensity of immunoexpression for these molecules (if Western blotting or ICC were used) after the cell attack. In canine ejaculates, a diminished number of sperm cells with low immunoreaction for HSP70 was found in semen of good quality. A reduction of the intensity of immunolabelling for this molecule was found after freezing/thawing (Figure 4), along with dislocation of the immunostaining from the acrosomal area to the sperm tail [30,31]. It has also been found a correlation between HSP70 immunoreaction in freshly ejaculated sperm and sperm damage after freezing/thawing procedures [30,31].

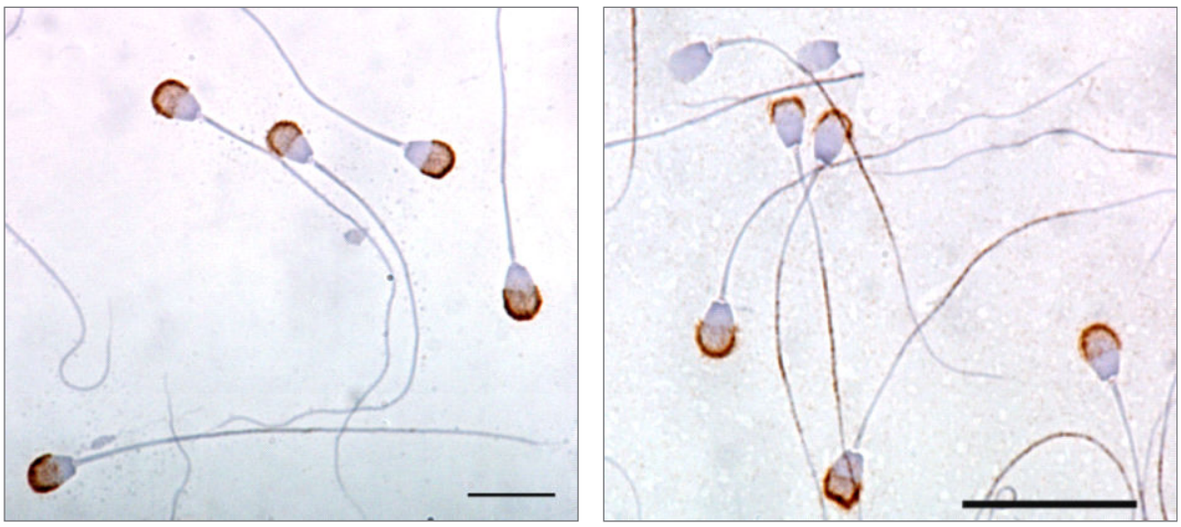

Figure 4. Canine sperm immunoreaction against HSP70 (Scale bar = 10 µm). In freshly ejaculated sperm (on the left), labelling for HSP70 is found over the acrosome region while the sperm tail is negative for this molecule. After freezing (on the right), a reduction of the intensity of immunostaining over the acrosome was found, with some negative sperm. In parallel, dislocation of the HSP immunoreactions to the sperm tail was observed.

3.3. Assessment of surface membrane integrity

Integrity of the sperm membrane is essential to sperm survival in the female genital tract and to fertilization [32]. Until placed in the female reproductive tract, spermatozoa are maintained in a hyperosmotic medium. Thereafter, it is passed into an iso-osmotic medium and contacts not only with the genital fluid, but also with the epithelia of the uterus and the uterine tubes, where it is stored. Further, molecules present on the sperm surface are of utmost significance for the spermatozoa interaction with the female local immune system, binding with the uterine tube epithelium, to cross the oocyte vestments (*cumulus* cells and *zona pellucida*) and to fertilize the oocyte. The molecular and hormonal local environment possess an important regulatory role on what concerns the sperm functions. However, to fulfil its role the sperm needs to acknowledge those influences and to react accordingly. Cell membrane

damages are one of the major side effects of cryopreservation and are irreversible [33]. It is due to changes in the membrane structure and lateral phase separation of the membrane components leading to focal aggregation of proteins, disarrangement of the membrane lipids and increased permeability to solutes [11,33].

When introduced in a hypo- or hypertonic environment, cells tend to adjust and reach osmotic equilibrium by allowing water and solutes to change across the cell membrane. Spermatozoa, among other cells, have the ability to maintain their volume after osmotic shock [1,34]. It has for long been proved that, for domestic species, cell volume control shows a close positive correlation with fertility [1]. In the ejaculate, there is usually sperm with different aptitudes and with differences in the ability to respond to osmotic stressors. This is often related with membrane deficiencies in ion channels or signalling pathways that control cell volume. The ability to adapt to osmotic changes can be tested by the hypoosmotic test (HOST), an indirect method to assess the membrane integrity, where sperm is incubated in hypoosmotic solutions between 1-60 minutes at 37ºC. Spermatozoa with intact plasmalemma become swollen and present coiled tails when incubated in a sucrose solution (ranging from 75 to 150 mOsm, according to the species) (Figure 5). After longer exposures, they recover the initial volume [34]. Although currently used for *in vitro* semen assessment, this evaluation is subjective and not quantitatively rigorous. It is also possible that a number of sperm cells may die if prolonged incubation periods are used, biasing the results. However, it becomes more precise if performed with the aid of an electronic cell counter. In this approach, known as the volume regulatory test, after the osmotic challenge, sperm passes through a capillary pore and cell volume is determined upon changes in the electric resistance to passage. The results are expressed as cell frequency distribution for the iso- and the hypoosmotic moments of the test and the amount of displacement of the distribution curve, which reflects the adaptability of the sampled cells [1].

Different combinations of fluorescent membrane-impermeable dyes may also be used to assess the sperm membrane integrity. Most commonly used ones, also show some degree of affinity for DNA, as for Hoechst 33258, propidium iodide (PI) or ethidium homodimer 1 [11]. Alternatively acylated membrane dyes are also used. These dyes can cross the intact cell membrane and be held in the viable spermatozoa. When the plasma membrane is damaged, the probe leak out of the cell. More recently, fluorescein diacetate (CFDA), carboxyl(methil)-derivates, such as carboxyl-SNARF and SYBR-14 have been used for this purpose (for more detail, see [11]). This sort of probes can be combined and used with flow cytometry. The combination of different patterns allows estimating different degrees of sperm viability [13]. When combined with PI, green fluorochromes such as CFDA (Carboxyfluorescein diacetate) or SYBER-14 are replaced in the dead spermatozoa by the red fluorescence, which is not found in the membrane intact sperm. Carboxyl-SNARF, a pH-indicator, stains the live spermatozoa in orange, whilst Hoechst 33258 stains the dead spermatozoa in bright-blue [11].

Sperm membrane integrity can also be assessed by the use of merocyanine 540 (MC540), a hydrophylic probe with highly disorganized lipids that shows a high affinity pattern for instable membranes. This probe allows to monitor the changes in the cell membrane lipid ar-

chitecture. Two sperm populations may be found under a fluorescent microscope: sperm with intact membranes devoid of fluorescence and sperm with disordered cell membranes that emit fluorescence [7,35]. This probe further labels sperm round, apoptotic bodies, which are more frequently found in men with decreased sperm quality [14]. Whether these structures are indicators of pathological or excessive apoptosis in the male genital tract or simply cell remnants of similar density to sperm heads is still to prove.

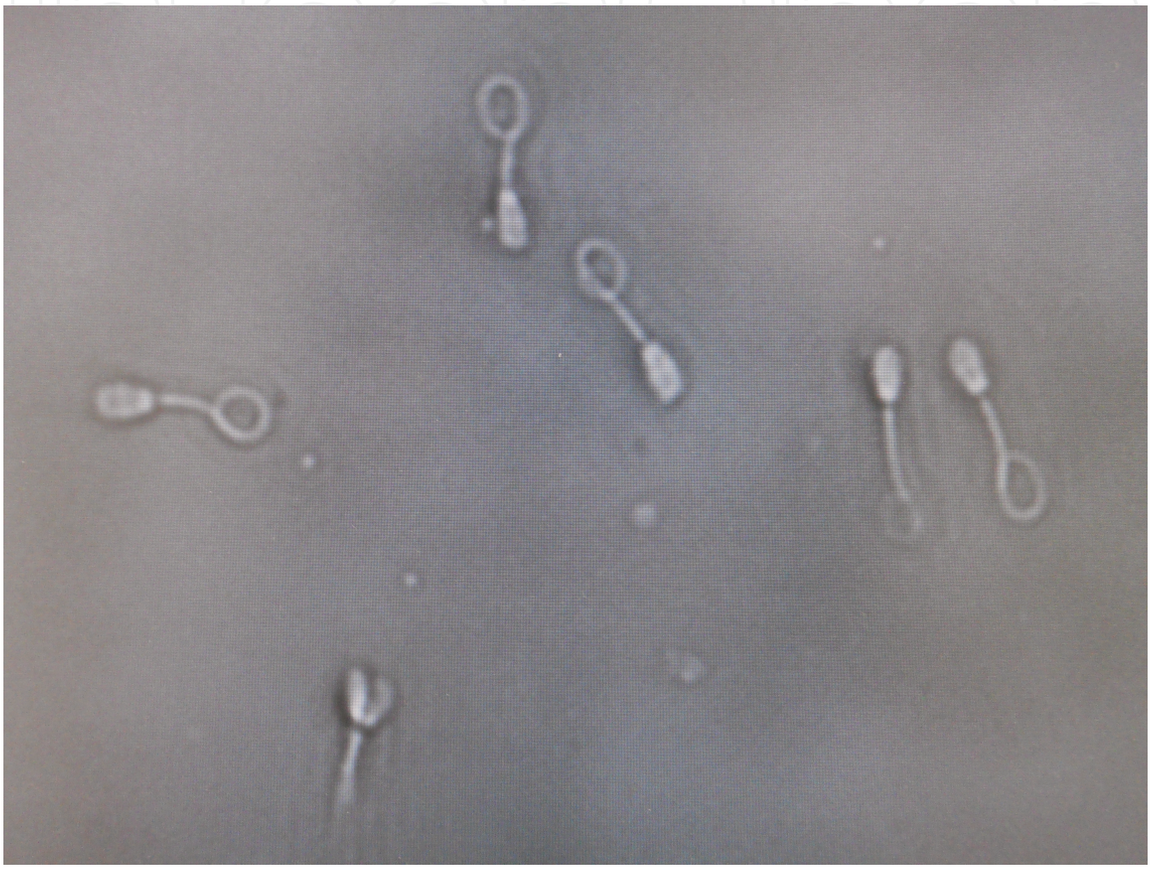

Figure 5. Canine spermatozoa in a HOST test (magnification 100x).

Besides the modifications on lipid arrangement in sperm plasma membrane, loss of membrane integrity also induces disorganization of the membrane proteins. In fact, in defective sperm or after cold-shock, the clustering of the membrane proteins is frequently observed. At fertilization, such modifications can interfere with the exposition of molecular epitopes and compromise receptor-ligand interactions between sperm and the oviductal cells or the oocyte [15,36]. A more conservative approach to test these changes includes the functional *in vitro* gamete interaction tests, such as the oocyte penetration test or the hemi-zona assay (for a quick review see [11]). The *zona pellucida* binding assay tests the ability of spermatozoa to interact with the *zona pellucida* of the oocytes. It is an assay with much variability and it tends to be replaced for the hemi-zona assay, which has the advantage of allowing the comparison between 2 sperm samples (one being used as control) on a single ovum. The oocyte penetration test assesses the fertilizing ability of spermatozoa by evaluating the presence of

fluorescent spermatozoa heads in the perivitelline space and in the ooplasm after several hours of sperm-oocyte co-incubation [37].

Further, ICC, Western blotting, Chromatography and ELISA (Enzyme-linked immunosorbent assay) techniques can be used to detect the immunoexpression of particular membrane proteins (like integrins, adhesins or membrane-anchored proteases- ADAM) and to assess possible changes in immunoexpression in defective sperm following a challenging stimulus.

Recently, some studies have been presented, concerning the water channels function in spermatozoa and their functions in the cell volume regulation and sperm adaptation to environmental changes in osmotic pressure. Aquaporins (AQPs) are a family of proteins highly specialized in water permeability and involved in water transport across membranes. It has been demonstrated that AQP3 is an important water channel localized on the principal piece of the sperm tail, which acts like a key-fluid regulator for sperm osmoadaptation, protecting the cell membrane from swelling and mechanical stretching damages [38]. By using a fluorescence immunocytochemistry approach and flow cytometry, it was found that in AQP3 defective sperm exist an increased proportion of tail bending at cytoplasmic droplet under osmotic stressor conditions, which were associated to membrane rupture and exaggerated cell swelling during HOST, along with decreased sperm motility and reduced fertilization [38]. Additional AQP's have been localized on the sperm of different species. AQP7 and AQP8 may play a role in the glycerol metabolism and water transport respectively, with AQP7 showing some association with sperm progressive motility [39].

3.4. Assessment of the oxidative stress and apoptosis

Sperm metabolism in aerobic conditions originates oxidative molecules (reactive oxygen species or ROS - short-lived reactive chemical intermediates), which are highly reactive and oxidize lipids, proteins and glycides. Cells contribute to the maintenance of the oxidative homeostasis by controlling the amount of ROS, converting them into less injuring molecules [40,41]. Excessive ROS production damages the sperm membrane, reduces motility (by decreasing membrane potential), induces irreparable DNA damage and is closely associated with apoptosis [42,43]. Oxidation reaction in the membranes increases ROS, changes membrane fluidity and compromises its integrity, impairs ion-gradients and lipid-protein interaction and causes changes in proteins [44,45]. The seminal plasma possesses various natural antioxidants that protect spermatozoa against the oxidative stress which are removed when sperm is diluted or submitted to a process for preservation. Spermatozoa are particularly susceptible to lipid peroxidation, and one should be aware that semen manipulation and cryopreservation-thaw procedures accelerate the production of reactive oxygen species. Within the spermatozoa, mitochondria and the plasma membrane are the most sensitive structures to ROS [45].

Lipid peroxidation (LPO) releases membrane polyunsaturated fatty acids that are used as substrates for ROS and hydroxyl radical generation. The most frequent product of LPO is malonaldehyde (MDA) [44]. LPO can be indirectly assessed using a spectrophotometer by measuring thiobarbituric acid reactive (TBAR) substances; the method is based on the measurement of the complex formed by the reaction of MDA with TBA under a temperature

stressor (incubation at 100ºC), which produce a pink-coloured chromogen and is readable at a wavelength of 532 nm. Also, the fluorescent probe BODIPY$^{581/591}$-C11 (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) is frequently used in association with flow cytometry to assess LPO in the sperm. BODIPY is a fatty acids sensitive fluorescent probe that changes fluorescence from red to green in the presence of lipid peroxidation. Its association with a vital probe further allows to evaluate the fluorescence emission ratio in living cells [44,46].

Additional, currently used methods also include the glutathione peroxidase reaction (where the hydrogen peroxide oxidizes GSH (reduced glutathione) into GSSG (oxidized glutathione) in the presence of glutathione reductase and NADPH results from the consumption of NADPH in proportion to the peroxide content), by flow cytometry measurement of the fluorescent intensity of the compounds oxidized by ROS (such as the dichlorofluorescin diacetate- DCFH-DA- or the Hydroethidine- HE), using the gas-liquid chromatography separation of lipid peroxides, followed by its identification by mass spectrometry and by measuring cytotoxic aldehydes through high performance liquid chromatography (HPLC) [44,45].

ROS production can be directly monitored by a luminol or a lucigenin-based chemilluminescence assay [43,45]. This assay does not distinguish between intracellular and extracellular ROS, but it differentiates between the production of superoxide and hydrogen peroxide according to the probe used (lucigenin and luminol, respectively for superoxide and hydrogen peroxide). Measurement of chemilluminescence is proportional to ROS accumulation [45].

An important side effect of the oxidative stress is apoptosis [42]. The most important changes associated to sperm apoptosis are the externalization of the phosphatidylserine (PS), a molecule usually confined to the inner leaflet of the plasma membrane, the caspase system activation, the DNA fragmentation, the lost of mitochondrial integrity and the increase of cell membrane permeability [41]. To assess sperm apoptosis it is frequently used the Annexin V, a Ca^{2+}-dependent PS-binding protein that reacts to the PS, which is translocated to the outer leaflet of the plasma membrane in damaged sperm. Annexin V can be conjugated to fluorochromes such as FITC (Fluorescein isothiocyanate) in flow cytometry analysis. If a vital staining is used, such as the propidium iodide, the combination allows to distinguish between three sperm sub-populations: viable (Annexin-FITC-PI-negative), early apoptotic (Annexin-FITC-positive and PI-negative) and late apoptotic (Annexin-FITC-PI-positive) [7,41].

Caspases are molecules associated with the apoptotic pathway and can be classified as initiators or executors; caspase 7 and 9 are initiators, while active caspase 3 is an executor. The determination of the caspase enzymatic activity in sperm extracts, in comparison to the one of neutrophils, can also be used to assess apoptosis in sperm, which may be completed by the semiquantitative determination of active caspase 3 and caspase 7 content, by Western blotting. Caspase activity has been shown to be consistently higher in low motility sperm, in particular, the active caspase 3 [47].

Assessment of additional molecules known to be involved in the apoptosis mechanism, which might work as possible biological markers, can be performed by ICC, Western blotting or even in proteomic studies. Some molecules participating or regulating apoptotic processes in cells have been analysed in sperm and in semen, and its concentration was found to correlate with sperm quality. Among these molecules, TNF localization in pig and canine sperm has been performed [48,49]. The immunolabelling is limited to the sperm mid-piece in the mitochondrial region (Figure 6) and it has been demonstrated that a decrease in TNF immunoreaction is observed in spermatozoa incubated in a capacitating medium. When exposed to TNF, spermatozoa showed decreased motility, increased PS externalization and chromatin and DNA damage, changes that are usually associated with apoptosis [50].

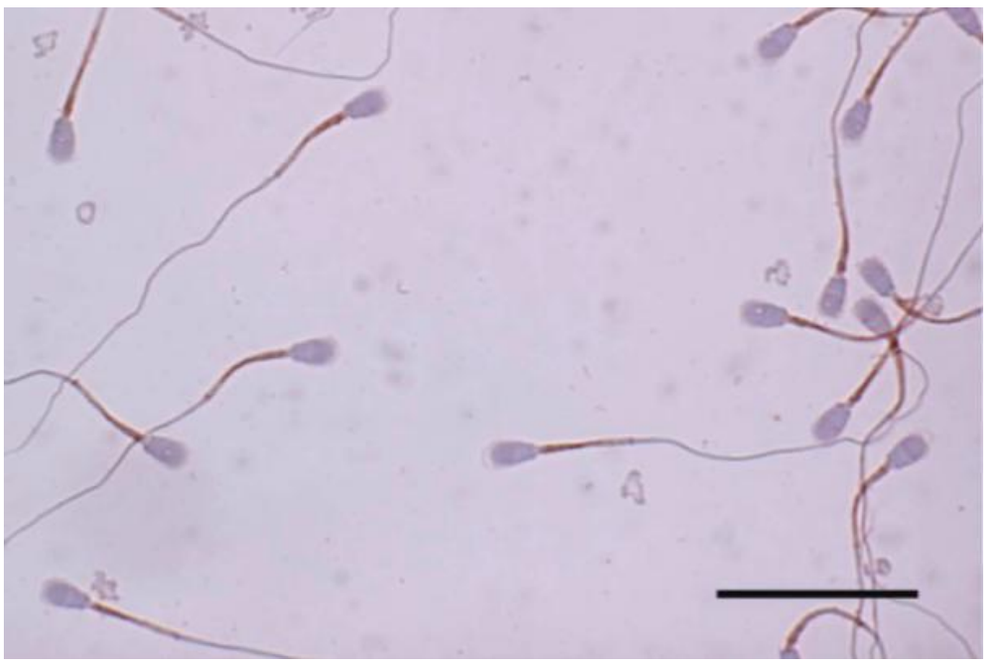

Figure 6. Sperm immunoreactions against TNF. In canine spermatozoa, strong immunolabelling for TNF was found in sperm mid–piece, in the mitochondrial region.

3.5. Assessment of DNA integrity

An association between infertility and the integrity of DNA content in sperm has been suggested. The integrity of male DNA is of utmost importance for embryo development and offspring production [13,41]. DNA damage is not usually perceived under classic or advanced semen assessment, but has been proposed to be at the origin of infertility in normospermic individuals. DNA damage (abnormal chromatin structure) may arise from different processes: deficient recombination or packaging during spermatogenesis, apoptosis and oxidative stress. DNA loss of integrity does not always impair fertilization, but compromises sustainable embryo development, predisposing to embryo losses and abortion [9,15]. DNA fragmentation may be associated with various pathological and environmental conditions [51,52], but also with endogenous mechanisms such as the oxidative stress and apoptosis.

Evaluation of sperm DNA integrity can be achieved by a variety of tests covering different aspects of the DNA damage. Unfortunately, most of the available techniques provide limited information regarding the nature of the DNA lesions evidenced, and do not allow to highlight the exact pathogenesis of disrupted sperm DNA [53,54].

Less expensive methods to assess the sperm chromatin structure uses chromatin structural probes or dyes, such as the acridine orange (measures the susceptibility to conformational changes), the aniline blue (that stains loosely condensed chromatin), chromomycin α (competing with protamine binding to DNA, it reveals protamination defects on sperm) and the toluidine blue (that stains phosphate residues of fragmented DNA). However, several factors modulate the DNA staining of chromatin, decreasing their specificity [52].

Nowadays, the most currently used tests of sperm DNA fragmentation are: the Comet assay (single cell gel electrophoresis), the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP (2′-deoxyuridine, 5′-triphosphate) nick end labelling) assay, the sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion (SCD) test. The first three assays focus on the DNA fragmentation detection, while the last assay is a sperm nuclear matrix assay detecting possible deficient DNA repair or chromatin disorganization [43]. On table 1 we compare these methods.

The Comet assay is a fluorescence microscopic test that identifies single (SS) and double-stranded (DS) DNA in single sperm. In this assay, sperm cells are mixed with low-to-moderate melting agarose and then placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis, the DNA being visualized with the aid of a fluorochrome dye. DNA damage is quantified by measuring the displacement between the genetic material of the comet nucleus (unbroken DNA) and the resulting tail (damaged DNA) [21,52,53]. The length of the tail is positively correlated with the percentage of DNA fragmentation. Although highly sensitive, this method is also labour intensive and the comet tail is of difficult standardization. Further, less apparent clinical association exists between the test results and clinical infertility [43], and clinical thresholds were yet to be established.

TUNEL assay is possibly the most common method used to assess sperm damage in sperm. It can be used as another ICC method, in both bright field and fluorescence microscopy, or associated with flow cytometry. In the TUNEL assay, terminal deoxynucleotidyl transferase (TdT) incorporates labelled nucleotides into 3'-OH at single- and double-strand DNA breaks, creating a signal of increasing intensity according to the number of DNA breaks. The fluorescence intensity of each analysed sperm is scored as a "positive" or "negative" on a microscope slide. When conjoined with a flow cytometer, precision of the method increases due to the increased number of cells analysed [43,53]. Proportion of TUNEL positive cells seems to be correlated with decreased pregnancy rates [13]. However, numerous variations for the test exist, which reduces its liability.

The sperm chromatin structure assay measures *in situ* DNA susceptibility to acid-induced DNA denaturation. It uses a flow cytometer and the acridine orange fluorescence, a traditional fluorescent dye that shows different colour when bonded to single- (red) or double-stranded (green) DNA [43]. The degree of red fluorescence in a sample (named DNA

fragmentation index - DFI) has been associated to male infertility [13,43,53]. It is possible to score different spermatozoa populations by using SCSA: the sperm without fragmented DNA, the sperm with moderate DFI and the sperm with high DFI.

The sperm chromatin dispersion test (SCD) is a method based on the principle that sperm with fragmented DNA fail to produce a halo, which is characteristically observed in sperm with non-fragmented DNA, when mixed in aqueous, low melting agarose followed by acid denaturation and removal of nuclear proteins [21,54]. Despite not being necessary, this test can be visualised using a fluorescent dye (such as propidium iodide, DAPI or ethidium bromide) or simply be stained with Diff-Quick® reagent. Halosperm® is a commercial kit to assess DNA fragmentation in sperm from different species, before or after semen manipulation. Regarding this kit, sperm presenting a large- and medium-sized halo is considered to have no fragmentation, while spermatozoa having a small halo or without halo is classified as having DNA fragmentation (Figure 7) [55].

Assay	Parameter	Principle	Detection method
TUNEL	Addition of labeled dUTP nucleotides with deoxynucleotidyl transferase to SS and DS DNA breaks Template independent	Cells with labelled DNA (%)	Microscopy (bright or fluorescence) Flow cytometry
Comet	Fragmented DNA in sperm cells is detected by eletrophoresis Alkaline conditions denature DNA and reveals SS and DS DNA breaks Neutral conditions reveal mostly DS breaks	% cells with migration tails (fragmented DNA) and also the length of the tail (% DNA in the tail)	Fluorescence microscopy
SCSA	Mild acid treatment denaturates and lyses DNA with SS or DS breaks Acridine orange differentially emits fluorescence with DS DNA (Green) or SS DNA (Red)	DFI (%) = cells with red fluorescence divided by the total of cells (red+green).	Flow cytometry
SCD	Mild acid denaturation of DNA and lysis of protamines induce a decondensation halo around sperm head if DNA is intact, and no halo is observed if DNA is damaged	% Cells with small or no halo	Microscopy (bright or fluorescence)

Table 1. Comparison of available methods for assessment of DNA fragmentation is spermatozoa (Adapted from [56]). (SS- Single-stranded; DS- Double-stranded; DFI-DNA fragmentation index)

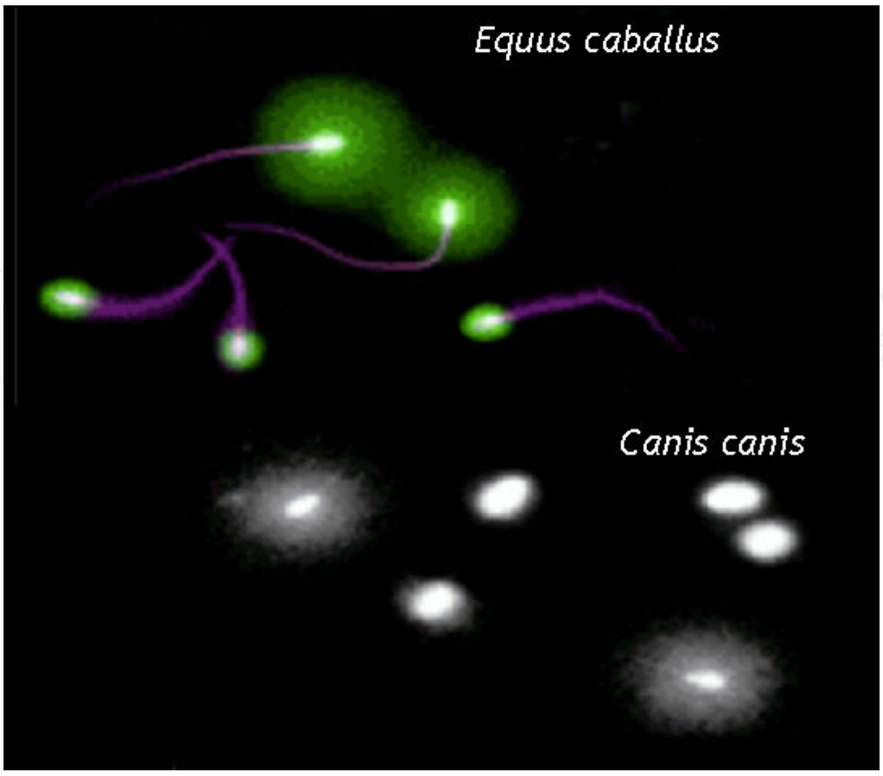

Figure 7. Image of the Halosperm® test for DNA fragmentation in horses and dogs. The existence of a large halo is indicative of DNA integrity (Adapted from [56]).

4. Concluding remarks

Conventional, currently methods used in sperm quality assessment are unsatisfactory to correctly predict sperm fertility potential and do not provide sufficient information for diagnosing and overcome some clinical infertility situations. The major advantages of biomarker approach over conventional semen analysis are the proficiency to accurately measure biomarker levels and to expose hidden sperm defects, which go undetected during current sperm morphology assessment. Newer, unconventional diagnostic tests of sperm function have the increased potential to deliver relevant information and to have an effective predictive role in male reproductive medicine. In the present work, several molecular markers have been presented for each of the sperm functions. Some are already used in human andrology, but are less used for the animals. Its use allows an increased efficiency in the identification of infertile individuals or to predict the sperm behaviour to manipulation, hence predicting the degree of damage to be expected for a given sperm sample. The development of test based on predicted sperm functions such as capacitation and in particular sperm–oocyte interaction will present increasing impact on the field of extenders research, as well as of semen banks implementation for both domestic and wild species. It is of utmost interest the characterization of a particular biomarker patterns/levels in fertile and infertile samples, with the subsequent ability to identify males with superior tolerance to semen cryopreserva-

tion. Nevertheless, putative molecular markers that may be used for sperm quality assessment were not exhausted in this review. Further efforts must be focused on understanding how these biomarkers correlate with transient impairments of male infertility caused by heat stress, malnutrition, diseases or trauma. Finally, the adjunctive evaluation of spermatozoa functions is particular important when considering sperm storage.

Acknowledgments

This work was supported by the project from CECAV/UTAD with the reference PEst-OE/AGR/UI0772/2011, by the Portuguese Science and Technology Foundation.

Author details

Rita Payan-Carreira[1*], Paulo Borges[1,2], Fernando Mir[2] and Alain Fontbonne[2]

*Address all correspondence to: rtpayan@gmail.com

1 CECAV [Veterinary and Animal Research Centre] – University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

2 CERCA (Centre d'Études en Reproduction des Carnivores), Animal Reproduction, National Veterinary School of Alfort, Paris-East University, France

References

[1] Petrunkina AM, Waberski D, Günzel-Apel AR, Töpfer-Petersen E. Determinants of sperm quality and fertility in domestic species. Reproduction. 2007, 134: 3-17

[2] Sutovsky P, Lovercamp K. Molecular markers of sperm quality. Soc Reprod Fertil Suppl. 2010, 67: 247-56.

[3] Dyck MK, Foxcroft GR, Novak S, Ruiz-Sanchez A, Patterson J, Dixon WT. Biological markers of boar fertility. Reprod Domest Anim. 2011, 46 (Suppl 2): 55-8.

[4] Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update. 1997, 3: 403-39.

[5] Payan-Carreira R, Miranda S and Nizanski W. Artificial Insemination in Dogs. In: Artificial Insemination in Farm Animals. Milad Manafi (Ed.), 2011. ISBN: 978-953-307-312-5, InTech, Available from: http://www.intechopen.com/books/artificial-insemination-in-farm-animals/artificial-insemination-in-dogs

[6] Lefièvre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GS, Chen Y, Kirkman-Brown JC, Afnan MA, Publicover SJ, Ford WC, Barratt CL. Counting sperm does not add up any more: time for a new equation? Reproduction. 2007, 133:675-84.

[7] Ramalho-Santos J, Amaral A, Sousa AP, Rodrigues AS, Martins L, Baptista M, Mota PC, Tavares R, Amaral S, Gamboa S. Probing the structure and function of mammalian sperm using optical and fluorescence microscopy. In: Modern Research and Educational Topics in Microscopy. A Mendez-Villas and J Diaz (Eds.), 2007. Badajoz, Spain: FORMATEX. Pp: 394-402.

[8] Heriberto Rodriguez-Martinez. Cryopreservation of Porcine Gametes, Embryos and Genital Tissues: State of the Art. In: Current Frontiers in Cryobiology, Igor I. Katkov (Ed.), 2012. ISBN: 978-953-51-0191-8, InTech, Available from: http://www.intechopen.com/books/current-frontiers-in-cryobiology/cryopreservation-of-pig-spermatozoa-oocytes-and-embryos-state-of-the-art.

[9] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci. 2000, 60-61:481-92.

[10] Pesch S, Hoffmann B. J. Reproduktionsmed. Cryopreservation of Spermatozoa in Veterinary Medicine. Endokrinol. 2007, 4: 101-105

[11] Partyka A, Niżański W and Ochota M. Methods of Assessment of Cryopreserved Semen, Current Frontiers in Cryobiology, Igor I. Katkov (Ed.), 2012. ISBN: 978-953-51-0191-8, InTech, Available from: http://www.intechopen.com/books/current-frontiers-in-cryobiology/methods-of-assessment-of-cryopreserved-semen

[12] Kim SH, Yu DH, Kim YJ. Apoptosis-like change, ROS, and DNA status in cryopreserved canine sperm recovered by glass wool filtration and Percoll gradient centrifugation techniques. Anim Reprod Sci. 2010, 119:106-14.

[13] Silva PF, Gadella BM. Detection of damage in mammalian sperm cells. Theriogenology. 2006, 65:958-78.

[14] Muratori M, Luconi M, Marchiani S, Forti G, Baldi E. Molecular markers of human sperm functions. Int J Androl. 2009, 32:25-45.

[15] Samplaski MK, Agarwal A, Sharma R, Sabanegh E. New generation of diagnostic tests for infertility: review of specialized semen tests. Int J Urol. 2010, 17:839-47.

[16] Rodriguez-Martinez H. Role of the oviduct in sperm capacitation. Theriogenology. 2007, 68 (Suppl 1):S138-46.

[17] Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S. Ca2+-stores in sperm: their identities and functions. Reproduction. 2009, 138:425-37.

[18] Graham JK.Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci. 2001, 68:239-47.

[19] Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature. 2011, 471:382-6.

[20] Makkar G, Ng EH, Yeung WS, Ho PC. The significance of the ionophore-challenged acrosome reaction in the prediction of successful outcome of controlled ovarian stimulation and intrauterine insemination. Hum Reprod. 2003, 18:534-9.

[21] Memili E, Dogan S, Rodriguez-Osorio E, Wang X, de Oliveira RV, Mason MC, Govindaraju A, Grant KE, Belser LE, Crate E, Moura A, Kaya A. Makings of the Best Spermatozoa: Molecular Determinants of High Fertility. In: Male Infertility, Anu Bashamboo and Kenneth David McElreavey (Ed.), 2012. ISBN: 978-953-51-0562-6, InTech,. Available from: http://www.intechopen.com/books/male-infertility/makings-of-the-best-spermatozoa-molecular-determinants-of-high-fertility

[22] Oliva R, de Mateo S, Estanyol JM. Sperm cell proteomics. Proteomics. 2009, 9:1004-17.

[23] Brewis IA, Gadella BM. Sperm surface proteomics: from protein lists to biological function. Mol Hum Reprod. 2010, 16:68-79.

[24] Berlinguer F, Madeddu M, Pasciu V, Succu S, Spezzigu A, Satta V, Mereu P, Leoni GG, Naitana S. Semen molecular and cellular features: these parameters can reliably predict subsequent ART outcome in a goat model. Reprod Biol Endocrinol. 2009, 7:125

[25] Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, Freitas A, Paiva A, Almeida-Santos T, Ramalho-Santos J. Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. PLoS One. 2011 6:e18112. doi:10.1371/journal.pone.0018112.

[26] Liu DY, Clarke GN, Baker HW. Hyper-osmotic condition enhances protein tyrosine phosphorylation and zona pellucida binding capacity of human sperm. Hum Reprod. 2006, 21: 745-752.

[27] Cole JA, Meyers SA. Osmotic stress stimulates phosphorylation and cellular expression of heat shock proteins in rhesus macaque sperm. J Androl. 2011, 32:402-10.

[28] Ecroyd H, Jones RC, Aitken RJ. Tyrosine phosphorylation of HSP-90 during mammalian sperm capacitation. Biol Reprod. 2003, 69:1801-7.

[29] Spinaci M, Vallorani C, Bucci D, Bernardini C, Tamanini C, Seren E, Galeati G. Effect of liquid storage on sorted boar spermatozoa. Theriogenology. 2010, 74:741-8.

[30] Borges P, Mir F, Fontbonne A, Carreira R, "Study of HSP70 expression in dog semen submitted to cold-thermal treatment". Book of Abstracts of the European Veterinary Conference - Voorjaarsdagen, 5 to 7 April 2012, Amsterdam, The Netherlands: 249-250

[31] Borges P, Mir F, Fontbonne A, Carreira R. [HSP70 Redistribution in canine spermatozoa after chilling and freezing] (In portuguese) Abstract book of the V Congresso da

Sociedade Portuguesa de Ciências Veterinárias, 13-15 October 2011, Fonte Boa, Portugal: 164.

[32] Holt, W.V. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 2000, 53: 47-58.

[33] Meyers SA. Spermatozoal response to osmotic stress. Anim Reprod Sci. 2005, 89:57-64.

[34] England GC, Plummer JM. Hypo-osmotic swelling of dog spermatozoa. J Reprod Fertil. 1993, Suppl. 47:261-70.

[35] Hallap T, Nagy S, Jaakma U, Johannisson A, Rodriguez-Martinez H. Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. Theriogenology. 2006, 65:1122-36.

[36] Rajeev SK, Reddy KV. Sperm membrane protein profiles of fertile and infertile men: identification and characterization of fertility-associated sperm antigen. Hum Reprod. 2004, 19:234-42.

[37] Hewitt, D.A., England, G.C.W. The canine oocyte penetration assay; its use as an indicator of dog spermatozoa performance in vitro. Anim Reprod Sci. 1997, 50:123-139.

[38] Chen Q, Peng H, Lei L, Zhang Y, Kuang H, Cao Y, Shi QX, Ma T, Duan E. Aquaporin3 is a sperm water channel essential for postcopulatory sperm osmoadaptation and migration.Cell Res. 2011, 21:922-33.

[39] Yeung CH, Callies C, Tüttelmann F, Kliesch S, Cooper TG. Aquaporins in the human testis and spermatozoa - identification, involvement in sperm volume regulation and clinical relevance. Int J Androl. 2010, 33:629-41.

[40] Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. Asian J Androl. 2004, 6:59-65.

[41] Agarwal A, Varghese AC, Sharma RK. Markers of oxidative stress and sperm chromatin integrity. Methods Mol Biol. 2009, 590:377–402

[42] Kim, S-H., Yu D-H. & KimY-J. Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. Theriogenology. 2010, 73:282–292.

[43] Natali A, Turek PJ. An assessment of new sperm tests for male infertility. Urology. 2011, 77:1027-34.

[44] Sanocka D, Kurpisz M. Reactive oxygen species and sperm cells. Reprod Biol Endocrinol. 2004, 2:12.

[45] Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol. 2008, 59:2-11.

[46] Gallardo Bolaños JM, Morán AM, Balao da Silva CM, Morillo Rodríguez A, Plaza Dávila M, Aparicio IM, Tapia JA, Ortega Ferrusola C, Peña FJ. Autophagy and Apoptosis Have a Role in the Survival or Death of Stallion Spermatozoa during Conservation in Refrigeration. 2012 PLoS ONE 7(1):e30688. doi:10.1371/journal.pone.0030688.

[47] Taylor SL, Weng SL, Fox P, Duran EH, Morshedi MS, Oehninger S, Beebe SJ. Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. Mol Hum Reprod. 2004, 10:825-34.

[48] Payan-Carreira R, Santana I, Pires MA, Holst BS, Rodriguez-Martinez H. Localization of tumor necrosis factor in the canine testis, epididymis and spermatozoa. Theriogenology. 2012, 77:1540-8

[49] Payan-Carreira R, Siqueira A, Gonzalez Herrero R "TNF immunolocalization in boar spermatozoa". Reprod Dom Anim. 2011, 46 (Suppl. 3): 72

[50] Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, Calogero AE. Effects of tumour necrosis factor-alpha on human sperm motility and apoptosis. J Clin Immunol. 2007, 27:152-62.

[51] Zini A, Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. CMAJ. 2006, 175:495-500.

[52] Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod Biomed Online. 2006, 12:466-72.

[53] Bungum M. Sperm DNA integrity assessment: a new tool in diagnosis and treatment of fertility. Obstet Gynecol Int. 2012, 2012:531042.

[54] Agarwal A, Said TM Sperm chromatin assessment. In Textbook of ART (2nd Ed.) DK Gardner, A Weissman, CM Howles, A Shoham (Edt), 2004. PLC (Taylor and Francis Group) London, UK: pp 93–106.

[55] Yılmaz S, Zergeroğlu AD, Yılmaz E, Sofuoglu K, Delikara N, Kutlu P. Effects of Sperm DNA Fragmentation on Semen Parameters and ICSI Outcome Determined by an Improved SCD Test, Halosperm. International Jourtal of Fertility and Sterility 2010 4(2): 73-78.

[56] Halotech DNA SL - www.halotechdna.com/products/halosperm [Assessed in 14-07-2012]

IntechOpen