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Flow cytometry in Spermatology: a bright future ahead

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ABSTRACT

Techniques such as mass spectrometry have led to unprecedented knowledge of the proteins that are present in the spermatozoa of humans and other mammals. However, in spite of their high-throughput and fractioning techniques, most of the techniques in use only offer average values for the entire sperm population. However, ejaculate is very heterogeneous, and average values may mask relevant biological information.

The application of flow cytometry may overcome this disadvantage, allowing proteomic analysis at the single-cell level. Moreover, recent advances in cytometry, allowing multiple analyses within a single cell combined with powerful statistical tools, as an expanding subfield in spermatology, are described. The increased use of advanced flow cytometers in andrology laboratories will allow the rapid development of multiparametric, multicolor flow cytometry in andrology that will expand the clinical applications and research possibilities of flow cytometry-based proteomic approaches, especially in the subfields of clinical andrology and sperm biotechnology

Key words: sperm, subpopulations, proteome, flow cytometry

INTRODUCTION

The spermatozoon is a highly differentiated cell that harbors the haploid male genome in a highly compact chromatin structure (Lindemann and Lesich 2016). The spermatozoon has a large tail with a midpiece rich in mitochondria, the organelles that provides energy via oxidative phosphorylation and have other important regulatory functions (Davila et al. 2016, Pena et al. 2015). Spermatozoa are generated in the seminiferous epithelium of the testis in a process called spermatogenesis. The initial cell in this process is the ad-basal spermatogonium, which experiences a series of mitotic divisions and commits to entering meiosis by becoming a spermatocyte (spermatocytogenesis). Meiotic recombination takes place through reductional divisions inside the adluminal protected compartment of the seminiferous tubule. When meiosis is complete, haploid, re-combined round spermatids are generated (Fraser and Lin 2016). These cells will experience dramatic changes in their transformation (spermiogenesis) to testicular spermatozoa that will be released to the seminiferous tubule lumen at spermiation (spermatoteleosis), after losing most of its cytoplasm when engulfed by the Sertoli cells. Spermiogenesis broadly implies the transformation of one of the centrioles into a flagellum, around which part of the mitochondria arranges to build the sperm tail together with specific columns of proteins. Transformation also implies remodeling of the cell nucleus, to which a modified lysosome is attached to form the acrosome, concomitant with extensive remodeling and compaction of the chromatin. This is accompanied by the acquisition of epigenetic marks such as DNA methylation, histone modifications and regulation by small RNAs (Fraser and Lin 2016). The intensive compaction and remodeling of the chromatin by replacement of

histones with protamines protects the paternal genome through its journey to fertilize the egg and silences all nuclear transcription. This implies that there is no protein synthesis in mature spermatozoa, which rely on post-translational modifications (PTM) to regulate their function (Fraser and Lin 2016). Additionally, spermatozoa experience further maturational processes as they transit through the epididymis, where further maturation of protamines is present. The final step of maturation in the female genital tract is called capacitation (Rodriguez-Martinez 1992). This later process implies intense redox-regulated PTM. Due to the inability of the spermatozoa to synthesize proteins *de novo*, major functions of the spermatozoa such as motility and capacitation are tightly regulated, involving redox-dependent tyrosine phosphorylation of sperm proteins (O'Flaherty 2015). However, dis-regulation of these systems leads to oxidative stress and cell death (Morielli and O'Flaherty 2015)

Mammalian ejaculate is composed of spermatozoa, caudal epididymis fluid and the secretions of the accessory sexual glands. Because dynamic spermatogenesis is a continuous process, the sperm reserve in the epididymis tail is composed of many generations of spermatogenesis. Thus, not all of the ejaculated spermatozoa is from the same generation or present the same physiological status. A well-known, but frequently overlooked fact is that only one spermatozoon will reach the goal of fertilizing the oocyte. The concept of sperm heterogeneity was introduced in the 1990s (Glassy et al. 1984, Paradisi et al. 1996, Thurston et al. 1999, Abaigar et al. 1999), with the beginning of the new century witnessing intense research into the subpopulation structure of the ejaculate in humans and other mammals (Santolaria et al. 2015, Unates et al. 2014, Abad et al. 2013, Thilagavathi et al. 2012, Mendoza et al. 2012, Marti et al. 2011, Dorado et al. 2010, Ortega-Ferrusola et al. 2009, Druart et al. 2009, Quintero-Moreno et al. 2007, Pena et al. 2006, Satake et al. 2006, Pena et al. 2005b, Pena et al. 2005a,

Martinez-Pastor et al. 2005, Chantler et al. 2004, Buffone et al. 2004, Correa-Perez et al. 2003, Thurston et al. 2001). The discovery of this ejaculate “heterogeneity” challenged the “competitive race model” of fecundation, in which large numbers of spermatozoa race towards the egg and compete to fertilize (Eisenbach and Giojalas 2006). In their journey to reach the oocyte, spermatozoa are exposed to different environments, and most spermatozoa succumb alongside sperm transport in the female. Only the best quality sperm reach the oviduct at the utero-tubal junction and form the sperm reservoir, where they are sequentially released in relation to ovulation (Hunter and Rodriguez-Martinez 2004, Hunter and Rodriguez-Martinez 2002). In fact, very few spermatozoa reach the oviduct; in humans, it is estimated that only 1 of every million ejaculated spermatozoa enter the Fallopian tubes (Eisenbach 2003, Eisenbach and Giojalas 2006), and only a small percentage of spermatozoa experience complete capacitation and are able to fertilize (Giojalas et al. 2004, Cohen-Dayag et al. 1995). The existence of redundant spermatozoa or spermatozoa with a function other than fertilization (perhaps assisting the fertilizing spermatozoa on their journey), is an intriguing hypothesis that is undermined by the fact that most spermatozoa are basically dying or already dead sperm (Roca et al. 2016). Research into sperm competition supports this line of reasoning. The study of sperm subpopulation structures includes morphological and kinematic studies. More recently, with the advent of the “omics” era to spermatology, detailed proteomic studies have been conducted (Agarwal et al. 2016, Bayram et al. 2016, Castillo et al. 2015, Huang et al. 2015, Kwon et al. 2014, Azpiazu et al. 2014, Aitken and Baker 2008). Proteomic approaches usually involve one- and two-dimensional electrophoresis (1/2-DE) for the separation of proteins, followed by mass spectrometry for peptide sequencing to facilitate protein identification. These techniques have yielded enormous advances in our knowledge of the sperm proteome in

human and mammals. However, this approach provides average values at single time points and masks sperm heterogeneity. Thus, techniques for single cell analysis are required to study the heterogeneous complexity of ejaculate. In this paper, we argue that flow cytometry can be a major tool for proteome research in spermatology in the coming years, complementing current large-scale identification techniques. To prepare the present review, we conducted an exhaustive literature search using the PubMed, Science Direct and Scopus databases to identify proteomic studies in spermatozoa, with a particular focus on flow cytometry in humans and pertinent animal models. Our search focused on papers that were published in the last 5 years (2012-2016). Particular attention was paid to single-cell analysis using flow cytometry because excellent reviews are already available for general sperm proteomics (Castillo et al. 2015, Amaral et al. 2014b, Castillo et al. 2014, Amaral et al. 2014a, Azpiazu et al. 2014, Swegen et al. 2015, Aitken and Baker 2008).

EJACULATED SPERMATOZOA ARE PHENOTIPICALLY DIFFERENT

The heterogeneous nature of the mammalian ejaculate has been recognized to include not only genotypic diversity but also differences in phenotype; different subpopulations of spermatozoa can be distinguished based on morphometric and kinetic analyses (Nunez-Martinez et al. 2007, Nunez-Martinez et al. 2006, Pena et al. 2006, Pena et al. 2005a, Ortega-Ferrusola et al. 2009). This complexity has been identified in many biological systems (Newman and Weissman 2006, Newman et al. 2006) and its importance is also stressed in different aspects of medicine (Saadatpour et al. 2015, Sigal et al. 2006, Cohen et al. 2008), even leading to a consensus in andrology related to the limited value of the report of average values in the spermiogramme . The guidelines

for the application of computer assisted sperm analysis (CASA) technology in the analysis of spermatozoa have indicated this fact and proposed that the spermatozoa should be classified into specific subpopulations related to relevant functional endpoint (Group 1998). More recently, flow cytometry has been used to identify specific sperm subpopulations capable of responding to specific stimuli (Escoffier et al. 2015) in relation to capacitation. These facts support the concept that only a small subpopulation of spermatozoa capacitates in humans (Gakamsky et al. 2008, Oren-Benaroya et al. 2008, Eisenbach and Giojalas 2006, Eisenbach 2003). This has profound implications for sperm proteomics, implying the existence of two distinct sperm subpopulations with potentially different proteomes: capacitated spermatozoa that can reach and fertilize the oocyte and spermatozoa that cannot capacitate properly and must be silently removed by phagocytosis in the absence of inflammatory reactions from the female genital tract (Eisenbach 2003). Spermatozoa experience selection in the female genital tract immediately after ejaculation (Sakkas et al. 2015). The deposition site varies with species; in humans, it occurs in the superior vagina near the cervical opening. Ejaculated spermatozoa encounter a hostile vaginal pH and an immediate inflammatory reaction response at the deposition site. In other species, the deposition site is located in the cervix or even the uterine lumen. These differences relate to the type, volume and anatomical characteristics of species. Ejaculates can have a single volume (as in ruminants) or be fractionated, as in human, canine, porcine or equine sperm. In fractionated ejaculate, the first portions of the ejaculate are always sperm-rich; the seminal plasma is protein-poor, while the next spurts of the ejaculate are sperm-poor but the seminal plasma is protein-rich. In humans, the first jets of ejaculate contain most of the spermatozoa bathed in prostate-dominated secretion. The secretion is acidic due to the epididymis fluid. Because of the prostate secretion, this portion does not coagulate

while bathing the os cervix. All these characteristics favor the immediate entry of a sub-population of spermatozoa into the cervical canal via capillarity. The rest of the ejaculate contains fewer spermatozoa and a dominance of secretion of the seminal vesicles. This portion of the ejaculate coagulates because it is more protein-rich. The coagulum traps spermatozoa in the vagina but also signals the female genital immune system. The coagulum often resorbs in minutes in vivo and readily egresses from the vagina by back-flow. Thus, this sperm subpopulation does not participate in fertilization. In vitro, praxis is to collect the entire ejaculate (all jets) into a single lab tube, which is a poor mimic of the physiological state. The spermatozoa that fortuitously escape this initial selection (e.g., those in the cervical canal) follow a new selection process. Spermatozoa with poor motility and abnormal morphology do not traverse the channels of sialic acid in the endocervix. Once in the uterus, spermatozoa rapidly progress upwards, due to contractions of the myometrium. In all species yet studied, there are strong, although transient, inflammatory vaginal, cervical and uterine reactions that are intended to eliminate foreign spermatozoa and the proteins of the seminal plasma, along with eventual microorganisms. This reaction is particularly strong in horses, a species in which the cervix does not constitute a major barrier to spermatozoa. Finally, the surviving (often vanguard) spermatozoa reach the utero-tubal junction, which exerts final sperm selection based on sperm morphology, motility and the battery of surface proteins exposed by the spermatozoa (Sakkas et al. 2015). These selection processes in the female reproductive tract all result in a spermatozoa reduction of 5 to 6 orders of magnitude in humans. This underlines sperm heterogeneity and the need for identifying the fertilizing sperm population in the whole ejaculate.

AVERAGE VALUES DO NOT PROVIDE ACCURATE INFORMATION OF SPERM BIOLOGY

When a semen sample is taken for sperm analysis or to study sperm biology, only average values are reported, and readouts are given at a single time point. Considering the heterogeneous nature of the mammalian ejaculate and strict selection in the female reproductive tract, it is clear that average values mask important information. Traditional whole-sperm proteomic approaches such as 2D polyacrylamide gel electrophoresis (2D-PAGE), mass spectrometry (MS), and differential in-gel electrophoresis (DIGE) have allowed for the identification of numerous sperm-specific proteins (Agarwal et al. 2016, du Plessis et al. 2011). Studies of the sperm proteome have demonstrated how post-translational modifications such as phosphorylation, glycosylation, proteolytic cleavage and mutation bring about physiological changes in spermatozoa function (Aitken and Baker 2008, Baker 2016). Furthermore, proteomic analysis has allowed for the study of spermatozoa in different functional states, e.g., immature vs. mature, uncapacitated vs. capacitated, normal vs. defective, all of which impact the reproductive potential of the male (Barazani et al. 2014, Gupta et al. 2014, Agarwal et al. 2014, du Plessis et al. 2011). More traditional techniques used to study sperm biology, such as western blotting, are limited by the reporting of average values (Hoppe et al. 2014) (see fig. 1). In addition, only a small percentage of ejaculated spermatozoa experience capacitation, and thus the fertilizing population is formed by a relative small sperm number that is easily masked by any technique other than single-cell analysis.

SINGLE CELL ANALYSIS IN SPERM PROTEOMICS: FLOW CYTOMETRY

Detailed descriptions of the sperm proteome of humans and other mammals are available (Swegen et al. 2015, Azpiazu et al. 2014, Amaral et al. 2014b, Amaral et al. 2014a) and provide useful information that can be used in single-cell analysis proteomic studies with the aid of flow cytometry. This approach allows consideration of the complex and heterogeneous nature of the ejaculate, thereby improving the quality of the information gathered. When signaling pathways in spermatozoa are considered, for example, only a fraction of the ejaculated spermatozoa undergoes capacitation (Escoffier et al. 2015). Due to the silencing of nuclear transcription in ejaculated spermatozoa, post-translational modifications of existing proteins are necessary for proper sperm function. These modifications include phosphorylation, glycosylation, disulfide cross-linking, ubiquitination, acetylation and methylation (Baker 2016).

Protein tyrosine phosphorylation plays an important role in capacitation. The oxidation of cholesterol facilitates its efflux from the plasma membrane, but oxidation also inactivates tyrosine phosphatases and the stimulation of cAMP production (Aitken 2011, Aitken and Curry 2011). The influx of calcium and bicarbonate ions also stimulates soluble adenylyl cyclase (sAC), which converts ATP to cAMP. In turn, sAC regulates protein kinase A (PKA), which phosphorylates proline-rich tyrosine kinase 2 (PYK2) and finally leads to a general increase in the tyrosine phosphorylation of many proteins (see (Baker 2016) for a detailed review).

Glycosylation of N-or O-linked residues is also a major post-translational modification in spermatozoa. This modification appears to play major roles in capacitation, together with a well-known role in the maturation of spermatozoa through epididymal transit and a means of spermatozoa to evade immune detection in the female reproductive tract

(Baker 2016). The formation of disulfide bonds, acetylation and ubiquitination are also important PTM in spermatozoa.

Techniques for single sperm cell analysis are required, ideally allowing the non-invasive quantification of molecular dynamics and sperm behavior over time. The dynamic and low-abundance nature of phosphorylated proteins makes the study of the phosphoproteome a challenge (Polat and Ozlu 2014). Miniaturized construction of analytical systems have been proposed to study the phosphoproteome at the single-cell level (Polat and Ozlu 2014).

Flow cytometry is now the central throughput method for simultaneous single-cell analysis of multiple proteins (Hoppe et al. 2014). Recent developments such as mass cytometry allow simultaneous detection of dozens of antigens in single cells (Nicholas et al. 2016, Spitzer and Nolan 2016). Although this technology is widely used in hematology, immunology and stem cell research, similar developments have not yet occurred in spermatology, even though similar developments can be envisioned. Similar to blood cells, spermatozoa are individual cells in suspension and are thus suitable for advanced flow cytometry. Phospho-proteomic approaches based on flow cytometry have been used in different fields of biology and medicine (Perez and Nolan 2006), and similar approaches are beginning to be used in spermatology (Gallardo Bolanos et al. 2014b, Pena et al. 2016, Perez and Nolan 2006, Krutzik et al. 2004). Flow cytometry is reinventing itself as a powerful tool for the study of sperm biology and clinical andrology, perhaps as a specific branch that could be termed *flow-spermetry*.

Single cell flow-proteomics and reproductive biotechnology

Phosphoflow cytometry has been defined as the use of fluorochrome-conjugated antibodies with molecules phosphorylated during signaling cascades, allowing the analysis of signaling networks within complex cell populations and providing quantitative data of phosphorylation events at the single-cell level (Davies et al. 2016, Krutzik and Nolan 2003, Hale and Nolan 2006, Oberprieler and Tasken 2011)

Phospho-proteomic approaches using flow cytometry have been used to disclose the role of the phosphorylation status of Akt on sperm survival after ejaculation (Gallardo Bolanos et al. 2014a, Gallardo Bolanos et al. 2014b). Because spermatozoa are transcriptionally silent cells that depend on post-translational modifications to regulate their function, the study of the sperm phospho-proteome is of utmost relevance. Proteins involved in apoptosis have been identified in proteomic studies (Amaral et al. 2014a), and flow cytometry has allowed the investigation of this pathway in spermatozoa. This particular area constitutes a good example on how large sets of proteomic data can be used in functional studies at the single flow-sperm cell level. In stallion spermatozoa, full-length and cleaved caspases 3 and 7 have been identified in our laboratory using western blotting, immunocytochemistry and flow cytometry (Ortega-Ferrusola et al. 2008). Functional studies have demonstrated that cleaved caspase 3 increases with cryopreservation, a procedure that is widely used in animal breeding but leads to accelerated sperm senescence. Experiments using multi-parametric flow cytometry have shed light on the mechanisms of this cryopreservation-induced premature sperm senescence, which at the same time demonstrates the value of this approach to complement proteomic studies in spermatology. Initial studies indicated that during cryopreservation, spermatozoa experience osmotic shock that leads to membrane

rupture and cell death, which results in an average 50% viability of spermatozoa during the procedure. The surviving spermatozoa experience further changes in physiology, resulting in a reduced lifespan in the female reproductive tract. Flow cytometry has demonstrated that this sperm subpopulation expresses active caspase 3, triggered by the loss of ROS homeostasis and the increased production of 4-hydroxynonenal (Martin Munoz et al. 2015). Other apoptotic proteins have been identified in mammalian spermatozoa, including cleaved poly ADP-ribose polymerase (Casao et al. 2015). Similar phenomena occur in other species, including humans (Thomson et al. 2009, Jeong et al. 2009, Martin et al. 2004, Pena et al. 2003). This approach also allows the identification of samples that will better survive cryopreservation (Martin Munoz et al. 2016), which is of great interest for animal breeding, for which the frozen semen market is a major component. This approach will greatly improve the profitability of the industry by reducing the number of low quality samples. Detailed research into the molecular and proteomic changes induced by cryopreservation is considered pivotal to improve this technology in humans and animals (Kopeika et al. 2015, Pena et al. 2011).

Single cell flow- proteomics and male fertility

Male factor infertility accounts for 30% of all infertility problems observed in couples requiring assisted reproduction, with 30% of the cases of male infertility classified as idiopathic. Moreover, delayed parenthood is linked to numerous problems in children born due to increased sensitivity to oxidative stress of spermatozoa in aged men (Charlton 2007, Tang et al. 2006, Tarin et al. 1998, Ozkosem et al. 2015, Morielli and O'Flaherty 2015). Traditional semen analysis is based on light microscopy and has been more recently aided by computerized systems and flow cytometry. Flow-proteomic

approaches have been recently incorporated to investigate the molecular basis of male infertility. Studies focused on the proteomics of asthenozoospermia have identified proteins associated with energetic metabolism, protein folding/degradation, vesicle traffic and the cytoskeleton (Amaral et al. 2014b). Detailed sperm-flow proteomics analysis has been conducted in this area; flow cytometry has allowed the study of α -tubulin acetylation in motile and immotile sperm fractions of normo- and asthenospermic individuals (Bhagwat et al. 2014). Whereas the subpopulation of immotile spermatozoa in normozoospermic patients showed no changes with respect to the motile population, the reduced levels of α -tubulin and acetylated α -tubulin were evident in asthenozoospermic patients. These observations suggest that this reduced acetylation is an inherent anomaly in these spermatozoa (Bhagwat et al. 2014), as tubulin acetylation is considered a PTM associated with microtubule stability. Flow-proteomic studies have also investigated proteomic changes related to asthenozoospermia in obese patients (Liu et al. 2015). Reduced expression of two proteins was evidenced in these patients: endoplasmic reticulum protein 57 (ERp57) and actin related protein (ACTRT2). Interestingly, fluorescence intensity of ERp57 and ACTRT2 (equivalent to protein expression level), was negatively correlated with Body Mass Index (BMI). At the same time, intensity was positively correlated with sperm motility. Angiotensin II type 2 receptor has been investigated using flow cytometry in human spermatozoa (Gianzo et al. 2016). The expression of this protein was reduced in asthenozoospermic patients. Sperm-borne oocyte activating factors (SOAF) are necessary to initiate intracellular calcium release and embryo development. Post-acrosomal WW binding protein (PAWP or WBP2NL) has recently been proposed as a major SOAF (Aarabi et al. 2015, Aarabi et al. 2014b), and its expression was studied using flow cytometry (Aarabi et al. 2014a). The levels of PAWP expression are

correlated with the fertilization rate and normal embryonic development after ICSI, which suggests that PAWP levels can be a valid biomarker for successful fertilization and preimplantation development after ICSI (Aarabi et al. 2014a). Interestingly, similar results have been reported in animals (Kennedy et al. 2014), and the development of a multiplex flow cytometry test detecting PAWP, ubiquitinated protein aggregates and acrosomal integrity has provided predictive values for conception rates (Kennedy et al. 2014).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is expressed as a type 2-membrane protein or a soluble cytokine. Although binding of TRAIL to its receptors induces an apoptotic response, other apoptosis-independent actions have been reported. The expression of TRAIL receptors (TRAIL1, TRAIL2, TRAIL3 and TRAIL4) was studied by flow cytometry (Zauli et al. 2014), and the presence of the soluble anti-inflammatory cytokine was investigated. This study provided evidence of pro-survival and anti-inflammatory actions for TRAIL. Studies from our laboratory support this finding, as TNF α was able to activate the phosphorylation of JNK, suggesting a pro-survival effect (Morillo Rodriguez 2015). Receptors implicated in the acquisition of sperm motility have also been detected using flow cytometry in epididymal spermatozoa. These include the expression of a forward motility stimulation factor (FMSF), and a glycoprotein that promotes progressive motility of spermatozoa characterized in the corpus, cauda and caput of the epididymis (Dey et al. 2014b, Dey et al. 2014a). Flow cytometry has been used to study other aspects of epididymal sperm maturation, such as the acquisition of hyaluronidase 2 from epididymal fluids, which, with its cofactor CD44, is necessary for fertilization. These factors permit the spermatozoon to penetrate extracellular matrix barriers containing hyaluronic acid

(Modelski et al. 2014). Other proteins detected using flow cytometry that are potential sperm biomarkers include a truncated form of KIT tyrosine kinase (Muciaccia et al. 2010), the spermatid specific thioredoxin-3 protein (SPTRX-3) (Buckman et al. 2009) other thioredoxins, peroxiredoxins, ubiquitin and ubiquitin-like modifier proteins (Sutovsky et al. 2015). Nuclear proteins have also been investigated using flow cytometry (Zhong et al. 2015). H2AX is a member of the H2A histone family of proteins; the phosphorylation of the Ser-139 of H2AX forming γ H2AX is an early cellular response to the induction of DNA double strand breaks. Measurement of γ H2AX using flow cytometry revealed that the levels of this protein were higher in male infertile patients, which indicated that this analysis can be a powerful assay for evaluating DNA double-strand breaks in human spermatozoa (Zhong et al. 2015). In conclusion, the use of flow cytometry is increasing in spermatology, from its initial use to analyze single or simple parameters, to current multi-parametric analyses and proteomic applications (see fig 2 as an example). The rapid advance of this technique will likely result in the development of more sophisticated assays that are able to provide information about the multiple compartments and functions of spermatozoa simultaneously in a single assay. Moreover, the development of computational flow cytometry (Saeys et al. 2016, Levine et al. 2015) applied to sperm analysis will facilitate the interpretation of data and the extraction of additional information from each particular analysis (fig 3), this approach has been recently applied to the study of the stallion ejaculate (Ortega Ferrusola et al. 2016).

The trade of semen is the basis of the animal breeding industry, and male factor infertility affects a large proportion of couples seeking reproductive medicine interventions. In human patients, only 20% of cases can be correctly diagnosed, while the rest are classified as idiopathic or of unknown origin (Amaral et al. 2014a).

However, semen analysis is still based on light microscopy to assess the sperm count, motility, morphology and membrane integrity based on dye exclusion assays. Many cases of male infertility remain undiagnosed. Recent developments in sperm biology are allowing new means to explore and diagnose this idiopathic infertility. The sperm proteome is being disclosed, and knowledge of the functionality of spermatozoa is also increasing. Discoveries in the sperm proteome using mass spectrometry approaches can be complemented with flow cytometry, allowing rapid assays to investigate sperm function at a single cell level that can be easily incorporated in andrology laboratories, facilitating the diagnosis of male infertility. In the animal breeding industry, flow cytometry assays based or completed with proteomic assays will largely improve the quality of doses, markedly increasing the profitability of the industry.

Implementation of these techniques (multiparametric and computational flow cytometry) combines the power of detection of multiple proteins with studies of protein functionality at the single cell level. This will lead to a rapid expansion of our knowledge of sperm biology, with applications to reproductive medicine and biotechnology. These facts will likely affect two major areas of development in spermatology; first diagnostic tests can be developed to target key proteins at a single-cell level in combination with functional assays. Second, our knowledge of sperm function will likely expand rapidly, resulting in better diagnostic assays, identification of therapeutically interesting targets and the potential development of treatments for male subfertility. Additionally, sperm biotechnologies will likely improve as a result of these developments.

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FIGURE LEGENDS

Fig 1. - Comparison of flow cytometry versus western blotting to identify individual proteins. In this experiment, four different expression levels of a protein are detected using western blotting. When the same protein is detected using flow cytometry, different situations can be detected; in one, the signal detected in WB can be due to a single population of spermatozoa expressing the protein of interest or to the presence of two populations of spermatozoa, one with high expression of the protein of interest and another population not expressing the protein; WB cannot discriminate between the two. Similar situations are depicted in 2-4 (Krutzik et al. 2004).

Fig 2.- Example of a multiparameter panel; in A spermatozoa are gated based in H33342 content and this gate is used to determine the integrity of the sperm membranes in B. In B three sperm subpopulations are easily gated representing live spermatozoa, and dead spermatozoa with a intermediate population classified as “?”. In C these three subpopulations are further investigated for the presence of active caspase 3 that only was present in the “?” population and in dead spermatozoa. In D three populations are classified in function of the presence of caspase 3, and the presence of 4-HNE in the is

investigated in D where overlay histograms show that 4-HNE is expressed in apoptotic spermatozoa.

Fig 3. - Example of computational cytometry applied to the analysis of protein expression at the single-cell level using Flow cytometry; data were analyzed using automatic classification of cellular expression by non linear stochastic embedding (ACCENSE). In figures A-F characteristics in raw semen are studied; in plots G-L characteristics of the same ejaculates after cryopreservation are depicted. A and F G represent t-SNE maps, each point represent an spermatozoa, B and H represent subpopulations identified in the ejaculate C and I represent the H33342 channel, D and J represent the Caspase 3 channel, E and K represent the ethidium channel, F and L represent the CellRox (identification of ROS) channel. A heat map at the right of each plot quantify the expression level of each marker.

Table 1. - Recent studies using flow cytometry to investigate sperm proteins in humans and animals

Study reference	Type of sperm	Protein studied	Main conclusions
Martin Muñoz et al 2015	Stallion Spermatozoa	Caspase 3	4-hydroxynonenal is related to caspase activation
Liu et al (2015)	Human spermatozoa (obese patients)	ERp57 and ACTRT2	Both proteins are reduced in obesity-induced asthenozoospermia
Bhagwat et al (2014)	Human spermatozoa, normozoospermic and asthenozoospermic patients	α -tubulin, acetylated α -tubulin and isoforms TUBA3C, TUBA4A and TUBA8	α -tubulin acetylation is associated with asthenozoospermia
Gallardo Bolaños et al (2014)	Stallion Spermatozoa	Phospho-Akt	De-phosphorylation of Akt leads to caspase 3 activation and sperm senescence
Ortega Ferrusola et al (2009)	Stallion Spermatozoa	Caspase 3	Caspase 3 is cleaved during

			cryopreservation
Yeung and Cooper (2007)	Human spermatozoa	K ⁺ Channels (KCNE1, KCNA5, KCNK5)	KCNE1 is the major channel regulating volume decrease in human spermatozoa
Gianzo et al (2016)	Human spermatozoa	Angiotensin II type 2 receptor (AT2R)	AT2R levels were lower in asthenozoospermic patients
Aarabi et al (2014)	Human spermatozoa	Postacrosomal WW binding protein (PAWP or WBP2NL)	PAWP expression levels were correlated with embryonic development after ICSI
Zauli et al (2014)	Human spermatozoa	Tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL1-4)	TRAIL 1-4 are expressed in human spermatozoa and soluble TRAIL may enhance sperm survival in capacitated spermatozoa
Kennedy et al (2014)	Bull spermatozoa	Postacrosomal WW binding protein	PAWN is a potential biomarker

		(PAWP or WBP2NL)	of bull fertility
Zhong et al 2016	Human spermatozoa	γ H2AX	γ H2AX levels are higher in the sperm of male infertility patients. Evaluation of this protein using FC can be a useful biomarker of double strand breaks in DNA
Casao et al 2015	Ram spermatozoa	Cleaved poly ADP ribose polymerase (cPARP)	Apoptosis inducers staurosporine or betulinic acid increase cPARP in ram spermatozoa