Counting sperm does not add up any more: time for a new equation?

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Abstract

Although sperm dysfunction is the single most common cause of infertility, we have poor methods of diagnosis and surprisingly no effective treatment (excluding assisted reproductive technology). In this review, we challenge the usefulness of a basic semen analysis and argue that a new paradigm is required immediately. We discuss the use of at-home screening to potentially improve the diagnosis of the male and to streamline the management of the sub-fertile couple. Additionally, we outline the recent progress in the field, for example, in proteomics, which will allow the development of new biomarkers of sperm function. This new knowledge will transform our understanding of the spermatozoon as a machine and is likely to lead to non-ART treatments for men with sperm dysfunction.

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Introduction

This review starts with the premise that there is a clear need to dramatically improve our understanding of the cellular and molecular basis of sperm function. This knowledge is fundamental for two key developments in male fertility: firstly, to provide the basis for effective diagnostic tools and, secondly, to facilitate the study of the physiology of abnormal/dysfunctional cells, which is central for developing rational, non-ART therapy. We initially discuss the developments in male fertility testing with particular reference to the potential role of at-home testing in the sub-fertile patient's pathway. Secondly, we discuss advances in potential markers involved in key physiological processes, such as capacitation and sperm proteomics, which are likely to transform our understanding of the normal functional cell and lead to improvements in diagnosis and developments of rational therapy.

Epidemiological data show that 1:7 couples are classed as sub-fertile (Hull *et al.* 1985, Templeton *et al.* 1990). Sperm dysfunction is the single most common cause of infertility and affects approximately 1:15 men (HFEA 2005, www.hfea.gov.uk). Studies using semen assessment as the

criteria for sub-fertility (sperm concentration $<20 \times 10^6/\text{ml}$) show that 1:5 18-year-olds are classed as sub-fertile (Andersen *et al.* 2000). This is a high proportion of the population compared with other prevalent diseases such as diabetes (2.8% of the population; Wild *et al.* 2004). Thus, male sub-fertility is a very significant global problem and, what is most worrying is that the recent reports suggest that its prevalence is increasing (Sharpe & Irvine 2004).

Improving and evaluating the diagnosis of male fertility, in particular, sperm dysfunction

There is an urgent requirement to develop new and robust tests of sperm function to accurately diagnose male infertility. The value of traditional semen parameters (concentration, motility and morphology) in the diagnosis and prognosis of male infertility has been debated for 60 years and, perhaps not surprisingly, the debate intensifies (see Bjorndahl & Barratt 2005 for detailed discussion). There are clear issues over concordance with standardized procedures (Holt 2005, Pacey 2006) with differences undoubtedly leading to uncritical reporting of results and thus precipitous

decline in usefulness of the tests. A stark warning presents itself: if the professionals cannot successfully turn around the current low performance in the majority of Andrology laboratories, semen analysis will become undervalued and a redundant procedure. Unquestionably, even with appropriate quality assurance, traditional semen parameters can only provide a limited degree of prognostic and diagnostic information for the infertile couple (Tomlinson *et al.* 1999), primarily at the lower ranges of the spectrum (Comhaire 2000). The insanity that surrounds perceived absolute values, e.g. 5% 'normal' forms is just that – insanity. Continual discussion and generation of putative cut-off values will lead to further irrelevant and misleading information.

It is, therefore, necessary to develop simple, robust and effective tests of sperm function. Yet, despite the plethora of potential assays available, results have been very disappointing (Muller 2000, Aitken 2006). Recent data suggest that only three potential tests of sperm function have sufficient data to support their routine use: penetration into cervical mucus (or e.g. hyaluronate; Ivic *et al.* 2002), measurement of reactive oxygen species production/lipid peroxidation (Ford 2004, Williams & Ford 2005) and estimate of sperm chromatin/DNA damage (Seli & Sakkas 2005); however, promising initial data for the latter is now being questioned (see Bungum *et al.* 2004, Gandini *et al.* 2004, Erenpreiss *et al.* 2006, Makhlof & Niederberger 2006).

After decades of research, the primary reason for the paucity of effective and robust estimate of sperm function is our limited basic understanding of the functioning of the spermatozoon (Ford 2001, Conner *et al.* 2007). Additionally, there has been inappropriate and uncritical use of the current tools (Barratt 1995, Tomlinson *et al.* 1999, Conner *et al.* 2007). However, there are a number of new developments which promise to transform our diagnostic and treatment pathways (for examples see Aitken 2006, Jimenez-Gonzalez *et al.* 2006, Publicover *et al.* 2007). Two of these are 1) development of at-home testing and 2) biomarkers for sperm function.

A new paradigm in male fertility diagnosis: the development of at-home testing

Many men find the production of a semen sample for infertility investigations an embarrassing, difficult and stressful experience. To add to this, there can be a significant waiting time for an initial appointment at the hospital and an additional time delay before the results are available. All these factors heighten the anxiety associated with infertility investigations, often delaying investigations and subsequent treatment and consequently reducing the chances of success (HFEA 2005, www.hfea.gov.uk). The development of an over-the-counter home sperm test, which would allow the patient to obtain an assessment of fertility potential at their own

convenience in the comfort of their home, has a number of benefits including potentially increasing the number of men who are tested and speeding up the diagnosis.

Several putative tests are available but the primary questions regarding home sperm testing are accuracy and reliability. In our laboratories, we have concentrated on developing a test (Fertell; Bjorndahl *et al.* 2006) based on assessing the concentration of progressively motile sperm, which is one of the most predictive parameters for estimating natural fertility in both sub-fertile (review Tomlinson *et al.* 1999) and normal couples (Larsen *et al.* 2000, Zinaman *et al.* 2000; Fig. 1). In addition, we wanted an assay that would provide an assessment of the functional capacity of the sperm as a small proportion of men with normal semen parameters (e.g. >10 million progressively motile sperm/ml semen) have dysfunctional sperm (see Barratt *et al.* 1989). Briefly, we mimicked penetration into human cervical mucus *in vitro* using hyaluronic acid, a known cervical mucus substitute for sperm function studies (Aitken *et al.* 1992). Our analysis show that the test is accurate (~95%) and patients find it easy to use (see Bjorndahl *et al.* 2006 for details).

Currently, we do not know what proportion of men with normal semen parameters but defective cells (so called hidden male factor patients – see Barratt & Publicover 2001) would be detected by our assay. However, as the number of these men is small, it is not a pivotal issue. What is a critical factor is where (and how) such home testing fits in the diagnostic pathway. Providing home testing is accurate then it should have a well-defined place in the screening of sub-fertile couples. The evaluation of diagnostic tests is now subject to renewed critical examination (Gluud & Gluud 2005). Diagnostic tests can be used as replacement, triage or add-on with their usefulness being dependent on a large number of factors (Bossuyt *et al.* 2006). Home sperm testing has not been evaluated in this critical manner and thus we are uncertain where in the pathways it fits best. Our analysis suggests it as a first-line initial investigation. Based on our preliminary data of ~95% accuracy, the likelihood is that men who test positive (red line – thus >10 million progressively motile sperm/ml semen) will not require a semen assessment unless specific, rare circumstances suggest otherwise. The couples (if the female is normal) may be encouraged to try longer (Steures *et al.* 2006). Men with a red line would certainly be suitable for intrauterine insemination and possible *in vitro* fertilization (IVF), but not intracytoplasmic sperm injection (ICSI). This paradigm would have a significant cost saving (~28% men in our tertiary referral centre would be positive) as no initial screening semen analysis would be required. Men who were negative (no red line) would urgently require a high-quality comprehensive semen assessment (WHO 1999). This diagnostic pathway requires validation to determine if home testing can be used to improve the pathway of

Figure 1 Diagrammatic representation of the home sperm test. Progressively motile sperm swim from semen (yellow) into hyaluronate (HA) solution (blue) via a mesh (red dotted line). Sperm are then released into capillary channel, labelled with anti-CD 59 colloidal gold conjugate (red sperm) and trapped on the nitrocellulose strip where a visible line is formed if sufficient numbers of progressively motile sperm have migrated into the HA solution. Red solid line around the green capsule is the heater to keep the cells at 37°C. A red line is visible if > 10 million motile cells/ml of semen are present in the ejaculate (Adapted from Bjorndahl *et al.* 2006).

infertility investigations and provide cost-effective analysis (Fig. 2).

Home fertility testing for the man is now a reality and combined with the plethora of tests available for women: urinary luteinizing hormone (Barratt *et al.* 1989, Robinson *et al.* 1992), follicle-stimulating hormone dipsticks (www.fertell.co.uk) and complex urinary monitors; there is the potential, as yet untested, to revolutionize the diagnostic and treatment pathways for the sub-fertile couple.

Biomarkers for sperm function

Events associated with sperm capacitation

After deposition in the female tract, mammalian sperm undergo a number of functional and structural changes termed 'capacitation', which render the cells competent to fertilize. Capacitation can also be induced *in vitro* by incubation, usually for some hours, in suitable medium. The most widely accepted functional definition of *in vitro* capacitation is acquisition of ability to undergo acrosome reaction in response to a biological agonist, such as zona pellucida or progesterone. Studies on sperm incubated under capacitating conditions have revealed numerous biochemical and physiological changes that accompany the process including an efflux of plasma membrane cholesterol, an increase in the activity of adenylate cyclase (both soluble and membrane localized), elevated levels of cAMP and protein kinase A (PKA) activity, a rise in intracellular pH, hyperpolarization of membrane potential and increased serine/threonine and tyrosine phosphorylation of some proteins (Tash & Means 1983, Leclerc *et al.* 1996, Cross 1998, Osheroff *et al.* 1999, Lefièvre *et al.* 2002, Visconti *et al.* 2002, O'Flaherty *et al.* 2004, Fraser *et al.* 2005, Moseley

et al. 2005). These observations provide useful indicators of the occurrence of capacitation in spermatozoa *in vitro*, but it is still far from clear how the various events relate to each other or whether all of them must occur for the acquisition of fertilization competence to occur. It is likely that sperm possess the ability to regulate the signalling pathways involved in capacitation, thus minimizing over-capacitation and premature acrosome reaction (Visconti *et al.* 2002, Ecroyd *et al.* 2004, De Jonge 2005, Fraser *et al.* 2005), but it is not clear to what extent the changes that have been observed in capacitating cells are reversible and whether they are capacitation endpoints or part of the capacitation process (such that reversal does not prevent subsequent induction of acrosome reaction).

We have recently demonstrated that upon transfer of cells from non-capacitating to capacitating media (CM), PKA activity, serine/threonine phosphorylation and $[Ca^{2+}]_i$ -signalling 'switch' between minimum and maximum rapidly (as fast as detectable; Fig. 3; Bedu-Addo *et al.* 2005, Moseley *et al.* 2005). In contrast, the occurrence of tyrosine phosphorylation of tail proteins requires up to 3 h to reach its maximum. Progesterone-induced acrosome reaction also rises much more slowly, possibly because of the latency of tyrosine phosphorylation of head proteins (Fig. 3; Bedu-Addo *et al.* 2005). We have also shown that different CM vary strikingly in their efficacy as IVF fertilization media was shown to accelerate sperm capacitation where both tyrosine phosphorylation and acrosome reaction reached a maximum after only 90 min (Moseley *et al.* 2005). This effect did not seem to be associated with enhanced activation of PKA or increased levels of serine/threonine phosphorylation suggesting factor(s) acting through signalling pathways other than the well-characterized

Figure 2 A new paradigm for sperm testing. The first step is a simple screening test (Fertell or similar), designed to distinguish men who are probably fertile from those where further investigation is indicated. If the man's semen passes the test and his partner is normal they can continue trying to conceive. The minority that experience prolonged infertility should be offered ART but in future improved tests that lead to other effective treatment. If the semen fails the screening test then the result will need to be confirmed in the laboratory, perhaps by a standard semen analysis supplemented by a sperm function test. If the screening test proves to be incorrect (i.e. the man has a large number of motile cells), the couple can continue trying. In some cases, men may have severe defects e.g. severe oligozoospermia, globozoospermia, in which case immediate referral for ICSI will usually be the best option. However, most men who reach this stage are likely to have low but adequate sperm numbers with impaired sperm function. Proteomics and other novel research tools will allow development of a battery of tests to diagnose the underlying cause of poor sperm function and to direct them to appropriate therapy (see section – Biomarkers of sperm function in text). It may be possible to treat the man, but it may be easier to develop ways to treat sperm *in vitro* to increase success rates in IUI. Only if appropriate therapy remains unsuccessful after a reasonable period (probably 6–12 cycles depending on age and other factors), would the couple be referred for IVF/ICSI.

activation of soluble adenylyl cyclase/cAMP/PKA (Moseley *et al.* 2005). Moreover, we have also demonstrated that, in human spermatozoa, progesterone-induced $[Ca^{2+}]_i$ signalling, protein serine/threonine phosphorylation, protein tyrosine phosphorylation and progesterone-induced acrosome reaction are all reversibly regulated by the external environment (Fig. 3; Bedu-Addo *et al.* 2005). Human spermatozoa are therefore capable of repeated and reversible cycles of many of the events that occur in response to capacitating conditions and have a high degree of plasticity and

adaptability in their responses to events which signal ovulation.

Tyrosine phosphorylation of sperm proteins is a well-established and widely-used marker for cells undergoing capacitation (Visconti *et al.* 1995a, 1995b, Leclerc *et al.* 1996, Osheroff *et al.* 1999). In our recent experiments, we showed that when spermatozoa were incubated in CM for 6 h and then resuspended in CM without bicarbonate or CM without albumin for an additional hour, the cells lost their ability to undergo acrosome reaction (Bedu-Addo *et al.* 2005). However, the absence of either albumin or

Figure 3 Proposed sequence of events during induction and reversal of capacitation *in vitro*. Under capacitating conditions, PKA activity (pink line), serine/threonine phosphorylation (green line) and $[Ca^{2+}]_i$ -signalling (blue line) 'switch' between minimum and maximum in minutes. In contrast, acquisition of competence to undergo progesterone induced AR (red line) and the occurrence of tyrosine phosphorylation (yellow line) of tail proteins requires up to 3 h to reach maximum. PKA levels drop over the subsequent 5 min before reaching a plateau at 30 min. Tyrosine phosphorylation (yellow line) and progesterone-induced acrosome reaction (AR, red line) rises more slowly, taking up to 3 h to reach a maximum. Upon return to NCM, serine/threonine tyrosine phosphorylation reverses slowly (approximately 3 h), but the progesterone-induced $[Ca^{2+}]_i$ signal falls to minimal levels within minutes. Progesterone-induced AR also falls to levels similar to those seen in uncapacitated cells in less than an hour of re-suspension. The kinetics of this change are shown to follow those of the $[Ca^{2+}]_i$ response such that AR is dependent on the development of tyrosine phosphorylation during capacitation and on the decay of the $[Ca^{2+}]_i$ signal during decapacitation. (Adapted from Bedu-Addo *et al.* 2005, see also Bedu-Addo *et al.* 2007).

bicarbonate did not prevent protein tyrosine phosphorylation in these cells. Levels of tyrosine phosphorylation were comparable to the ones observed in CM medium (Bedu-Addo *et al.* 2005). These results support the use of sperm protein tyrosine phosphorylation as a marker of cells undergoing capacitation (Visconti *et al.* 1995a, 1995b, Leclerc *et al.* 1996, Osheroff *et al.* 1999), but suggest that tyrosine phosphorylation is not a diagnostic marker of capacitated cells.

There is little doubt that failure of capacitation is a cause of infertility in a subset of patients. However, the complexity of the process involved is such that it is not yet possible to identify a reliable marker of capacitated (competent to fertilize) sperm. The complexity of 'capacitation' may even be such that no single reliable marker exists.

Calcium oscillations in sperm – a new signalling system?

We have recently reviewed and discussed at length the exciting developments in calcium (Ca^{2+}) signalling in the spermatozoon (Harper & Publicover 2005, Jimenez-Gonzalez *et al.* 2006, Publicover *et al.* 2007). We believe that sperm intracellular calcium ($[Ca^{2+}]_i$) oscillations are functionally significant, modulating flagellar activity in a way that promotes functional sperm motility changes (Harper & Publicover 2005). Intriguingly, the ability of cells to generate these complex $[Ca^{2+}]_i$ signals does not seem to be noticeably related to capacitation. Perhaps, this should not be surprising. The prime marker of capacitation is acrosome reaction, and the Ca^{2+} store

mobilization that occurs during oscillations raises $[Ca^{2+}]_i$ in the neck and midpiece regions of the sperm and affects the flagellum but does not induce acrosome reaction (Harper *et al.* 2004, Bedu-Addo *et al.* 2007). Similarly, hyperactivation, though often associated with capacitation, can be induced separately and occurs by a separate, Ca^{2+} -dependent, signalling pathway (Marquez & Suarez 2004). Our recent findings suggest that two factors, prior filling of the Ca^{2+} store in the sperm neck region and nitrosylation of sperm proteins, predispose the sperm to generate $[Ca^{2+}]_i$ oscillations and consequent effects on flagellar activity. Thus, the ability of the sperm to generate complex Ca^{2+} signals is apparently regulated, but by mechanisms separate to those that underlie classical 'capacitation'. Identification of features that are diagnostic of this regulation may provide a new way to identify sperm that are capable of generating complex patterns of flagellar activity and provide the first step to being able to manipulate these patterns using specific drugs.

The sperm proteome: the potential for new biomarkers of male fertility and a transformation in our understanding of the spermatozoon as a machine

The comprehensive and systematic identification and quantification of proteins expressed in cells and tissues are providing important and fascinating insights into the dynamics of cell function (Chu *et al.* 2006, Rifai *et al.* 2006). Yet, although spermatozoa are ideal to study from a proteomic perspective, until very recently, there have

been relatively few studies examining the proteome of the spermatozoa (Ainsworth 2005, Conner *et al.* 2007). By virtue of the rudimentary technology available, initial human sperm proteomic studies were relatively crude. Many studies used antispermatid antibody sera in an attempt to detect potential novel (and functional) sperm targets for male contraception. Unfortunately this rational approach met with limited success (Naaby-Hansen *et al.* 1997, Shetty *et al.* 1999, Shibahara *et al.* 2002; review in Conner *et al.* 2007). Perhaps surprisingly, recent studies employing more sophisticated proteomic profiling have similarly failed to identify interesting targets (Bhande & Naz 2007). It is likely that we need to redefine the nature of immunological infertility before this strategy will yield meaningful results. The more recent experiments have followed four main themes: (1) identification of proteins associated with specific events in sperm function, (2) examination of specific structures and associated signalling complexes, (3) whole proteome investigation and (4) comparison with normal versus abnormal (fertile versus sub-fertile).

Identification of proteins associated with specific events in sperm function. It has been 12 years since the discovery of tyrosine phosphorylation as a putative marker of capacitation (Visconti *et al.* 1995a, 1995b; see above), but the role of these proteins and their sequence of activation is still very sketchy with the exception of only a small number of candidate proteins (see Naz & Rajesh 2004). Until recently, we were a long way from obtaining even a minimal 'picture' of events. However, several recent proteomic studies have provided a wealth of information which has the capacity to transform our potential understanding of protein changes associated with capacitation. For example, examining the phosphoproteome of human sperm during capacitation has provided some interesting novel targets. In addition to A-kinase-anchoring proteins (AKAPs; AKAP3 and AKAP4) and calcium-binding tyrosine-phosphorylation regulated protein, several other interesting proteins: heat shock protein 70 and 90 α suggested as being involved in sperm egg interaction and valosin-containing protein (VCP/p97) a member of the ATPases associated with various cellular activities were detected (Ficarro *et al.* 2003). Interestingly, tyrosine phosphorylation of VCP/p97 regulates its sub-cellular localization and its localization changes from the neck to the head after capacitation (Ficarro *et al.* 2003). Several other groups of proteins such as enzymes, structural proteins and channels were also identified. Moreover, with the pivotal role of tyrosine phosphorylation in sperm capacitation it is important to identify the kinases involved. Lalancette *et al.* (2006) used an affinity chromatography approach combined with proteomic analysis on the cytosolic fraction of bovine sperm to enrich tyrosine kinase activity. Tyrosine kinases from three families were identified: Src (Lyn), Csk and Tec (bmX, Btx). In addition, other important proteins involved in different cellular events associated with sperm, such as capacitation and acrosome reaction were also identified

(a total of 126 proteins). These proteins include: actin polymerization, chaperones, proteases, protease inhibitors, metabolism, vesicle transport/membrane fusion, signal transduction, sperm-egg interaction and structural proteins. Surprisingly, a number of sperm RNA binding/transport proteins were also identified. In view of the recent suggestions of the potential role of RNA in early embryo development, these could be an important surreptitious finding (Miller *et al.* 2005).

Stein *et al.* (2006) performed a selected proteomic analysis of mouse sperm of the regions that are likely to be involved in mediating sperm-egg interactions. Sub-cellular fractionation included enrichment of acrosomal contents and plasma membrane. In the acrosomal contents, 166 proteins were identified and, as expected, two of the largest groups were enzymes and their inhibitors and secretory proteins. Twenty-five percent of the proteins were novel. Two hundred twenty-nine proteins were identified from the vesicle fraction and 171 from biotinylation fraction. Interestingly, a significant number of the biotinylated proteins were ion channels, most of which had unknown function.

Examination of specific structures and associated signalling complexes. Nomura & Vacquier (2006) performed immunoprecipitation experiments for soluble adenylate cyclase (sAC) to determine the proteins associated with sAC in the sea urchin flagellum. Ten proteins were tightly associated with sAC including guanylyl cyclase and cGMP specific phosphodiesterase (PDE5A). This approach provides a critical starting point demonstrating the potential role in protein phosphorylation and ion channel activities in sperm motility.

There are a number of studies examining the proteomic composition of the 9+2 arrangement of the cilium. For example, Pazour *et al.* (2005) performed a proteomic analysis of *Chlamydomonas reinhardtii* flagella documenting the basic building blocks (for updates go to <http://labs.umassmed.edu/chlamyfp/index.php>). As the 9+2 structure is relatively conserved throughout evolution, you would expect a similar proteome across species. Yet, although there is remarkable conservation across the phyla, detailed proteomic studies on, for example, the flagella of trypanosomes show surprising diversity among the 300 or so proteins forming the axoneme (Broadhead *et al.* 2006). Interestingly, comparing the proteomes of *Trypanosoma brucei* with *C. reinhardtii* classified only 49 proteins to be common. 249 proteins were present but not in *C. reinhardtii* and 203 proteins present in *C. reinhardtii* but not in *T. brucei*. Further studies are required to determine the role of 'organism specific elaborations'. Sperm proteomic techniques are still refining the composition of the axonemal structures, e.g. outer dynein arms (Hozumi *et al.* 2006). Such studies, used in comparisons across species, are likely to provide a detailed understanding of the basic molecular motor apparatus. For example, comparisons between mutants of *Chlamydomonas* can

provide a powerful analysis of the defects present in the sperm of sub-fertile men and identify potential interactions of novel signalling complexes (see Zhang *et al.* 2004). Detailed imaging of the flagella motion (Ohmuro & Ishijima 2006) will allow a clear understanding of how the dynamics of the signalling complexes affect waveform. In addition to the studies on the axoneme, there is also data on the sperm accessory structures (fibrous sheath, outer dense fibres). For example, Cao *et al.* (2006) identified 50 proteins associated with these structures dramatically enhancing our knowledge of localization of both expected and unexpected complexes. This study confirmed that the fibrous sheath is a dynamic structure having a fundamental role in signalling, metabolism and oxidative stress. A proteomic study of the human fibrous sheath identified a unique ADP/ATP carrier protein and seven glycolytic enzymes previously unreported in the human fibrous sheath confirming that the fibrous sheath is a complex structure intimately involved in energy transduction (Kim *et al.* 2007). Such studies will lead to a new understanding of sperm metabolism and open exciting avenues for research potentially addressing 'old' questions such as the role of glucose in sperm energy metabolism (Ford 2006).

Whole proteome investigation. So far, there are only two studies addressing the complete human sperm proteome (Johnston *et al.* 2005, Martinez-Heredia *et al.* 2006). Using a two-dimensional gel analysis followed by mass spectrometry, Martinez-Heredia *et al.* (2006) identified 98 different proteins. A significant number was associated with proteasome turnover providing further evidence for the existence of the proteasome in human sperm function (Sutovsky *et al.* 2004). Interestingly, the second most abundant group of proteins were those involved with transcription, protein synthesis and turnover and protein transport/folding. As the spermatozoon is supposed to be transcriptionally and translationally silent, this may reflect a role for sperm RNA in the egg (see above and Lalancette *et al.* 2006, Martinez-Heredia *et al.* 2006). Alternatively, the dogma that the sperm are translationally dormant may need to be reassessed in the light of recent preliminary evidence that they can both synthesize and turnover proteins (Gur & Breitbart 2006).

Johnston *et al.* (2005) adopted a direct one-dimensional SDS-PAGE approach with liquid chromatography tandem mass spectrometry performed on the various protein bands from detergent soluble and insoluble fractions of human spermatozoa. They identified 1760 proteins with high confidence – representing 76% of the proteins predicted to be in sperm. This represents the most comprehensive sperm proteome to date. Interestingly, the greatest number of proteins characterized was either novel proteins or proteins for which no ontology was available providing a wealth of new information. Of particular interest was the significant number of tissue-specific proteins as these could potentially be used as

targets for male contraception. It is likely that a number of studies reporting/refining the whole-sperm proteome will become available in the next 12 months allowing a comprehensive first draft of the mature cell to be available. In addition to studies on the sperm proteome, a number of authors have examined the protein profile of seminal plasma. In the most comprehensive study to date, Pilch & Mann (2006) identified 923 proteins with high confidence. Not surprisingly, the most abundant proteins were those associated with involvement in clot formation, metabolism and protection of the sperm cell. Potentially, this dataset could be used for biomarker discovery for testicular cancer, prostate cancer and for male infertility. Once a subset of protein markers is available, it may be feasible to perform protein chip diagnoses as used for cancer (Ciordia *et al.* 2006).

Comparison with normal versus abnormal (fertile versus sub-fertile). In our laboratory, we have been using proteomic strategies to identify defects in sperm function responsible for fertilization (Lefièvre *et al.* 2003, Pixton *et al.* 2004, reviewed Conner *et al.* 2007). Specifically, we are interested in identifying differences in sperm protein expression between control (fertile) men and patients with spermatozoa that failed to fertilize oocytes *in vitro*. Our initial studies showed, surprisingly, relatively little intra- and inter-donor variation and we have categorized one man (Pixton *et al.* 2004) where we identified 20 differences from the control that we are confident would represent true differences. Examination of five more men has found several consistent differences in protein expression levels (Conner *et al.* 2007) but further studies are required to determine if these differences relate to sperm function and where in the pathways these defects are manifested. Interestingly, with the clear need to identify (and improve) the fertility potential of animals in the agricultural industry, there is a concentration of effort to identify factors responsible for high fertility. For example, a study in bulls showed a significant effect of seminal plasma proteins on the function of the sperm, i.e. seminal plasma from high fertility bulls can act to improve the fertilizing potential of low-fertility bulls (Henault *et al.* 1995). Detailed proteomic studies of accessory gland fluids have shown consistent differences in high-/low-fertility groups, where four interesting proteins were identified: bovine seminal plasma 30 kDa (sperm capacitation), osteopontin (sperm-oolema interaction), phospholipase A2 (acrosome reaction) and spermadhesin Z13 (sperm motility) as contributing to the higher fertility (Moura *et al.* 2006, 2007a, 2007b). Examination of fertility in bulls is a very powerful approach as it allows *in vitro* studies to be complemented with data *in vivo*. Such complementary experiments cannot be performed in humans. The identification of proteins in the accessory glands of bulls contributing to fertility will allow a detailed understanding of how the sperm cell is modified by extrinsic factors.

How can all this detailed information be used to improve diagnosis and treatment of male infertility?

There has been a dramatic increase in our knowledge of the protein composition of the spermatozoon, its structures and the surrounding fluids (epididymal, vesicular, prostate) contributing to its function. Additionally, we have preliminary insight into the signalling complexes involved in key physiological processes, e.g. capacitation and the regulation of sperm motility. The difficulty is that this knowledge represents only but a starting point, somewhat analogous to knowing all the genes in the human genome but uncertain of their role and interactions. Detailed physiological experiments are required to fully understand the biological function of these proteins (and complexes) in the mature cell and its interaction with its functional environment – the female tract and human egg. The data generated by proteomic analysis is leading to a dramatic increase in our understanding (and by its nature re-evaluation) of sperm cell function. As the current methods to assess sperm function are inadequate, a key objective will be to develop more effective biomarkers as has been done in other diseases (reviewed in Rifai *et al.* 2006). For example, if we can successfully adopt redox proteomics to sperm then we may be better able to identify men who will benefit from antioxidant therapy. If we apply critical validation methods to evaluate these new biomarkers, we can expect a dramatic change in the way we diagnose male fertility.

In addition, knowledge of the cell proteome (combined with metabolomics) will allow us, for the first time, to be able to develop rational therapies for sperm dysfunction. Remarkably, although, male infertility is an important health issue, there are no drug treatments to enhance sperm function that have been shown to be effective in randomized controlled trials (Kamischke & Nieschlag 2002, cf. Greco *et al.* 2005). The only treatment option for the sub-fertile man is IVF/ICSI. ART is very expensive, invasive, has limited success, a number of side effects, is not widely available, and poses significant concerns about the long-term health of children (Maher 2005; review Hansen *et al.* 2005). This means that, put simply, as a result of our ignorance of the causes of sperm dysfunction, we are currently subjecting an increasing proportion of women to inappropriate invasive therapy in order to treat their partners. Hopefully, in the near future, perhaps using high throughput screening tools, we will be able to exploit our new knowledge base to develop and test new drug regimes for the enhancement of sperm function and avoid unnecessary ART treatment.

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