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REVIEW

Proteomics, oxidative stress and male infertility

Ashok Agarwal [a,*], Damayanthi Durairajanayagam [a,b], Jacques Halabi [a], Jason Peng [a], Monica Vazquez-Levin [c]

[a] *Center for Reproductive Medicine, Glickman Urological and Kidney Institute, Cleveland, OH, USA;* [b] *Faculty of Medicine, MARA University of Technology, Sungai Buloh, Selangor, Malaysia;* [c] *Institute of Biology and Experimental Medicine, National Research Council of Argentina, CONICET, Buenos Aires, Argentina*
* Corresponding author. *E-mail address:* agarwaa@ccf.org (A Agarwal).

Ashok Agarwal is a Professor at the Lerner College of Medicine, Case Western Reserve University and the Head of the Andrology Center and Director of Research at the Center for Reproductive Medicine, Cleveland Clinic, USA. He has researched extensively on oxidative stress and its implications on human fertility. He serves on the editorial boards of several key journals in human reproduction. Ashok's current research interests are the study of molecular markers of oxidative stress, DNA fragmentation and apoptosis using proteomics and bioinformatics tools, as well as fertility preservation in patients with cancer, and the efficacy of certain antioxidants in improving male fertility.

Abstract   Oxidative stress has been established as one of the main causes of male infertility and has been implicated in many diseases associated with infertile men. It results from high concentrations of free radicals and suppressed antioxidant potential, which may alter protein expression in seminal plasma and/or spermatozoa. In recent years, proteomic analyses have been performed to characterize the protein profiles of seminal ejaculate from men with different clinical conditions, such as high oxidative stress. The aim of the present review is to summarize current findings on proteomic studies performed in men with high oxidative stress compared with those with physiological concentrations of free radicals, to better understand the aetiology of oxidative stress-induced male infertility. Each of these studies has suggested candidate biomarkers of oxidative stress, among them are DJ-1, PIP, lactotransferrin and peroxiredoxin. Changes in protein concentrations in seminal plasma samples with oxidative stress conditions were related to stress responses and to regulatory pathways, while alterations in sperm proteins were mostly associated to metabolic responses (carbohydrate metabolism) and stress responses. Future studies should include assessment of post-translational modifications in the spermatozoa as well as in seminal plasma proteomes of men diagnosed with idiopathic infertility.
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Introduction

Infertility is defined as the inability to achieve a clinical pregnancy after 12 months or more of regular, unprotected and well-timed intercourse (Practice Committee of American Society for Reproductive Medicine, 2013). Infertility affects around 15% of all couples of reproductive age, with about 50% being associated with abnormalities in the male, called male factor infertility (Sabanegh and Agarwal, 2012). A recent study using the current duration approach to assess the prevalence of infertility estimated that 9 to 14% of American men within reproductive age (i.e. 15 to 44 years old) will probably experience difficulties to conceive (Louis et al., 2013). Male infertility could result from dysfunction at various levels along the hypothalamic-pituitary-gonadal axis: pre-testicular (damage at the hypothalamus or pituitary level), testicular (failure of the testis), post-testicular (normal testicular function but with obstruction or inflammation that leads to infertility) or a combination of these. Causes of male infertility include hypogonadotrophic hypogonadism and Kallmann syndrome, direct trauma, inflammation or infection of the testis, varicocele, cryptorchidism, Y-chromosome microdeletions, testicular cancer and chemotherapy, erectile dysfunction, infrequent or retrograde ejaculation, epididymitis, congenital bilateral absence of the vas deferens, Klinefelter's syndrome (47,XXY), and Sertoli-cell only syndrome (Wiser et al., 2012).

The aetiology of male factor infertility, although multifactorial, remains largely idiopathic (Sabanegh and Agarwal, 2012). Reactive oxygen species (ROS)-induced oxidative stress is well-known to play a major role in male factor infertility (Hamada et al., 2012; Tremellen, 2008). Excess ROS concentrations and oxidative stress in the male reproductive tract are detrimental to spermatozoa (Aziz et al., 2004) and have been associated with negative changes in sperm concentration, motility and morphology, leading to poor semen parameters and eventually to infertility (Khosrowbeygi and Zarghami, 2007). In fact, oxidative stress has been implicated in several male infertility-associated pathologies, including leukocytospermia and varicocele as well as idiopathic infertility (Pasqualotto et al., 2000).

The diagnosis of male infertility routinely begins with a basic semen analysis, which measures various semen parameters including semen volume, colour, pH, liquefaction time, viscosity, sperm count and motility, sperm morphology, concentration of round cells and polymorphonucleocytes, sperm agglutination and sperm viability (if required). Two or more of these basic semen analyses are used to identify abnormalities in: sperm concentration (oligozoospermia or azoospermia), motility (asthenozoospermia) and morphology (teratozoospermia), based on reference values established by the World Health Organization (WHO, 1999, 2010). In addition to the routine evaluation, several advanced tests can be performed to establish the causes(s) of infertility, among them are the assessment of ROS levels, total antioxidant capacity and sperm DNA fragmentation level, DNA compaction and apoptosis, as well as presence and localization of antisperm antibodies and genetic testing (Kovac et al., 2013). However, results of these tests typically either fall within the normal range or do not help determine an exact aetiology of infertility, leading to a classification of 'idiopathic infertility' (Kovac et al., 2013). While assisted reproductive technology (ART) may increase the chances of conception, it does not ensure the genomic integrity of the embryo (Tremellen, 2008). In fact, high ROS concentrations in infertile men have been associated with DNA fragmentation and poor chromatin packing (Tremellen, 2008). Damaged DNA in spermatozoa is indicative of poor cellular health. Sperm DNA damage reduces semen quality and is the cause of infertility in many men (Lewis et al., 2013; Simon et al., 2013). In assisted reproduction, spermatozoa with damaged DNA lower fertilization and pregnancy rates, impair embryo development and quality and increase the risk of spontaneous miscarriage, birth defects and childhood diseases such as cancer (Aitken et al., 2013; Lewis, 2014). The level of DNA damage is suggestive of clinical outcome in assisted reproduction: idiopathically infertile couples with higher levels of sperm DNA fragmentation were found to have lower live-birth rates following IVF (Simon et al., 2013).

Highly specialized techniques such as proteomics allow characterization of the semen profile at a molecular level, proving useful in the assessment of proteins and the understanding of biological pathways that play a key role in male infertility (du Plessis et al., 2011). Advances in this rapidly-evolving field have allowed researchers to better identify seminal plasma and sperm proteins and to determine how their presence or concentration may differ in fertile versus infertile patients (Mitulovic and Mechtler, 2006). Studies looking at the sperm and seminal plasma protein profiles of men with oxidative stress-induced infertility would help in identifying alterations in the protein expression and/or translational modifications that may occur during sperm maturation and functions of proteins involved. Moreover, these studies may be extended to the characterization of other pathologies associated with male infertility at the molecular level.

Despite the established role of oxidative stress in the aetiology of male infertility, there are, as of yet, relatively few studies that have investigated the correlation between ROS-induced oxidative stress and a differential protein expression profile in the human ejaculate using proteomic analysis. Our laboratory has recently published a series of studies on patients diagnosed with primary and secondary infertility and elevated ROS concentrations using proteomic approaches (Hamada et al., 2013; Sharma et al., 2013a, 2013b). Using similar strategies, other laboratories have also studied the proteomic profile of infertile patients with poor semen quality who were also affected with oxidative stress (Herwig et al., 2013; Wang et al., 2009).

Thus, in this review, we aim to summarize and compare the findings of these initial studies that have utilized proteomic analysis to look into the differential expression of proteins in the seminal ejaculate of infertile men with high oxidative stress and fertile men with physiological levels of ROS. In this review, we only included proteomic studies in which the oxidative status of infertile men was measured. Our review begins with an overview of oxidative stress and its impact on male infertility as well as the methodologies and general work flow utilized in proteomic studies, in order to provide some basis to readers less familiar with the field.

In addition, this review highlights seminal plasma and spermatozoa proteins identified using proteomic analysis that are likely to play a major role in oxidative stress-induced male infertility and subsequently it lists proteins that have the potential to serve as diagnostic biomarkers of male infertility. To conclude, current limitations of these research studies as well as some perspectives in this area of research are highlighted. It is hoped that proteomic studies in men with different diagnosis of infertility will eventually lead to the discovery of biomarkers for idiopathic male infertility, which would help with the diagnosis and better management of male factor infertility.

Oxidative stress and male infertility

Oxidative stress occurs when there is an imbalance between ROS and the antioxidants that scavenge surplus free radicals (Hwang and Lamb, 2012; Sharma and Agarwal, 1996; Sikka, 2001; Sikka et al., 1995). ROS are natural products of cellular metabolism which, in physiological amounts, are essential requirements of spermatozoa for sperm processes leading to successful fertilization, such as capacitation, hyperactivated motility and acrosomal reaction (Agarwal et al., 2004). However, studies have shown that 30—80% of male factor infertility cases are due to ROS—mediated sperm damage (Iwasaki and Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz et al., 1995a, 1995b; Tremellen, 2008; Zini et al., 1993).

There are two principal methods in which ROS can cause male infertility: through damage of the sperm membrane and damage of the sperm DNA. Sperm membranes have large amounts of polyunsaturated fatty acids, making them susceptible to oxidative stress. This can then affect sperm motility as well as their ability to fertilize oocytes. Furthermore, DNA fragmentation may harm the paternal genetic contribution to the developing embryo (Tremellen, 2008).

ROS are considered a class of free radicals because they contain oxygen molecules with one or more unpaired electrons. This makes them highly reactive and susceptible to radical formation, potentially altering cellular function and ultimately endangering cell survival (de Lamirande and Gagnon, 1993; Hwang and Lamb, 2012). In turn, reactions such as oxidation of membrane lipids, carbohydrates related to DNA and amino acids may occur (Ochsendorf, 1999). However, not all ROS are free radicals (Cheeseman and Slater, 1993). There are three different general forms of the ROS (Figure 1): (i) the primary form of ROS, the superoxide anion radical from which secondary ROS can be derived either directly or indirectly (Hwang and Lamb, 2012; Tremellen, 2008); (ii) the secondary form of ROS, hydrogen peroxide (an example of a ROS that is not a free radical), hydroxyl radical and peroxyl radical; and (iii) the tertiary form of ROS, a class of free radicals that are nitrogenous compounds: peroxynitrous acid, nitroxyl anion, peroxynitrile and nitrous oxide (Hwang and Lamb, 2012).

Sources of oxidative stress

The origins of oxidative stress may vary from lifestyle choices and environmental factors (exogenous), to testicular (endogenous) sources, in addition to idiopathic causes.

Lifestyle, for example, is a major contributor to ROS production; smokers see a 107% increase in ROS concentrations in the semen, increased leukocytes and a greater likelihood of DNA fragmentation compared with non-smokers (Saleh and Agarwal, 2002; Sepaniak et al., 2004; Trummer et al., 2002). Alcohol abuse induces systemic oxidative stress and reduces antioxidant defences, which is likely further exacerbated by an antioxidant deficient diet (Hwang and Lamb, 2012; Mostafa et al., 2006; Villalta et al., 1997). Diet and exercise are also important factors in oxidative stress: improper diet and sedentary lifestyles can lead to obesity. Obesity in general increases the risk of co-morbidities, such as hypertension, dyslipidaemia, type 2 diabetes, coronary heart disease, stroke, non-alcoholic fatty liver disease, osteoarthritis, sleep apnoea and several types of cancers (Savini et al., 2013). These obesity-linked systemic inflammatory conditions upset the redox balance and contribute to seminal oxidative stress, which causes detriment to sperm function (Palmer et al., 2012). The accumulation of
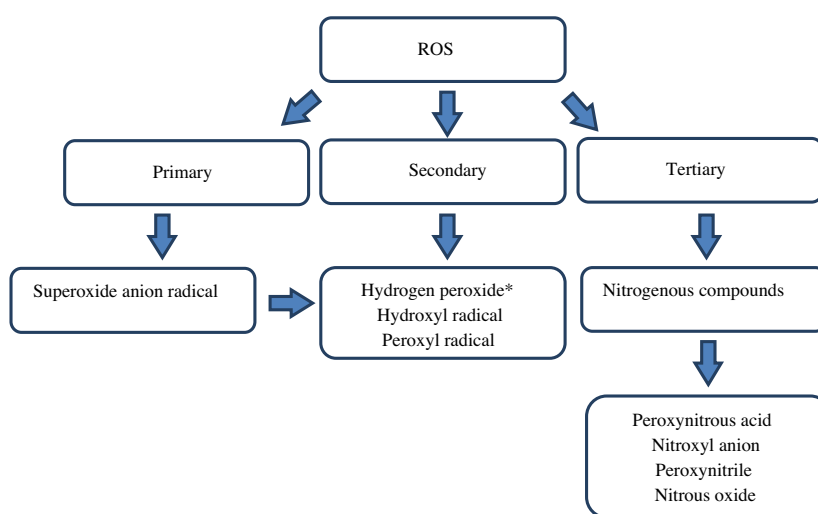
* Not a free radical

Figure 1 Primary, secondary and tertiary forms of ROS and different types of free radicals.

adipose tissue, which causes obesity, may increase ROS levels through the release of pro-inflammatory cytokines, increased ROS production in leukocytes and heating of the testicles (Banks et al., 2005; Ishii et al., 2005; Perez-Crespo et al., 2008; Singer and Granger, 2007). Conversely, intensive exercise has been linked to increased ROS concentrations, regardless of the type of exercise, because of an increased demand for energy in the muscles (Peake et al., 2007).

Environmental factors also have an impact in ROS production. Phthalates are chemicals that are added to plastics to increase its flexibility (plasticizers), and are used in food packaging, medical devices and personal care products. They have been linked to increased generation of ROS and reduction in antioxidants, leading to testicular oxidative stress (Agarwal et al., 1985; Hauser et al., 2007; Kasahara et al., 2002). Pesticide and heavy metal exposure are associated with diminished antioxidant levels and elevated 8-hydroxy-2′-deoxyguanosine levels in sperm DNA, indicative of increased oxidative DNA damage in spermatozoa (Chitra et al., 2001; Latchoumycandane et al., 2003; Xu et al., 2003). Other studies have detailed how both drug administration (such as aspirin and acetaminophen) and even assisted reproduction treatment (such as IVF and intrauterine insemination) increase oxidative stress (Agarwal and Said, 2005; Iwasaki and Gagnon, 1992; Shekarriz et al., 1995a, 1995b). This is either through increasing enzymic activity in the case of drugs or through assisted reproduction practices such as centrifugation or removal of antioxidants from semen.

ROS can also be produced as a result of infection and inflammation. Generally, pathogens will elicit a natural immune response such as an acute inflammatory response from leukocytes and macrophages (Mazzilli et al., 1994; Roos, 1991). For example, genitourinary tract infections cause elevated levels of sperm oxidative stress (Brackett et al., 2008; Padron et al., 1997), while a history of or a current *Chlamydia* infection is linked to an increase in sperm oxidative damage (Segnini et al., 2003).

In terms of testicular sources of oxidative stress, varicocele is believed to be the principal underlying pathology for the infertile male (Agarwal et al., 2006a; Barbieri et al., 1999; Hendin et al., 1999; Nallella et al., 2004; Saleh et al., 2003; Smith et al., 2007). Varicocele is the abnormal dilation and tortuosity of the pampiniform plexus veins within the spermatic cord and is the cause for nearly 35% of male factor infertility. Clinical varicocele can be found in 35% of men with primary infertility and 80% of men with secondary infertility. Infertile men with varicocele have high levels of seminal ROS and oxidative stress, which causes significant sperm DNA damage (Agarwal et al., 2006a, 2009; Pasqualotto et al., 2008).

Cryptorchidism is also a common testicular cause of oxidative stress; this pathology is the result of a deficient maturation of gonocytes into type A spermatogonia, which causes hypospermatogenesis (Huff et al., 1991). If there is prolonged ischaemia followed by restoration of the blood flow (spontaneous or surgical), an influx of activated leukocytes follows into both testicles (Turner et al., 2004). This causes an increase in ROS production, leading to necrosis of germinal cells and ultimately subfertility or infertility (Filho et al., 2004; Tremellen, 2008).

In the seminal ejaculate, the principal sources of free radical production come from either the leukocytes or the spermatozoa themselves (Tremellen, 2008). However, it is implied that leukocytes contribute the most to oxidative stress because compared with spermatozoa, the rate of ROS production in leukocytes is 1000-times greater (Plante et al., 1994). This is considered an 'extrinsic source' of ROS as opposed to the 'intrinsic' sources from sperm. In cases of idiopathic causes of male infertility, patients may have normozoospermia and yet are infertile, showing high ROS production and reduced antioxidant levels when compared with fertile men (Agarwal et al., 2006b; Pasqualotto et al., 2001).

Effects of oxidative stress on semen parameters

Low levels of ROS are generated naturally during processes such as spermatogenesis. Indeed, ROS are necessary, in balance with antioxidants, for proper spermatogenic mechanisms to occur. For example, hydrogen peroxide, a secondary form of ROS, stimulates acrosomal reaction, hyperactivation, and tyrosine phosphorylation in the sperm (Aitken et al., 1995; de Lamirande and Gagnon, 1993). Eventually, hydrogen peroxide leads to sperm binding to the zona pellucida (Hwang and Lamb, 2012). The antioxidant catalase, on the other hand, decomposes hydrogen peroxide and is also produced to preserve sperm motility (Tremellen, 2008). This delicate balance between hydrogen peroxide and its antioxidant, catalase, illustrates the subtle equilibrium that both facilitates proper function and prevents oxidative stress.

ROS are generally produced as a byproduct of enzymic reactions in oxidative phosphorylation, which is used to produce energy in the form of ATP. These reactions that involve the reduction of oxygen usually take place in the mitochondria (Tremellen, 2008; Valko et al., 2007). In the sperm cell, mitochondria are located on the midpiece. Studies show that mitochondrial DNA is more susceptible to mutations than nuclear DNA, increasing the production of ROS during this process (Bogenhagen, 1999; Liu et al., 2004; Taylor and Turnbull, 2005). In fact, elevated ROS levels have been linked to release of cytochrome C, a protein that activates apoptotic reactions, which is increased in patients with male factor infertility (Wang et al., 2003).

While mutations are less likely in nuclear DNA than in mitochondrial DNA, prior studies have shown that intrinsic ROS production is highly related to DNA fragmentation (Henkel et al., 2005). Free radicals can attack the purine and pyrimidine bases and the deoxyribose backbone. DNA damage may ultimately lead to poor blastocyst formation *in vitro* (Meseguer et al., 2008; Zorn et al., 2003).

Finally, sperm membranes contain large amounts of unsaturated fatty acids which provide fluidity, a process that is necessary for membrane fusion (Hwang and Lamb, 2012). However, this also makes spermatozoa vulnerable to ROS attack. Seminal fluid is an important source of antioxidants in semen, as the lack of cytoplasm and DNA compaction in spermatozoa leaves very little room for translation or for antioxidant defenses (Jeulin et al., 1989; Zini et al., 1993). Lipid peroxidation has also been associated with a decrease in sperm motility (Agarwal et al.,
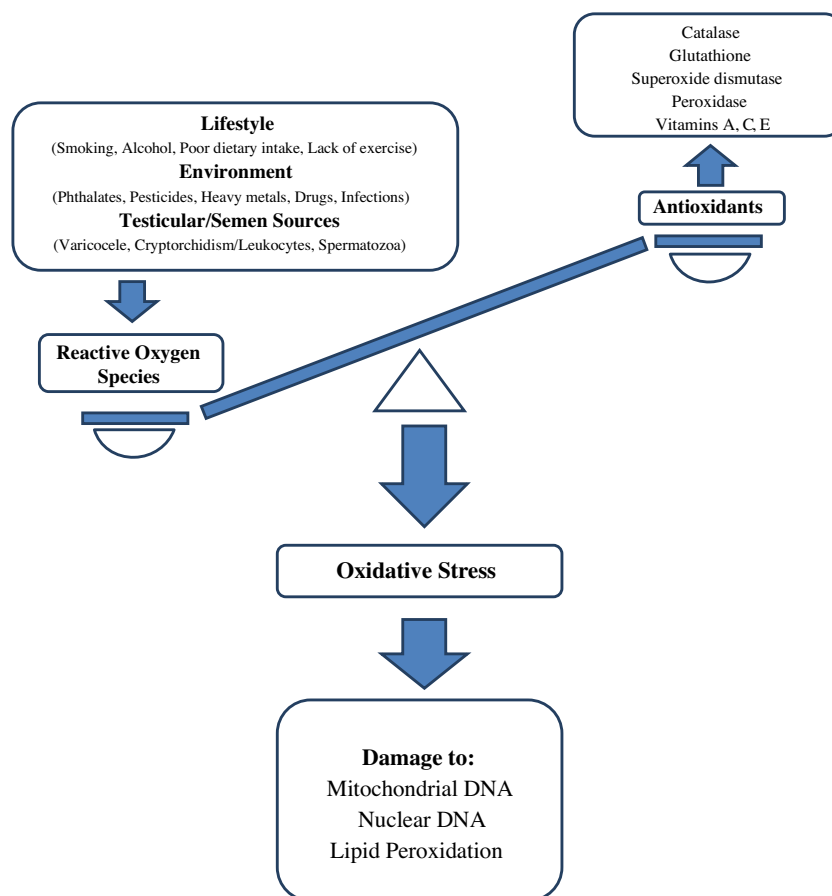
Figure 2   Model of the build up of oxidative stress in the semen. The model highlights the imbalance caused by accumulating ROS and depleting antioxidant, which brings about a state of oxidative stress. Various lifestyle and environmental factors along with testicular and seminal sources cause the generation of ROS. Antioxidants comprise both enzymatic and non-enzymatic types.

2008). The main concepts of oxidative stress are summarized in **Figure 2**.

Assessment of oxidative stress

Using the seminal ejaculate of a patient consulting for infertility, a clinical diagnosis of oxidative stress can be made using two alternative approaches: (i) measurement of ROS generated by spermatozoa; or (ii) measurement of either the amount of protein that is oxidized due to the presence of ROS (such as protein carbonyl) or the concentrations of antioxidant enzymes present (such as gluthathione peroxidase, superoxide dismutase and catalase).

Measurement of intra- and extracellular ROS generated by spermatozoa in a semen sample is performed using a luminol-mediated chemiluminescence assay. This method measures ROS concentrations in a sperm suspension (Kobayashi et al., 2001), where horseradish peroxidase is added in order to sensitize the assay to hydrogen peroxide. A chemical called luminol is then added, which is extremely sensitive to oxidation by a number of ROS at normal pH, producing luminescence and this signal is then measured by a luminometer (Saleh and Agarwal, 2002). This method is used in our clinical laboratory as part of the advanced tests for seminal ejaculates of infertile patients and we

have previously published reference levels used as cut-off values to determine levels of seminal ROS in patients: physiological ROS concentrations are <20 relative light units (RLU)/s/10 million spermatozoa while pathological ROS concentrations (i.e. oxidative stress) are ≥ 20 RLU/s/10 million spermatozoa (Benjamin et al., 2012).

For the measurement of ROS levels in a semen sample, products of oxidation such as protein carbonyls, which are chemically stable, are useful for detection purposes (Dalle-Donne et al., 2003) and are a more reliable and commonly used marker of protein oxidation (Stadtman and Berlett, 1991). Protein carbonyl content in the seminal plasma can be quantitated by enzyme-linked immunosorbent assay (ELISA; El-Taieb et al., 2009) by using a colourimetric assay. In contrast, measurement of unstable antioxidants may be subjected to variability handling and processing (Herwig et al., 2013).

Total antioxidant capacity measures the overall antioxidant capacity present in the seminal plasma. This includes: enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase; non-enzymatic antioxidants such as ascorbic acid (vitamin C) and alpha-tocotrienol (vitamin E); and molecules such as albumin, ceruloplasmin, ferritin, bilirubin, uric acid and reduced glutathione. Together, these antioxidants represent the cumulative

effect of the antioxidants present in the seminal plasma (Kashou et al., 2013).

Proteomics as a tool to study protein functions

For a long time, it was thought that sequencing of the human genome would be the ultimate strategy for unravelling the different diseases expressed by the human body. Along with advances in technology, this strategy culminated in a complete sequencing of the human genome, which was then placed on a database for public consultation (Levy et al., 2007). As many as 31,000 genes were identified (Baltimore, 2001); however, this number did not seem to accurately account for the total number of proteins in the body (Anderson and Anderson, 1998). The one-gene-one-polypeptide theory, dominant in the past, was found to be far too simplistic to explain the relationship between the genotype and phenotype. This discrepancy in explaining the phenotype by solely examining the genotype further increased with studies that claimed the presence of around a million proteins in the human body (Wilkins et al., 1996). These findings then brought about the idea of a single gene encoding multiple proteins with common or even different functions.

Proteins are more dynamic and intricate than the genome, varying with the state of the cell rather than remaining relatively fixed (May et al., 2011). Protein diversity may be derived through three main processes: at the DNA level (gene polymorphisms), the pre-mRNA or mRNA level (alternative splicing) or the protein level subsequent to RNA translation (post-translational modification and specific proteolytic cleavages) (Casado-Vela et al., 2011). Post-translational modifications may involve glycosylation, phosphorylation and ubiquitination. Translation usually occurs in the cytoplasm and involves activation, initiation, elongation and termination of the polypeptide chain. After translation, proteins undergo chemical modification, by the addition of a functional chemical group (glycosylation or phosphorylation) or other proteins (ubiquitination), or undergo structural changes (proteolytic cleavage, protein folding), which modifies the immature protein before it turns into a mature protein product. This important aspect of protein development shows how sequencing the human genome alone is not enough. Proteins play a major role in the understanding of the body and the diseases that affect it.

In the 1990s, researchers came to realize that the biochemical role of proteins needed to be explored to explain what genomics could not, which pushed researchers to work towards filling this gap in scientific knowledge (du Plessis et al., 2011). This endeavour was supplemented with the advancement in techniques such as 2D-differential in-gel electrophoresis (2D-DIGE) and mass spectroscopy. These technical advancements helped molecular biologists to identify protein—protein interactions and gain understanding of the cell phenotype. Proteomic approaches, such as labelling and fractioning techniques, are powerful tools for the quantitative and qualitative measurement of the total proteins in a cell, where different techniques can be used to elucidate differentially expressed proteins when comparing various tissue types or the organism in different states (Wilkins et al., 1996).

Proteomics continues to rapidly evolve and it is currently considered a promising field with many applications in the future. While basic proteomic analysis helps in the identification of proteins present in a particular tissue, quantitative proteomics deals with relative quantification of proteins present in different physiological or pathological conditions to identify differentially expressed genes in order to unravel the cellular processes and their biological significance (Zhou et al., 2013). Bioinformatics helps connect these initial protein lists to its biological significance in various states of disease, which is resourceful in the discovery of biomarkers of fertility (Zhou et al., 2013).

Specific proteins that are differentially expressed in diseased states may be used as biomarkers, which can act as a highly important non-invasive diagnostic tool (Milardi et al., 2013). The development of proteomics-based therapy may also prove more effective than therapies presently used. However, leaps in the field are not made without challenges. Factors such as lifestyle and nutrition, environment, race and population differences cause diversity in protein expression. Further, factors such as ageing may affect post-translational modifications in a cell, making measurements less precise and difficult to combine (Kashou et al., 2011). Advancements in technology and an increase in knowledge of post-transcriptional modification may, in the future, be able to eliminate such drawbacks.

Methods used in proteomics

The evolution of proteomics parallels the development of the techniques involved. Currently, several methods are employed to identify thousands of differentially expressed proteins.

Differences in amino acid content dictate variation in functional and chemical properties of proteins. Characteristics such as size and charge help molecular biology studies, as many separation techniques rely on these properties. For example, 2D-gel electrophoresis (2D-GE) is a method where proteins from cell lysates or fluids are run on an immobilized pH gradient that facilitates their separation in the first dimension based on their isoelectric point. Proteins resolved after the first run are then separated on the second dimension based on their apparent molecular weight, using classic polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS—PAGE; May et al., 2011). SDS is a denaturing agent (detergent) that imposes a negative charge on all proteins, overriding the original charge which no longer affects protein separation. At the end of SDS—PAGE, proteins have migrated at different distances depending on their molecular size. In order to determine the proteins that are differentially expressed, their expression is compared between treated and control conditions and a cut-off value is established to take into account the presence of artefacts.

One disadvantage of 2D-GE is its inability to resolve hydrophobic molecules and its low load—separation capacity (Bunai and Yamane, 2005). In addition, 2D-GE is insensitive to proteins present in low abundance and therefore it is not a completely reliable technique. However, it does provide a general overview of protein expression and concentrations in the cell (Wilkins et al., 1998). More
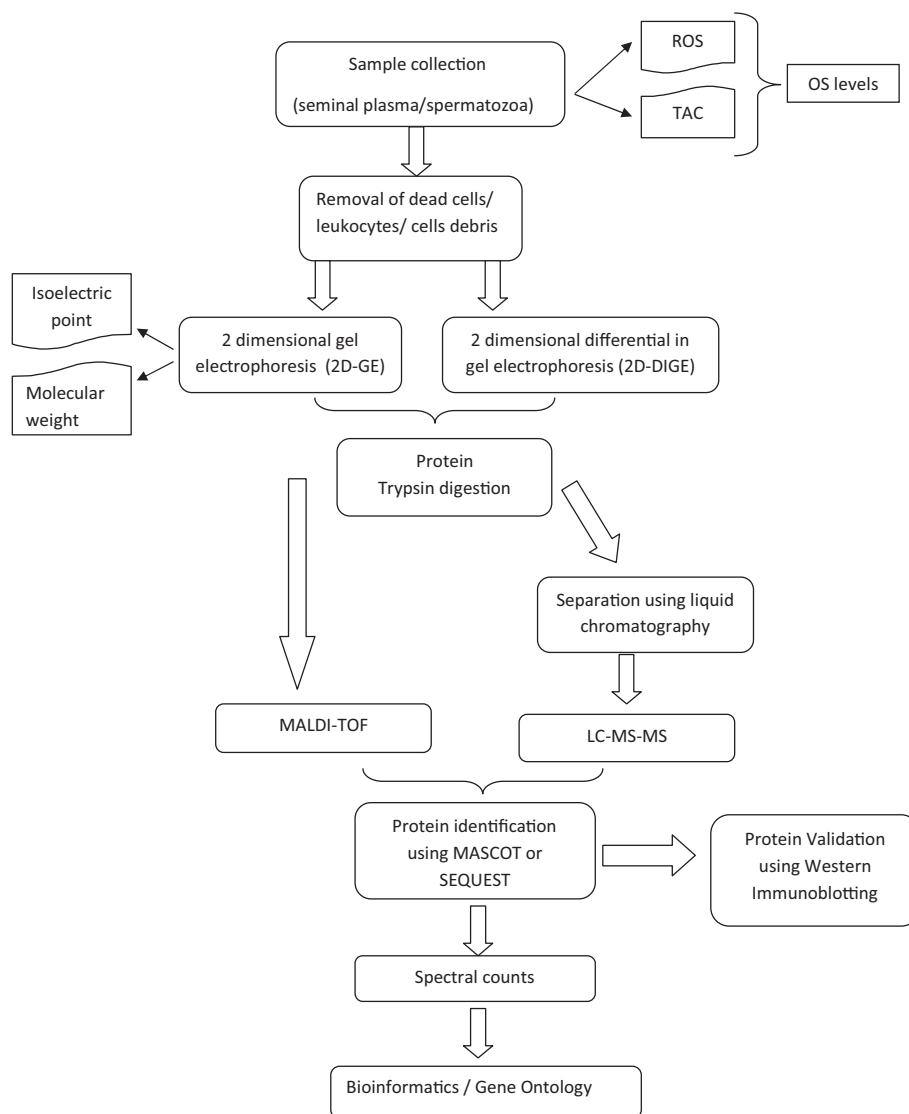
Figure 3 Overview of the general methods used for protein isolation and identification in seminal plasma and spermatozoa. Levels of oxidative stress (OS) in the semen sample are determined by measuring the reactive oxygen species (ROS) and/or the total antioxidant capacity (TAC). Protein separation can be performed using either 2D gel electrophoresis (2D-GE) or 2D differential in-gel electrophoresis (2D-DIGE). Next, protein identification can be done using liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) method. Isolated proteins are identified using tandem MS data analysis programs such as MASCOT or SEQUEST. Proteins of interest can be validated using Western blotting. Based on spectral counts, protein expression is quantified. Protein lists are generated and analysed using bioinformatics. Gene ontology (GO) is then used to determine the specific gene function in cellular pathways.

sophisticated techniques such as 2D-DIGE are available for more accurate results. This technique eliminates gel—gel differences (Baker and Aitken, 2009) and involves labelling extracted proteins from the control and experimental samples with different dyes before allowing them to run on separate gels, which are subsequently overlapped for better comparison (Rozanas and Loyland, 2008). However, this method has technical limitations, as the Cy3 and Cy5 fluorescent dyes used for protein identification and quantification in the 2D-DIGE can in fact alter the protein profile obtained, depending on the lysine content, molecular mass, abundance and acidity/hydrophobicity of the proteins present in a sample (Vazquez-Levin, 2013).

Mass spectrometry

Mass spectrometry (MS) is an automated process that has revolutionized protein detection and identification in cells. In sperm protein analysis, protein bands generated through gel electrophoresis are excised and identified via either one of two approaches. The first method, liquid chromatography-tandem MS (LC-MS/MS), involves digestion of proteins with trypsin, followed by high-performance liquid chromatography (HPLC), to separate polypeptides depending on hydrophobicity, charge and size (Mitulovic and Mechtler, 2006). After this procedure, repeated MS with an interface is performed to analyse and identify complex extracts by

resolving the charge-to-mass ratio of molecules ionized for detection. LC-MS/MS is a highly sensitive and selective technique that allows for the recognition of primary peptide sequences from complex proteins (Oliva et al., 2008).

The second method is called matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), and involves trypsin digestion of protein bands collected from 2D-GE but excludes HPLC. After protein processing, MS is performed to identify the molecules. One advantage of this technique is the pulsatile nature of MALDI, which allows for parallel ionization and mass analysis, resulting in the detection of greater portions of the sample. TOF is a mass analyser that separates ions formed at the same time: its role is to accelerate the different particles through a fixed distance, from a starting point to the detector. The time of flight of each ion is inversely correlated to the root square of mass-to-charge ratio (m/z). By determining particles' TOF properties, the m/z can be established and consequently the protein can be identified (Glish and Vachet, 2003).

The peptide mass-to-charge ratio that is determined through MS is then compared with set masses from previously sequenced and isolated proteins loaded in a database. Proteins are identified when a minimum of three of its peptide fragment masses match its homologous peptide masses in the protein database (Oliva et al., 2008). A major advantage of MS is its ability to recognize proteins that have undergone post-translational modification. Indeed, alterations in the initial structure of the protein results in a change of its molecular mass, which is reflected by the mass of the peptide where the modification has occurred (Baldwin, 2004). Finally, proteins that are differentially expressed are selected. Spectral count is a technique by which the relative protein concentration in pre-digested proteins are analysed by MS for quantification of its expression (Carvalho et al., 2008).

Western immunoblotting

In proteomics, Western immunoblotting is an important step to verify the presence and, in some cases, to quantify a protein of interest in a complex sample. In this technique, proteins are separated using 2D-GE and individual protein spots are seen along the length of the gel. A replica of the protein profile is obtained on a specific blotting support (typically nitrocellulose or PVDF, polyvinylidene difluoride membranes) using a perpendicularly-directed electric field to achieve protein transfer. Proteins on the membrane can be developed using immunodetection with specific antibodies followed by incubation with a system composed of a secondary antibody coupled to an enzyme and a substrate and a reaction detection system to visualize antigen-antibody complexes (Western immunoblotting; Mahmood and Yang, 2012). In some cases, proteins immobilized on the membrane can be subjected to interaction with other proteins, after which immunodetection of the added protein is carried out to evaluate protein–protein interaction (far-Western immunoblotting; Edmondson and Dent, 2001).

Bioinformatics

Bioinformatics describes the scientific field dealing with the overlap between biology, engineering and computer science.

It links the computer to genetics and molecular biology through the creation of a software program. Each protein detection method has its own programs that use specific algorithms. This technology is used by a bioinformatician to derive meaning from the large amount of information collected through proteomics studies (Lan et al., 2003).

Proteins lists that are generated from proteomic analysis are converted to gene names for functional annotations. Annotations for undefined genes or biological functions that are not listed in a particular database can be functionally annotated using prediction tools (Zhou et al., 2013). Once the annotations are obtained for the list of genes, a gene ontology (GO) analysis is conducted. GO is the study of the number of genes involved and its correlation between protein localization, structure, function and involvement in cellular biochemical pathways. In GO analysis, functions of gene products are classified using structured and controlled vocabularies, consisting of: (i) cellular components (giving functional meaning to the intracellular/extracellular localization of the gene product); (ii) biological processes (defining the molecular events taking place in the cell); and (iii) molecular functions (basic molecular activities of a gene product and its regulatory activities on the process studied (Zhou et al., 2013). GO studies are complemented by a pathway analysis, which centres the evaluation on how a group of genes in a defined biological process interrelate to form a complex network, and the result of this analysis is commonly visualized as a pathway map (Zhou et al., 2013). An overview of the general workflow involved in protein quantification and identification of semen samples with oxidative stress is shown in Figure 3.

Proteomic studies on seminal ejaculate with oxidative stress

Spermatogenesis is the biological process involving a series of successive divisions and cellular modifications of germ cells that results in the formation of mature and functional spermatozoa. The main function of spermatozoa is to deliver the haploid paternal genome to the female gamete. Although spermatozoa are very nearly transcriptionally inactive, sperm DNA goes through numerous modifications (such as methylation) and the majority of histones are replaced by small basic proteins, called protamines. It is believed that these changes are meant to transfer epigenetic factors to the female pronucleus in order to produce a viable embryo (Oliva et al., 2009). Previous studies have also demonstrated the involvement of proteins in various molecular events, such as acrosomal reaction and penetration of the egg's extracellular matrix, the zona pellucida (Chakravarty et al., 2008). These findings have encouraged and stimulated further exploration of proteins and the pathways involved in sperm maturation and capacitation (Lefievre et al., 2003). Sperm cell exploration has been facilitated by the ease of non-invasive collection of a sizable number of cells in each semen sample; in this regard, simple laboratory techniques such as centrifugation can be applied to separate a sufficient number of cells for analysis. However, a drawback in studying sperm proteins is the small size of the cell and the low amount of protein recovered from each cell. This can be overcome by combining semen samples

from men with a similar diagnosis and then analysing the sample.

Today, thousands of proteins have been profiled in human semen, and scientists have been working on comparing protein expression in fertile and infertile men using different approaches. However, notwithstanding its relevance, until the present time, there are only a few studies that have focused on oxidative stress and its ability to alter the protein expression in the semen of patients with high ROS levels. In those studies, researchers have identified several proteins that are differentially expressed, which may play a role in the regulation and response of cells with high ROS levels.

This review includes a set of proteomic studies that assessed oxidative stress levels in their subjects as quantitative proof of oxidative stress status in seminal ejaculate. The studies selected in this review are three by our group from the Centre for Reproductive Medicine, Glickman Urological and Kidney Institute, Cleveland Clinic (Hamada et al., 2013; Sharma et al., 2013a, 2013b) and two by other research teams (Herwig et al., 2013; Wang et al., 2009) (Table 1). Among these five studies in men who were mostly infertile with high levels of oxidative stress, three looked into seminal plasma proteins (Herwig et al., 2013; Sharma et al., 2013a; Wang et al., 2009) whereas two of our studies analysed sperm protein mixtures (Hamada et al., 2013; Sharma et al., 2013b). Patient populations in these studies are characterized as males with asthenozoospermia, idiopathic oligoasthenoteratozoospermia (OAT) or primary or secondary infertility (a majority of which have varicocele). The donors used as controls were normozoospermic (according to WHO, 1999 criteria) males with either proven or unproven fertility. Donors with proven fertility are men who have established a clinical pregnancy that resulted in a live birth.

The studies in this review pertain to men with clinical oxidative stress versus those with physiological ROS levels. To support our discussion in this review, references are occasionally made to studies performed on a similar group of subjects (i.e. infertile men with various semen parameters), although there was no assessment of oxidative stress levels in that study group (Sharma et al., 2013c). ROS levels in the seminal ejaculate are independent of semen parameters (Agarwal et al., 2006b), as the seminal constituents are mainly added to the spermatozoa at ejaculation. Semen samples used in studies from our team as well as from Herwig's group were negative for leukocytospermia (negative Endtz test), to rule out oxidative stress originating from ROS produced by leukocytes (Hamada et al., 2013; Herwig et al., 2013; Sharma et al., 2013a, 2013b). Other exclusion criteria observed in each study are mentioned in Table 1.

Oxidative stress was measured in these studies either by chemiluminescence assay (for ROS; Hamada et al., 2013; Sharma et al., 2013a, 2013b; Wang et al., 2009) or by ELISA (for carbonyl proteins; Herwig et al., 2013). In our latest study (Sharma et al., 2013a), total antioxidant capacity and sperm DNA fragmentation were also measured as a further indication of the oxidative status of the sample. Oxidative stress is likely to result in an abundance of immature proteins in their precursor or preprotein forms, which entails a deficiency of mature functional proteins (Martinez-Heredia et al., 2008). At the same time, presence of the immature proteins may be indicative of insufficient post-translational processing (Martinez-Heredia et al., 2008) that could result from high ROS levels. Protein oxidation may inhibit enzymatic and protein-binding activities as well as increase molecular weight, aggregation or proteolysis (Shacter, 2000), which may affect the number of identified proteins.

The methodology employed in these studies played a part in the number of proteins that were identified (Table 1). Protein separation in these studies was performed by SDS-PAGE, except for the study by Hamada et al. (2013), which used the 2D-DIGE method. Most of the studies ran samples in replicates. All the studies used the LC-MS/MS method (either the linear trap quadrupole Orbitrap (Hamada et al., 2013; Sharma et al., 2013a, 2013b; Wang et al., 2009), or the Hybrid Velos (Herwig et al., 2013). The largest pool of proteins identified was 2489 proteins in the seminal plasma (Herwig et al., 2013) and 1343 proteins in spermatozoa (Hamada et al., 2013).

The database used for protein identification searches was comparable and most studies employed more than one database search to reduce false positives. As is commonly used in quantitative proteomics, the relative protein abundance of the proteins expressed was measured by the intensity of the spectral count. As an overall, the cut-off values for over- and under-expressed proteins were 1.5 to 3-fold, respectively. The protein(s) identified were validated by Western immunoblotting in studies reported by Wang et al. (2009). To determine whether the pooled sample groups used in their study were truly representative of the infertile/fertile individual, the study by Herwig et al. (2013) evaluated individual samples alongside the pooled samples. Based on the results of the study, the authors reported a lower number of unique proteins in an individual idiopathic OAT sample compared with the pooled idiopathic OAT group. All the studies had performed enrichment analysis in the GO categories of the genes expressed except for our first paper on sperm proteins (Hamada et al., 2013). This was followed by pathway and network analysis and protein-protein interaction analysis to determine the processes involved when the seminal plasma or the spermatozoa is in a state of oxidative stress. In the next sections, a brief summary of reported findings of proteomics studies on seminal ejaculate with oxidative stress is presented.

Seminal plasma proteins and oxidative stress

Seminal plasma is the fluid in the semen that contains secretions from the testis, epididymis, prostate, seminal vesicles and Cowper's glands. Seminal plasma plays an important role in providing nourishment and protection to spermatozoa and acts as a buffer as well as a medium for sperm motility. Human semen is composed of lipids, ions (such as citrate, calcium, magnesium, potassium, sodium, zinc and chloride), fructose, ascorbic acid, proteins (such as semenogelin and fibronectin), albumin and globulins, amino acids and amines, cytokines and hormones. It also contains numerous enzymatic (gluthathione peroxidase, superoxide dismutase, catalase) and non-enzymatic antioxidants (vitamins C and E, zinc) that protect spermatozoa from oxidative stress (Pahune et al., 2013). Protein concentration in human seminal plasma has been estimated to be 45 mg/ml (Tomar et al., 2012a).

Table 1 Summary of proteomic studies in patients with oxidative stress.

Study	Source of proteins	Subjects	Exclusion criteria	Indication of oxidative stress	Proteomic analysis strategy	Main findings	Potential biomarkers of oxidative stress
Wang et al. (2009)	Seminal plasma	38 infertile patients with AS (motility a + b: 8.7 ± 20.4%)	Liquefaction time >30 min	Chemiluminescent measurement of ROS: patients had 3.3-times higher ROS than controls ($P < 0.01$)	SDS—PAGE, LC-MS/MS, LTQ Orbitrap, Peptide-Prophet, Protein-Prophet ($P > 0.5$), SEQUEST, Western blot (SDS—PAGE, DJ-1 antibody)	741 proteins identified	DJ-1 (about 50% lower in seminal plasma of AS than controls; $P < 0.05$)
		20 normal donors (normozoospermic; motility a + b 57.4 ± 9.7%)	Sperm density $<20 \times 10^6$ spermatozoa/ml			101 differentially expressed proteins: 45 over-expressed ($\geq$3-fold) in AS samples; 56 underexpressed ($\leq$3-fold) in AS samples	
			Abnormal morphology >10% of total in AS samples			DJ-1 the most down-regulated protein in AS	
Hamada et al. (2013)	Spermatozoa	32 infertile patients (25 primary infertility + 7 secondary infertility; 21/32 had clinical varicocele of grades 1—2)	Leukocytospermia (Endtz positive >0.1 million/ml)	Chemiluminescent measurement of ROS: ROS+ (31 samples) $\geq$20 RLU/s/10^6 spermatozoa; ROS— (21 samples) <20 RLU/s/10^6 spermatozoa	2D-DIGE, LC-MS/MS, Finnigan, LTQ Orbitrap, NCBI, MASCOT, SEQUEST, BLAST	1343 proteins identified in ROS— gel	Lactotransferrin-2 and peroxiredoxin-1 (increased in ROS— group)
		20 healthy donors with unproven fertility (normozoospermic)				1265 proteins identified in ROS+ gel	
Sharma et al. (2013b)	Spermatozoa	32 infertile patients (25 primary infertility + 7 secondary infertility; 21/32 had clinical varicocele of grades 1—2)	Leukocytospermia (Endtz positive >0.1 million/ml)	Chemiluminescent measurement of ROS: ROS+ (31 samples) $\geq$20 RLU/s/10^6 spermatozoa; ROS— (21 samples)<20 RLU/s/10^6 spermatozoa	In-solution digestion, LC-MS/MS, Finnigan, LTQ Orbitrap, NCBI, MASCOT, SEQUEST	74 proteins identified	HIST1H2BA, MDH2, TGM4, GPX4, GLUL, HSP90B1, HSPA5 (all higher in seminal plasma of ROS+ versus ROS—)
		20 healthy donors with unproven fertility (normozoospermic)				20 differentially expressed proteins: 15 over-expressed (>2-fold) in ROS+ samples; 5 underexpressed (<0.5-fold) in ROS+ samples ODF1 and ACE1 underexpressed; LDHC over-expressed; all modulated by CREM activators	

Table 1 (continued)

Study	Source of proteins	Subjects	Exclusion criteria	Indication of oxidative stress	Proteomic analysis strategy	Main findings	Potential biomarkers of oxidative stress
Herwig et al. (2013)	Seminal plasma	11 infertile patients with idiopathic OAT	<5 days of sexual abstinence	Quantification of carbonyl protein using ELISA	In-solution digestion, LC-MS/MS, LTQ Orbitrap, Hybrid Velos, SwissProt, MASCOT, Phenyx	2489 proteins identified	TBCB, AACT, ALDR (possibly connecting idiopathic OAT and oxidative stress/ inflammation)
		11 fertile donors (normozoospermic)	Known cause of idiopathic OAT, positive culture for any organism		Individual samples representing pooled samples for each group	46 proteins indicative of infertility: 27 proteins common to all idiopathic OAT patients; 24 proteins over-expressed (≥1.5-fold) in idiopathic OAT samples; 5 proteins were both common to all idiopathic OAT samples and over-expressed (≥1.5-fold) in idiopathic OAT samples TBCB, AACT, ALDR up-regulated in idiopathic OAT samples	
			Samples with leukocyte-spermia (Endtz positive, ≥0.1 M/ml)				
Sharma et al. (2013a)	Seminal plasma	32 infertile patients (25 primary infertility + 7 secondary infertility; 21/32 had clinical varicocele of grades 1—2); 10/32 had a history of smoking	Donor samples with leukocytospermia (Endtz positive, >0.1 M/ml)	Chemiluminescence measurement of ROS: ROS+ (31 samples) = ≥20 RLU/s/10^6 spermatozoa; ROS— (21 samples) <20 RLU/s/10^6 spermatozoa	In-solution digestion, LC-MS/MS, Finnigan, LTQ Orbitrap, NCBI, MASCOT, SEQUEST	14 proteins identified	PIP (higher in seminal plasma of ROS+ versus ROS—)
		20 healthy donors with unproven fertility (normozoospermic)				7 uniquely expressed proteins: 3 proteins expressed only in ROS— samples; 4 proteins expressed only in ROS+ samples 7 differentially expressed proteins: 4 over-expressed (>2-fold) in ROS+ samples; 3 underexpressed (<0.5-fold) in ROS— samples AZGP1, CLU, KLK3, PIP and ACPP transcriptionally regulated by the androgen receptor	

Proteomic analysis strategy included information on protein separation and sequencing, peptide identification, database search software and validation studies. $P < 0.05$ was used for protein identification. Motility grade a + b indicates rapid and slow progression; the cut off for normal progressive motility (grade a + b) is ≥50% within 60 min of ejaculation (WHO, 1999). The cut off for normal ROS concentration is <20 RLU/s/million spermatozoa (Benjamin et al., 2012). AACT = anti-a1-antichymotrypsin; ACE1 = antihuman angiotensin-converting enzyme; ACPP = prostatic acid phosphatase/ prostatic-specific acid phosphatase; ALDR = alcohol dehydrogenase; AS = asthenozoospermia; AZGP1 = zinc-alpha-2-glycoprotein 1; BLAST = Basic Local Alignment Search Tool; CLU = clusterin; CREM = cAMP-responsive element modulator; DIGE = differential in-gel electrophoresis; ELISA = enzyme-linked immunosorbent assay; GLUL = glutamine synthetase; GPX4 = glutathione peroxidase 4; HIST1H2BA = histone H2B type 1-A; HSP90B1 = heat shock protein HSP90 β; HSPA5 = heat shock 70 kDa protein 5; KLK3 = isoform I preprotein; LC MS/MS = liquid chromatography—tandem mass spectrometry; LDHC = lactate dehydrogenase C; LTQ = linear ion trap; MASCOT = a tandem MS data analysis program for protein identification from Matrix Science; MDH2 = malate dehydrogenase 2; NCBI = National Centre for Biotechnology Information; OAT = oligoasthenozoospermia; ODF1 = outer dense fibre; Phenyx = software platform used for identification and characterization of proteins and peptides from MS data; PIP = prolactin-inducing protein; RLU = relative light units; ROS+= high reactive oxygen species concentration (≥20 RLU/s/million spermatozoa); ROS— = low reactive oxygen species concentration (<20 RLU/s/million spermatozoa); SDS—PAGE = sodium dodecyl sulphate—polyacrylamide gel electrophoresis; SEQUEST = a tandem MS data analysis program used for protein identification; SwissProt = manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB); TBCB = tubulin-folding cofactor B; TGM4 = transglutaminase 4 (prostate).

The study of these proteins can provide a basis for the identification of biomarkers for the assessment of male infertility disorders. Tables 2—5 give an overview of seminal fluid proteins that have been associated with elevated levels of oxidative stress.

Seminal plasma protein expression was analysed in patients with primary and secondary infertility who presented high levels of oxidative stress (ROS+; $\geq$20 RLU/s/10 million spermatozoa) versus those with physiological levels of oxidative stress (ROS−; <20 RLU/s/10 million spermatozoa) (Sharma et al., 2013a). Using LC-MS/MS, the study resulted in the identification of 14 proteins: seven commonly present in both ROS+ and ROS− samples, three exclusively present in ROS− samples (fibronectin I isoform 3 preprotein (FN1), macrophage migration inhibitory factor-1 peptide (MIF) and galectin 3 binding protein (G3BP)) and four expressed solely in ROS+ samples (cystatin S precursor, albumin preprotein, lactotransferrin precursor 1 peptide and prostate-specific antigen isoform 4 preprotein) (Sharma et al., 2013a).

Semenogelin 2 precursor was found to be 2-fold up-regulated in the ROS+ group, whereas semenogelin 1 isoform a was found to be down-regulated in the ROS+ group (Sharma et al., 2013a). Both semenogelin 1 (50 kDa) and semenogelin 2 (63 kDa, present in lesser abundance) are secreted from the seminal vesicle and represent the most abundant components (about 20—40%) of the human semen coagulum (de Lamirande and Lamothe, 2010). Semenogelin are responsible for the coagulation of the gel matrix that encloses the spermatozoa and it helps prevent the capacitation process (the initial part of sperm activation) by inhibiting the formation of ROS (de Lamirande and Lamothe,

2010). A small amount of ROS, generated by the spermatozoa itself, is required to facilitate the initiation of the capacitation process and the subsequent hyperactivated motility of the spermatozoa. However, a premature onset of capacitation leads to poor fertility outcomes (de Lamirande and Gagnon, 1993).

Semenogelin decreases the formation of ROS through several pathways: (i) by reducing sperm motility and energy consumption (de Lamirande et al., 2001); (ii) by indirectly interacting with sperm NADH-oxidase to block superoxide radical generation (Bonilha et al., 2008); and (iii) by binding the antioxidant zinc ion (Zn^{2+}) and allowing it to function inside the spermatozoa (Bonilha et al., 2008). In the report by Wang et al. (2009), the quantity of semenogelin in the seminal plasma of fertile donors (sperm motility grades A + B i.e. rapid + slow progression of 48—67%) versus asthenozoospermic patients (sperm motility 6—11%) with high levels of oxidative stress was not significantly different. The results suggested that seminal vesicle proteins such as semenogelin were less likely to be associated with the regulation of sperm motility in asthenozoospermic patients compared with proteins of the epididymis and prostate, such as DJ-1 (Wang et al., 2009).

Seminal plasma motility inhibitors (SPMI) are degradatory products or proteinase-resistant fragments of semenogelin 1 and 2 (Terai et al., 2010). Terai et al. (2010) studied the association of SPMI and spermatozoa in asthenozoospermic infertile patients (sperm motility <50%) by labelling washed sperm cells with anti-SPMI antibody, followed by flow cytometry analysis and Western immunoblotting. Although spermatozoa from both asthenozoospermic patients and normal subjects showed similar labelling patterns, both

Table 2 Seminal plasma proteins over-expressed in semen samples with oxidative stress compared to semen samples without oxidative stress.

Protein	UniProt ID	Function	Reference
Semenogelin-2 (SEMG2)	Q02383	Coagulation of semen	Sharma et al. (2013a)
Prolactin-induced protein (PIP)	P12273	Breakdown of fibronectin during semen liquefaction	Sharma et al. (2013a)
Prostatic acid phosphatase (PAP or ACPP)	P15309	Liquefaction of semen	Sharma et al. (2013a)
Epididymal secretory protein E1 (NPC2)	P61916	Transport of intracellular cholesterol Egress of cholesterol from endosomal/lysosomal compartment	Wang et al. (2009)
Epididymal secretory protein E4 (WFDC2)	Q14508	Serine protease inhibitor, though function is still debated	Wang et al. (2009)
Immunoglobulin kappa chain C protein (IGKC)	P01834	Immune response	Wang et al. (2009)
Pyruvate kinase M2 (PKM2)	P14618	Phosphotyrosine-binding protein	Wang et al. (2009)
Cathepsin H (CTSH)	P09668	Lysosomal cysteine proteinase	Wang et al. (2009)
L-lactate dehydrogenase B chain (LDHB)	P07195	Catalyses conversion of lactate and NAD to pyruvate and NADH (glycolysis)	Wang et al. (2009)
Legumain precursor (LGMN)	Q99538	Hydrolysis of asparaginyl bonds	Wang et al. (2009)
Delta aminolevulinic acid dehydratase (ALAD or ALADH)	P13716	Catalyse the second step in haeme synthesis to form porphobilinogen	Wang et al. (2009)
Alpha-1-antichymotrypsin (AACT)	P01011	Inactivate serine proteases Control oxidative damage, anti-inflammatory and is involved in defence mechanism	Wang et al. (2009)
Aldose reductase (ALDR or AKR1B1)	P15121	Catalyse the reduction of aldehydes and carbonyl	Herwig et al. (2013)

Table 3 Seminal plasma proteins underexpressed in semen samples with oxidative stress compared to semen samples without oxidative stress.

Protein	UniProt ID	Function	Reference(s)
Semenogelin 1 isoform (SEMG1)	P04279	Coagulation of semen	Sharma et al. (2013a)
Prostate specific antigen isoform 1 preprotein (KLK3)	P07288	Hydrolysis of semenogelin 1 and liquefaction of seminal coagulum Remodelling of extracellular matrix Degradation of connective tissue	Sharma et al. (2013a)
Zinc alpha 2 glycoprotein 1 (AZGP1)	P25311	Plays a role in signal transduction	Sharma et al. (2013a)
Clusterin preprotein (CLU)	P10909	Protection against oxidative reactions, protein denaturation and aggregation of abnormal spermatozoa and controls complement-induced sperm lysis	Sharma et al. (2013a), Wang et al. (2009)
Alpha-2-macroglobulin (A2 M)	P01023	Antiprotease activity	Wang et al. (2009)
Fructose-biphosphate aldolase A (ALDOA)	P04075	Glycolytic enzyme converting fructose 1,6 biphosphate to GAPH and DHAP	Wang et al. (2009)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P04406	Enzyme in glycolysis pathway	Wang et al. (2009)
Dipeptidyl peptidase 4 (DPP4)	P27487	Fused to spermatozoa, induce sperm motility and prevent premature capacitation	Wang et al. (2009)
DJ-1 (PARK 7)	Q99497	Antioxidant protein that protects cells against oxidative stress and cell death	Sharma et al. (2013a), Wang et al. (2009)
Laminin subunit alpha-5 (LAMA5) precursor	O15230	Binds to cells, acting as part of laminin, which is found in basement membranes	Wang et al. (2009)
Keratin, type 1 cytoskeletal 9 (KRT9)	P35527	Part of keratin filament assembly; function in skin tissue	Wang et al. (2009)
Rab GDP dissociation inhibitor beta (GDI2)	P50395	Inhibits dissociation of GDP from Rab proteins and the subsequent binding of GTP to them	Wang et al. (2009)
Annexin 6 isoform 2 (ANXA6)	P08133	It may associate with CD21 and regulate calcium release from intracellular stores, cell receptor for chondroitin sulphate chain	Wang et al. (2009)
Attractin precursor isoform 2 (ATRN)	O75882	Inflammatory response; involved in immune cell clustering and possibly regulates the chemotactic activity of chemokines	Wang et al. (2009)
Alpha-actinin-4 (ACTN4)	O43707	Actin-binding protein (a bundling protein)	Wang et al. (2009)
Nesprin-2 isoform 1 (SYNE2)	Q8WXH0	Links organelles to actin cytoskeleton for organization in the cell	Wang et al. (2009)
Transitional endoplasmic reticulum ATPase (VCP)	P55072	Involved in fragmenting the Golgi stacks during mitosis and reassembling afterwards	Wang et al. (2009)
SNC66 protein	Q8WY24	Involved in membranes; function is not well known	Wang et al. (2009)
Alpha-N-acetyl glucosaminidase precursor (NAGLU)	P54802	Located in lysosomes, breaks down glycosaminoglycans	Wang et al. (2009)

labelling intensity and the number of labelled spermatozoa were higher in patient samples compared with normal subjects. Further, a marked negative correlation was found between labelled sperm cells and gamete motility and viability. Based on these findings, Terai's group postulated that the presence of membrane surface-bound SPMI on the sperm head and tail was the basis for poor motility in asthenozoospermic patients rather than the presence of semenogelin in their seminal plasma (Terai et al., 2010).

Prostate-specific antigen (PSA, or human kallikrein 3 (hK3)) is a serine protease that is synthesized in prostate tissue and involved in semenogelin breakdown, causing liquefaction of the semen coagulum (Jansen et al., 2009; Robert and Gagnon, 1999). PSA isoforms were found to be differentially expressed between patients with high ROS

levels and donors with physiological ROS levels: PSA isoform 1 preprotein was down-regulated in ROS+ patients, while PSA isoform 4 preprotein was unique to ROS+ samples (Sharma et al., 2013a). The identification of these precursor forms of incompletely modified proteins can be explained by faulty post-translational modifications, which result in a decrease in the presence of the mature form of the protein and thereby the lack of its function (Martinez-Heredia et al., 2008).

Prolactin-induced protein (PIP; ~17 kDa) is secreted from the prostate gland, seminal vesicles and testis (Yamakawa et al., 2007) and constitutes about 1% of seminal plasma (Lilja, 1993). PIP has attracted much attention when it comes to seminal fluid studies. This protein plays various roles, such as fibronectin degradation during semen

Table 4 Seminal plasma proteins expressed uniquely in semen samples with oxidative stress compared to semen samples without oxidative stress.

Protein	UniProt ID	Function	Reference
Cystatin-S precursor (CST4)	P01036	Inhibits cysteine proteases which degrades tissues	Sharma et al. (2013a)
Albumin preprotein (ALB)	P02768	Involved in reservoir of cholesterol in spermatozoa	Sharma et al. (2013a)
Lactotransferrin precursor 1 peptide (LTF)	P02788	Forms sperm-coating antigen	Sharma et al. (2013a)
Prostate specific antigen isoform 4 preprotein (KLK3)	P07288	Hydrolysis of semenogelin 1 and liquefaction of seminal coagulum Remodelling of extracellular matrix Degradation of connective tissue	Sharma et al. (2013a)
Tubulin-folding cofactor B (TBCB)	Q99426	Is involved in assembly of alpha beta tubulin heterodimer Regulates tubulin heterodimer dissociation	Herwig et al. (2013)

Table 5 Seminal plasma proteins absent from semen samples with oxidative stress.

Protein	UniProt ID	Function	Reference
Fibronectin 1 isoform b precursor (FN1)	P02751	Help formation of seminal gel Help select abnormal spermatozoa	Sharma et al. (2013a)
Macrophage migration inhibitory factor-1 protein (MIF)	P14174	Affect sperm motility by maintaining thiol protein oxidoreductase status of sperm cell	Sharma et al. (2013a)
Galectin-3-binding protein (LGALS3)	P17931	Immunomodulation Cell–cell and cell–matrix interaction Pathogen–host interaction	Sharma et al. (2013a)

liquefaction, immunoregulation, antimicrobial activity, apoptosis and tumour progression (Tomar et al., 2012b). It also interacts with numerous proteins such as fibrinogen, actin, keratin, myosin, tropomyosin (Schenkels et al., 1994), human zinc-alpha-2 glycoprotein (Hassan et al., 2008) and human serum albumin (Kumar et al., 2012). Although PIP has been linked to male infertility, its exact physiological function remains unclear (Tomar et al., 2012b). The expression of PIP appears to vary between different studies. In a study on men with various semen parameters, we found that PIP was commonly expressed in groups that differed in sperm concentration and morphology parameters (Sharma et al., 2013c). However, in our subsequent study, PIP concentrations were found to be highly increased in the ROS+ group compared with the ROS− group (Sharma et al., 2013a). On the other hand, PIP was reported to be lowered in asthenozoospermic patients compared with donors (Martinez-Heredia et al., 2008). In azoospermic patients, PIP is either absent or decreased in expression compared with fertile men (Yamakawa et al., 2007). However, PIP was found to be over-expressed in non-obstructive azoospermic patients when compared with oligozoospermic patients (Davalieva et al., 2012). These findings support the use of PIP as a potential biomarker for azoospermia, although additional studies using a larger number of samples are warranted (Davalieva et al., 2012; Kumar et al., 2012; Yamakawa et al., 2007).

In the human testis, DJ-1 is found in spermatids, spermatogonia, spermatocytes, Sertoli cells and Leydig cells. In Sertoli and Leydig cells, DJ-1 colocalizes with the androgen receptor, suggesting a role for this protein in the regulation of spermatogenesis via the receptor (Yoshida et al., 2003). DJ-1 is secreted by the testis, the epididymis and the prostate (Utleg et al., 2003; Yoshida et al., 2003) and serves as a highly conserved antioxidant protein to support antioxidative stress reactions (Yasuda et al., 2013). DJ-1 self-oxidizes three cysteine residues in order to quench ROS (Yasuda et al., 2013). Thus, DJ-1 plays a role in controlling oxidative stress and helps to protect the sperm cell from the detrimental effects of high ROS levels (Wang et al., 2009). DJ-1 was found to be present on the surface of the sperm head, midpiece and flagella, suggesting an additional role in fertilization (Yoshida et al., 2003). Interestingly, DJ-1 was found to be present in the seminal plasma of fertile men (Yoshida et al., 2003) and down-regulated in asthenozoospermic patients, in whom oxidative stress were about 3-times higher than those in normal fertile patients (Wang et al., 2009). We found that DJ-1 was over-expressed in patients (whose oxidative stress were not assessed) with: (i) normal sperm count but with abnormal morphology; and (ii) oligozoospermia but with normal sperm morphology (Sharma et al., 2013c). However, in the same study, DJ-1 was not detected in oligozoospermic samples with abnormal sperm morphology (Sharma et al., 2013c). Therefore, when under

stressful conditions, it seems that DJ-1 expression increases. As stress concentrations increase, ROS concentrations increase and antioxidant concentrations fall, leading to a decline in DJ-1 expression (Sharma et al., 2013c). GO analysis showed that proteins in patients with normal sperm count and abnormal morphology were involved in pathways for scavenging free radicals (Sharma et al., 2013c). These findings further substantiate the functions attributed to DJ-1.

Clusterin (also referred to as apolipoprotein or sulphated glycoprotein 2; 70–80 kDa) is a heterodimeric glycoprotein that is produced by the Sertoli cells (Morales et al., 1987) and secreted by the epididymis and prostate. It has a widespread distribution in human tissues and it is involved in a number of biological functions including cell-to-cell interaction, apoptosis, sperm maturation and degradation of extracellular matrix (Hosseinifar et al., 2013). Moreover, clusterin protects against harmful ROS reactions, protein precipitation and aggregation of defective spermatozoa, as well as controlling complement-induced cell lysis (Hosseinifar et al., 2013). In infertile men, clusterin is a major antigen for sperm agglutination auto-antibodies (Carlsson et al., 2004). Clusterin has been proposed as a sensitive cellular biosensor of oxidative stress, since it possesses a chaperone activity that functions to protect from the harmful effects of free radicals and oxidative stress (Trougakos, 2013; Trougakos and Gonos, 2006, 2009). In our latest study, down-regulation of clusterin preprotein in seminal ejaculates was found with increased ROS levels (Sharma et al., 2013a). However, Wang et al. (2009) reported an increased clusterin precursor expression in asthenozoospermic patients with 3.3-fold higher ROS levels when compared with fertile men with physiological ROS levels, although the clusterin concentration in the asthenozoospermic ejaculate was reduced. A GO analysis performed in our study demonstrated the transcriptional regulation of the clusterin gene by the androgen receptor as well as activation of prostate induction by the androgen receptor signalling pathway (Sharma et al., 2013a).

Prostatic acid phosphatase (PAP, or prostatic-specific acid phosphatase (PSAP)) is an enzyme produced in the prostate gland that has been extensively studied as a biomarker and negative growth regulator for prostate cancer (Watson and Tang, 1980). Serum concentrations of PAP are especially increased in men with metastasized prostate cancer and it served as an important tumour marker and diagnostic indicator of prostate cancer, prior to the use of PSA (Muniyan et al., 2013). PSA is a chymotrypsin-like serine protease that is produced and secreted by the prostate gland (Veveris-Lowe et al., 2007). PSA cleaves semenogelins and fibronectin that form the seminal coagulum, causing liquefaction to occur (Lilja, 1993).

Previous reports have shown seminal PAP concentrations to be increased in azoospermic (Vaubourdolle et al., 1985) and severely oligozoospermic (Singh et al., 1996) men compared with normal controls, suggesting an inverse relationship between PAP concentrations and sperm concentration. Similarly, Davalieva et al. (2012) reported higher concentrations of PAP (using colourimetric assay) in seminal plasma of azoospermic patients compared with those in normozoospermic, asthenozoospermic and oligozoospermic patients, although the differences between the groups were not statistically significant. In one of our other proteomic studies (oxidative stress levels not measured), PAP concentrations were down-regulated in patients with normozoospermia and abnormal sperm morphology, as well as patients with oligozoospermic semen and normal sperm morphology (Sharma et al., 2013c). In our subsequent study (with oxidative stress levels measured), acid phosphatase and the prostate-specific antigen isoform I preprotein (KLK3) were both present in semen ejaculates with both increased and normal ROS levels (Sharma et al., 2013a). However, acid phosphatase concentration was up-regulated in seminal plasma with increased ROS levels while isoform I preprotein was down-regulated in seminal plasma with physiological ROS levels (Sharma et al., 2013a). Further, the prostate-specific antigen isoform 4 preprotein was found to be uniquely expressed in seminal plasma with increased ROS levels (Sharma et al., 2013a). The differential expression of these biomarkers of prostate cancer in infertile patients with oxidative stress may help elucidate the aetiology of prostate cancer (Sharma et al., 2013a). Similar to clusterin, PAP is controlled by the androgen receptor and is involved in prostate induction through the androgen receptor signalling pathway (Sharma et al., 2013b).

Identifying differentially expressed proteins between abnormal and normal semen samples is only the first step in understanding the mechanisms of male infertility at the molecular level. Protein structure, localization and involvement in biological pathways all need to be analysed to determine the dynamic cellular processes that occur. We studied the function and distribution of proteins that are commonly or differentially expressed between ROS+ and ROS− groups and found that most of the common proteins are present in the extracellular compartment (Sharma et al., 2013a). Proteins unique to seminal plasma with increased ROS, such as cystatin S precursor and albumin preprotein, are restricted to the extracellular matrix. Polypeptides unique to seminal ejaculates with normal ROS concentrations, such as fibronectin 1, are considered to aid in the process of endocytosis due to their presence in the vesicular lumen region. It could be proposed that the absence of certain proteins in the ROS+ group make these individuals more prone to infection and inflammatory responses (Sharma et al., 2013a).

Most of the proteins found were involved in stress and regulatory pathways. These proteins were also found to play an important role in catalytic activities. Analysis of biochemical processes revealed the involvement of proteins common to ROS+ and ROS− groups in major pathways such as regulation, response to stress, interaction with neighbouring cells and organisms. On the other hand, proteins solely expressed in oxidative stress were assumed to be involved in sperm interaction, apoptosis, necrosis and cell death because of their role in cell cycling, ageing, morphogenesis and motility. Finally, proteins restricted to semen with physiological ROS concentrations were involved in enzymic reactions such as antioxidant activities, DNA binding, serine hydrolase and serine endopeptidase activity (Sharma et al., 2013a).

Similarly, Herwig et al. (2013) determined the protein profile of seminal fluid in idiopathic OAT patients with high levels of oxidative stress compared with normal donors.

Their proteomics analysis identified 46 proteins related to infertility. GO analysis determined that the protein processes of the 27 proteins common in all idiopathic OAT patients are focused on cellular organization and modification. Our results in samples with elevated levels of oxidative stress concur with their findings (Sharma et al., 2013a). In the study by Herwig et al. (2013), the 24 proteins highly expressed in ROS+ idiopathic OAT patients were involved in biological processes that centre on metabolism, inflammation, immunity and stress response. Pathway analysis of the proteins identified uniquely in idiopathic OAT had an enrichment of the glycerolipid metabolism pathway only (Herwig et al., 2013).

Sperm proteins and oxidative stress

Seminal proteins are not the only proteins that are of interest as potential biomarkers of oxidative stress. Proteins expressed by the spermatozoa themselves are equally as important, as they help scientists identify their roles in the spermatozoon's various metabolic processes, capacitation reactions and oocyte fertilization. Alterations in testicular ROS can disrupt the internal milieu of the cell, resulting in sperm dysfunction and impaired viability, motility and fertilization capacity (Aitken and Clarkson, 1987; Jones et al., 1979; Tremellen, 2008). Few studies have been reported regarding protein expression in spermatozoa and how they correlate with oxidative stress. Some of the important sperm proteins in relation to oxidative stress that have been isolated from infertile patients are summarized in Tables 6—8.

Our earlier paper (Hamada et al., 2013), we studied proteins expressed in spermatozoa from semen with and without oxidative stress and found that a total of 1343 protein spots were identified in ROS— samples and 1265 protein spots were identified in ROS+ samples. The majority of protein spots did not show any differential expression between ROS+ and ROS— samples, although when bands were compared in a gel, six spots were found to be decreased and 25 spots were increased.

Among the differentially expressed proteins, A kinase anchor protein kinase A 4 (AKAP4) was found to be over-expressed in ROS+ patients (Hamada et al., 2013). This cytoskeletal glycoprotein is the major protein in the fibrous sheath of the spermatozoon. AKAP4 plays a role in sperm motility because it anchors cAMP-dependent protein kinase, which phosphorylates neighbouring proteins in the presence of a threshold concentration of cAMP in the cell (Brown et al., 2003; Edwards and Scott, 2000). Elevated ROS concentrations increase cAMP concentrations, inhibiting tyrosine phosphatase and activating tyrosine kinase. This results in an increase in tyrosine phosphorylation of AKAP4 (Ford, 2004), which might explain the overexpression of AKAP4 in ROS+ patients. Furthermore, Ficarro et al. (2003) reported an increase in AKAP4 phosphorylation due to premature capacitation induced by elevated ROS concentrations.

In the same study (Hamada et al., 2013), AKAP3, another member of the A kinase anchoring protein family, was similarly found to be over-expressed. AKAP3 regulates sperm motility by forming complexes with different proteins. It anchors testis-specific protein ropporin (a sperm-specific binding protein of rhophilin localized in the sperm fibrous sheath) and AKAP-associated sperm protein and also interacts with AKAP4. Premature capacitation resulting from oxidative stress increases, in the same fashion as AKAP4, the concentrations of AKAP3 (Ficarro et al., 2003). However, our following study did not find any differential expression of both AKAP3 and AKAP4 proteins in spermatozoa from men with and without oxidative stress (Sharma et al., 2013b).

Heat shock proteins (HSP) play an important role in protein assembly, stabilization, folding and translocation of oligomeric proteins in physiological cellular conditions (Pockley, 2003). HSP are present in different cellular compartments. Under stressful conditions that cause protein unfolding and aggregation, HSP expression increases considerably (Pockley, 2003), which may explain the increase of HSP90 β and endoplasmin HSP90 β1 expression in spermatozoa of ROS+ patients (Hamada et al., 2013). In our subsequent study (Sharma et al., 2013b), we found that heat shock proteins (heat shock 70 kDa protein 5 (HSPA5) and heat shock protein 90 kDa β member 1 (HSP90B1)) were over-expressed in ROS+ samples. HSPA5, known also as glucose-regulated protein 78 or binding immunoglobulin protein, is a chaperone protein that is present in the cytoplasmic droplet of abnormal spermatozoa (Marin-Briggiler et al., 2010). In normozoospermic men, HSPA5 is localized either in the whole flagellum or confined to the midpiece or neck region in both ejaculated and capacitated spermatozoa (Marin-Briggiler et al., 2010). HSPA5 is associated to the sperm acrosomal cap and modulates the spermatozoon—zona pellucida interaction in a calcium-dependent manner (Marin-Briggiler et al., 2010). HSP90 β takes part in the reduction of cytochrome C (which serves as an apoptotic signal) through its sulphydryl groups and defends the cell against apoptosis (Nardai et al., 2000). However, increased oxidation of the chaperone's thiol group following the elevation of ROS concentrations, resulted in the inactivation of HSP90 β activity (Burton and Jauniaux, 2011; Gao et al., 2005).

Oxidative stress is detrimental to sperm DNA because oxygen radicals react with the nucleic content during spermiogenesis, an important step in sperm development and maturation. During this process, histones (which anchor DNA in somatic cells) are replaced by protamines (major nuclear sperm proteins) for greater condensation of the sperm genetic material (Oliva, 2006). Oxidative stress-induced defects in this pathway lead to an increase in histone concentrations inside the cell, which has been associated with infertility (Boissonnas et al., 2013). We found histone cluster 1 to be over-expressed in ROS+ samples (Sharma et al., 2013b).

Antioxidants play an essential role in maintaining an appropriate intracellular concentration of ROS. Suppression of antioxidants present in the cell result in oxidative stress. Peroxiredoxins are antioxidant enzymes that scavenge ROS and act as regulators of redox homeostasis and ROS-dependent signalling (O'Flaherty and de Souza, 2011). Each of our studies that peroxiredoxin 1 (PRDX1; Hamada et al., 2013) and peroxiredoxin 6 (PRDX6; Sharma et al., 2013b) have reduced expresssion in samples with oxidative stress compared with those without oxidative stress. O'Flaherty and de Souza (2011) reported that members of the

Table 6 Sperm proteins overexpressed in semen samples with oxidative stress compared to semen samples without oxidative stress.

Protein	UniProt ID	Subcellular location in spermatozoa	Function	Reference(s)
A-kinase anchor protein 4 (AKAP4)[a]	Q5JQC9	Fibrous sheath that provides mechanical support of the flagellum Testis-specific; only expressed in spermatid cells; post-meiotic phase of spermatogenesis	Structural protein, involved in sperm motility: anchors protein kinase A which can phosphorylate (under high cAMP stimulation) other functional proteins	Hamada et al. (2013)
Heat shock protein HSP 90-beta (HSP90AB1 or HSP90B)[a]	P08238	Endoplasmic reticulum	Molecular chaperone, reduces cytochrome C (apoptotic signal) and protects against apoptosis	Hamada et al. (2013)
Endoplasmin (heat shock protein 90 kDa β member 1 (HSP90B1))	P14625	Lumen of endoplasmic reticulum	Molecular chaperone, ATP binding, helps in translocation of secreted proteins, protein folding and assembly under normal conditions Increases in response to cellular stressors (e.g. free radical attack, heat and infection)	Hamada et al. (2013), Sharma et al. (2013b)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH or GAPD)[b]	P04406	Cytoplasm	Oxidoreductase, key enzyme in glycolytic pathway Binds to and activates the androgen receptor	Sharma et al. (2013b)
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific (GAPDHS or GAPD2)	O14556	Cytoplasm	Oxidoreductase, essential enzyme for male fertility — required for sperm motility and male fertility	Sharma et al. (2013b)
Fructose-biphosphate aldolase A (ALDOA)	P04075	Cytosol	Lyase, plays an important role in glycolysis and gluconeogenesis	Sharma et al. (2013b)
Malate dehydrogenase, mitochondrial (MDH2) precursor	P40926	Mitochondrial matrix	Oxidoreductase; produces ROS; catalyses the last step of the Krebs cycle	Sharma et al. (2013b)
Triosephosphate isomerase (isoform 1 (TPI1))	P60174	Cytosol	Isomerase, catalyses the isomerization of G3P and DHAP in glycolysis	Sharma et al. (2013b)
Histone H2B type 1-A (testis-specific histone H2B) (HIST1H2BA)	Q96A08	Nucleus, chromosome	Transcribed exclusively in testis, corresponding protein present in mature spermatozoa; core part of nucleosomes that bind and compact DNA into chromatin	Sharma et al. (2013b)
Heat shock 70 kDa protein 5 (HSPA5)	Q24JP5	Lumen of endoplasmic reticulum	Under normal conditions, plays a role in protein folding and assembly in the endoplasmic reticulum	Sharma et al. (2013b)
Glutamine synthetase (glutamate-ammonia ligase (GLUL))	P15104	Exists as a transcript	Ligase, catalyses the synthesis of glutamine from glutamate and ammonia	Sharma et al. (2013b)
Olfactomedin-4 (OLFM4) precursor	Q6UX06	Extracellular matrix	Strongly expressed in the prostate; facilitates cell adhesion	Sharma et al. (2013b)
Sperm acrosome membrane-associated protein 4 (sperm acrosomal membrane protein 14 (SPACA4))	Q8TDM5	Sperm acrosomal matrix and inner and outer acrosome membranes Testis-specific	Sperm surface membrane protein with a possible role in sperm—egg plasma membrane adhesion and fusion during fertilization	Sharma et al. (2013b)
Adipocyte plasma membrane-associated protein (APMAP) (chromosome 20 open reading frame 3 (C20orf3))	Q9HDC9	Membrane, also found on the cell surface of monocytes	Possible role in adipocyte differentiation	Sharma et al. (2013b)

Table 6 (continued)

Protein	UniProt ID	Subcellular location in spermatozoa	Function	Reference
Transglutaminase 4 (TGM4)	P49221	Prostate-specific; Sperm cytoplasm	Transferase, catalyses protein cross-linking and polyamine-conjugation to specific proteins in the seminal tract; Catalyse post-translational modifications of glutamine residues on proteins	Sharma et al. (2013b)
Glutathione peroxidase 4 (GPX4) isoform A precursor	P36969	Mainly in testis, also in sperm cytosol and mitochondria	Oxidoreductase and peroxidase; reduces H_2O_2, protects from oxidative damage, lipid peroxidation and apoptosis; required for normal sperm development	Sharma et al. (2013b)
l-Lactate dehydrogenase (LDHC)	P07864	Sperm cytoplasm	Involved in cAMP responsive element modulator pathway, which is essential for normal spermatogenesis	Sharma et al. (2013b)

Location and function are based on NCBI and UniProt databases.
[a]No differential expression between ROS+ and ROS− (Sharma et al., 2013b).
[b]Abundant in ROS compared to ROS+: enzyme may have been degraded by ROS (Hamada et al., 2013).

peroxiredoxin family, namely PRDX 1, PRDX4, PRDX5 and PRDX6, are each present in different locations of human spermatozoa as well as in seminal plasma. Both PRDX1 and PRDX6 were found in the sperm tail and in the equatorial segment and post-acrosomal region of the sperm head. The authors suggested that when a human spermatozoon is faced with high ROS concentrations, both isoforms 1 and 6 of peroxiredoxin are converted into complexes with high molecular mass to avoid protein modification or become themselves molecular chaperones (Kumsta and Jakob, 2009) that guard unmodified proteins.

Lactotransferrin (80 kDa) or lactoferrin is a glycoprotein that coats the sperm surface to act as an antigen with antimicrobial activities. It also serves as an antioxidant and chelates iron to prevent lipid peroxidation (Hamada et al., 2013). Lactotransferrin is increased in samples without oxidative stress (Hamada et al., 2013). A recent study that isolated lactotransferrin from *in vitro* secretions of human oviductal tissue, reported that lactotransferrin inhibits the spermatozoon-oocyte interaction in a dose-dependent manner (Zumoffen et al., 2013). The study also demonstrated that lactotransferrin concentrations increase during inflammatory processes, which may indicate its role in certain reproductive disorders that are inflammatory in nature (Zumoffen et al., 2013).

Several isoforms of histones were present in higher abundance in ROS+ compared with ROS− groups, signifying dysfunctional sperm chromatin packaging leading to DNA damage (Sharma et al., 2013b), which is common during an oxidative stress state (Sharma and Agarwal, 2011).

GO analysis was performed in order to further understand the protein profiles generated. In our second study, Sharma et al. (2013b), we found that a larger distribution of the proteins present in both ROS+ and ROS− groups are located in the intracellular region as compared with the extracellular millieu and that both over- and underexpressed proteins occupy predominantly cytoplasm, intracellular and organelle locations. Over-expressed proteins solely occupied membrane-bound organelles while underexpressed proteins largely occupied the cytosol (Sharma et al., 2013b).

Among the common biological processes for which both over- and underexpressed proteins are associated include cellular, metabolic and regulatory processes (Sharma et al., 2013b). However, over-expressed proteins are solely involved in cellular component biogenesis, homeostatic processes and gluconeogenesis as well as cytoskeleton organization, while only underexpressed proteins are involved in cellular component movement, carbohydrate catabolic processes, glycolysis and response to unfolded protein. When compared with common processes, more over-expressed proteins are involved in metabolic processes while more underexpressed proteins are involved in response to stress. The principal function of the differentially expressed proteins is carbohydrate metabolism, comprising pathways of gluconeogenesis and glycolysis. Based on this, we suggested that while oxidative phosphorylation is important, energy production for sperm motility is mainly through the glycolytic pathway (Sharma et al., 2013b).

We also conducted a transcriptional network analysis and found 21 proteins that seemed to be transcriptionally

Table 7 Sperm proteins underexpressed in semen samples with oxidative stress compared to semen samples without oxidative stress.

Protein	UniProt ID	Subcellular location in spermatozoa	Function	Reference
Semenogelin II precursor (SEMG2)	Q02383	Main protein in semen	Forms the semen coagulum and is degraded by prostate-specific antigen	Sharma et al. (2013b)
Peroxiredoxin-6 (PRDX6)	P30041	Post-acrosomal region and equatorial segment and sperm tail	Antioxidant, detoxifies H_2O_2	Sharma et al. (2013b)
Peroxiredoxin-1 (PRDX1)	Q06830	Post-acrosomal region and equatorial segment and sperm tail	Antioxidant, detoxifies H_2O_2	Hamada et al. (2013)
Clathrin heavy chain 1 (CLTC)	Q00610	Cytoplasmic face of intracellular organelles	Binds and activates androgen receptor (required for normal spermatogenesis)	Sharma et al. (2013b)
Eukaryotic translation elongation factor 2 (EEF2)	Q6PK56	Cytoplasm	Plays an important role in protein synthesis	Sharma et al. (2013b)
			Allows translocation of the nascent protein chain from the A site to the P site of the Ribosome	
Enolase 1 (ENO1)	P06733	Cytosol	Involved in energy production Enzyme of the glycolytic pathway	Sharma et al. (2013b)
Angiotensin 1 converting enzyme 1 isoform 1 precursor (ACE)	P12821	Membrane	Involved in cAMP responsive element modulator pathway, which is essential for normal spermatogenesis	Sharma et al. (2013b)
Outer dense fibre of sperm tails 1 (ODF1)	Q5BJF6	Cytoskeleton	Involved in cAMP responsive element modulator pathway, which is essential for normal spermatogenesis	Sharma et al. (2013b)
Phosphoglycerate kinase 2 (PGK2)	P07205	Cytoplasm	Involved in energy production	Sharma et al. (2013b)
			Enzyme of the glycolytic pathway	
Heat shock protein beta-1 (HSPB1)	P04792	Mainly in cytosol	Binds and activates androgen receptor (required for normal spermatogenesis)	Sharma et al. (2013b)

Location and function are based on NCBI and UniProt databases.

Table 8 Sperm proteins over-expressed in semen samples without oxidative stress compared to semen samples with oxidative stress.

Protein	UniProt ID	Subcellular location in spermatozoa	Function	Reference(s)
Lactotransferrin isoforms 2 and 1	P02788	Sperm membrane	Antioxidant, antibacterial, immune modulating agent	Hamada et al. (2013), Sharma et al. (2013b)
Peroxiredoxin-1 (PRDX1)	Q06830	Post-acrosomal region and equatorial segment and sperm tail	Antioxidant, detoxifies H_2O_2	Hamada et al. (2013), Sharma et al. (2013b)
Mitochondrial superoxide dismutase (SOD)	P04179	Spermatozoal mitochondria	Scavenges superoxide ions and converts it to H_2O_2	Hamada et al. (2013)
Glyceraldehyde-3-phosphate dehydrogenase (GADH)	P04406	Testis specific	Plays a role in the glycolysis pathway	Hamada et al. (2013)
			Binds to androgen receptor and activates it	

Location and function are based on NCBI and UniProt databases.

activated by the androgen receptor (Sharma et al., 2013b). Androgen receptors are present in spermatids and Sertoli and Leydig cells, through which androgens mediate various developmental processes during spermatogenesis (Mostafa et al., 2012; Zhou et al., 2002). Infertile men have been reported to have decreased androgen receptor expression

compared with fertile normozoospermic men (Zalata et al., 2013). Androgen receptor expression has a positive correlation with serum testosterone, sperm count and normal sperm morphology (Zalata et al., 2013). Oxidative stress-induced DNA damage has been shown to occur in spermatocytes when androgen is suppressed (Stanton et al., 2012).

The spermatogenic process is dependent on the cAMP-responsive element modulator (CREM) signalling pathway in the testis, which is regulated by repressors and activators (Tamai et al., 1997). In that regard, our study (Sharma et al., 2013b) revealed underexpression of ODF1 (outer dense fibre) and ACE1 (antihuman angiotensin-converting enzyme) and overexpression of LDHC (lactate dehydrogenase C) in the ROS+ group, and these three proteins were regulated by CREM activators.

Biomarkers of oxidative stress

An ideal biomarker can be detected with ease and is able to accurately indicate an abnormal state at early onset. In addition, its evaluation should have minimal associated side effects and it should be available at an affordable price (Kovac et al., 2013). In general clinical terms, biomarkers would serve to screen for, diagnose or monitor disease activity, while therapeutically, they would serve as a guide to targeted therapy or would assess therapeutic response (Etzioni et al., 2003). In the studies reviewed in this paper, several proteins may be selected as putative biomarkers that are indicative of a state of oxidative stress. A summary of possible biomarkers that are indicative of oxidative stress-induced male infertility are provided in Table 9.

From the seminal plasma studies, six proteins have been suggested: i.e. DJ-1, TBCB (tubulin-folding cofactor B), AACT (anti-a1-antichymotrypsin), ALDR (alcohol dehydrogenase), DGK (diacylglycerol kinase) and PIP. All of these proteins are highly expressed in seminal plasma with high oxidative stress except for DJ-1, which decreases in samples with oxidative stress. PIP has also been suggested as a biomarker of azoospermia and asthenozoospermia and its concentrations are decreased in both conditions; Martinez-Heredia et al., 2008; Yamakawa et al., 2007; Tomar et al., 2012b). Sperm proteins such as MDH2 (malate dehydrogenase 2), TGM4 (transglutaminase 4 (prostate)), GPX4 (glutathione peroxidase 4), GLUL (glutamine synthetase), HSP90B1 and HSPA5 are suggested as possible biomarkers due to their increased expression in ROS+ samples compared with those without oxidative stress. Heat shock proteins such as HSP90B1 and HSPA5 are reported to be highly related to varicocele (Ferlin et al., 2010), which was a cause of oxidative stress and infertility in our patient population. Most of these candidate proteins or enzymes seem to have a general function of reducing oxidation or to play a role in anti-apoptosis and regulatory mechanisms.

Challenges faced in proteomic studies

The proteomics road ahead is long, windy and strewn with obstacles. Studies characterizing protein profiles result in the generation of enormous amounts of information, complex protein compositions and a large number of isolated proteins whose actions or functions are not yet discovered. These limiting factors could potentially restrict the use of proteomic approaches to elucidate biological markers solely for the purpose of research (Sánchez et al., 2013).

Proteomics is a highly technical and user-based field that is of growing interest. In order to apply this technology towards the understanding of the molecular basis of male factor infertility associated with high oxidative stress, researchers and clinicians are bound to be challenged in various areas. To begin with, patient selection will require on-site advanced tests for the measurement of oxidative stress in freshly collected semen samples — a facility that many clinical laboratories may not currently have. Moreover, in order to conduct proteomic analysis in these samples, laboratories will need to have access to proteomic research facilities or services and, along with that, adequate funding to support the expense and the technical know-how to adequately process the samples. Statistical evaluations and interpretation of the protein lists generated by these studies are key elements in making sense of the data generated. In this regard, the role of bioinformaticians is vital in describing a meaningful and valid protein pathway and protein–protein interactions for completion of the project.

In addition to these aspects, researchers and clinicians would have to take in to account that proteomic profiles from patients with a particular infertility diagnosis are subjected to a great deal of variability, not only between experimental settings but also amongst individuals themselves. Differences in the proteomic strategy employed, such as the LC-MS/MS analysis, and the database searches used add to the variability of the proteins that are identified (Macleod and Varmuza, 2013). Further, infertile individuals may have multiple underlying conditions or contributing factors, which could add to a great variation in protein expression. In that regard, fertile individuals have been found to share very little common proteome (Milardi et al., 2012). Specifically in this study, the authors reported a common pool of only 83 seminal plasma proteins between five healthy fertile men, who each generated between 919–1487 unique proteins per sample (Milardi et al., 2012).

Regarding data interpretation, researchers would have to keep in mind that if any particular protein does not turn up on the list of proteins identified from the particular tissue, cell or fluid being studied, it cannot be taken for granted that the protein of interest is not represented in that sample. Instead, this absence could be attributed to that the protein expression may have been too low to be detected, that the proteomic coverage may have been incomplete or that the protein was inadvertently missed (Baker and Aitken, 2009).

The dynamic nature of the proteomic profile is further complicated by post-translational modifications that may occur in the cell or tissue of interest. As technology advances and MS techniques develop further, hopefully the complexity when dealing with translated proteins that continue to be intracellularly modified can be toned down (Macleod and Varmuza, 2013).

All these factors should be kept in mind, as we continue to apply proteomic techniques are applied to clinical

Table 9 Candidate protein biomarkers that may be indicative of oxidative stress-induced male infertility.

Suggested protein biomarker	UniProt ID	Function	Expression with pathological ROS or oxidative stress	Expression with physiological ROS or without oxidative stress	Reference
Seminal plasma					
DJ-1	Q99497	Protects against oxidative stress and apoptosis, eliminates H_2O_2, positive regulator of androgen receptor-dependent transcription	Decreased	Identified	Wang et al. (2009)
Tubulin-folding cofactor B (TBCB)	Q99426	Aids in the regulation of tubulin heterodimer dissociation	Increased	Not stated	Herwig et al. (2013)
Alpha-1-antichymotrypsin (AACT)	P01011	Physiological role undefined, may be involved in controlling oxidative damage, highly anti-inflammatory, significant role in defence mechanism against pathological processes	Increased	Identified	Herwig et al. (2013)
Aldose reductase (ALDR)	P15121	Catalyses the reduction of aldehydes and carbonyls	Increased	Identified	Herwig et al. (2013)
Diacylglycerol kinase (DGK) eta	Q86XP1	Upstream regulator of oxidative stress-induced activation of PKD signalling pathway	Increased	Not identified	Herwig et al. (2013)
Prolactin-inducible protein (PIP)	P12273	Involved in immunoregulation, antimicrobial activity, apoptosis and tumour progression	Increased	Identified (very low amount)	Sharma et al. (2013a)
Spermatozoa					
Lactotransferrin-2	P02788	Antioxidant Chelates iron (an essential catalyst for ROS production)	Not identified	Increased	Hamada et al. (2013)
Peroxiredoxin-1 (PRDX1)	Q06830	Detoxifies H_2O_2	Decreased	Increased	Hamada et al. (2013)
Histone cluster 1, H2ba (HIST1H2BA)	Q96A08	Core component of nucleosome which wrap and compact DNA into chromatin	Increased	Identified	Sharma et al. (2013b)
Malate dehydrogenase 2, NAD (mitochondrial) (MDH2) precursor	P40926	Catalyses the reversible oxidation of malate to oxaloacetate (citric acid cycle)	Increased	Not identified	Sharma et al. (2013b)
Transglutaminase 4 (TGM4)	P49221	Catalyses post-translational cross-linking of proteins, part of a calcium-dependent enzyme	Increased	Identified	Sharma et al. (2013b)
Glutathione peroxidase 4 (GPX4) isoform A precursor	P36969	Catalyses the reduction of hydrogen peroxide, protects against oxidative damage	Increased	Identified	Sharma et al. (2013b)
Glutamate-ammonia ligase (GLUL)	P15104	Catalyses the synthesis of glutamine (from glutamate and ammonia), which plays a role in inhibition of apoptosis, cell proliferation and signalling, aids in control of body pH	Increased	Identified	Sharma et al. (2013b)
Heat shock protein 90 kDa beta (HSP90B1)	P14625	ATP-metabolizing molecular chaperone involved in stabilizing and folding other proteins, expressed in formation of tumours and other pathogenic states	Increased	Identified	Sharma et al. (2013b)
Heat shock protein 70 kDa protein 5 (HSPA5)	Q24JP5	Plays a role in the folding and assembly of proteins in the endoplasmic reticulum; possible important role in monitoring protein transport through the cell	Increased	Not identified	Sharma et al. (2013b)

Function is based on NCBI and UniProt databases.

samples in the search for plausible male infertility biomarkers of clinical relevance. In future, if and when an assay is successfully developed and adopted in andrology laboratories for use in detecting biomarkers of male infertility, other considerations come into play. For example, the cost of the new 'test' should be maintained at an affordable rate in line with the battery of other tests in the diagnosis of male infertility. This would allow for its wider use among patients from different socioeconomic backgrounds. This new test will have to be carried out according to a standardized measurement methodology and test procedural protocols across different laboratories in order to maintain uniformity and minimize variability. The use of internal and external controls is required to provide the quality control measure to ensure that the test is valid, highly repeatable and robust.

The way forward

The goal of studying the proteomic profiles of infertile patients with signs of oxidative stress would be to identify from the lengthy protein lists generated, several components of interest that may represent a particular disease process, a 'protein signature' that could be used in the clinic to help in the diagnosis of male factor infertility. As researchers delve deeper into the proteome, experimental techniques should look into deciphering the post-translational modifications that would better explain the dynamic nature and function of the proteins of interest. This is particularly important when characterizing the proteome of mature ejaculated spermatozoa, as the male gamete does not undergo further transcription or translation (Macleod and Varmuza, 2013). As previously reported (Kamath et al., 2011), post-translational modifications may be reversible or irreversible, which adds complexity and diversity to the proteins in the tissue studied and warrants further studies to look into uncovering protein functions, activities and networks in a given physiological or pathological status.

Conclusion

In this review, we have compared findings from studies on protein samples from seminal plasma and spermatozoa that evaluated the protein profile of seminal ejaculate with oxidative stress and with physiological oxidative status. Proteins from both spermatozoa and seminal plasma components that may play a role in the response to prevent oxidative stress have been listed and putative biomarkers indicative of oxidative stress have been identified.

Considering all these studies altogether, pathway analyses reveal proteins involved in stress response and cellular, metabolic and regulatory pathways. Studies such as the ones compiled in this report will contribute towards elucidating the main causes of idiopathic male infertility. Ultimately, it is hoped that once the aetiology of male infertility is known at a molecular level, its findings will contribute towards an improved diagnosis, treatment and prevention of male infertility.

Acknowledgement

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