Human Reproduction Update, Vol.12, No.4 pp. 417–435, 2006 doi:10.1093/humupd/dml009
Advance Access publication March 31, 2006

Protamines and male infertility

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Protamines are the major nuclear sperm proteins. The human sperm nucleus contains two types of protamine: protamine 1 (P1) encoded by a single-copy gene and the family of protamine 2 (P2) proteins (P2, P3 and P4), all also encoded by a single gene that is transcribed and translated into a precursor protein. The protamines were discovered more than a century ago, but their function is not yet fully understood. In fact, different hypotheses have been proposed: condensation of the sperm nucleus into a compact hydrodynamic shape, protection of the genetic message delivered by the spermatozoa, involvement in the processes maintaining the integrity and repair of DNA during or after the nucleohistone–nucleoprotamine transition and involvement in the epigenetic imprinting of the spermatozoa. Protamines are also one of the most variable proteins found in nature, with data supporting a positive Darwinian selection. Changes in the expression of P1 and P2 protamines have been found to be associated with infertility in man. Mutations in the protamine genes have also been found in some infertile patients. Transgenic mice defective in the expression of protamines also present several structural defects in the sperm nucleus and have variable degrees of infertility. There is also evidence that altered levels of protamines may result in an increased susceptibility to injury in the spermatozoan DNA causing infertility or poor outcomes in assisted reproduction. The present work reviews the articles published to date on the relationship between protamines and infertility.

Key words: chromatin/genome/mutation/protamine/spermatozoa

Introduction

Protamines and DNA were isolated and discovered from the sperm more than a century ago by Friedrich Miescher (Miescher, 1874; Kossel, 1928; Felix, 1960; Dixon and Smith, 1968; Dahm, 2005). They are the most abundant sperm nuclear proteins in many species and act by packaging the paternal genome (Bloch, 1969; Ando *et al*., 1973; Calvin, 1976; Mezquita and Teng, 1977, 1978; Subirana, 1983; Oliva and Dixon, 1991a; Aoki and Carrell, 2003; Lewis *et al*., 2003a). They are proteins with a high content of positively charged amino acids, particularly arginine (48% in human protamines; Figure 1).

In mammals, two types of protamines are known: the P1 protamine and the family of P2 proteins. The P1 protamine is present in all species of vertebrates studied (McKay *et al*., 1985, 1986; Gusse *et al*., 1986; Balhorn *et al*., 1987; Bellvé *et al*., 1988; Oliva and Dixon, 1991a; Chauvière *et al*., 1992; Yoshii *et al*., 2005). Protamine P2 is formed by the P2, P3 and P4 components, and it is only present in some mammalian species including human and mouse (Balhorn *et al*., 1977, 1987; McKay *et al*., 1985, 1986; Gusse *et al*., 1986; Bélaïche *et al*., 1987; Bower *et al*., 1987; Bellvé *et al*., 1988; Oliva and Dixon, 1991a; Yoshii *et al*., 2005).

Several functions have been proposed for the protamines (reviewed by Oliva and Dixon, 1991a). The most obvious would be:

(i) Generation of a condensed paternal genome with a more compact and hydrodynamic nucleus. The spermatozoa with the most hydrodynamic nucleus would move faster, being able to fertilize the oocyte first. Therefore, the most condensed and hydrodynamic sperm would transmit the advantageous trait to future generations through a marked Darwinian selection.

(ii) Protecting the paternal genetic message delivered by the spermatozoa through making it inaccessible to nucleases or mutagens potentially present in the internal or in the external media. This hypothesis could be supported by recent observations in assisted reproduction linking defects in protamination with injured spermatozoal DNA, compatible with fertilization of the oocyte but precluding subsequent embryo development.

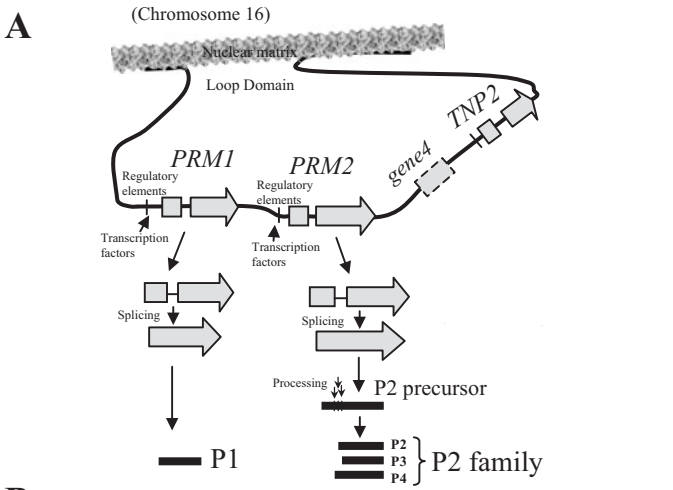

Figure 1. Transcription of the protamine genes and translation and processing of human protamines. (**A**) Schematic representation of the genomic structure of protamine genes (*PRM1* and *PRM2*) and the transcription, translation and processing involved in the synthesis of mature protamine. Protamine 1 (P1) is synthesized as a mature precursor, whereas the protamine 2 (P2) family is generated by partial processing of a single P2 precursor (see text for further details). *TNP2*, gene-encoding transition protein 2. (**B**) Amino acid sequences for human P1 and for the main components (P2, P3 and P4) of the protamine 2 family. It should be noted that P2 is the most abundant component, while P3 and P4 are minor components of the P2 family. The arginine, histidine and lysine residues are shown in bold. Cysteines are underlined.

However, of relevance to the understanding of the mechanisms leading to infertility, the presence of protamines may also recruit and/or potentiate the effect of certain toxins or heavy metals in the testis or spermatozoa.

(iii) Competition and removal of transcription factors and other proteins from the spermatid resulting in a blank paternal genetic message, devoid of epigenetic information, therefore allowing its reprogramming by the oocyte.

(iv) Involvement in the imprinting of the paternal genome during spermatogenesis. Also protamines themselves could confer an epigenetic mark on some regions of the sperm genome, affecting its reactivation upon fertilization.

In addition to the above potential functions, it has also been proposed that (v) protamines could be part of a checkpoint during spermiogenesis and (vi) they could have a role in the fertilized ova.

The present review focuses on the available evidence between protamines and male infertility. Thus, it complements and updates more extensive previous reviews on the nucleohistone–nucleoprotamine transition (Mezquita, 1985; Poccia, 1986; Ward and Coffey, 1991; Oliva and Dixon, 1991a; Dadoune, 1995, 2003; Wouters-Tyrou *et al*., 1998; Raukas and Mikelsaar, 1999; Braun, 2001; Aoki and Carrell, 2003; Meistrich *et al*., 2003; Kierszenbaum and Tres, 2004; Hogarth *et al*., 2005). The role of histones (His), histone modifications, remodelling factors and epigenetic changes during spermatogenesis have also been elegantly reviewed by different groups (Sassone-Corsi, 2002; Lewis *et al*., 2003b; Govin *et al*., 2004; Caron *et al*., 2005; Horsthemke and Ludwig, 2005; Kimmins and Sassone-Corsi, 2005; Morgan *et al*., 2005; Rousseaux *et al*., 2005). To cover the subject of the DNA-repair mechanisms, oxidative stress and sperm DNA integrity and male infertility, the reader is referred to recent articles and reviews (McPherson and Longo, 1993; Aitken and Krausz, 2001; Baarends *et al*., 2001; Kierszenbaum, 2001; Oehninger *et al*., 2003; Sakkas *et al*., 2003; O'Brien and Zini, 2005; Seli and Sakkas, 2005; Silva and Gadella, 2005; Erenpreiss *et al*., 2006; Muratori *et al*., 2006).

The following section of this review has been included to provide a brief synthetic summary of the protamine genes, their evolution, expression and involvement in the nucleohistone–nucleoprotamine transition. This initial section is not comprehensive but has been included to focus the subject and to facilitate reading of the rest of the review. In contrast, the rest of the review is intended to be comprehensive, for all articles published to date concerning protamines and infertility in man. The articles considered for inclusion were selected from the results of Medline and Journal Citation Report (ISI Web of Knowledge) searches with the keyword 'protamine' alone or combined with other keywords ('infertility', 'human' + 'sperm' and 'human' + 'testis').

Summary of protamine structure and function

Evolution of the protamines

Protamines are proteins that have increased the number of positively charged residues in evolution allowing the formation of a highly condensed complex with the paternal genomic DNA, which has a strong negative charge (Oliva and Dixon, 1990, 1991a; Retief *et al*., 1993; Oliva, 1995; Queralt *et al*., 1995; Lewis *et al*., 2003a). In addition, protamines of different species incorporate cysteines (Cys) in their sequence allowing the formation of disulphide bonds between adjacent protamine molecules, therefore strongly stabilizing the nucleoprotamine complex (Saowaros and Panyim, 1979; Balhorn *et al*., 1992; Lewis *et al*., 2003a; Vilfan *et al*., 2004). Evidence already exists that protamines may have evolved from histone H1 ancestors (Ausió, 1999; Lewis *et al*., 2004; Eirin-Lopez *et al*., 2006). Another characteristic of the protamines is that they are among those proteins with one of the highest rates of evolutionary variation (Oliva and Dixon, 1991a; Oliva, 1995; Lewis *et al*., 2003a). It has been proposed that one cause of this rapid evolution rate could be a positive Darwinian selection (Rooney and Zhang, 1999; Clark and Civetta, 2000; Wyckoff *et al*., 2000). This proposal is supported by the observation, when comparing the sequence of protamines from different species, that the ratio of non-synonymous substitutions (the nucleotide changes resulting in a change of amino acid) per residue to synonymous substitutions is greater than 1 and also that the protamine exons evolve faster than the protamine intron (Rooney and Zhang, 1999; Wyckoff *et al*., 2000). However, a closer examination revealed an unusual form of purifying selection, where the overall number of arginine residues is maintained at about 50% in mammals, but the total number of amino acids and the positions of the arginine residues have changed considerably (Rooney *et al*., 2000). It has been proposed that the driving forces for this arginine-rich selection could be (i) the DNA-binding function of the protamine P1 resulting

in a more compact sperm nucleus and (ii) the interaction and strong activation of oocyte creatine kinase II by protamine (Ohtsuki *et al*., 1996; Rooney and Zhang, 1999). While the evolution of protamines is providing important clues towards the understanding of their function, this aspect is not covered further here, so the reader is referred to other reviews and articles for a more in-depth analysis of this topic (Oliva and Dixon, 1991a; Ausió, 1999; Clark and Civetta, 2000; Wyckoff *et al*., 2000; Torgerson *et al*., 2002; Lewis *et al*., 2003a; Eirin-Lopez *et al*., 2005).

Genomic organization and transcription of the protamine genes

Humans have one copy of the protamine 1 gene (*PRM1*) and one copy of the protamine 2 gene (*PRM2*) per haploid genome, located on chromosome 16 (Figure 1; Krawetz *et al*., 1989; Reeves *et al*., 1989; Domenjoud *et al*., 1990; Oliva and Dixon, 1990, 1991a; Engel *et al*., 1992; Nelson and Krawetz, 1993, 1994; Queralt *et al*., 1993; Schlüter *et al*., 1996). Both genes contain a single intron (Figure 1). The genomic sequences of the *PRM1* and *PRM2* genes are organized in the form of a loop domain together with the transition protein 2 gene (*TNP2*) and a sequence called *gene4* (Figure 1; Engel *et al*., 1992; Choudhary *et al*., 1995; Schlüter and Engel, 1995; Schlüter *et al*., 1996; Kramer and Krawetz, 1998; Wykes and Krawetz, 2003; Martins *et al*., 2004). This spatial organization may allow a co-ordinated expression of these genes during spermiogenesis. However, while the protamine (*PRM1* and *PRM2*) and transition protein (*TNP2*) genes are expressed at high levels and their function has been extensively studied, the potential role of *gene4* is more controversial and is expressed at very low levels, if at all, in humans (Schlüter and Engel, 1995; Schlüter *et al*., 1996; Kramer and Krawetz, 1998). Further studies should clarify whether or not *gene4* is a pseudogene in humans. The *gene4* sequence has also been called protamine 3 (*Prm3; or gene4/Prm3*), based on some evidence that it may have originated by duplication of the *PRM1* gene (Schlüter *et al*., 1996; Kramer and Krawetz, 1998). However, the name *Prm3* is misleading since its predicted amino-acid sequence is not at all related to protamines, as it lacks arginine clusters and, instead, is rich in glutamic acid. Therefore, *gene4/Prm3* is not likely to bind DNA and should not be called protamine.

The positioning of nucleosomes in the protamine 1 gene has been assessed *in vivo* and *in vitro* using the rat as a model (Adroer and Oliva, 1998). The identification of regulatory elements and the expression of the protamine genes have been studied using a variety of approaches including homology comparisons, transgenic or knockout mice and different *in vivo* and *in vitro* approaches (Tamura *et al*., 1992; Queralt and Oliva, 1993, 1995; Zambrowicz *et al*., 1993; Nelson and Krawetz, 1994; Choi *et al*., 1997; Stewart *et al*., 1999; Giorgini *et al*., 2001; Hummelke and Cooney, 2004; Aleem *et al*., 2005). For further information on this subject, the reader is referred to excellent reviews and articles on the transcriptional, molecular and cellular mechanisms in spermatogenesis (Iatrou and Dixon, 1978; Mezquita, 1985; Hecht, 1988, 1993; Perreault, 1992; Braun *et al*., 1995; Dadoune, 1995, 2003; Kramer and Krawetz, 1997; Siffroi *et al*., 1999; Steger, 1999, 2001; Steger *et al*., 2000, 2002; Grootegoed *et al*., 2000; Aoki and Carrell, 2003; Hebbar and Archer, 2003; Kleene, 2003; Dadoune *et al*., 2004; Kierszenbaum and Tres, 2004; Kimmins *et al*., 2004; Rockett *et al*., 2004; Krawetz, 2005; Miller *et al*., 2005; Tanaka and Baba, 2005). Despite substantial knowledge available on the fundamental aspects of the transcriptional mechanisms, so far there have been relatively few studies assessing the potential involvement of changes in protamine gene transcription factors in human male infertility (Sassone-Corsi, 2002; Blocher *et al*., 2003; Kimmins *et al*., 2004; Krausz and Sassone-Corsi, 2005). Because of the extensive evidence for deregulation of protamine expression in male infertility, this issue would deserve further attention in the future.

Synthesis of protamines

The protamine P1 is synthesized as a mature protein, whereas the components of the P2 family are generated by proteolysis from a precursor encoded by a single gene (Figure 1A and B; Mckay *et al*., 1986; Yelick *et al*. 1987; Sautière *et al*., 1988; Chauvière *et al*., 1992; Green *et al*., 1994; Queralt *et al*., 1995; Wouters-Tyrou *et al*., 1998). Members of the P2 family differ only by the N-terminal extension of 1–4 residues, although the P2 component is the most abundant (Figure 2; Gusse *et al*., 1986; McKay *et al*., 1986; Sautière *et al*., 1988; Martinage *et al*., 1990; Arkhis *et al*., 1991; Oliva and Dixon, 1991a; Bianchi *et al*., 1992; Alimi *et al*., 1993; Yoshii *et al*., 2005). The content of protamine P1 in the human sperm nucleus is similar to the content of protamine P2 (P1/P2 ratio of approximately 1; Balhorn *et al*., 1988; de Yebra *et al*., 1993; Bench *et al*., 1996; Corzett *et al*., 2002; Mengual *et al*., 2003a; Aoki *et al*., 2005a). However, despite this, their functions may differ. Arguments in favour of the hypothesis of a different function for P1 and P2 protamines could be that (i) unlike P1 protamine, P2 protamines are zinc-finger proteins with one Cys2–His2 motif (Bianchi *et al*., 1992), (ii) P2 proteins are expressed only in some mammals whereas P1 is invariably present in all mammals, indicating a more basic and conserved function for P1 and an accessory function for P2 protamines in some species and (iii) alterations of P1 or P2 protamines in infertile patients impact differently on the integrity of the DNA and in the assisted reproduction outcome (Aoki *et al*., 2005b).

Both protamines will undergo post-transcriptional modifications before binding to the DNA and generating the highly compact nucleoprotamine complex.

The nucleohistone–nucleoprotamine transition

In the final stage of spermatogenesis, the nucleosomal structure is progressively disassembled, then replaced by TNPs and finally by protamines (Figure 2; reviewed by Mezquita, 1985; Poccia, 1986; Oliva and Dixon, 1991a; Hecht, 1993; Green *et al*., 1994; Dadoune, 1995; Grootegoed *et al*., 2000; Meistrich *et al*., 2003; Kierszenbaum and Tres, 2004; Rousseaux *et al*., 2005). This transition is preceded by extremely marked changes in many chromatin activities (Puwaravutipanich and Panyim, 1975; Oliva *et al*., 1982; Mezquita, 1985; Oliva and Dixon, 1991a; Dadoune, 1995, 2003; Wouters-Tyrou *et al*., 1998; Fuentes-Mascorro *et al*., 2000; Braun, 2001; Govin *et al*., 2004; Kierszenbaum and Tres, 2004). One of the initial chromatin changes is the incorporation of histone variants (Prigent *et al*., 1996, 1998; reviewed by Churikov *et al*., 2004; Govin *et al*., 2004; Loppin *et al*., 2005; Tanaka *et al*., 2005). Another important early event is histone hyperacetylation that occurs during spermiogenesis before the nucleosome disassembly *in vivo* (Candido and Dixon, 1972; Oliva and Mezquita,

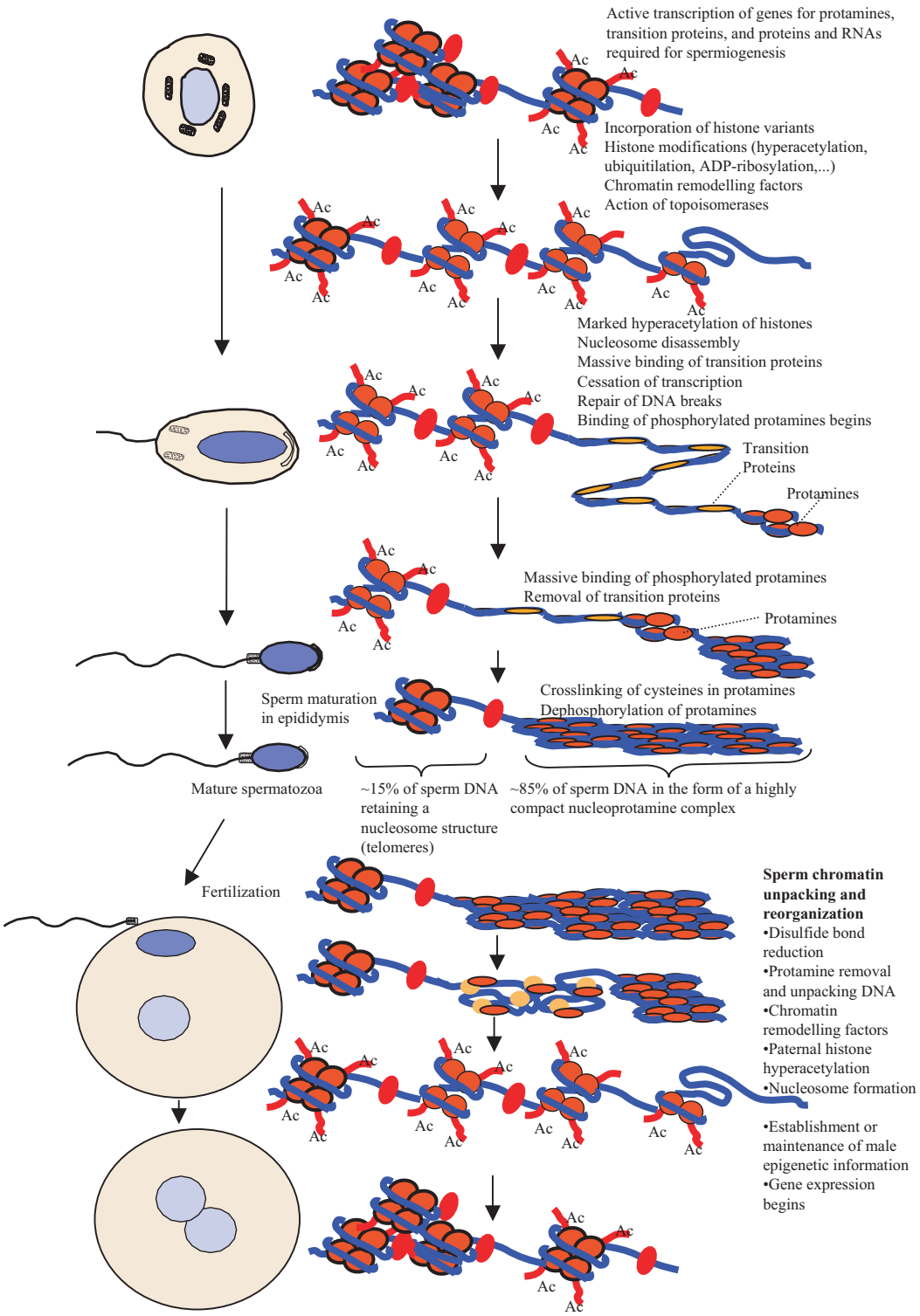

Figure 2. Schematic representation of the major chromatin changes occurring during the nucleohistone–nucleoprotamine transition in spermiogenesis and the subsequent nucleoprotamine unpacking and nucleohistone structure reconstitution at fertilization. The round spermatid (top left) has a chromatin structure similar to that present in all somatic cells, with the DNA organized in nucleosomes and many genes being actively transcribed. During the initial stages of spermiogenesis, histones are hyperacetylated and undergo other modifications, nucleosomes are disassembled, topoisomerase II unwinds superhelicity of the DNA, transcription ceases and transition proteins (TNPs) bind the DNA. At the final stage of spermiogenesis, TNPs are removed and protamines progressively bind the DNA. During sperm maturation in the epididymis, the formation of disulphide bonds in protamines further stabilizes the nucleoprotamine complex. At fertilization, the highly compact nucleoprotamine structure must be unpacked and reorganized into a nucleosomal structure. Histones are represented in red and DNA is represented by blue lines. The presence of hyperacetylation in the N-terminal histone tails is indicated by 'Ac'. Transition proteins are represented as orange elongated ovals. Protamines are represented as red elongated ovals.

1982; Grimes and Henderson, 1984; Meistrich *et al*., 1992; Hazzouri *et al*., 2000; Marcon and Boissonneault, 2004). It was postulated that histone hyperacetylation and rapid turnover of acetyl groups could rapidly and reversibly expose binding sites in chromatin for subsequent binding of chromosomal proteins (Oliva and Mezquita, 1982). More recently, it was also shown *in vitro* that histone hyperacetylation facilitated nucleosome disassembly and histone displacement by protamines (Oliva and Mezquita, 1986; Oliva *et al*., 1987). Also, hyperacetylated nucleosomes were shown to appear in a more relaxed structure upon binding to electron microscopy grids (Oliva *et al*., 1990). It has been shown that the testis-specific bromodomain-containing protein (BRDT) binds to hyperacetylated histone 4 (H4) triggering a reorganization of the chromatin (Pivot-Pajot *et al*., 2003). Impaired H4 hyperacetylation has been detected in infertile patients (Sonnack *et al*., 2002; Faure *et al*., 2003).

Concomitant with nucleosome disassembly, the sperm DNA is extensively complexed with TNPs (Figure 2; Kierszenbaum, 2001; Meistrich *et al*., 2003). Transition proteins are then finally replaced by protamines to form a highly compact nucleoprotamine complex (Figure 2). It is known that protamines are phosphorylated before binding to DNA and that a substantial dephosphorylation takes place concomitant with nucleoprotamine maturation (Ingles and Dixon, 1967; Marushige and Marushige, 1978; Oliva and Dixon, 1991a; Papoutsopoulou *et al*., 1999). The dynamics of protamine binding to DNA have also been studied (Prieto *et al*., 1997; Brewer *et al*., 1999, 2003). After binding to the DNA, the formation of disulphide bonds between protamines further stabilizes the nucleoprotamine complex (Balhorn *et al*., 1992). Different models for the structure of the nucleoprotamine have been proposed (Balhorn, 1982; Allen *et al*., 1993, 1997; Hud *et al*., 1993; Raukas and Mikelsaar, 1999; Vilfan *et al*., 2004; Biegeleisen, 2006). However, despite the substantial amount of information available, our understanding of the molecular mechanisms governing the nucleohistone–nucleoprotamine transition is still in its infancy. For example, little information is available on what other proteins or structures interact with protamines and what their function is (Kierszenbaum and Tres, 2004; Mylonis *et al*., 2004).

Organization of DNA in the sperm nucleus

It is important to note that not all of the DNA in the sperm nucleus is organized into a nucleoprotamine structure, but some regions retain a nucleosomal structure (Figure 2). It has been shown that approximately 85% of the DNA in the sperm nucleus is associated with protamines and that 15% remains associated with histones or other proteins (Figure 2; Tanphaichitr *et al*., 1978; Ammer *et al*., 1986; Gusse *et al*., 1986; Gatewood *et al*., 1987, 1990; de Yebra *et al*., 1993; Zalensky *et al*., 2002). In addition, human sperm DNA has a heterogeneous structure with some regions and genes remaining associated with histones or with other proteins (Zalensky *et al*., 1995, 2002; Gardiner-Garden *et al*., 1998; Kramer *et al*., 2000; Zalenskaya *et al*., 2000; Zalenskaya and Zalensky, 2002; Wykes and Krawetz, 2003). It will be interesting to determine how these heterogeneous structures in the sperm nucleus relate to the establishment of epigenetic information in the male gamete and how they may affect subsequent embryo development (Rousseaux *et al*., 2005). The spatial architecture of chromosomal DNA has also been studied with data, supporting that the centromeres are organized in a chromocentre, positioned well inside the nucleus, whereas the telomeres forming dimers are positioned in the nuclear periphery (Zalensky *et al*., 1995; Solov'eva *et al*., 2004). For further information on the nucleohistone–nucleoprotamine transition, the reader is referred to different reviews (Ward and Coffey, 1991; Oliva and Dixon, 1991a; Dadoune, 1995, 2003; Wouters-Tyrou *et al*., 1998; Raukas and Mikelsaar, 1999; Braun, 2001; Aoki and Carrell, 2003; Meistrich *et al*., 2003; Kierszenbaum and Tres, 2004; Rousseaux *et al*., 2005). The extent to which the structural organization of the sperm DNA is altered in infertile patients remains relatively unexplored.

After fertilization, the highly packaged nucleoprotamine sperm genome must be decondensed (Figure 2). One of the first steps must be reduction of the protamine disulphide bonds to allow protamine removal and subsequent organization of the DNA in a nucleosomal structure (Figure 2). The chromatin changes and unpacking after fertilization potentially relevant to the function of protamines are reviewed elsewhere (Griveau *et al*., 1992; Perreault, 1992; Poccia and Collas, 1996; Colleu *et al*., 1997; Shimada *et al*., 2000, 2002; Braun, 2001; Esterhuizen *et al*., 2002; Nakazawa *et al*., 2002; Schultz, 2002; Lefievre *et al*., 2003; McLay and Clarke, 2003; Mudrak *et al*., 2005; Romanato *et al*., 2005). It is possible that differential marking of different sperm genomic DNA regions with P1 or P2 protamines or with histones, histone variants or with other proteins could contribute, after fertilization, to establish the order of paternal gene reactivation or even could be involved in setting up the appropriate imprinting of different paternal genes.

Transgenes and knockout models

A great deal of information relevant to the function and involvement of protamines in male infertility has been obtained from transgenes and knockout models for protamines and TNPs. The first transgenic model for a protamine corresponded to the homologous mouse protamine 1 gene (Peschon *et al*., 1987). The resulting mice correctly expressed the protamine construction in round spermatids, indicating the good recognition of the regulatory elements in the transgene (Peschon *et al*., 1987; Zambrowicz *et al*., 1993). In addition, the mice were fertile indicating that small variations in the levels of expression of the protamine 1 were compatible with an apparently normal function of the spermatozoa (Peschon *et al*., 1987). Lines of transgenes, generated using the promoter of the mouse protamine 2 gene coupled to a reporter gene, also supported the idea that the regulatory elements were correctly recognized by the endogenous factors resulting in the correct expression at round spermatid stage (Stewart *et al*., 1988). Subsequent models designed to express the protamine 1 gene prematurely or in excess resulted in premature condensation of the nuclear chromatin, anomalies in the morphology of the sperm head and incomplete processing of protamine P2 (Peschon *et al*., 1989; Lee *et al*., 1995). The first heterologous expression of a protamine corresponded to over-expression of the chicken protamine gene in transgenic mice, which resulted in a disruption of the chromatin in spermatozoa (Oliva and Dixon, 1989; Rhim *et al*., 1995). Unexpectedly, however, these mice turned out to be fertile, suggesting that a very precise packaging of the DNA in the germinal cell line was not essential for decondensation and pronuclear

formation in the fertilized oocytes (Rhim *et al*., 1995). Subsequent studies characterized in detail the presence of different anomalies in the spermatozoa of these transgenic mice, also confirming their relative fertility (Maleszewski *et al*., 1998). In a different type of experiment, over-expression of the human protamine cluster in transgenic mice demonstrated a conservation of the temporal expression pattern, indicating that the human regulatory elements were recognized by the mouse transcription factors (Stewart *et al*., 1999). Also, transgenic mice containing the complete human protamine domain flanked by different configurations of nuclear matrix attachment regions (MARs) demonstrated the importance of the overall chromatin structure for correct expression and function of the domain (Martins *et al*., 2004).

Of importance, it was found that knockout mice for only one of the P1 or P2 alleles were sufficient to result in infertility (Braun *et al*., 1989; Oliva and Dixon, 1991a; Cho *et al*., 2001). Since protamines are expressed in the haploid phases of spermatogenesis (Hecht, 1988; Oliva *et al*., 1988; Choudhary *et al*., 1995; Steger 1999, 2001; Dadoune *et al*., 2004), it could be thought that the disruption of only one allele should not affect the expression of the protamine gene in the other half of cells having the normal gene. But it is also known that cytokinesis is incomplete in the spermatogenic cells, which are connected by cytoplasmic bridges that can allow spermatids to share mRNA (Braun *et al*., 1989; Oliva and Dixon, 1991b). A few years later, the presence of damaged DNA in sperm cells of these knockout infertile mice was detected (Cho *et al*., 2003). Of relevance, these authors also observed that, if ICSI was used, it was possible to activate the oocytes but that few could progress to the blastocyst stage (Cho *et al*., 2003). It is also important to note that a similar phenomenon has been described in many infertile patients, with injured DNA under ICSI treatment (Tesarik *et al*., 2004; Greco *et al*., 2005).

Another extensively studied model is the knockout mouse for TNP1 or TNP2 (Yu *et al*., 2000; Adham *et al*., 2001; Zhao *et al*., 2001, 2004a,b; Meistrich *et al*., 2003; Shirley *et al*., 2004; Suganuma *et al*., 2005). In the double-knockout mice (for both TNPs), the remodelling of nuclear morphology, the repression of transcription, the disappearance of histones and the deposition of protamines were relatively normal. However, it was observed that condensation of the chromatin was irregular, that protamine P2 was not processed and that many of the elongated spermatids had DNA breaks (Zhao *et al*., 2004a). Interestingly, it has been found that there is an increase in structural anomalies in these mice, as revealed by acridine orange (AO) staining, during epididymal passage and that fertility declines, as revealed by ICSI (Suganuma *et al*., 2005).

Alterations in protamine content of spermatozoa in infertile patients

Direct determination of protamines by electrophoresis

The first evidence of anomalies in the protamine content of spermatozoa was described in a study, which did not detect protamines, but did detect histones, in the spermatozoon of diverse infertile patients (Table I; Silvestroni *et al*., 1976). Subsequently, an independent group described an anomalous protein pattern in different patients, which was characterized by the presence of additional proteins (Chevaillier *et al*., 1987). However, in this work no reference was made to the protamines. One of the first complete studies that analysed the protamines in a series of fertile controls ($n = 17$) and compared the data with that of patients ($n = 7$) detected an increased P1/P2 ratio in six of the seven patients studied (Balhorn *et al*., 1988). A more heterogeneous protamine fraction was also observed in patients with altered seminal parameters as compared with samples with normal parameters (Lescoat *et al*., 1988). Subsequently, it was found that the percentage of protamines in fertile men was the same as that in infertile patients with normal seminal parameters, but that it varied in the patients with abnormal seminal parameters (Bach *et al*., 1990). Another independent group found that in patients with morphologic anomalies in the spermatozoa, characterized by the presence of a round head, the spermatozoa contained less protamines and more histones than normal spermatozoa (Blanchard *et al*., 1990).

The decrease in protamine P2 level and the increased P1/P2 ratio were confirmed a few years later (Belokopytova *et al*., 1993). But it was not until a report of the first extended series of patients ($n = 116$) that it was recognized that an important proportion of the patients (3.4%; $n = 4$) had a marked reduction in protamine P2 (de Yebra *et al*., 1993; de Yebra and Oliva, 1993), whereas the rest of the patients had a normal P1/P2 ratio (22.4%) or a slightly altered ratio (74.1%). In addition, it was noticed that a large proportion of the samples with an altered P1/P2 ratio also had increased levels of proteins with a mobility similar to histones and to intermediate proteins (de Yebra *et al*., 1993). More recently, an increase in histone H2B in infertile patients has been confirmed using immunocytochemistry (Zhang *et al*., 2006).

All these observations raised the question of the origin of the reduction of protamine P2 levels relative to those of protamine P1 in some of the patients. The detection of increased protamine P2 precursors in patients with an increased P1/P2 ratio narrowed the possible origin to an abnormal processing of the protamine P2 precursor (de Yebra *et al*., 1998). It should be noted that detectable levels of P2 precursors are also present in the mature sperm nucleus in the mouse and rat (Stanker *et al*., 1992; Debarle *et al*., 1995). This reduction in protamine content in patients was consistent with results of the analysis of the phosphorus and sulphur contents in individual spermatozoa by particle-induced X-ray emission (PIXE; Bench *et al*., 1998). In addition, the protamine P1/P2 ratio varied in samples taken from the same patients at different times (Bench *et al*., 1998). Another explanation for the altered P1/P2 ratio detected in different infertile patients is that it could be the consequence of a general failure in the replacement of histones by protamines during spermiogenesis. The detection of increased amounts of histones and intermediate proteins in patients with decreased protamines or altered P1/P2 ratio would support this hypothesis (Blanchard *et al*., 1990; de Yebra *et al*., 1993; Zhang *et al*., 2006).

All these initial works were carried out by analysing the semen samples without fractionation. It is well known that, even in a normal human ejaculate, populations of abnormal spermatozoa coexist with morphologically normal spermatozoa. Therefore, it was considered if the anomalies detected in the P1/P2 ratio affected all the cells in the sample or, instead, reflected a mixture of a normal population plus a population with an altered P1/P2 ratio. Percoll gradient centrifugation allowed separation of spermatozoa according to morphology and mobility, and fractions with a higher density were shown to be enriched in less-intermediate proteins and

Table I. Studies in infertile patients where protamines were detected directly after extraction from sperm samples and separated by polyacrylamide gel electrophoresis

Reference	Main findings
Silvestroni *et al*., 1976	Protamines not detected in the spermatozoon of infertile patients
Chevaillier *et al*., 1987	Proteins additional to the normal ones were found in infertile patients
Balhorn *et al*., 1988	P1/P2 ratio = 0.98 ± 0.12 in normal samples (*n* = 17) P1/P2 ratio = 1.58 ± 0.24 in infertile patients (*n* = 7) Increased P1/P2 ratio in six of the seven patients studied
Lescoat *et al*., 1988	Heterogeneous protamine fraction observed in patients with altered seminal parameters (*n* = 11) compared with samples with normal parameters (*n* = 11)
Bach *et al*., 1990	Percentage of protamines is different in the patients with abnormal seminal parameters compared to patients with normal parameters
Blanchard *et al*., 1990	Round-headed spermatozoa from patients (*n* = 2) contain less protamines and more histones and intermediate proteins than the normal spermatozoa (*n* = 2) Expression of P2 proteins is lower in round-headed sperm
Belokopytova *et al*., 1993	P1/P2 ratio = 0.99 ± 0.06 in normal samples (*n* = 20) P1/P2 ratio = 1.50 ± 0.05 in infertile patients (*n* = 10)
de Yebra and Oliva, 1993	Description of an optimized method to extract and analyse protamines by gel electrophoresis to allow easier and faster clinical application
de Yebra *et al*., 1993	P1/P2 ratio = 1.10 ± 0.08 (normal) in 22.4% of infertile patients (*n* = 26) P1/P2 ratio = 3.00 ± 2.84 (abnormal) in 74.1% of infertile patients (*n* = 86) Absence of detectable P2 in 3.4% of the patients (*n* = 4)
Colleu *et al*., 1996	The densest Percoll gradient fractions were enriched in less-intermediate proteins and more P2 in patient samples with normal count and motility (*n* = 12)
Khara *et al*., 1997	P1/P2 ratio = between 0.55 and 1.29 in patients with FI ≥50% (*n* = 18) P1/P2 ratio = outside the 0.55–1.29 range in patients with FI <50% (*n* = 3)
de Yebra *et al*., 1998	Detection of increased protamine P2 precursors by western analysis in patients with an increased P1/P2 ratio
Bench *et al*., 1998	P1/P2 ratio varied in patients' samples obtained at different times
Carrell *et al*., 1999	Differences in protamine content and sperm ultrastructure found in two siblings associated with different ICSI outcomes
Evenson *et al*., 2000	Appearance of protamine P2 precursors detected by electrophoresis between 33 and 39 days post-hyperthermia in one patient
Carrell and Liu, 2001	12 of 13 patients without detectable P2 had a reduction in the sperm penetration assay in comparison with the patients with P2 P2 precursor bands associated with reduction in the penetration capacity
Mengual *et al*., 2003a	P1/P2 ratio = 1.01 ± 0.15 in control fertile men (*n* = 10) P1/P2 ratio = 1.51 ± 0.48 in oligozoospermic patients (*n* = 12) P1/P2 ratio = 1.23 ± 0.65 in asthenozoospermic patients (*n* = 13) Little heterogeneity between Percoll fractions from individual samples and marked differences between patients and controls
Nasr-Esfahani *et al*., 2004b	Negative significant correlation of fertilization rate with protamine deficiency and P1/P2 ratio
Chen *et al*., 2005	Altered levels of protamines present in infertile patients are shown to improve upon patient treatment
Aoki *et al*., 2005a	P1/P2 ratio = 1.06 ± 0.01 in fertile donors (*n* = 87) P1/P2 ratio < 0.8 in 13.6% of the patients (*n* = 37) P1/P2 ratio = between 0.8 and 1.2 in 46.7% of the patients (*n* = 127) P1/P2 ratio > 1.2 in 39.7% of the patients (*n* = 108) P1/P2 ratio correlates with sperm penetration score and fertilization rate
Aoki *et al*., 2005b	DNA fragmentation raised in low P1/P2 samples versus normal/high P1/P2 ratio
Aoki *et al*., 2006	Correlations between P1 and P2 proteins and mRNA detected by real-time PCR
Zhang *et al*., 2006	Increased proportion of H2B to protamine in infertile men
Torregrosa *et al*., 2006	P2 precursors related to protamine content and DNA integrity

FI, fertilization index; P1, protamine 1; P2, protamine 2; H2B, histone 2B.

contain more mature protamine 2 (Colleu *et al*., 1996). However, the separation of cells in individual ejaculates from infertile patients and controls using a Percoll gradient, and the subsequent determination of the P1/P2 ratio in each of the fractions, detected only small differences in P1/P2 ratio between fractions despite the presence of marked differences in the morphology and mobility (Mengual *et al*., 2003a). Nevertheless, marked differences in the P1/P2 ratio were detected when comparing oligozoospermic and asthenozoospermic patients to controls (Mengual *et al*., 2003a). It will be interesting to test other separation methods, such as swim-up (Colleu *et al*., 1996; Sakkas *et al*., 2000), electrophoresis (Ainsworth *et al*., 2005) or cell sorting (Ziyyat *et al*., 1999), and the use of immunocytochemical methods (Zhang *et al*., 2006) to test whether levels of the protamines and other proteins do indeed vary among the different cells of an ejaculate and may correlate with DNA integrity or assisted reproduction outcomes.

Radical differences in protamine content in two siblings associated with different ICSI outcomes were also reported (Carrell *et al*., 1999). A recent article reporting the analysis of 272 infertile patients and 87 donors described a new type of anomaly in some patients, characterized by the presence of a decreased P1/P2 ratio (Aoki *et al*., 2005a). A summary of all articles measuring protamines directly after extraction and electrophoresis is given in Table I.

In addition to the above studies in infertile patients, the expression of protamines has also been determined in response to thermal stress in normal testicles (Love and Kenney, 1999; Evenson

et al., 2000). Thermal stress in stallion testicle is associated with decreased formation of disulphide bridges in protamines (Love and Kenney, 1999). This aspect has also been studied in humans by Evenson *et al*. (2000), who measured protamine levels in a patient just after an episode of hyperthermia, induced by the influenza, and reported the appearance of protamine P2 precursors, detected by electrophoresis, between 33 and 39 days post-hyperthermia. These authors also showed that the P1/P2 ratio remained within the normal range, whereas the ratio between histones and protamines increased slightly between 33 and 39 days post-hyperthermia. Expression of the gene-encoding protamine P2 was also altered concomitant to induced thermal stress in the mouse testicle (Iuchi *et al*., 2003).

Indirect assessment of sperm chromatin structure by histochemical procedures

In all of the above studies, the protamine content was measured directly through protamine extraction and polyacrylamide gel electrophoresis (PAGE). Indirect methods of assessing the amount of protamines or measuring chromatin structure based on different staining procedures or fluorochromes have also been used (Bianchi *et al*., 1996; Lolis *et al*., 1996; Bizzaro *et al*., 1998; Sakkas *et al*., 1998; Franken *et al*., 1999; Esterhuizen *et al*., 2002; Zubkova *et al*., 2005). For example, *in situ* competition between protamine and chromomycin A3 (CMA3) indicated that CMA3 staining inversely correlated with the protamination state of spermatozoa (Bizzaro *et al*., 1998). Interestingly, CMA3 staining has been shown to be increased in the sperm cells of infertile patients (Lolis *et al*., 1996; Franken *et al*., 1999; Razavi *et al*., 2003; Nasr-Esfahani *et al*., 2004a,b, 2005). Correlations between CMA3 staining in sperm and assisted reproduction outcome have also been found (Nasr-Esfahani *et al*., 2004a, 2005). However, CMA3 staining cannot distinguish whether the potential protamine deficiency is due to a lack of P1, P2 or a combination of both. Another very popular test has been the sperm chromatin structure assay (SCSA) based on the AO red–green shift to differentiate double- versus single-stranded DNA (Evenson *et al*., 1980; Virro *et al*., 2004; Evenson and Wixon, 2005). A large amount of information correlating results from this indirect test, mainly intended to infer the presence of DNA breaks, with infertility or assisted reproduction outcome has accumulated over the years (Virro *et al*., 2004; Evenson and Wixon, 2005).

Another indirect approach has been the use of aniline blue staining to detect the presence of histones and therefore indirectly infer the presence of lower amounts of protamines in the sperm nucleus (Chevaillier *et al*., 1987; Colleu *et al*., 1988). An increase in the percentage of aniline blue cells was found in asthenozoospermic as compared with normozoospermic samples (Colleu *et al*., 1988). Acidic aniline blue was also correlated with differences in sperm nuclear morphology in sperm donors and in infertile patients (Auger *et al*., 1990). A decreased resistance to chromatin decondensation by treatment with sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) in abnormal sperm compared with normal sperm has also been taken as evidence for lower protamine S–S stability and chromatin packaging (Bustos-Obregón and Leiva, 1983; Le Lannou *et al*., 1986; Jager, 1990). The accessibility of the fluorescent dye ethidium bromide to DNA has also been correlated to IVF outcomes (Filatov *et al*., 1999).

Other new sperm chromatin structure tests based on sperm chromatin dispersion are also being proposed (Silvestroni *et al*., 2004; Evenson and Wixon, 2005; Fernández *et al*., 2005; Schlegel and Paduch, 2005). The interpretation of the results of all these indirect tests is difficult since they depend on the sperm chromatin composition, structure, accessibility and integrity of the DNA (Schlegel and Paduch, 2005; Erenpreiss *et al*., 2006). Thus, changes in the overall amount of protamines, degree of protamine cross-linking, P1/P2 ratio, presence of P2 precursors, proportion of histones and other proteins, protein modifications, topological state of the DNA and double- or single-DNA breaks may all result in measurable changes. So, at present, direct protamine extraction and electrophoresis are still the gold standard to directly quantify protamines (Balhorn *et al*., 1988; de Yebra *et al*., 1993; de Yebra and Oliva, 1993; Mengual *et al*., 2003a; Aoki *et al*. 2005a). However this direct approach was more complex and time consuming that indirect staining procedures (Mckay *et al*., 1986; Yelick *et al*., 1987; Sautière *et al*., 1988). A systematic assessment of the factors involved in protamine recovery led to drastic reduction in the time involved and complexity of the methods used, so that routine clinical application is now easier (de Yebra *et al*., 1993; de Yebra and Oliva, 1993; Mengual *et al*., 2003a).

The use of antibodies to P1, P2 or to the protamine P2 precursor increases the sensitivity but should be further elaborated to allow fast routine clinical use (Stanker *et al*., 1992, 1993; Le Lannic *et al*., 1993; de Yebra *et al*., 1998). Also, because of the clinical use of protamines as drugs, there is pharmaceutical interest in developing more sensitive protamine detection methods (Lochmann *et al*., 2004; Shvarev and Bakker, 2005) and new proteomic approaches based on liquid fractionation mass spectrometry or new fluidic devices that have the potential to make protamine quantification even easier and faster in the near future.

Anomalies in protamine content and IVF potential

The first evidence that an altered expression of protamines could be related to IVF capacity came from a comparison of the P1/P2 ratio in two groups of infertile patients classified on the basis of their fertilization index (FI), either above or below 50% (Khara *et al*., 1997; Table I). Specifically, these authors found a P1/P2 ratio between 0.55 and 1.29 in the group with a FI ≥50%, whereas three of the infertile patients who had a FI below 50% had a ratio outside this range (Khara *et al*., 1997). However, these authors did not support the idea that the altered P1/P2 ratio detected was the primary cause of the reduction in FI.

A few years later in a larger series of patients, 12 of the 13 patients without detectable protamine P2 were found to have a significant reduction in the sperm penetration assay compared with the patients with protamine P2 (Carrell and Liu, 2001). In this work, an unusually high proportion of patients without detectable P2 was considered (17%; 13 of 75), in contrast with articles published by other groups (de Yebra *et al*., 1993; Mengual *et al*., 2003a) or in recent studies published by the same group (Aoki *et al*., 2005a,b). In this initial work, the detection of bands corresponding to protamine precursors was also associated with a reduction in the penetration capacity (Carrell and Liu, 2001). This fact was consistent with the previous observation that patients with an increased P1/P2 ratio also have increased levels of protamine 2 precursors (de Yebra *et al*.,

1998). However, Carrell and Liu (2001) did not find any significant difference in the results of the treatment by ICSI when comparing the groups of patients with and without detectable P2.

More recently, it has been shown that spermatozoa staining with CMA3, which indirectly indicates a possible deficiency in protamines, has an IVF percentage of 36.8%, which is below the index reached (64.6%) with the negative spermatozoa after using this dye (Nasr-Esfahani *et al*., 2004a). Subsequent work using this approach demonstrated the presence of increased DNA fragmentation in, presumably, protamine-deficient spermatozoa (Nasr-Esfahani *et al*., 2005). This group also measured the protamines P1 and P2 directly by gel electrophoresis and found a significant negative correlation of the fertilization rate with the protamine deficiency and the P1/P2 ratio (Nasr-Esfahani *et al*., 2004b).

The expression of the gene-encoding protamines 1 and 2 in testicular spermatids of azoospermic patients biopsied during ICSI has also been studied (Steger *et al*., 2003; Mitchell *et al*., 2005), and a lower expression of protamine P1 mRNA in couples that did not achieve a pregnancy was found compared with the couples that did.

In a recent and an extensive work, it has been reported that the reduction in P1/P2 ratio results in a marked reduction of the IVF index in comparison with the patients with a normal or an increased P1/P2 ratio (Aoki *et al*., 2005a). Of relevance, this group has also reported that altered levels of protamines are correlated with a decreased integrity of the DNA (Aoki *et al*., 2005b). Thus, many independent laboratories confirmed that altered protamine ratios are related to infertility. Also, protamine-deficient animal models indicate that integrity of the DNA decreases upon spermatozoan passage from the epididymis, affecting subsequent embryo development (Suganuma *et al*., 2005).

It is also interesting to note that variation over time of protein and DNA contents in sperm from an infertile human male possessing protamine defects has been described (Bench *et al*., 1998). Moreover, altered levels of protamines in infertile patients have been shown to improve upon patient treatment (Chen *et al*., 2005). Thus, another potential aspect of the protamines in clinical practice could be their use as a marker to follow-up infertility treatments.

Part of the explanation of the correlation between low IVF rates and protamine deficiency could come from a series of IVF experiments using spermatozoa damaged with DTT, to break the disulphide bridges that normally stabilize the nucleoprotamine structure (Ahmadi and Ng, 1999a). These authors found that the damaged spermatozoa had a normal IVF rate, but there was a reduction in post-implantation development (Ahmadi and Ng, 1999a). The same authors also described that in spermatozoa treated with DTT, the binding and penetration of the oocyte in the hamster assay are markedly reduced. However, if ICSI is used, the DTT-damaged spermatozoa reach an even higher rate of pronuclear formation and decondensation of the sperm head in comparison with the controls (Ahmadi and Ng, 1999b). However, the subsequent development of the embryos was not studied. Of course, these experiments must be interpreted with caution as DTT may affect, in addition to protamines, many additional sperm proteins and structures involved in sperm function.

Variations in protamine transcripts and infertility

It is generally well justified to consider altered mRNA levels as a potential origin of altered protein levels. This point could be even more important in this model because the protamine genes must be transcribed and stored in spermatocytes and round spermatids for later translation in elongating spermatids when transcription is no longer active (Mezquita, 1985; Oliva and Dixon, 1991a,b; Hecht, 1993; Steger, 2001; Kleene, 2003; Tanaka and Baba, 2005). In one of the first studies measuring the expression of protamines in testicular cells isolated by flow cytometry, a complete absence of the expression of the P2 gene in round spermatids was reported (Ziyyat *et al*., 1999). A reduction in the protamines 1 and 2 mRNA levels was also found in round spermatids of infertile patients using testicular biopsies and in situ hybridisation (Steger *et al*., 2001). Also a correlation between the protamine 1 to protamine 2 mRNA ratio in round spermatids was found to be related to successful fertilization (Steger *et al*., 2001). The same group produced similar results using real-time PCR (Steger *et al*., 2003). Furthermore, ISH also showed a significant reduction in expression of P1, which could be associated with the outcome of assisted reproduction (Mitchell *et al*., 2005). Another independent group also identified anomalies in the expression of protamines in biopsies of azoospermic patients (Friel *et al*., 2002). Analysis of the expression of P2 mRNA in patients with non-obstructive azoospermia by RT–PCR found increased expression in the biopsies, where testicular sperm were present (Qiu *et al*., 2005).

The presence of mRNAs corresponding to the protamine genes can be detected not only in the mature testicle but also in the mature spermatozoa, either by microarray techniques (Miller *et al*., 1999; Dadoune *et al*., 2004; Ostermeier *et al*., 2004; Miller *et al*., 2005) or by RT–PCR (Lambard *et al*., 2004). An interesting finding is that differences in the expression of the P1 gene were detected in fractions from spermatozoa with different mobility and density obtained from normozoospermic donors (Lambard *et al*., 2004). This fact is consistent with previous data indicating that, even within a normal ejaculate, there are differences in the expression of protamines in the different cells (Colleu *et al*., 1996; Mengual *et al*., 2003a). Of importance, a potential mechanism for protamine expression deregulation has been highlighted by the detection of abnormal protamine transcript retention in infertile human males with sperm protamine deficiency (Aoki *et al*., 2006).

Protamines and integrity of the DNA in sperm cells

One of the hypotheses for the function of protamines is that they could be involved in the protection of the genetic message delivered by the spermatozoa (Oliva and Dixon, 1991a; Mengual *et al*., 2003a). Incomplete protamination could render the spermatozoa more vulnerable to attack by endogenous or exogenous agents, such as nucleases (Szczygiel and Ward, 2002; Sotolongo *et al*., 2003), free radicals (Irvine *et al*., 2000; Alvarez *et al*., 2002) or mutagens. However, it is also important to keep in mind that other potential reasons for decreased DNA integrity could be the presence of altered recombination, abortive apoptosis, abnormal action of topoisomerases and abnormal DNA repair during spermatogenesis (Roca and Mezquita, 1989; McPherson and Longo, 1993; Baarends *et al*., 2001; Sakkas *et al*., 2003; Laberge and Boissonneault, 2005; Erenpreiss *et al*., 2006; Muratori *et al*., 2006). The potential relation between protamination defects and decreased DNA integrity has been assessed by different groups using a variety of direct or indirect approaches. A lot of evidence links high-DNA fragmentation indexes obtained with the SCSA with lower

ICSI or IVF rates (Evenson *et al*., 1980; Evenson and Wixon, 2005). Of importance, a negative significant correlation between fertilization rate and CMA3 staining or P1/P2 ratio measured directly by electrophoresis has been reported (Nasr-Esfahani *et al*., 2004b). Subsequently, this group also demonstrated using single cell gel electrophoresis (the comet assay) that the results correlated with embryo cleavage score and with CMA3 staining, suggesting that DNA fragmentation is more frequent in protamine-deficient spermatozoa (Nasr-Esfahani *et al*., 2005). A quite good direct proof that DNA integrity is compromised in protamine-deficient human sperm has been obtained by direct measurement of protamines by electrophoresis (Aoki *et al*., 2005b). Consistent with this observation we have found that the proportion of protamine 2 precursors also correlates with decreased DNA integrity (Torregrosa *et al*., (2006), submitted for publication).

The correlation between protamines and DNA integrity in sperm cells is also supported by animal models. By using transgenic knockout mice for TNPs, it has been demonstrated that the sperm genomic integrity deteriorates and that fertility declines during epididymal passage, as revealed by ICSI. AO fluorescence also suggests incomplete disulphide bond formation (Suganuma *et al*., 2005). This loss of genomic integrity during passage from the caput to the cauda epididymis in these mice has been related to abnormalities in the protection of DNA by protamine, since only 11% of the protamine 2 is processed to the mature form, potentially reducing intermolecular disulphide bond formation (Yelick *et al*., 1987; Shirley *et al*., 2004; Suganuma *et al*., 2005). Furthermore, in these mice, the developmental defects appeared at implantation, as has been described in clinical reports from infertile patients with decreased DNA integrity (Tesarik *et al*., 2004; Suganuma *et al*., 2005; Lewis and Aitken, 2005).

The use of ICSI with testicular sperm has been demonstrated to improve pregnancy rates in patients with poor pregnancy rates and decreased DNA integrity of ejaculated spermatozoa (Greco *et al*., 2005). Thus, a reasonable explanation could be that incomplete or abnormal protamination, as observed in many studies (Table I), could lead to incomplete disulphide bond formation and incomplete DNA protection during epididymal passage in these patients.

Polymorphisms and mutations in the protamine genes

As soon as marked differences in the protamine content were identified in the sperm cells of some infertile patients, it was postulated that potential mutations in the corresponding genes could be present (Belokopytova *et al*., 1993; de Yebra *et al*., 1993). This idea was additionally supported by the fact that the lack of protamine P2 in the sperm nucleus of some mammals, such as the pig or the bull, was due to mutations in the corresponding genes (Maier *et al*., 1990). However, preliminary mutation analysis of the protamine 2 gene did not identify the presence of pathogenic mutations in any of the four patients with a markedly altered P1/P2 ratio (de Yebra *et al*., 1993), although this approach did lead to the identification of several polymorphisms in the protamines genes (Queralt *et al*., 1993; Schnulle *et al*., 1994). Subsequent complete mutation analyses in 36 infertile patients with evidence of anomalies of the sperm chromatin did not detect any pathogenic mutation in the gene-encoding protamines P1, P2 or the TNP1 (Schlicker *et al*., 1994). In another study, a role for a candidate mutation in a region of contact in the nuclear MAR close to the protamine genes was presented in two of five individuals with reduced sperm counts and abnormally low protamine levels (Kramer *et al*., 1997). Subsequently, transgenic mice with the human *PRM1–PRM2–TNP2* domain with different configurations of MARs demonstrated that these attachment regions may convey a selective reproductive advantage for transgene passage (Martins *et al*., 2004).

More recently, mutations in the protamine P1 (*PRM1*) and P2 (*PRM2*) genes have been studied in Japan in 226 sterile patients and in 270 males with proven fertility (Tanaka *et al*., 2003). In this case, four synonymous single-nucleotide polymorphisms (SNPs) were found in the coding region of the P1 gene, and one SNP (*c248t*) in the P2 gene, causing the appearance of a stop codon. These authors proposed that premature termination of the protamine P2 mRNA would cause the infertility in the patient with the *c248t* change in the P2 gene (Tanaka *et al*., 2003). Also in this work, one SNP in the 3′ region of the P1 gene and 2 SNPs in the intron of the gene P2 were identified.

All the above mutational studies suggested that protamine gene mutations were a rare cause of infertility in man (Schlicker *et al*., 1994; Tanaka *et al*., 2003). However, recently one SNP (*G197T*) resulting in an arginine to a serine change in the protamine 1 gene has been detected in 3 out of 30 unrelated infertile patients (Iguchi *et al*., 2005). It is interesting to note that these patients were selected based on a spermatozoan phenotype similar to that present in protamine P1 or P2 knockout mice (Cho *et al*., 2001, 2003; Iguchi *et al*., 2005). The change detected in the three patients would destroy one of the arginine clusters and create a new phosphorylation site in protamine 1 (Iguchi *et al*., 2005). So, in the light of this latest report, protamine gene mutations causing infertility are infrequent but not so rare as previously thought.

Several amino acid substitutions of the *TNP1* gene and a deletion in the promoter region of the *TNP2* gene have been identified in several infertile patients (Miyagawa *et al*., 2005). Mouse models have already demonstrated that alteration in TNPs results in altered protamine structure and decreased integrity in the DNA (Shirley *et al*., 2004; Suganuma *et al*., 2005). Thus, it will be interesting to determine how these mutations in the TNPs of infertile patients alter their sperm chromatin and protamine content.

Disulphide bonds in protamines

The nucleoprotamine structure is strongly stabilized in the sperm nucleus through the formation of intermolecular disulphide bonds between cysteine residues (Saowaros and Panyim, 1979). In addition, intramolecular disulphide bonds stabilize the folding of different protamine domains (Vilfan *et al*., 2004), and glutathione peroxidase activity could be involved in disulphide cross-linking in protamines (Pfeifer *et al*., 2001; Conrad *et al*., 2005). A model for the bull protamine has been recently proposed, which provides an explanation for the positions of cysteine residues that form the intermolecular disulphide bonds (Vilfan *et al*., 2004).

There are many data indicating that the sperm protein thiols are oxidized upon passage from caput to the cauda epididymis (Shalgi *et al*., 1989; Rufas *et al*., 1991; Seligman and Shalgi, 1991). When comparing thiol labelling patterns, oligospermic or infertile samples were found to have a higher SH content (fewer disulphide bonds) compared with normozoospermic samples (Rufas *et al*., 1991; Lewis *et al*., 1997; Zini *et al*., 2001). The level of sperm SH groups also correlated positively with DNA

denaturation (Zini *et al*., 2001). The sperm thiol status has been found to correlate with tyrosine phosphorylation of sperm proteins (Seligman *et al*., 2004).

Animal models also support a correlation between disulphide bond formation and integrity of the DNA (Bennetts and Aitken, 2005). Mice with a targeted deletion of glutathione peroxidase exhibited abnormal toluidine blue and AO staining, abnormal sperm heads and altered thiol status (Conrad *et al*., 2005).

A significant increase in thiol quantity was found in spermatozoa from older rats as compared with young controls, which correlated with increased susceptibility to oxidative damage (Zubkova *et al*., 2005). Recent results obtained in the clinical setting or with animal models suggest that decreased DNA integrity associated with the epididymal passage could be related to the disulphide content (Shirley *et al*., 2004; Greco *et al*., 2005; Suganuma *et al*., 2005; Aoki *et al*., 2005b). It will be interesting to look at the protamine content and thiol status in vasectomized men undergoing ICSI who have decreased pregnancy rates (McVicar *et al*., 2005; Steger *et al*., 2005).

Phosphorylation of protamines

Protamine phosphorylation was first described during trout spermatogenesis (Ingles and Dixon, 1967; Marushige *et al*., 1969; Sanders and Dixon, 1972; Louie and Dixon, 1973) and subsequently in mammals (Marushige and Marushige, 1978). Protamines are phosphorylated as soon as they are synthesized and phosphorylation may be required for the proper binding to DNA (reviewed in Oliva and Dixon, 1991a). Recently, it has been found that protamine phosphorylation is required for protamine binding to laminin B receptor, suggesting that docking of the protamine to the nuclear envelope could be an important intermediate step (Mylonis *et al*., 2004). Kinases involved in protamines 1 and 2 phosphorylation have been described (Pirhonen *et al*., 1994b; Papoutsopoulou *et al*., 1999; Wu *et al*., 2000). Also, mice lacking Camk4, which phosphorylates protamine 2 *in vitro*, are infertile with impaired spermiogenesis, specific loss of protamine 2 and retention of TNP2 (Wu *et al*., 2000). After binding of the protamine to DNA, a substantial dephosphorylation occurs before the spermatozoa enter the epididymis. In humans, it has been shown that phosphorylated protamines are still present in mature spermatozoa and the corresponding phosphorylation sites of P1 and P2 have been determined (Gusse *et al*., 1986; Pruslin *et al*., 1987; Bellvé *et al*., 1988; Arkhis *et al*., 1991; Chirat *et al*., 1993; Pirhonen *et al*., 1994a; Papoutsopoulou *et al*., 1999). It has been shown in mice that pesticides may alter chromatin structure by phosphorylating protamines (Piña-Guzman *et al*., 2005). Given the importance of phosphorylation in regulating protein function, the possibility that altered protamine phosphorylation could also be associated with infertility or assisted reproduction outcomes deserves to be evaluated.

Interaction of protamines with metals and effect on reproductive function

Due to their nature, protamines not only form electrostatic interactions with the DNA but also have the potential to bind metals or other agents, either as part of the normal physiology or involved in potential alterations of the chromatin. One of the first observations that stimulated the study of possible associations between protamines and metals was the observation that zinc is very abundant in the sperm nucleus (Morisawa and Mori, 1972). Subsequent studies corroborated these observations, proposing that zinc in the spermatozoa could stabilize the chromatin through its binding to thiol groups not participating in the formation of disulphide bridges. The observation that P2 protamines could be zinc-finger proteins with one Cys2/His2 motif opened new perspectives in understanding their function (Bianchi *et al*., 1992, 1994a,b; Bal *et al*., 2001). The quantification by PIXE of zinc levels in the spermatozoa of different species demonstrated that the content of zinc is proportional to the amount of P2 protamine indicating that this metal would bind to it stoichiometrically in a 1:1 ratio (Bench *et al*., 2000). The interaction between zinc and the P2 protamine would therefore have a role in the normal function of the spermatozoon, and a deficiency of zinc, or its excess, could cause alterations (Bedwal and Bahuguna, 1994; El-Tawil, 2003; Matsuda and Watanabe, 2003; Piao *et al*., 2003). A reduction in zinc content concomitant to the increase in disulphide bonding of protamines, which occurs during maturation of the spermatozoa in the epididymis, has also been reported (Dias *et al*., 2006).

However, in addition to the physiological presence of zinc in the spermatozoon, there is also clear evidence for the presence of toxic heavy metals, such as the lead, copper or nickel (Johansson and Pellicciari, 1988; Bal *et al*., 1997; Liang *et al*., 1999; Quintanilla-Vega *et al*., 2000; Massanyi *et al*., 2004; Hernandez-Ochoa *et al*., 2005). The toxicity in these cases could either be direct or mediated through an interaction with the P2 protamine. The association between the presence of these heavy metals and infertility in man is clear, and the mechanisms involved in their toxicity are being investigated very actively.

Other contaminants, such as pesticides, also have the potential to alter the structure of the sperm chromatin. In the case of the organophosphate pesticide Diazinon, it has been found that the toxic mechanism could be mediated through alteration of the phosphorylation of protamines (Sánchez-Peña *et al*., 2004). It has also been reported that acrylamide-induced genetic damage in spermatogenic cells could be mediated by protamine alkylation (Sega *et al*., 1989; Sega, 1991; Xie *et al*., 2006).

Antibodies to protamines

Protamine sulphate from salmon sperm (salmine) has been widely used in clinical practice as a heparin antagonist (Portmann and Holden, 1949; Carr and Silverman, 1999; Liang *et al*., 2005). Also, protamine-containing insulin preparations have become very popular (Raap *et al*., 2005). More recently, protamines or protamine-like polypeptides are being used as carriers to deliver gene therapy constructs (Lanuti *et al*., 1999; Arangoa *et al*., 2003; Park *et al*., 2003). Adverse reactions concomitant to the use of protamine in clinical practice were described early and included allergy, the generation of antibodies to protamine or the formation of strong interactions with other proteins or factors (Weiler *et al*., 1985; Porsche and Brenner, 1999; Park, 2004; Raap *et al*., 2005). While the potential for life-threatening acute and allergic reactions is the highest concern in these patients, the potential effects on reproduction also deserve to be investigated.

A completely different issue is the generation of autoantibodies to protamines in subjects not exposed to protamine-containing drugs. Autoantibodies to human sperm protamines 1 and 2 have been detected in infertile and vasectomized men (Samuel, 1977; Hellema *et al*., 1979; Naz *et al*., 1989; Rousseaux-Prevost *et al*., 1992).

Protamine-reactive natural immunoglobulin M (IgM) antibodies are present in human sera of normal fertile male and female individuals (Rodman *et al*., 1988). There has been a case report of a vasectomized man who reacted with shock to i.v. protamine (Adourian *et al*., 1993). Antibodies present in infertile human sera reduced fertility in female rabbits (Naz, 1990). The chromatin status in infertile patients with immunological male infertility has been studied (Molina *et al*., 2001). It could be expected that, since protamine is a nuclear protein, antibodies to it should not attach to the sperm surface and interfere with sperm function (Naz *et al*., 1989, 1992). However, sensitization against protamine could reduce fertility through induction of cell-mediated immune factors resulting in spermicidal effects (Naz and Mehta, 1989).

Future perspectives

Research in the field of the relationship between protamines and infertility is now at an exciting point, but many questions still remain to be solved. From the fundamental perspective, it still must be clarified what the mechanism and the proteins involved in the nucleohistone–nucleoprotamine transition are and what other proteins, in addition to protamines and histones, remain in the sperm nucleus and why. There is also a relative lack of data to better understand the function of protamines. From the applied perspective, it will be necessary to clarify the mechanism by which alterations of protamines in infertile patients may lead to decreased integrity of the DNA and whether they relate to other factors affecting sperm fertility and assisted reproduction outcomes. From the etiological perspective, it will be important to find out what causes altered levels of protamines to appear. As part of these questions, it will also be necessary to explain what the relationship is between the presence of DNA breaks, alterations in protamines, epigenetic changes in the spermatozoa and infertility. This aspect is especially important because of the potential transmission of a damaged or altered genome to future generations.

Also, it will be necessary to clarify the potential role of the mutations and polymorphisms in the protamine genes, TNPs and MARs, found in some patients and whether these alter the protamine ratio and level. It will also be interesting to determine to what extent the presence of genetic mutations or genetic risk factors in other genes associated to infertility alters the expression of protamines (Oliva *et al*., 1998; Egozcue *et al*., 2000; Huynh *et al*., 2002; Mengual *et al*., 2003b; de Llanos *et al*., 2005; Vogt, 2005). In this review, it has also been mentioned how environmental or exogenous factors, such as the presence of polluting agents or thermal stress, can affect sperm chromatin structure in a process involving the protamines. Therefore, it seems logical to also study the interrelation between genetic and environmental factors in the determination of the molecular maturity and normality of the spermatozoon nucleus. It is likely that the present genomic, transcriptomic and proteomic tools will contribute to the detection of proteins and factors involved in the normal remodelling of the sperm nucleus and in the identification of the pathogenic mechanisms involved in infertility.

Acknowledgements

The author thanks Prof Dr Cristóbal Mezquita for critical review, supported by grants from Ministerio de Ciencia y Tecnología BMC2003–03937, fondos FEDER, Ministerio de Sanidad y Consumo V-2003-REDC07A-O and by Generalitat de Catalunya 2001SGR00382.

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Submitted on November 23, 2005; resubmitted on February 8, 2006; accepted on February 15, 2006