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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.

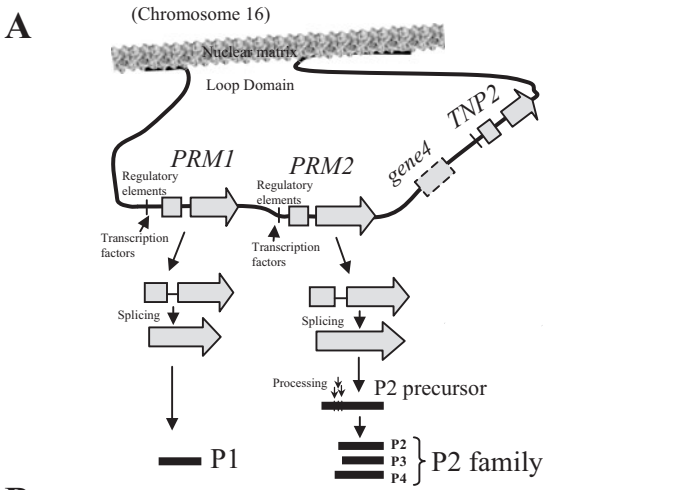

Figure 1. Transcription of the protamine genes and translation and processing of human protamines. (**A**) Schematic representation of the genomic structure of protamine genes (*PRM1* and *PRM2*) and the transcription, translation and processing involved in the synthesis of mature protamine. Protamine 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,

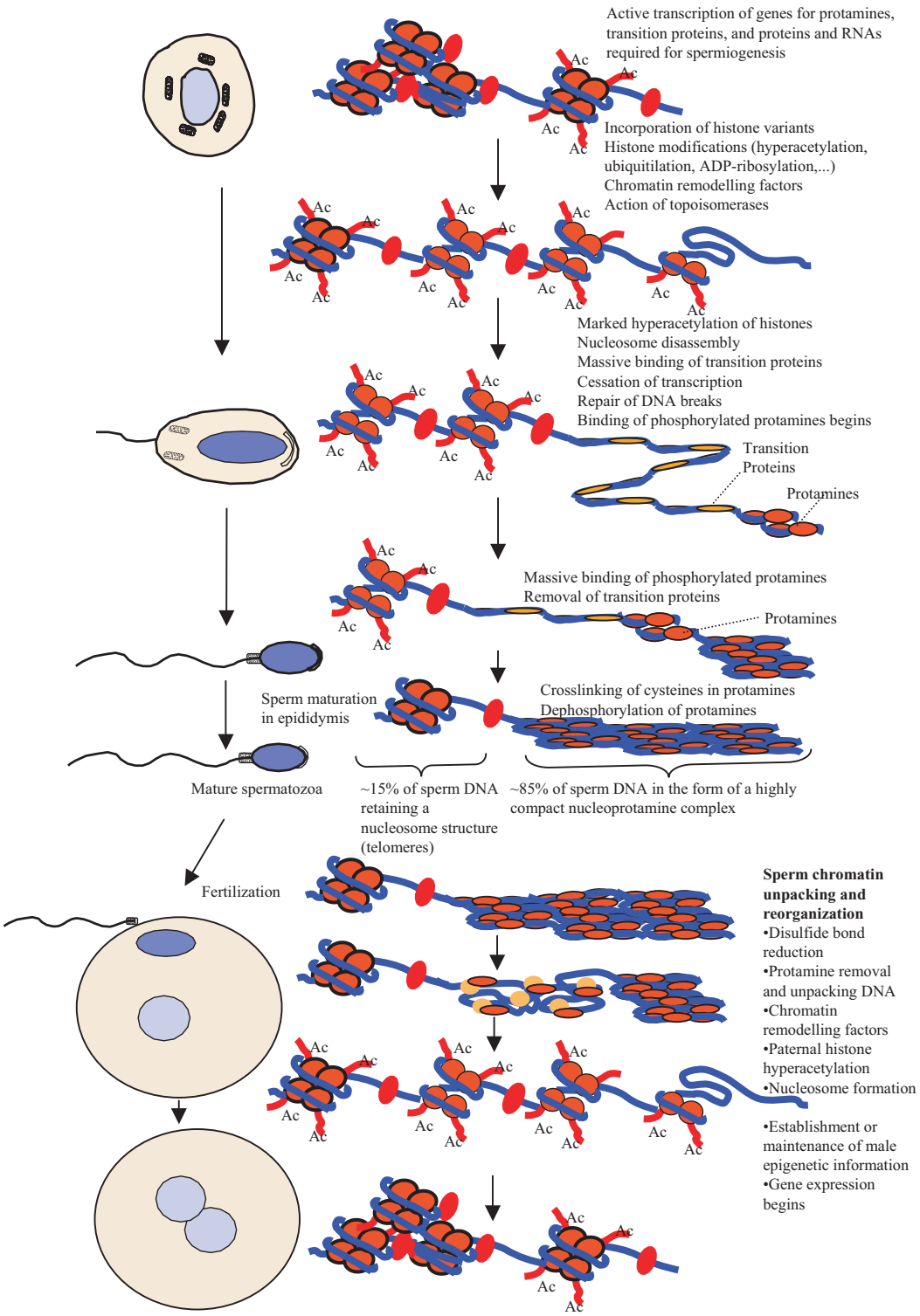

Figure 2. Schematic representation of the major chromatin changes occurring during the nucleohistone–nucleoprotamine transition in spermiogenesis and the subsequent nucleoprotamine unpacking and nucleohistone structure reconstitution at fertilization. The round spermatid (top left) has a chromatin structure 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.

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References

Adham IM, Nayernia K, Burkhardt-Gottges E, Topaloglu O, Dixkens C, Holstein AF and Engel W (2001) Teratozoospermia in mice lacking the transition protein 2 (Tnp2). Mol Hum Reprod 7,513–520.

Adourian U, Shampaine EL, Hirshman CA, Fuchs E and Adkinson NF Jr (1993) High-titer protamine-specific IgG antibody associated with anaphylaxis: report of a case and quantitative analysis of antibody in vasectomized men. Anesthesiology 78,368–372.

Adroer R and Oliva R (1998) Nucleosome positioning in the rat protamine 1 gene in vivo and *in vitro*. Biochim Biophys Acta 1442,252–260.

Ahmadi A and Ng SC (1999a) Developmental capacity of damaged spermatozoa. Hum Reprod 14,2279–2285.

Ahmadi A and Ng SC (1999b) Destruction of protamine in human sperm inhibits sperm binding and penetration in the zona-free hamster penetration test but increases sperm head decondensation and male pronuclear formation in the hamster-ICSI assay. J Assist Reprod Genet 16,128–132.

Ainsworth C, Nixon B and Aitken RJ (2005) Development of a novel electrophoretic system for the isolation of human spermatozoa. Hum Reprod 20,2261–2270.

Aitken RJ and Krausz C (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122,497–506.

Aleem M, Padwal V, Choudhari J, Balasinor N, Parte P and Gill-Sharma M (2005) Effects of tamoxifen citrate on gene expression during nuclear chromatin condensation in male rats. Asian J Androl 7,311–321.

Alimi E, Martinage A, Arkhis A, Belaiche D, Sautiere P and Chevaillier P (1993) Amino acid sequence of the human intermediate basic protein 2 (HPI2) from sperm nuclei. Structural relationship with protamine P2. Eur J Biochem 214,445–450.

Allen MJ, Lee C, Lee JD, Pogany GC, Balooch M, Siekhaus WJ and Balhorn R (1993) Atomic force microscopy of mammalian sperm chromatin. Chromosoma 102,623–630.

Allen MJ, Bradbury EM and Balhorn R (1997) AFM analysis of DNA-protamine complexes bound to mica. Nucleic Acids Res 25,2221–2226.

Alvarez JG, Sharma RK, Ollero M, Saleh RA, Lopez MC, Thomas AJ Jr, Evenson DP and Agarwal A (2002) Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. Fertil Steril 78,319–329.

Ammer H, Henschen A and Lee CH (1986) Isolation and amino-acid sequence analysis of human sperm protamines P1 and P2. Occurrence of two forms of protamine P2. Biol Chem Hoppe Seyler 367,515–522.

Ando T, Yamasaki M and Suzuki K (1973) Protamines. Isolation, characterization, structure and function. Mol Biol Biochem Biophys 12,1–114.

Aoki VW and Carrell DT (2003) Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. Asian J Androl 5,315–324.

Aoki VW, Liu L and Carrell DT (2005a) Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 20,1298–1306.

Aoki VW, Moskovtsev SI, Willis J, Liu LH, Mullen JBM and Carrell DT (2005b) DNA integrity is compromised in protamine-deficient human sperm. J Androl 26,741–748.

Aoki VW, Liu L and Carrell DT (2006) A novel mechanism of protamine expression deregulation highlighted by abnormal protamine transcript retention in infertile human males with sperm protamine deficiency. Mol Hum Reprod 12,41–50.

Arangoa MA, Duzgunes N and Tros de Ilarduya C (2003) Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes. Gene Ther 10,5–14.

Arkhis A, Martinage A, Sautiere P and Chevaillier P (1991) Molecular structure of human protamine P4 (HP4), a minor basic protein of human sperm nuclei. Eur J Biochem 200,387–392.

Auger J, Mesbah M, Huber C and Dadoune JP (1990) Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. Int J Androl 13,452–462.

Ausió J (1999) Related histone H1 and evolution of sperm nuclear basic proteins. J Biol Chem 274,31115–31118.

Baarends WM, van der Laan R and Grootegoed JA (2001) DNA repair mechanisms and gametogenesis. Reproduction 121,31–39.

Bach O, Glander HJ, Scholz G and Schwarz J (1990) Electrophoretic patterns of spermatozoal nucleoproteins (NP) in fertile men and infertility patients and comparison with NP of somatic cells. Andrologia 22,217–224.

Bal W, Lukszo J and Kasprzak KS (1997) Mediation of oxidative DNA damage by nickel (II) and copper (II) complexes with the N-terminal sequence of human protamine HP2. Chem Res Toxicol 10,915–921.

Bal W, Dyba M, Szewczuk Z, Jezowska-Bojczuk M, Lukszo J, Ramakrishna G and Kasprzak KS (2001) Differential zinc and DNA binding by partial peptides of human protamine HP2. Mol Cell Biochem 222,97–106.

Balhorn R (1982) A model for the structure of chromatin in mammalian sperm. J Cell Biol 93,298–305.

Balhorn R, Gledhill BL and Wyrobek AJ (1977) Mouse sperm chromatin proteins: quantitative isolation and partial characterization. Biochemistry 16,4074–4080.

Balhorn R, Corzett M, Mazrimas J, Stanker LH and Wyrobek A (1987) High-performance liquid chromatographic separation and partial characterization of human protamines 1, 2, and 3. Biotechnol Appl Biochem 9,82–88.

Balhorn R, Reed S and Tanphaichitr N (1988) Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. Experientia 44,52–55.

Balhorn R, Corzett M and Mazrimas JA (1992) Formation of intraprotamine disulfides *in vitro*. Arch Biochem Biophys 296,384–393.

Bedwal RS and Bahuguna A (1994) Zinc, copper and selenium in reproduction. Experientia 50,626–640.

Bélaïche D, Loir M, Kruggle W and Sautière P (1987) Isolation and characterization of two protamines St1 and St2 from stallion spermatozoa, and amino-acid sequence of the major protamine St1. Biochim Biophys Acta 913,145–149.

Bellvé AR, McKay DJ, Renaux BS and Dixon GH (1988) Purification and characterization of mouse protamines P1 and P2. Amino acid sequence of P2. Biochemistry 27,2890–2897.

Belokopytova IA, Kostyleva EI, Tomilin AN and Vorob'ev VI (1993) Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. Mol Reprod Dev 34,53–57.

Bench GS, Friz AM, Corzett MH, Morse DH and Balhorn R (1996) DNA and total protamine masses in individual sperm from fertile mammalian subjects. Cytometry 23,263–271.

Bench G, Corzett MH, De Yebra L, Oliva R and Balhorn R (1998) Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. Mol Reprod Dev 50,345–353.

Bench G, Corzett MH, Kramer CE, Grant PG and Balhorn R (2000) Zinc is sufficiently abundant within mammalian sperm nuclei to bind stoichiometrically with protamine 2. Mol Reprod Dev 56,512–519.

Bennetts LE and Aitken RJ (2005) A comparative study of oxidative DNA damage in mammalian spermatozoa. Mol Reprod Dev 71,77–87.

Bianchi F, Rousseaux-Prevost R, Sautiere P and Rousseaux J (1992) P2 protamines from human sperm are zinc-finger proteins with one CYS2/HIS2 motif. Biochem Biophys Res Commun 182,540–547.

Bianchi F, Rousseaux-Prevost R, Bailly C and Rousseaux J (1994a) Interaction of human P1 and P2 protamines with DNA. Biochem Biophys Res Commun 201,1197–1204.

Bianchi F, Rousseaux-Prevost R, Hublau P and Rousseaux J (1994b) Interaction of mammalian sperm nuclear protamines and peptides derived thereof with immobilized zinc. Int J Pept Protein Res 43,410–416.

Bianchi PG, Manicardi GC, Urner F, Campana A and Sakkas D (1996) Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. Mol Hum Reprod 2,139–144.

Biegeleisen K (2006) The probable structure of the protamine complex DNA. J Theor Biol [Epub ahead of print 25 January 2006].

Bizzaro D, Manicardi GC, Bianchi PG, Bianchi U, Mariethoz E and Sakkas D (1998) In-situ competition between protamine and fluorochromes for sperm DNA. Mol Hum Reprod 4,127–132.

Blanchard Y, Lescoat D and Le Lannou D (1990) Anomalous distribution of nuclear basic proteins in round-headed human spermatozoa. Andrologia 22,549–555.

Bloch DP (1969) A catalog of sperm histones. Genetics 61 (Suppl.),93–111.

Blocher S, Behr R, Weinbauer GF, Bergmann M and Steger K (2003) Different CREM-isoform gene expression between equine and human normal and impaired spermatogenesis. Theriogenology 60,1357–1369.

Bower PA, Yelick PC and Hecht NB (1987) Both P1 and P2 protamine genes are expressed in mouse, hamster, and rat. Biol Reprod 37,479–488.

Braun RE (2001) Packaging paternal chromosomes with protamine. Nat Genet 28,10–12.

Braun RE, Behringer RR, Peschon JJ, Brinster RL and Palmiter RD (1989) Genetically haploid spermatids are phenotypically diploid. Nature 337,373–376.

Braun RE, Lee K, Schumacher JM and Fajardo MA (1995) Molecular genetic analysis of mammalian spermatid differentiation. Recent Prog Horm Res 50,275–286.

Brewer LR, Corzett M and Balhorn R (1999) Protamine-induced condensation and decondensation of the same DNA molecule. Science 286,120–123.

Brewer L, Corzett M, Lau EY and Balhorn R (2003) Dynamics of protamine 1 binding to single DNA molecules. J Biol Chem 278,42403–42408.

Bustos-Obregón E and Leiva S (1983) Chromatin packing in normal and teratozoospermic human ejaculated spermatozoa. Andrologia 15,468–478.

Calvin HI (1976) Comparative analysis of the nuclear basic proteins in rat, human, guinea pig, mouse and rabbit spermatozoa. Biochim Biophys Acta 434,377–389.

Candido EP and Dixon GH (1972) Trout testis cells. 3. Acetylation of histones in different cell types from developing trout testis. J Biol Chem 247,5506–5510.

Caron C, Govin J, Rousseaux S and Khochbin S (2005) How to pack the genome for a safe trip. Prog Mol Subcell Biol 38,65–89.

Carr JA and Silverman N (1999) The heparin-protamine interaction. A review. J Cardiovasc Surg (Torino) 40,659–666.

Carrell DT and Liu L (2001) Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 22,604–610.

Carrell DT, Emery BR and Liu L (1999) Characterization of aneuploidy rates, protamine levels, ultrastructure, and functional ability of round-headed sperm from two siblings and implications for intracytoplasmic sperm injection. Fertil Steril 71,511–516.

Chauvière M, Martinage A, Debarle M, Sautière P and Chevaillier P (1992) Molecular characterization of six intermediate proteins in the processing of mouse protamine P2 precursor. Eur J Biochem 204,759–765.

Chen S, Cao J, Fei RR, Mao QZ and Li HZ (2005) Analysis of protamine content in patients with asthenozoospermia. Zhonghua Nan Ke Xue 11,587–589.

Chevaillier P, Mauro N, Feneux D, Jouannet P and David G (1987) Anomalous protein complement of sperm nuclei in some infertile men. Lancet 2,806–807.

Chirat F, Arkhis A, Martinage A, Jaquinod M, Chevaillier P and Sautière P (1993) Phosphorylation of human sperm protamines HP1 and HP2: identification of phosphorylation sites. Biochim Biophys Acta 1203,109–114.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB and Eddy EM (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28,82–86.

Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB and Eddy EM (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 69,211–217.

Choi YC, Aizawa A and Hecht NB (1997) Genomic analysis of the mouse protamine 1, protamine 2, and transition protein 2 gene cluster reveals hypermethylation in expressing cells. Mamm Genome 8,317–323.

Choudhary SK, Wykes SM, Kramer JA, Mohamed AN, Koppitch F, Nelson JE and Krawetz SA (1995) A haploid expressed gene cluster exists as a single chromatin domain in human sperm. J Biol Chem 270,8755–8762.

Churikov D, Zalenskaya IA and Zalensky AO (2004) Male germline-specific histones in mouse and man. Cytogenet Genome Res 105,203–214.

Clark AG and Civetta A (2000) Evolutionary biology. Protamine wars. Nature 403,261–263.

Colleu D, Lescoat D, Boujard D and Le Lannou D (1988) Human spermatozoal nuclear maturity in normozoospermia and asthenozoospermia. Arch Androl 21,155–162.

Colleu D, Lescoat D and Gouranton J (1996) Nuclear maturity of human spermatozoa selected by swim-up or by Percoll gradient centrifugation procedures. Fertil Steril 65,160–164.

Colleu D, Lescoat D, Thomas D and Gouranton J (1997) Changes in protamine 1 distribution in human sperm nucleus during *in vitro* sperm–oocyte interaction: an immunoelectron microscopic study. Fertil Steril 67,123–128.

Conrad M, Moreno SG, Sinowatz F, Ursini F, Kolle S, Roveri A, Brielmeier M, Wurst W, Maiorino M and Bornkamm GW (2005) The nuclear form of phospholipid hydroperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability. Mol Cell Biol 25,7637–7644.

Corzett M, Mazrimas J and Balhorn R (2002) Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 61,519–527.

Dadoune JP (1995) The nuclear status of human sperm cells. Micron 26,323–345.

Dadoune JP (2003) Expression of mammalian spermatozoal nucleoproteins. Microsc Res Tech 61,56–75.

Dadoune JP, Siffroi JP and Alfonsi MF (2004) Transcription in haploid male germ cells. Int Rev Cytol 237,1–56.

Dahm R (2005) Friedrich Miescher and the discovery of DNA. Dev Biol 278,274–288.

de Llanos M, Ballescà JL, Gázquez C, Margarit E and Oliva R (2005) High frequency of gr/gr chromosome Y deletions in consecutive oligospermic ICSI candidates. Hum Reprod 20,216–220.

de Yebra L and Oliva R (1993) Rapid analysis of mammalian sperm nuclear proteins. Anal Biochem 209,201–203.

de Yebra L, Ballescà JL, Vanrell JA, Bassas L and Oliva R (1993) Complete selective absence of protamine P2 in humans. J Biol Chem 268,10553–10557.

de Yebra L, Ballescà JL, Vanrell JA, Corzett M, Balhorn R and Oliva R (1998) Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. Fertil Steril 69,755–759.

Debarle M, Martinage A, Sautière P and Chevaillier P (1995) Persistence of protamine precursors in mature sperm nuclei of the mouse. Mol Reprod Dev 40,84–90.

Dias GM, Retamal CA, Tobella L, Arnholdt AC and Lopez ML (2006) Nuclear status of immature and mature stallion spermatozoa. Theriogenology [Epub ahead of print 16 January 2006].

Dixon GH and Smith M (1968) Nucleic acids and protamine in salmon testes. Prog Nucleic Acid Res Mol Biol 8,9–34.

Domenjoud L, Nussbaum G, Adham IM, Greeske G and Engel W (1990) Genomic sequences of human protamines whose genes, PRM1 and PRM2, are clustered. Genomics 8,127–133.

Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, Barri PN, Vidal F and Egozcue J (2000) Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Reprod Update 6,93–105.

Eirin-Lopez JM, Frehlick LJ and Ausió J (2006) Protamines, in the footsteps of linker histone evolution. J Biol Chem 281,1–4.

El-Tawil AM (2003) Zinc deficiency in men with Crohn's disease may contribute to poor sperm function and male infertility. Andrologia 35,337–341.

Engel W, Keime S, Kremling H, Hameister H and Schluter G (1992) The genes for protamine 1 and 2 (PRM1 and PRM2) and transition protein 2 (TNP2) are closely linked in the mammalian genome. Cytogenet Cell Genet 61,158–159.

Erenpreiss J, Spano M, Erenpreisa J, Bungum M and Giwercman A (2006) Sperm chromatin structure and male fertility: biological and clinical aspects. Asian J Androl 8,11–29.

Esterhuizen AD, Franken DR, Becker PJ, Lourens JG, Muller II and van Rooyen LH (2002) Defective sperm decondensation: a cause for fertilization failure. Andrologia 34,1–7.

Evenson DP and Wixon R (2005) Comparison of the Halosperm test kit with the sperm chromatin structure assay (SCSA) infertility test in relation to patient diagnosis and prognosis. Fertil Steril 84,846–849.

Evenson DP, Darzynkiewicz Z and Melamed MR (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210,1131–1133.

Evenson DP, Jost LK, Corzett M and Balhorn R (2000) Characteristics of human chromatin structure following an episode of influenza and high fever: a case study. J Androl 21,739–746.

Faure AK, Pivot-Pajot C, Kerjean A, Hazzouri M, Pelletier R, Peoc'h M, Sele B, Khochbin S and Rousseaux S (2003) Misregulation of histone acetylation in Sertoli cell-only syndrome and testicular cancer. Mol Hum Reprod 9,757–763.

Felix K (1960) Protamines. Adv Protein Chem 15,1–56.

Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, LaFromboise M and De Jonge C (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 84,833–842.

Filatov MV, Semenova EV, Vorob'eva OA, Leont'eva OA and Drobchenko EA (1999) Relationship between abnormal sperm chromatin packing and IVF results. Mol Hum Reprod 5,825–830.

Franken DR, Franken CJ, de la Guerre H and de Villiers A (1999) Normal sperm morphology and chromatin packaging: comparison between aniline blue and chromomycin A3 staining. Andrologia 31,361–366.

Friel A, Houghton JA, Glennon M, Lavery R, Smith T, Nolan A and Maher M (2002) A preliminary report on the implication of RT-PCR detection of DAZ, RBMY1, USP9Y and Protamine-2 mRNA in testicular biopsy samples from azoospermic men. Int J Androl 25,59–64.

Fuentes-Mascorro G, Serrano H and Rosado A (2000) Sperm chromatin. Arch Androl 45,215–225.

Gardiner-Garden M, Ballesteros M, Gordon M and Tam PP (1998) Histone- and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. Mol Cell Biol 18,3350–3356.

Gatewood JM, Cook GR, Balhorn R, Bradbury EM and Schmid CW (1987) Sequence-specific packaging of DNA in human sperm chromatin. Science 236,962–964.

Gatewood JM, Cook GR, Balhorn R, Schmid CW and Bradbury EM (1990) Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem 265,20662–20666.

Giorgini F, Davies HG and Braun RE (2001) MSY2 and MSY4 bind a conserved sequence in the 3′ untranslated region of protamine 1 mRNA *in vitro* and in vivo. Mol Cell Biol 21,7010–7019.

Govin J, Caron C, Lestrat C, Rousseaux S and Khochbin S (2004) The role of histones in chromatin remodelling during mammalian spermiogenesis. Eur J Biochem 271,3459–3469.

Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C and Tesarik J (2005) Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 20,226–230.

Green GR, Balhorn R, Poccia DL and Hecht NB (1994) Synthesis and processing of mammalian protamines and transition proteins. Mol Reprod Dev 37,255–263.

Grimes SR Jr and Henderson N (1984) Hyperacetylation of histone H4 in rat testis spermatids. Exp Cell Res 152,91–99.

Griveau JF, Charbonneau M, Blanchard Y, Lescoat D and Le Lannou D (1992) Decondensation of human sperm nuclei and HP1 protamine degradation from normospermia and asthenospermia in Xenopus egg extracts. Arch Androl 29,127–136.

Grootegoed JA, Siep M and Baarends WM (2000) Molecular and cellular mechanisms in spermatogenesis. Baillieres Best Pract Res Clin Endocrinol Metab 14,331–343.

Gusse M, Sautiere P, Belaiche D, Martinage A, Roux C, Dadoune JP and Chevaillier P (1986) Purification and characterization of nuclear basic proteins of human sperm. Biochim Biophys Acta 884,124–134.

Hazzouri M, Rousseaux S, Mongelard F, Usson Y, Pelletier R, Faure AK, Vourc'h C and Sele B (2000) Genome organization in the human sperm nucleus studied by FISH and confocal microscopy. Mol Reprod Dev 55,307–315.

Hebbar PB and Archer TK (2003) Chromatin remodelling by nuclear receptors. Chromosoma 111,495–504.

Hecht NB (1988) Post-meiotic gene expression during spermatogenesis. Prog Clin Biol Res 267,291–313.

Hecht NB (1993) Gene expression during male germ cell development. In Desjardins C and Ewing LL (eds), Cell and Molecular Biology of the Testis. Oxford University Press, New York, pp. 400–432.

Hellema HW, Samuel T and Rumke P (1979) Sperm autoantibodies as a consequence of vasectomy. II. Long-term follow-up studies. Clin Exp Immunol 38,31–36.

Hernandez-Ochoa I, Garcia-Vargas G, Lopez-Carrillo L, Rubio-Andrade M, Moran-Martinez J, Cebrian ME and Quintanilla-Vega B (2005) Low lead environmental exposure alters semen quality and sperm chromatin condensation in northern Mexico. Reprod Toxicol 20,221–228.

Hogarth C, Itman C, Jans DA and Loveland KL (2005) Regulated nucleocytoplasmic transport in spermatogenesis: a driver of cellular differentiation? Bioessays 27,1011–1025.

Horsthemke B and Ludwig M (2005) Assisted reproduction: the epigenetic perspective. Hum Reprod Update 11,473–482.

Hud NV, Allen MJ, Downing KH, Lee J and Balhorn R (1993) Identification of the elemental packing unit of DNA in mammalian sperm cells by atomic force microscopy. Biochem Biophys Res Commun 193,1347–1354.

Hummelke GC and Cooney AJ (2004) Reciprocal regulation of the mouse protamine genes by the orphan nuclear receptor germ cell nuclear factor and CREMtau. Mol Reprod Dev 68 (4),394–407.

Huynh T, Mollard R and Trounson A (2002) Selected genetic factors associated with male infertility. Hum Reprod Update 8,183–198.

Iatrou K and Dixon GH (1978) Protamine messenger RNA: its life history during spermatogenesis in rainbow trout. Fed Proc 37,2526–2533.

Iguchi N, Yang S, Lamb DJ and Hecht NB (2005) A protamine SNP: one genetic cause of male infertility. J Medical Genet [Advanced online publication 30 September 2005].

Ingles CJ and Dixon GH (1967) Phosphorylation of protamine during spermatogenesis in trout testis. Proc Natl Acad Sci USA 58,1011–1018.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA and Aitken RJ (2000) DNA integrity in human spermatozoa: relationships with semen quality. J Androl 21,33–44.

Iuchi Y, Kaneko T, Matsui S, Sasagawa I and Fijii J (2003) Concerted changes in the YB2/RYB-a Protein and Protamine 2 messenger RNA in the mouse testis under heat stress. Biol Reprod 68,129–135.

Jager S (1990) Sperm nuclear stability and male infertility. Arch Androl 25,253–259.

Johansson L and Pellicciari CE (1988) Lead-induced changes in the stabilization of the mouse sperm chromatin. Toxicology 51,11–24.

Khara KK, Vlad M, Griffiths M and Kennedy CR (1997) Human protamines and male infertility. J Assist Reprod Genet 14,282–290.

Kierszenbaum AL (2001) Transition nuclear proteins during spermiogenesis: unrepaired DNA breaks not allowed. Mol Reprod Dev 58,357–358.

Kierszenbaum AL and Tres LL (2004) The acrosome-acroplaxome-manchette complex and the shaping of the spermatid head. Arch Histol Cytol 67,271–284.

Kimmins S and Sassone-Corsi P (2005) Chromatin remodelling and epigenetic features of germ cells. Nature 434,583–589.

Kimmins S, Kotaja N, Davidson I and Sassone-Corsi P (2004) Testis-specific transcription mechanisms promoting male germ-cell differentiation. Reproduction 128,5–12.

Kleene KC (2003) Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. Cytogenet Genome Res 103,217–224.

Kossel A (1928) The Protamines and Histones. Longmans Green, London.

Kramer JA and Krawetz SA (1998) Genesis of a novel human sequence from the protamine PRM1 gene. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 120,467–473.

Kramer JA and Krawetz SA (1997) RNA in spermatozoa: implications for the alternative haploid genome. Mol Hum Reprod 3,473–478.

Kramer JA, Zhang S, Yaron Y, Zhao Y and Krawetz SA (1997) Genetic testing for male infertility: a postulated role for mutations in sperm nuclear matrix attachment regions. Genet Test 1,125–129.

Kramer JA, McCarrey JR, Djakiew D and Krawetz SA (2000) Human spermatogenesis as a model to examine gene potentiation. Mol Reprod Dev 56 (Suppl.),254–258.

Krausz C and Sassone-Corsi P (2005) Genetic control of spermiogenesis: insights from the CREM gene and implications for human infertility. Reprod Biomed Online 10,64–71.

Krawetz SA (2005) Paternal contribution: new insights and future challenges. Nat Rev Genet 6,633–642.

Krawetz SA, Herfort MH, Hamerton JL, Pon RT and Dixon GH (1989) Chromosomal localization and structure of the human P1 protamine gene. Genomics 5,639–645.

Laberge RM and Boissonneault G (2005) On the nature and origin of DNA strand breaks in elongating spermatids. Biol Reprod 73,289–296.

Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A and Carreau S (2004) Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. Mol Hum Reprod 10,535–541.

Lanuti M, Kouri CE, Force S, Chang M, Amin K, Xu K, Blair I, Kaiser L and Albelda S (1999) Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene Ther 6,1600–1610.

Le Lannic G, Arkhis A, Vendrely E, Chevaillier P and Dadoune JP (1993) Production, characterization, and immunocytochemical applications of monoclonal antibodies to human sperm protamines. Mol Reprod Dev 36,106–112.

Le Lannou D, Colleu D, Boujard D, Le Couteux A, Lescoat D and Segalen J (1986) Effect of duration of abstinence on maturity of human spermatozoa nucleus. Arch Androl 17,35–38.

Lee K, Haugen HS, Clegg CH and Braun RE (1995) Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci USA 92,12451–12455.

Lefievre L, Barratt CL, Harper CV, Conner SJ, Flesch FM, Deeks E, Moseley FL, Pixton KL, Brewis IA and Publicover SJ (2003) Physiological and proteomic approaches to studying prefertilization events in the human. Reprod Biomed Online 7,419–427.

Lescoat D, Colleu D, Boujard D and Le Lannou D (1988) Electrophoretic characteristics of nuclear proteins from human spermatozoa. Arch Androl 20,35–40.

Lewis SE and Aitken RJ (2005) DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 322,33–41.

Lewis SE, Sterling ES, Young IS and Thompson W (1997) Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 67,142–147.

Lewis JD, Song Y, de Jong ME, Bagha SM and Ausio J (2003a) A walk though vertebrate and invertebrate protamines. Chromosoma 111,473–482.

Lewis JD, Abbott DW and Ausio J (2003b) A haploid affair: core histone transitions during spermatogenesis. Biochem Cell Biol 81,131–140.

Lewis JD, Saperas N, Song Y, Zamora MJ, Chiva M and Ausio J (2004) Histone H1 and the origin of protamines. Proc Natl Acad Sci USA 101,4148–4152.

Liang JF, Yang VC and Vaynshteyn Y (2005) The minimal functional sequence of protamine. Biochem Biophys Res Commun 336,653–659.

Liang R, Senturker S, Shi X, Bal W, Dizdaroglu M and Kasprzak KS (1999) Effects of Ni(II) and Cu(II) on DNA interaction with the N-terminal sequence of human protamine P2: enhancement of binding and mediation of oxidative DNA strand scission and base damage. Carcinogenesis 20,893–898.

Lochmann D, Stadlhofer S, Weyermann J and Zimmer A (2004) New protamine quantification method in microtiter plates using o-phthaldialdehyde/N-acetyl-L-cysteine reagent. Int J Pharm 283,11–17.

Lolis D, Georgiou I, Syrrou M, Zikopoulos K, Konstantelli M and Messinis I (1996) Chromomycin A3-staining as an indicator of protamine deficiency and fertilization. Int J Androl 19,23–27.

Loppin B, Bonnefoy E, Anselme C, Laurencon A, Karr TL and Couble P (2005) The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature 437,1386–1390.

Louie AJ and Dixon GH (1973) Kinetics of phosphorylation and dephosphorylation of testis histones and their possible role in determining chromosomol structure. Nat New Biol 243,164–168.

Love CC and Kenney RM (1999) Scrotal stress induces altered sperm chromatin structure associated with a decrease in protamine disulfide bonding in the stallion. Biol Reprod 60,615–620.

Maier WM, Nussbaum G, Domenjoud L, Klemm U and Engel W (1990) The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. Nucleic Acids Res 18,1249–1254.

Maleszewski M, Kuretake S, Evenson D, Yanagimachi H, Bjordahl J and Yanagimachi R (1998) Behavior of transgenic mouse spermatozoa with galline protamine. Biol Reprod 58,8–14.

Marcon L and Boissonneault G (2004) Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. Biol Reprod 70,910–918.

Martinage A, Arkhis A, Alimi E, Sautière P and Chevaillier P (1990) Molecular characterization of nuclear basic protein HPI1, a putative precursor of human sperm protamines HP2 and HP3. Eur J Biochem 191,449–451.

Martins RP, Ostermeier GC and Krawetz SA (2004) Nuclear matrix interactions at the human protamine domain: a working model of potentiation. J Biol Chem 279,51862–51868.

Marushige K, Ling V and Dixon GH (1969) Phosphorylatiion of chromosomol basic proteins in maturing trout testis. J Biol Chem 244,5953–5958.

Marushige Y and Marushige K (1978) Phosphorylation of sperm histone during spermiogenesis in mammals. Biochim Biophys Acta 518,440–449.

Massanyi P, Trandzik J, Nad P, Korenekova B, Skalicka M, Toman R, Lukac N, Halo M and Strapak P (2004) Concentration of copper, iron, zinc, cadmium, lead, and nickel in bull and ram semen and relation to the occurrence of pathological spermatozoa. J Environ Sci Health Part A Tox Hazard Subst Environ Eng 39,3005–3014.

Matsuda Y and Watanabe T (2003) Effects of oyster extract on the reproductive function of zinc-deficient mice: bioavailability of zinc contained in oyster extract. Congenit Anom Kyoto 43,271–279.

McKay DJ, Renaux BS and Dixon GH (1985) The amino acid sequence of human sperm protamine P1. Biosci Rep 5,383–391.

McKay DJ, Renaux BS and Dixon GH (1986) Human sperm protamines. Amino-acid sequences of two forms of protamine P2. Eur J Biochem 156,5–8.

McLay DW and Clarke HJ (2003) Remodelling the paternal chromatin at fertilization in mammals. Reproduction 125,625–633.

McPherson SM and Longo FJ (1993) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol 158,122–130.

McVicar CM, O'Neill DA, McClure N, Clements B, McCullough S and Lewis SE (2005) Effects of vasectomy on spermatogenesis and fertility outcome after testicular sperm extraction combined with ICSI. Hum Reprod 20,2795–2800.

Meistrich ML, Trostle-Weige PK, Lin R, Bhatnagar YM and Allis CD (1992) Highly acetylated H4 is associated with histone displacement in rat spermatids. Mol Reprod Dev 31,170–181.

Meistrich ML, Mohapatra B, Shirley CR and Zhao M (2003) Roles of transition nuclear proteins in spermiogenesis. Chromosoma 111,483–488.

Mengual L, Ballescà JL, Ascaso C and Oliva R (2003a) Marked differences in protamine content and P1/P2 ratios in sperm cells from Percoll fractions between patients and controls. J Androl 24,438–447.

Mengual L, Oriola J, Ascaso C, Ballescà JL and Oliva R (2003b) An increased CAG repeat length in the androgen receptor gene in azoospermic ICSI candidates. J Androl 24,279–284.

Mezquita C (1985) Chromatin composition, structure and function in spermatogenesis. Revis Biol Celular 5,1–124.

Mezquita C and Teng CS (1977) Studies on sex-organ development. Changes in nuclear and chromatin composition and genomic activity during spermatogenesis in the maturing rooster testis. Biochem J 164,99–111.

Mezquita C and Teng CS (1978) Studies on sex-organ development. Changes in chromatin structure during spermatogenesis in maturing rooster testis as demonstrated by the initiation pattern of ribonucleic acid synthesis *in vitro*. Biochem J 170,203–210.

Miescher F (1874) Das Protamin – Eine neue organische Basis aus denSamenf – den des Rheinlachses. Ber Dtsch Chem Ges 7,376.

Miller D, Briggs D, Snowden H, Hamlington J, Rollinson F, Lilford R and Krawtz SA (1999) A complex population of RNAs exists in human ejaculated spermatozoa: implications for understanding molecular aspects of spermatogenesis. Gene 237,385–392.

Miller D, Ostermeier GC and Krawetz SA (2005) The controversy, potential and roles of spermatozoal RNA. Trends Mol Med 11,156–163.

Mitchell V, Steger K, Marchetti C, Herbaut JC, Devos P and Rigot JM (2005) Cellular expression of protamine 1 and 2 transcripts in testicular spermatids from azoospermic men submitted to TESE-ICSI. Mol Hum Reprod 11,373–379.

Miyagawa Y, Nishimura H, Tsujimura A, Matsuoka Y, Matsumiya K, Okuyama A, Nishimune Y and Tanaka H (2005) Single-nucleotide polymorphisms and mutation analyses of the TNP1 and TNP2 genes of fertile and infertile human male populations. J Androl 26,779–786.

Molina J, Castilla JA, Castano JL, Fontes J, Mendoza N and Martinez L (2001) Chromatin status in human ejaculated spermatozoa from infertile patients and relationship to seminal parameters. Hum Reprod 16,534–539.

Morgan HD, Santos F, Green K, Dean W and Reik W (2005) Epigenetic reprogramming in mammals. Hum Mol Genet 14 (Spec 1),R47–R58.

Morisawa M and Mori H (1972) Heavy metals and spermatozoan motility. I. Distribution of iron, zinc and copper in sea urchin spermatozoa. Exp Cell Res 70,311–316.

Mudrak O, Tomilin N and Zalensky A (2005) Chromosome architecture in the decondensing human sperm nucleus. J Cell Sci 118,4541–4550.

Muratori M, Marchiani S, Maggi M, Forti G and Baldi E (2006) Origin and biological significance of DNA fragmentation in human spermatozoa. Front Biosci 11,1491–1499.

Mylonis I, Drosou V, Brancorsini S, Nikolakaki E, Sassone-Corsi P and Giannakouros T (2004) Temporal association of protamine 1 with the inner nuclear membrane protein lamin B receptor during spermiogenesis. J Biol Chem 279,11626–11631.

Nakazawa Y, Shimada A, Noguchi J, Domeki I, Kaneko H and Kikuchi K (2002) Replacement of nuclear protein by histone in pig sperm nuclei during *in vitro* fertilization. Reproduction 124,565–572.

Nasr-Esfahani MH, Razavi S, Mozdarani H, Mardani M and Azvagi H (2004a) Relationship between protamine deficiency with fertilization rate and incidence of sperm premature chromosomal condensation post-ICSI. Andrologia 36,95–100.

Nasr-Esfahani MH, Salehi M, Razavi S, Mardani M, Bahramian H, Steger K and Oreizi F (2004b) Effect of protamine-2 deficiency on ICSI outcome. Reprod Biomed Online 9,652–658.

Nasr-Esfahani MH, Salehi M, Razavi S, Anjomshoa M, Rozbahani S, Moulavi F and Mardani M (2005) Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. Reprod Biomed Online 11,198–205.

Naz RK (1990) Effects of sperm-reactive antibodies present in human infertile sera on fertility of female rabbits. J Reprod Immunol 18,161–177.

Naz RK and Mehta K (1989) Cell-mediated immune responses to sperm antigens: effects on mouse sperm and embryos. Biol Reprod 41,533–542.

Naz RK, Deutsch J, Phillips TM, Menge AC and Fisch H (1989) Sperm antibodies in vasectomized men and their effects on fertilization. Biol Reprod 41,163–173.

Naz RK, Barad D, Barg P, Ahmad K and Bhargava K (1992) Antigenic differences in human sperm samples related to various morphological abnormalities. Arch Androl 29,117–126.

Nelson JE and Krawetz SA (1993) Linkage of human spermatid-specific basic nuclear protein genes. Definition and evolution of the P1→P2→TP2 locus. J Biol Chem 268,2932–2936.

Nelson JE and Krawetz SA (1994) Characterization of a human locus in transition. J Biol Chem 269,31067–31073.

O'Brien J and Zini A (2005) Sperm DNA integrity and male infertility. Urology 65,16–22.

Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H and Beebe S (2003) Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. Reprod Biomed Online 7,469–476.

Ohtsuki K, Nishikawa Y, Saito H, Munakata H and Kato T (1996) DNA-binding sperm proteins with oligo-arginine clusters function as potent activators for egg CK-II. FEBS Lett 378,115–120.

Oliva R (1995) Sequence, evolution and transcriptional regulation of mammalian P1 type protamines. In Jamieson BGM, Ausió J and Justine JL (eds), Advances in Spermatozoal Phylogeny and Taxonomy, Vol. 166. Mémoires du Muséum National d'Histoire Naturelle, Paris, pp. 537–548.

Oliva R and Dixon GH (1989) Chicken protamine genes are intronless: the complete nucleotide sequence and organization of the two loci. J Biol Chem 264,12472–12481.

Oliva R and Dixon GH (1990) Vertebrate protamine gene evolution I. Sequence alignments. J Mol Evol 30,333–346.

Oliva R and Dixon GH (1991a) Vertebrate protamine genes and the histone-to-protamine replacement reaction. Prog Nucleic Acid Res Mol Biol 40,25–94.

Oliva R and Dixon GH (1991b) Expression and processing of the rooster protamine mRNA. Ann NY Acad Sci 637,289–299.

Oliva R and Mezquita C (1982) Histone H4 hyperacetylation and rapid turnover of acetyl groups in transcriptionally inactive rooster testis spermatids. Nucleic Acids Res 10,8049–8059.

Oliva R and Mezquita C (1986) Marked differences in the ability of distinct protamines to disassemble nucleosomal core particles '*in vitro*'. Biochemistry 25,6508–6511.

Oliva R, Vidal S and Mezquita C (1982) Cellular content and biosynthesis of polyamines during rooster spermatogenesis. Biochem J 208,269–273.

Oliva R, Bazett-Jones D, Mezquita C and Dixon GH (1987) Factors affecting nucleosome disassemble by protamines '*in vitro*'. Histone hyperacetylation and chromatin structure, time dependence, and the size of the sperm nuclear proteins. J Biol Chem 262,17016–17025.

Oliva R, Mezquita J, Mezquita C and Dixon GH (1988) Haploid expression of the rooster protamine mRNA in the postmeiotic stages of spermatogenesis. Dev Biol 125,332–340.

Oliva R, Bazett-Jones D, Locklear B and Dixon GH (1990) Histone Hyperacetylation can induce unfolding of the nucleosomal core particles. Nucleic Acids Res 188,2739–2747.

Oliva R, Margarit E, Ballescà JL, Carrió A, Sánchez A, Milà M, Ballesta F and Alvarez-Vijande JR (1998) Prevalence of Y chromosome microdeletions in consecutive oligospermic and azoospermic ICSI candidates. Fertil Steril 70,506–510.

Ostermeier GC, Miller D, Huntriss JD, Diamond MP and Krawetz SA (2004) Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429,154.

Papoutsopoulou S, Nikolakaki E, Chalepakis G, Kruft V, Chevaillier P and Giannakouros T (1999) SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1. Nucleic Acids Res 27,2972–2980.

Park KW (2004) Protamine and protamine reactions. Int Anesthesiol Clin 42,135–145.

Park YJ, Liang JF, Ko KS, Kim SW and Yang VC (2003) Low molecular weight protamine as an efficient and nontoxic gene carrier: *in vitro* study. J Gene Med 5,700–711.

Perreault SD (1992) Chromatin remodeling in mammalian zygotes. Mutat Res 296,43–55.

Peschon JJ, Behringer RR, Brinster RL and Palmiter RD (1987) Spermatid-specific expression of protamine 1 in transgenic mice. Proc Natl Acad Sci USA 84,5316–5319.

Peschon JJ, Behringer RR, Palmiter RD and Brinster RL (1989) Expression of mouse protamine 1 genes in transgenic mice. Ann NY Acad Sci 564,186–197.

Pfeifer H, Conrad M, Roethlein D, Kyriakopoulos A, Brielmeier M, Bornkamm GW and Behne D (2001) Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. FASEB J 15,1236–1238.

Piao F, Yokoyama K, Ma N and Yamauchi T (2003) Subacute toxic effects of zinc on various tissues and organs of rats. Toxicol Lett 145,28–35.

Piña-Guzman B, Solis-Heredia MJ and Quintanilla-Vega B (2005) Diazinon alters sperm chromatin structure in mice by phosphorylating nuclear protamines. Toxicol Appl Pharmacol 202,189–198.

Pirhonen A, Linnala-Kankkunen A and Maenpaa PH (1994a) Identification of phosphoseryl residues in protamines from mature mammalian spermatozoa. Biol Reprod 50,981–986.

Pirhonen A, Linnala-Kankkunen A and Menpaa PH (1994b) P2 protamines are phosphorylated *in vitro* by protein kinase C, whereas P1 protamines prefer cAMP-dependent protein kinase. A comparative study of five mammalian species. Eur J Biochem 223,165–169.

Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S and Khochbin S (2003) Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. Mol Cell Biol 23,5354–5365.

Poccia D (1986) Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. Int Rev Cytol 105,1–65.

Poccia D and Collas P (1996) Transforming sperm nuclei into male pronuclei in vivo and *in vitro*. Curr Top Dev Biol 34,25–88.

Porsche R and Brenner ZR (1999) Allergy to protamine sulfate. Heart Lung 28,418–428.
Portmann AF and Holden WD (1949) Protamine sulphate, heparin, and blood coagulation. J Clin Invest 28,1451–1458.
Prieto MC, Maki AH and Balhorn R (1997) Analysis of DNA-protamine interactions by optical detection of magnetic resonance. Biochemistry 36,11944–11951.
Prigent Y, Muller S and Dadoune JP (1996) Immunoelectron microscopical distribution of histones H2B and H3 and protamines during human spermiogenesis. Mol Hum Reprod 2,929–935.
Prigent Y, Troalen F and Dadoune JP (1998) Immunoelectron microscopic visualization of intermediate basic proteins HPI1 and HPI2 in human spermatids and spermatozoa. Reprod Nutr Dev 38,417–427.
Pruslin FH, Imesch E, Winston R and Rodman TC (1987) Phosphorylation state of protamines 1 and 2 in human spermatids and spermatozoa. Gamete Res 18,179–190.
Puwaravutipanich T and Panyim S (1975) The nuclear basic proteins of human testes and ejaculated spermatozoa. Exp Cell Res 90,153–158.
Qiu XD, Yang YR, Li X, Li YG and Li CH (2005) Determination of expression of protamine-2 mRNA in different positions of the testis of patients with nonobstructive azoospermia. Zhonghua Nan Ke Xue 11,590–593.
Queralt R and Oliva R (1993) Identification of conserved potential regulatory sequences of the protamine-encoding P1 genes from ten different mammals. Gene 133,197–204.
Queralt R and Oliva R (1995) Demonstration of trans-acting factors binding to the promoter region of the testis-specific rat protamine P1 gene. Biochem Biophys Res Com 208,802–812.
Queralt R, de Fabregues-Boixar O, Adroer R, Gene M, Gomez-Catalan J, Huguet E and Oliva R (1993) Direct sequencing of the human protamine P1 gene and application in forensic medicine. J Forensic Sci 38,1491–1501.
Queralt R, Adroer R, Oliva R, Winkfein RJ, Retief JD and Dixon GH (1995) Evolution of protamine P1 genes in mammals. J Mol Evol 40,601–607.
Quintanilla-Vega B, Hoover DJ, Bal W, Silbergeld EK, Waalkes MP and Anderson LD (2000) Lead interaction with human protamine (HP2) as a mechanism of male reproductive toxicity. Chem Res Toxicol 13,594–600.
Raap U, Liekenbrocker T, Kapp A and Wedi B (2005) Delayed-type hypersensitivity to protamine as a complication of insulin therapy. Contact Dermatitis 53,57–58.
Raukas E and Mikelsaar RH (1999) Are there molecules of nucleoprotamine? Bioessays 21,440–448.
Razavi S, Nasr-Esfahani MH, Mardani M, Mafi A and Moghdam A (2003) Effect of human sperm chromatin anomalies on fertilization outcome post-ICSI. Andrologia 35,238–243.
Reeves RH, Gearhart JD, Hecht NB, Yelick P, Johnson P and O'Brien SJ (1989) Mapping of PRM1 to human chromosome 16 and tight linkage of Prm-1 and Prm-2 on mouse chromosome 16. J Hered 80,442–446.
Retief JD, Winkfein RJ, Dixon GH, Adroer R, Queralt R, Ballabriga J and Oliva R (1993) Evolution of protamine P1 genes in primates. J Mol Evol 37,426–434.
Rhim JA, Connor W, Dixon GH, Harendza CJ, Evenson DP, Palmiter RD and Brinster RL (1995) Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. Biol Reprod 52,20–32.
Roca J and Mezquita C (1989) DNA topoisomerase II activity in nonreplicating, transcriptionally inactive, chicken late spermatids. EMBO J 8,1855–1860.
Rockett JC, Patrizio P, Schmid JE, Hecht NB and Dix DJ (2004) Gene expression patterns associated with infertility in humans and rodent models. Mutat Res 549,225–240.
Rodman TC, Pruslin FH, Chauhan Y, To SE and Winston R (1988) Protamine-reactive natural IgM antibodies in human sera. Characterization of the epitope demonstrates specificity of antigenic recognition; occurrence indicates obscurity of origin and function. J Exp Med 167,1228–1246.
Romanato M, Regueira E, Cameo MS, Baldini C, Calvo L and Calvo JC (2005) Further evidence on the role of heparan sulfate as protamine acceptor during the decondensation of human spermatozoa. Hum Reprod 20,2784–2789.
Rooney AP and Zhang J (1999) Rapid evolution of a primate sperm protein: relaxation of functional constraint or positive Darwinian selection? Mol Biol Evol 16,706–710.
Rooney AP, Zhang J and Nei M (2000) An unusual form of purifying selection in a sperm protein. Mol Biol Evol 17,278–283.
Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK and Khochbin S (2005) Establishment of male-specific epigenetic information. Gene 345,139–153.
Rousseaux-Prevost R, de Almeida M, Jouannet P, Hublau P, Sautière P and Rousseaux J (1992) Auto-antibodies to human sperm basic nuclear proteins in infertile and vasectomized men: characterization of antigens and epitopes recognized by antibodies. Mol Immunol 29,895–902.
Rufas O, Fisch B, Seligman J, Tadir Y, Ovadia J and Shalgi R (1991) Thiol status in human sperm. Mol Reprod Dev 29,282–288.
Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y and Campana A (1998) Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod 13 (Suppl. 4),11–19.
Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG and Bianchi U (2000) The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 15,1112–1116.
Sakkas D, Seli E, Bizzaro D, Tarozzi N and Manicardi GC (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. Reprod Biomed Online 7,428–432.
Samuel T (1977) Antibodies reacting with salmon and human protamines in sera from infertile men and from vasectomized men and monkeys. Clin Exp Immunol 30,181–187.
Sánchez-Peña LC, Reyes BE, Lopez-Carrillo L, Recio R, Moran-Martinez J, Cebrián ME and Quintanilla-Vega B (2004) Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol Appl Pharmacol 196,108–113.
Sanders MM and Dixon GH (1972) The biosynthesis of protamine in trout testis. IV. Sites of phosphorylation. J Biol Chem 247,851–855.
Saowaros W and Panyim S (1979) The formation of disulfide bonds in human protamines during sperm maturation. Experientia 35,191–192.
Sassone-Corsi P (2002) Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 296,2176–2178.
Sautière P, Martinage A, Bélaïche D, Arkhis A and Chevaillier P (1988) Comparison of the amino acid sequences of human protamines HP2 and HP3 and of intermediate basic nuclear proteins HPS1 and HPS2. Structural evidence that HPS1 and HPS2 are pro-protamines. J Biol Chem 263,11059–11062.
Schlegel PN and Paduch DA (2005) Yet another test of sperm chromatin structure. Fertil Steril 84,854–859.
Schlicker M, Schnulle V, Schneppel L, Vorob'ev VI and Engel W (1994) Disturbances of nuclear condensation in human spermatozoa: search for mutations in the genes for protamine 1, protamine 2 and transition protein 1. Hum Reprod 9,2313–2317.
Schlüter G and Engel W (1995) The rat Prm3 gene is an intronless member of the protamine gene cluster W and is expressed in haploid male germ cells. Cytogenet Cell Genet 71 (4),352–355.
Schlüter G, Celik A, Obata R, Schlicker M, Hofferbert S, Schlung A, Adham IM and Engel W (1996) Sequence analysis of the conserved protamine gene cluster shows that it contains a fourth expressed gene. Mol Reprod Dev 43,1–6.
Schnulle V, Schlicker M and Engel W (1994) A (GA)n repeat polymorphism in the human protamine 2 (PRM 2) gene. Hum Mol Genet 3,1445.
Schultz RM (2002) The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 8,323–331.
Sega GA (1991) Adducts in sperm protamine and DNA vs. mutation frequency. Prog Clin Biol Res 372,521–530.
Sega GA, Alcota RP, Tancongco CP and Brimer PA (1989) Acrylamide binding to the DNA and protamine of spermiogenic stages in the mouse and its relationship to genetic damage. Mutat Res 216,221–230.
Seli E and Sakkas D (2005) Spermatozoal nuclear determinants of reproductive outcome: implications for ART. Hum Reprod Update 11,337–349.
Seligman J and Shalgi R (1991) Protein thiols in spermatozoa and epididymal fluid of rats. J Reprod Fertil 93,399–408.
Seligman J, Zipser Y and Kosower NS (2004) Tyrosine phosphorylation, thiol status, and protein tyrosine phosphatase in rat epididymal spermatozoa. Biol Reprod 71,1009–1015.
Shalgi R, Seligman J and Kosower NS (1989) Dynamics of the thiol status of rat spermatozoa during maturation: analysis with the fluorescent labeling agent monobromobimane. Biol Reprod 40,1037–1045.
Shimada A, Kikuchi K, Noguchi J, Akama K, Nakano M and Kaneko H (2000) Protamine dissociation before decondensation of sperm nuclei during *in vitro* fertilization of pig oocytes. J Reprod Fertil 120,247–256.
Shimada M, Kawano N and Terada T (2002) Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of *in vitro* maturation media. Reproduction 124,557–564.
Shirley CR, Hayashi S, Mounsey S, Yanagimachi R and Meistrich ML (2004) Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. Biol Reprod 71,1220–1229.

Shvarev A and Bakker E (2005) Response characteristics of a reversible electrochemical sensor for the polyion protamine. Anal Chem 77,5221–5228.
Siffroi JP, Alfonsi MF and Dadoune JP (1999) Co-localization of HP1 and TP1 transcripts in human spermatids by double electron microscopy *in situ* hybridization. Int J Androl 22,83–90.
Silva PF and Gadella BM (2006) Detection of damage in mammalian sperm cells. Theriogenology 65,958–978.
Silvestroni L, Frajese G and Fabrizio M (1976) Histones instead of protamines in terminal germ cells of infertile, oligospermic men. Fertil Steril 27,1428–1437.
Silvestroni L, Mantovani A and Palleschi S (2004) The partial head decondensation test is a new, quick method to assess acrosome status in human spermatozoa. Fertil Steril 81,1007–1012.
Solov'eva L, Svetlova M, Bodinski D and Zalensky AO (2004) Nature of telomere dimers and chromosome looping in human spermatozoa. Chromosome Res 12,817–823.
Sonnack V, Failing K, Bergmann M and Steger K (2002) Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis. Andrologia 34,384–390.
Sotolongo B, Lino E and Ward WS (2003) Ability of hamster spermatozoa to digest their own DNA. Biol Reprod 69,2029–2035.
Stanker LH, McKeown C, Balhorn R, Lee C, Mazrimas J, Goralka M and Wyrobek A (1992) Immunological evidence for a P2 protamine precursor in mature rat sperm. Mol Reprod Dev 33,481–488.
Stanker LH, Wyrobek A, McKeown C and Balhorn R (1993) Identification of the binding site of two monoclonal antibodies to human protamine. Mol Immunol 30,1633–1638.
Steger K (1999) Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol (Berl) 199,471–487.
Steger K (2001) Haploid spermatids exhibit translationally repressed mRNAs. Anat Embryol (Berl) 203,323–334.
Steger K, Pauls K, Klonisch T, Franke FE and Bergmann M (2000) Expression of protamine-1 and -2 mRNA during human spermiogenesis. Mol Hum Reprod 6,219–225.
Steger K, Failing K, Klonisch T, Behre HM, Manning M, Weidner W, Hertle L, Bergmann M and Kliesch S (2001) Round spermatids from infertile men exhibit decreased protamine-1 and -2 mRNA. Hum Reprod 16,709–716.
Steger K, Fink L, Klonisch T, Bohle RM and Bergmann M (2002) Protamine-1 and -2 mRNA in round spermatids is associated with RNA-binding proteins. Histochem Cell Biol 117,227–234.
Steger K, Fink L, Failing K, Bohle RM, Kliesch S, Weidner W and Bergmann M (2003) Decreased protamine-1 transcript levels in testes from infertile men. Mol Hum Reprod 9,331–336.
Steger K, Slavov M, Failing K, Weidner W and Bergmann M (2005) Effect of vasectomy on sperm nuclear chromatin condensation in the rabbit. J Androl 26,289–295.
Stewart TA, Hecht NB, Hollingshead PG, Johnson PA, Leong JA and Pitts SL (1988) Haploid-specific transcription of protamine-myc and protamine-T-antigen fusion genes in transgenic mice. Mol Cell Biol 8,1748–1755.
Stewart KS, Kramer JA, Evans MI and Krawetz SA (1999) Temporal expression of the transgenic human protamine gene cluster. Fertil Steril 71,739–745.
Subirana JA (1983) The sperm cell. In Andre J (eds), Proceedings of the Forth International Symposium of Spermatogly. Martinus-Nijhoff Publications, The Hague, The Netherlands, pp. 197–213.
Suganuma R, Yanagimachi R and Meistrich ML (2005) Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. Hum Reprod 20,3101–3108.
Szczygiel MA and Ward WS (2002) Combination of dithiothreitol and detergent treatment of spermatozoa causes paternal chromosomal damage. Biol Reprod 67,1532–1537.
Tamura T, Makino Y, Mikoshiba K and Muramatsu M (1992) Demonstration of a testis-specific trans-acting factor Tet-1 *in vitro* that binds to the promoter of the mouse protamine 1 gene. J Biol Chem 267,4327–4332.
Tanaka H and Baba T (2005) Gene expression in spermiogenesis. Cell Mol Life Sci 62,344–354.
Tanaka H, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A and Nishimune Y (2003) Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. Mol Hum Reprod 9,69–73.
Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, Onishi M, Masai K, Maekawa M, Toshimori K et al. (2005) HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. Mol Cell Biol 25,7107–7119.
Tanphaichitr N, Sobhon P, Taluppeth N and Chalermisarachai P (1978) Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. Exp Cell Res 117,347–356.
Tesarik J, Greco E and Mendoza C (2004) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum Reprod 19,611–615.
Torgerson DG, Kulathinal RJ and Singh RS (2002) Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. Mol Biol Evol 19,1973–1980.
Torregrosa N, Domínguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Ballescà JL and Oliva R (2006) Protamine 2 (P2) precursors, P1/P2 ratio, DNA integrity and other sperm parameters in infertile patients. Hum Reprod in press.
Vilfan ID, Conwell CC and Hud NV (2004) Formation of native-like mammalian sperm cell chromatin with folded bull protamine. J Biol Chem 279,20088–20095.
Virro MR, Larson-Cook KL and Evenson DP (2004) Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 81,1289–1295.
Vogt PH (2005) AZF deletions and Y chromosomal haplogroups: history and update based on sequence. Hum Reprod Update 11,319–336.
Ward WS and Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 44, 569–574.
Weiler JM, Freiman P, Sharath MD, Metzger WJ, Smith JM, Richerson HB, Ballas ZK, Halverson PC, Shulan DJ, Matsuo S et al. (1985) Serious adverse reactions to protamine sulfate: are alternatives needed? J Allergy Clin Immunol 75,297–303.
Wouters-Tyrou D, Martinage A, Chevaillier P and Sautiere P (1998) Nuclear basic proteins in spermiogenesis. Biochimie 80,117–128.
Wu JY, Ribar TJ, Cummings DE, Burton KA, McKnight GS and Means AR (2000) Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. Nat Genet 25,448–452.
Wyckoff GJ, Wang W and Wu CI (2000) Rapid evolution of male reproductive genes in the descent of man. Nature 403,304–309.
Wykes SM and Krawetz SA (2003) The structural organization of sperm chromatin. J Biol Chem 278,29471–29477.
Xie Q, Sun H, Liu Y, Ding X, Fu D and Liu K (2006) Adduction of biomacromolecules with acrylamide (AA) in mice at environmental dose levels studied by accelerator mass spectrometry. Toxical Lett 163,101–108.
Yelick PC, Balhorn R, Johnson PA, Corzett M, Mazrimas JA, Kleene KC and Hecht NB (1987) Mouse protamine 2 is synthesized as a precursor whereas mouse protamine 1 is not. Mol Cell Biol 7,2173–2179.
Yoshii T, Kuji N, Komatsu S, Iwahashi K, Tanaka Y, Yoshida H, Wada A and Yoshimura Y (2005) Fine resolution of human sperm nucleoproteins by two-dimensional electrophoresis. Mol Hum Reprod 11,677–681.
Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR and Meistrich ML (2000) Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc Natl Acad Sci USA 97,4683–4788.
Zalenskaya IA and Zalensky AO (2002) Telomeres in mammalian male germline cells. Int Rev Cytol 218,37–67.
Zalenskaya IA, Bradbury EM and Zalensky AO (2000) Chromatin structure of telomere domain in human sperm. Biochem Biophys Res Commun 279,213–218.
Zalensky AO, Allen MJ, Kobayashi A, Zalenskaya IA, Balhorn R and Bradbury EM (1995) Well-defined genome architecture in the human sperm nucleus. Chromosoma 103,577–590.
Zalensky AO, Siino JS, Gineitis AA, Zalenskaya IA, Tomilin NV, Yau P and Bradbury EM (2002) Human testis/sperm-specific histone H2B (hTSH2B). Molecular cloning and characterization. J Biol Chem 277,43474–43480.
Zambrowicz BP, Harendza CJ, Zimmermann JW, Brinster RL and Palmiter RD (1993) Analysis of the mouse protamine 1 promoter in transgenic mice. Proc Natl Acad Sci USA 90,5071–5075.
Zhang X, San Gabriel M and Zini A (2006) Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. J. Androl [Published ahead of print 1 February 2006].
Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM et al. (2001) Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol 21,7243–7255.

Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R and Meistrich ML (2004a) Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. Genesis 38,200–213.

Zhao M, Shirley CR, Mounsey S and Meistrich ML (2004b) Nucleoprotein transitions during spermiogenesis in mice with transition nuclear protein Tnp1 and Tnp2 mutations Biol Reprod 71,1016–1025.

Zini A, Kamal KM and Phang D (2001) Free thiols in human spermatozoa: correlation with sperm DNA integrity. Urology 58,80–84.

Ziyyat A, Lasalle B, Testard J, Briot P, Amar E, Finaz C and Lefevre A (1999) Flow cytometry isolation and reverse-transcriptase chain reaction characterization of human round spermatids in infertile patients. Hum Reprod 14,379–387.

Zubkova EV, Wade M and Robaire B (2005) Changes in spermatozoal chromatin packaging and susceptibility to oxidative challenge during aging. Fertil Steril 84 (Suppl. 2),1191–1198.

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