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Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients

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BACKGROUND: The protamine 1-to-protamine 2 ratio (P1/P2) is altered in the sperm cells of some infertile patients. Also, evidence for increased protamine 2 precursors (pre-P2) in a few patients has been reported. But so far, there have been no studies measuring simultaneously these two variables in a large number of patients. METHODS: We measured the P1/P2 ratio and the presence of pre-P2 using, for the first time, an antibody specific to the precursor pre-P2, together with other sperm parameters in 224 infertile patients. Additionally, the DNA integrity was assessed by terminal transferase dUTP nick-end labelling (TUNEL) in a subset of the samples. RESULTS: Pre-P2 levels show a significant positive correlation with the P1/P2 ratio, with the presence of other proteins and, at low pre-P2 levels, with TUNEL-positive sperm. An inverse correlation with sperm count, normal morphology and motility was detected. CONCLUSIONS: The levels of pre-P2 may provide clues into the pathogenic mechanisms of infertility. The increased proportion of pre-P2 in some patients with increased P1/P2 ratio suggests an involvement of pre-P2 processing. The positive correlation between TUNEL-positive sperm and pre-P2 at low pre-P2/P2 ratios also suggests a link between deficient protamine processing and decreased DNA integrity.

Key words: DNA integrity/infertility/precursor/protamine/sperm

Introduction

Protamine 1 (P1) and protamine 2 (P2) are the most abundant nuclear proteins in the sperm nucleus packaging the human male genome (Gusse *et al*., 1986; Oliva and Dixon, 1991; Bianchi *et al*., 1992; Aoki and Carrell, 2003; Lewis *et al*., 2003). P1 is synthesized as a mature protein, whereas P2 is synthesized as a precursor (pre-P2) (Ammer *et al*., 1986; Gusse *et al*., 1986; McKay *et al*., 1986; Balhorn *et al*., 1987; Sautière *et al*., 1988; Queralt *et al*., 1995). The P2 content in the nucleus of human sperm cells is similar to that of P1 (P1/P2 ratio of approximately 1) (Balhorn *et al*., 1988; de Yebra *et al*., 1993; Yoshii *et al*., 2005), and an elevated P1/P2 ratio has been taken as evidence of nuclear immaturity (Belokopytova *et al*., 1993; Colleu *et al*., 1996). A reduction in P2 protamine content has been reported in different studies in infertile patients (Chevaillier *et al*., 1987; Balhorn *et al*., 1988; Bach *et al*., 1990; Blanchard *et al*., 1990; Belokopytova *et al*., 1993; de Yebra *et al*., 1993, 1998; Bench *et al*., 1998; Mengual *et al*., 2003; Aoki *et al*., 2005a). The evidence for increased levels of pre-P2 concomitant with an increased P1/P2 ratio in some infertile patients has also been reported (Bench *et al*., 1998; de Yebra *et al*., 1998).

Despite all the above reports describing the presence of pre-P2, changing levels of protamines and other sperm and seminal parameters in infertile patients or animal models, there is a relative lack of studies measuring all these parameters in the same set of infertile patients. Therefore, our objective in the present study has been to determine all these data in the same set of sperm samples from infertile patients.

The methods for determination of the P1/P2 levels have been reported (de Yebra and Oliva, 1993; de Yebra *et al*., 1993; Mengual *et al*., 2003). However, the presence of protamine precursors in infertile patients had been measured previously in a limited number of samples using antibodies against P2 (Bench *et al*., 1998; de Yebra *et al*., 1998) but not specific to the precursor peptide. Therefore, in this work, we also report, as a technical improvement and for the first time, the detection of pre-P2 using an antibody specific to the precursor domain of P2.

Methods

Subjects and sample collection

Sperm samples (ejaculates) from 224 infertile patients collected at their first visit to the Assisted Reproduction Unit of the Hospital Clinic of Barcelona were included in this study. Fourteen sperm samples from normozoospermic fertile sperm donors were also included. The samples were collected in specific sterile containers after at least 3 days of sexual abstinence and were allowed to liquefy. After liquefaction of the semen, sperm parameters (volume, sperm concentration, percentage of motility and motion characteristics) were evaluated according to the published recommendations (World Health Organization, 1999) using a computer-assisted semen analyser and a Makler counting chamber (Sefi Medical Instruments, Hainfa, Israel). Individual sperm were categorized as having rapid progressive motility, slow or sluggish motility, non-progressive motility or being non-motile. For the examination of sperm morphology, smears of the samples were fixed with Labofix (Labonort, Templemars, France), stained using the Diff-Quik kit (Baxter Healthcare Corporation, McGraw, IL, USA) and rinsed immediately with water after staining to remove the excess dye and air-dried. Sperm morphology was evaluated using strict criteria (Kruger *et al*., 1987), and at least 100 cells were examined per slide. This project was approved by the bioethics committee of the hospital, and informed consent was obtained from the participants.

Extraction of sperm proteins

An aliquot of the semen sample containing 14×10^6 spermatozoa was washed twice with Ham's F10 supplemented at 3 with 7.5% $NaHCO_3$. The sediment was resuspended in 200 µl of 20 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (Sigma Chemicals, St Louis, MO, USA) and 100 mM Tris–HCl (pH 8) and then processed as described (de Yebra and Oliva, 1993), with the exception that no iodoacetate treatment was performed (an equal volume of H_2O was added instead followed by a 10-min incubation). Finally, each sample was resuspended in 20 µl buffer of 5.5 M urea, 20% β-mercaptoethanol and 5% acetic acid.

Separation and analysis of proteins

Nuclear proteins were analysed in acid-urea polyacrylamide gels. Electrophoresis was performed on a Miniprotean System (Bio-Rad, Life Science Group, Hercules, CA, USA) using gels containing (final concentrations) 0.9 M acetic acid, 2.5 M urea, 15% acrylamide, 0.09% bis-acrylamide, 0.53% ammonium persulfate and 0.53% TEMED (N,N,N,N-Tetramethyl-Ethylenediamine) (Amersham Biosciences, Uppsala, Sweden). Following polymerization, the gels were pre-electrophoresed for 1 h at 150 V before loading 2.5 µl of each of the samples. In addition to the samples under analysis, different quantities of a human protamine standard (0.435, 0.87, 1.74 and 2.61 µg) were loaded. This standard had been previously prepared and quantified by amino acid analysis using an Alpha Plus autoanalyser (Pharmacia LKB Biotechnology, USA) (Mengual *et al*., 2003) from a pool of human normozoospermic sperm samples. After loading, the gel was electrophoresed for 50 min at 150 V in 0.9 M acetic acid buffer.

The gels were stained with a filtered solution of 1.1 g of Coomassie Blue R-250 (Bio-Rad) dissolved in 250 ml of methanol, 250 ml of H_2O and 50 ml of acetic acid for 60 min and destained first for 10 min in 50% methanol, 10% acetic acid and then for 1 h in 10% methanol and 10% acetic acid. The gels were then scanned (Figure 1A) and the intensity of the bands quantified with the QUANTITY ONE software (Bio-Rad). Finally, the gels were soaked in 10% methanol, 10% acetic acid and 5% glycerol for 5 min and dried between two sheets of cellophane film for 48 h and stored.

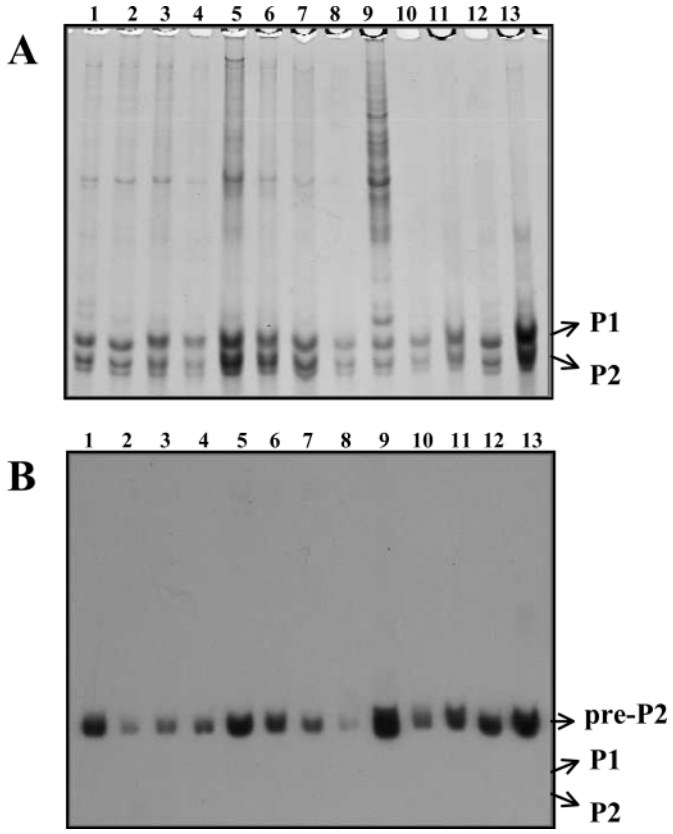

Figure 1. Analysis of protamine 1 (P1), protamine 2 (P2) and P2 precursors (pre-P2) in infertile patients. (**A**) Proteins extracted from spermatozoa, separated on a polyacrylamide-acetic-urea gel and stained with Coomassie Blue. Lanes 1–9 correspond to different sperm samples from infertile patients. In each lane (1–9), the protein extracted from 1.75×10^6 spermatozoa has been loaded. Lanes 10–13 correspond to increasing amounts of a human protamine standard (0.435, 0.87, 1.74 and 2.61 µg) included in each gel. (**B**) Western blot, corresponding to a replica of the gel shown in **A**, using an antibody specific to the pre-P2 showing variations in the levels of pre-P2 in the different patients. Also, note that no signal is present in the region where P2 migrates, indicating the specificity of the antibody.

The Coomassie Blue-stained gels (Figure 1A) were also used to quantify the proportion of other proteins present in each lane over the total protein. To do this, the entire region spanning from the top of each lane to the beginning of the P1 protamine band (but not including it) was integrated. Thus, the proportion of 'other proteins/total' was calculated as follows: optical density (OD) for other proteins/OD for other proteins + OD for protamines.

Antiserum production and detection of pre-P2

A replica of each gel was transferred at 75 V for 1 h to an Immobilon-P membrane (Millipore, Bedford, Massachusetts, USA) and analysed by Western blot using a novel anti-mouse P2 precursor polyclonal antiserum prepared for these studies. A peptide sequence with a predicted high antigenicity and homology with the human sequence but no homology with other proteins in the database was selected from the portion of the precursor region of mouse P2 that is common to the full-length and partially processed precursors. The peptide QGLSPERVEDYGRTHR (amino acids 29-44) was synthesized, conjugated to keyhole limpet haemocyanin and used to immunize two New Zealand rabbits (BioSynthesis, Lewisville, TX, USA). Booster injections were

given at week 4 and then every 2 weeks. Serum was drawn periodically, and the immunoglobulin G (IgG) fraction was purified.

As a secondary antibody, we used a donkey anti-rabbit IgG, horseradish peroxidase-linked whole antibody (Amersham Biosciences, Buckinghamshire, England). Thus, proteins on the blots were detected by chemiluminescence developed by a peroxidase substrate (ECL™ Western Blotting Analysis System, Amersham Biosciences). Western blots performed with proteins extracted from mature mouse and human sperm and with proteins extracted from human and mouse testicles demonstrated the specificity of the antibody and its ability to recognize human pre-P2 (not shown). The film obtained was scanned (Figure 1B), and the bands were quantified with the QUANTITY ONE Software (Bio-Rad). The pre-P2 signals from the different gels were normalized using the signal from the standard protamine included in each gel. Finally, the pre-P2 signal was expressed relative to the average signal present in the normozoospermic fertile donors. Thus, a pre-P2 value of 1 means that a particular sample has a pre-P2 signal equal to the average present in normal samples, and a pre-P2 value of 2 means that a particular sample has twice the pre-P2 signal present in normal samples.

Terminal transferase dUTP nick-end labelling assay

Terminal transferase dUTP nick-end labelling (TUNEL) has been carried out as previously described (Barroso *et al*., 2000; Ollero *et al*., 2001; Alvarez *et al*., 2002; Sakkas *et al*., 2002; Agarwal and Said, 2003) using the *in situ* cell death detection kit from Roche (Ref. 11684795910, Roche Diagnostic Corp. Indianapolis, IN, USA). A semen aliquot containing 2.5×10^6 spermatozoa was washed by centrifugation (800 *g*) at room temperature for 5 min with phosphate-buffered saline (PBS; BioMérieux, Marcy l'Etoile, France). After removal of the seminal plasma, the pellet was washed twice in PBS with 1% bovine serum albumin (BSA; Sigma Chemicals). Then, the pellet was resuspended in 100 µl of PBS/1% BSA and fixed in 100 µl of 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature with agitation. After fixation, one wash was performed with PBS/1% BSA. The pellet was permeabilized with 100 µl of 0.1% Triton X-100 in 0.1% sodium citrate on PBS for 2 min on ice. Permeabilization solution was removed by centrifugation, and two additional washes were performed with PBS/1% BSA. The pellet was resuspended in 50 µl of the staining solution for 1 h at 37°C in the dark and agitating each 15 min. The staining solution contained terminal deoxynucleotidyltransferase (TdT). The negative and the positive controls were performed, respectively, by omitting the TdT enzyme following the kit instructions and by pre-incubating fixed and permeabilized sperm samples with DNase I (40 IU/ml) for 10 min at room temperature to produce DNA breaks.

After the staining, two washes with 1% BSA in PBS were performed, and the spermatozoa were resuspended in PBS and analysed by flow cytometry. A minimum of 10 000 events were examined for each measurement at a flow rate of about 200 events on a flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA). Data were processed using CELLQUEST (Becton Dickinson) and WINMDI v2.8 software. Green fluorescence (TUNEL-positive cells) was measured using a 530 ± 30 nm band-pass filter. Spermatozoa were gated by using forward-angle light scatter and side-angle light scatter dot plot to gate out debris, aggregates and cells other than spermatozoa. Then, TUNEL-positive spermatozoa in this population were measured.

Statistical analysis

Statistical analyses were performed by using the Statistics Package for the Social Sciences software, version 12.0 (SPSS, Chicago, IL, USA), and statistical tests have been evaluated by using at least a significance level of 0.05. To identify significant correlations between variables, we performed Spearman test, except where specifically indicated otherwise. Lines in the dispersion plots were calculated by linear regression. Data in tables are expressed as mean ± SD.

Results

Determination of P2 precursors, P1/P2 ratio and other proteins

Running each sample in duplicate polyacrylamide gels and subsequent Coomassie Blue staining of one of the gels and Western blotting the other has allowed simultaneous determination of the P1/P2 ratio and the presence of pre-P2 in each sample (Figure 1). The P1/P2 ratio in the patient samples averaged 1.11 and ranged from 0.03 to 19.5 (Table I). The antibody also detected varying amounts of a pre-P2 band in human sperm protein extracts from the patients as well as control samples (Figure 1B). The average proportion of P2 present as pre-P2/P2 in the normozoospermic fertile donor samples was arbitrarily defined as 1 and ranged from 0.02 to 2.42 with an SD of 0.70. The proportion of pre-P2/P2 in the patient samples, relative to the average pre-P2/P2 present in the normozoospermic fertile donors, averaged 1.43 and ranged from 0.0 to 73.9 (Table I). The average pre-P2 ratio in infertile patients was not significantly different from that of controls (T-test).

The Coomassie Blue-stained gels (Figure 1) were also used to quantify the proportion of other proteins present in each lane

Table I. Basic semen parameters and average results in the samples from infertile patients

	Average	SD	Minimum	Maximum	Samples with data (*n*)
Total number of samples included					224
Age of patients (year)	35.0	5.2	19.0	50.0	224
Sperm count ($\times 10^6$ sperm/ml)	51.2	39.3	0.9	178.5	224
Volume (ml)	3.5	1.7	0.6	9.0	224
Motility 'c' (non-progressive motility, %)	15.7	8.2	0.0	50.0	224
Motility 'b' (slow or sluggish motility, %)	22.17	11.6	0.0	63.4	224
Motility 'a' (rapid progressive motility, %)	20.2	18.0	0.0	87.8	224
Spermatozoa with normal morphology (%)	16.2	8.5	2	40	139
P1/P2 ratio	1.11	1.33	0.03	19.5	224
Pre-P2/P2	1.43	5.18	0.0	73.9	224
Proteins other than protamines/total	0.51	0.23	0.0	0.97	207
TUNEL-positive sperm by cytometry (%)	37.9	21.3	8.9	91.8	41

P1, protamine 1; P2, protamine 2; TUNEL, terminal transferase dUTP nick-end labelling.

over the total protein (region spanning from the top of each lane to the beginning of the P1 protamine band, but not including it). The values obtained averaged 0.51 and ranged from 0.0 to 0.97. Because of the large region analysed from each lane, these proteins could be histones, intermediate proteins, pre-P2, other proteins or, most likely, a combination of all of these.

TUNEL-positive spermatozoa

The percentage of TUNEL-positive spermatozoa as determined by cytometry averaged 37.9 ± 21.3% and ranged from a minimum of 8.9% to a maximum of 91.8% (Table I).

Correlation between presence of pre-P2, conventional sperm parameters, P1/P2 ratio and other proteins

The levels of pre-P2/P2 correlate negatively with the sperm count (Figure 2; $r = –0.196$, $P < 0.005$), with normal morphology ($r = –0.257$, $P < 0.005$) and with the rapid progressive motility ($r = –0.240$, $P < 0.001$). The amount of pre-P2/P2 also correlated positively with the P1/P2 ratio (Figure 2; $r = 0.283$, $P < 0.001$) and with the presence of other proteins ($r = 0.172$, $P < 0.05$). Samples with a pre-P2 value over 6 ($n = 5$) were excluded from the scatter plots (but not from the statistical analysis) to show the details in the range up to 6 on the y-axis. Also in the P1/P2 ratio plot, one point (P1/P2 of 19.49 and pre-P2 of 73.9) was excluded from the graph to show the details in the range up to 3.0 on the x-axis.

Analysis of the proportion of TUNEL-positive spermatozoa and presence of pre-P2

A linear correlation is not detected in the overall analysis between the presence of TUNEL-positive spermatozoa and pre-P2/P2 (Figure 3A). However, a detailed observation of Figure 3A suggested a correlation at low values of the pre-P2/P2 variable. Therefore, we tested the exclusion of the samples

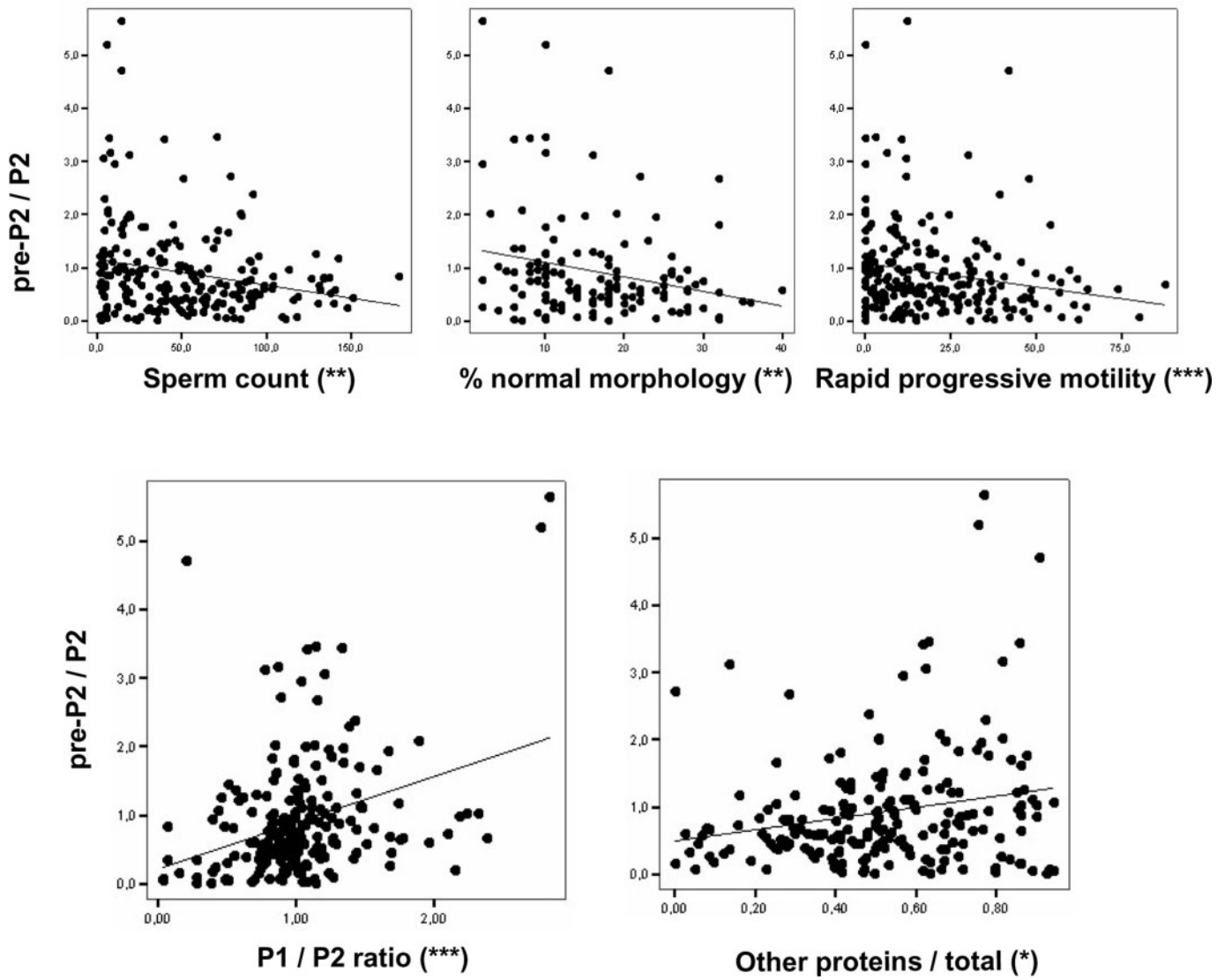

Figure 2. Correlation between the presence of protamine 2 (P2) precursors and basic seminal parameters, P1-to-P2 ratio and other proteins present in the spermatozoa. All correlations shown are statistically significant (Spearman; *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$). Regression lines have been calculated by linear regression.

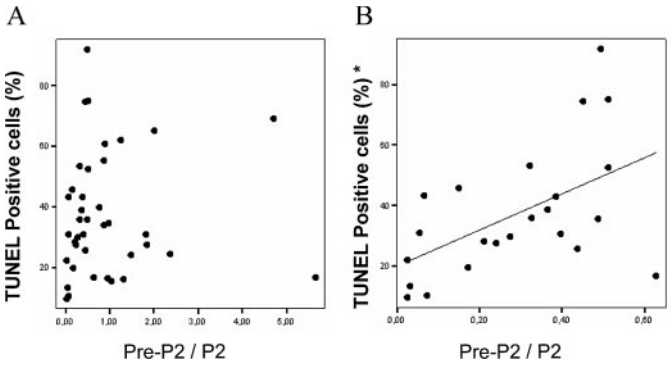

Figure 3. Scatter plot of the proportion of terminal transferase dUTP nick-end labelling (TUNEL)-positive sperm cells and the presence of protamine 2 precursor (pre-P2)/P2. (**A**) Dispersion plot of the TUNEL results versus the pre-P2/P2 ratio. The Spearman test did not detect a correlation in this overall analysis. (**B**) Dispersion plot of the TUNEL results versus the pre-P2/P2 ratio, excluding the samples with a pre-P2/P2 value over 0.7 (Spearman $r = 0.399$, $*P = 0.05$).

with values of pre-P2/P2 above 0.7, which resulted in the detection of a significant positive correlation (Figure 3; Spearman $r = 0.399$, $P = 0.05$). This correlation turned out to be even more marked and significant for samples with pre-P2/P2 under 0.4 (Spearman $r = 0.518$, $P < 0.005$; not shown).

Discussion

In this work, we measured pre-P2 in the sperm cells from infertile patients and controls and have found that a substantial amount of pre-P2 is present in normal sperm samples and that in samples from infertile men this variable correlates significantly with different sperm and seminal parameters, with the P1/P2 ratio and with other proteins present in the sperm cells. Additionally, the presence of pre-P2 also correlates with decreased DNA integrity at low pre-P2 ratio.

The evidence for an association between the presence of pre-P2 and the P1/P2 ratio had been previously presented in a limited number of samples using a P2 antibody, which was not specific to the P2 precursor (Bench *et al*., 1998; de Yebra *et al*., 1998). In this work, we have used an antibody that was generated using a peptide corresponding to the precursor sequence of the P2 molecule. Therefore, we have been able to specifically detect, for the first time, only the P2 precursor without any confounding signals arising from the mature P2 molecules. The evidence for the presence of pre-P2 in normal mature sperm had been previously reported in the rat (Stanker *et al*., 1992; Unni *et al*., 1994). Also, in humans, small amounts of proteins compatible with pre-P2 have been detected by mass spectrometry (Yoshii *et al*., 2005). Thus, these observations are consistent with the observation in the present work of a substantial amount of pre-P2 present in normal human samples detected using the specific pre-P2 antibody.

The correlation detected between pre-P2 and the P1/P2 ratio in the patient samples is consistent with the hypothesis that incomplete processing of pre-P2 may result in lower levels of mature P2 and therefore an increased P1/P2 ratio (Bench *et al*., 1998; de Yebra *et al*., 1998). However, it is also interesting to note the presence of a large dispersion in the scatter plot data. This heterogeneity may reflect the existence of different independent causes for an altered P1/P2 ratio and increased pre-P2.

The correlation between pre-P2 and the presence of other proteins could indicate that a general failure in the histone-to-protamine replacement occurs in the samples with higher pre-P2. This hypothesis would be consistent with the inverse correlation of pre-P2 levels and sperm count, normal morphology and motility.

The range of TUNEL-positive spermatozoa detected here is consistent with that described in other articles (Barroso *et al*., 2000; Sakkas *et al*., 2002). The overall analysis of TUNEL versus pre-P2 did not detect a significant correlation. However, when the analysis was limited to pre-P2/P2 values <0.7, these variables correlated significantly. This result could indicate that very low pre-P2 levels could be associated with a preserved DNA integrity but that at higher pre-P2 the DNA could be more vulnerable to attack by multiple factors, some related to pre-P2 processing and others unrelated. Several hypotheses have been proposed to explain the origin of decreased DNA integrity in the sperm cells of infertile patients, which include (1) incomplete repair during meiosis (Baarends *et al*., 2001), (2) incomplete repair of DNA breaks arising from the action of topoisomerase II during spermiogenesis (Roca and Mezquita, 1989; Marcon and Boissonneault, 2004), (3) incomplete removal of apoptotic cells (Sakkas *et al*., 2002; Weng *et al*., 2002), (4) incomplete protamination resulting in increased susceptibility of DNA (Oliva and Dixon, 1991; Sakkas *et al*., 2002; Cho *et al*., 2003; Aoki *et al*., 2005b), (5) defects in the expression of transition proteins (Adham *et al*., 2001; Meistrich *et al*., 2003; Shirley *et al*., 2004; Zhao *et al*., 2004; Suganuma *et al*., 2005), (6) damage mediated by heavy metals or toxins interacting with protamines (Bal *et al*., 1997; Quintanilla-Vega *et al*., 2000), (7) increased ageing and oxidation of the spermatozoa during passage and storage in the male tract (Aitken *et al*., 1998; Ollero *et al*., 2001; Suganuma *et al*., 2005) and (8) increased action of exogenous factors such as infection and increased oxidant action of leukocytes (Alvarez *et al*., 2002). Our data do not support a general correlation between DNA integrity and defects in protamine processing but instead, consistent with all proposed hypotheses, suggest that there are multiple, independent causes for lower DNA integrity in the different patients.

The present results demonstrate that sperm cells of infertile patients and controls contain different levels of pre-P2. The presence of pre-P2 in infertile patients correlates negatively with sperm count, with normal morphology and with motility of the cells and positively with the P1/P2 ratio, with the presence of other proteins and, at low pre-P2 levels, with decreased DNA integrity.

The opportunity is now open to determine whether differences in P2 precursors exist in subpopulations of spermatozoa separated by motility, centrifugation on density gradients, sorted by TUNEL positivity or detected by immunohistochemical methods. Also, it will be interesting to determine whether genetic variation can be detected in protamines, P2 precursor-processing enzymes, transition proteins or other regulatory proteins in the samples with altered levels of pre-P2.

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