WILEY-BLACKWELL

Asian J Androl 2008; 10 (6): 865–871
DOI: 10.1111/j.1745-7262.2008.00415.x

AJA

·*Original Article*·

Sperm nuclear histone H2B: correlation with sperm DNA denaturation and DNA stainability

Armand Zini, Xiaoyang Zhang, Maria San Gabriel

Division of Urology, Department of Surgery, Royal Victoria Hospital, McGill University, Montreal, Quebec H3T 1M5, Canada

Abstract

Aim: To examine the relationship between sperm DNA damage and sperm nuclear histone (H2B) staining. **Methods:** We evaluated sperm samples from 14 consecutive asthenoteratozoospermic infertile men and six consecutive fertile controls. Sperm nuclear histone (H2B) staining and sperm chromatin integrity (assessed by sperm chromatin structure assay and expressed using the percentage of (i) DNA fragmentation index [%DFI] and (ii) high DNA stainability [%HDS)]) were evaluated. **Results:** Histone H2B immunocytochemistry demonstrated two nuclear staining patterns: (i) focal punctate staining; and (ii) diffuse staining. Infertile men had a higher mean percentage of spermatozoa exhibiting diffuse H2B staining than did fertile men (7.7% ± 4.6% *vs.* 1.6% ± 1.2%, respectively, $P < 0.01$). We observed significant relationships between the proportion of spermatozoa with diffuse nuclear histone staining and both sperm %DFI ($r = 0.63$, $P < 0.01$) and sperm %HDS ($r = 0.63$, $P < 0.01$). **Conclusion:** The data demonstrate that infertile men have a higher proportion of spermatozoa with diffuse histone H2B than do fertile men and suggest that sperm DNA damage might, at least in part, be due to abnormally high histone H2B levels. *(Asian J Androl 2008 Nov; 10: 865–871)*

Keywords: spermatozoa; sperm DNA; histones; male infertility; DNA fragmentation

1 Introduction

Sperm chromatin is very tightly compacted as a result of the associations between the DNA and the sperm nuclear proteins [1, 2]. During the later stages of spermatogenesis (spermiogenesis), spermatid nuclear remodeling and condensation is associated with histone modifications and the sequential displacement of histones by transition proteins and then by protamines [1].

In humans, up to 15% of the sperm DNA remains packaged by histones in sequence-specific areas [3, 4]. Several histone isoforms (H2A, H2B, H3 and H4) and isoform variants are present in human spermatozoa, with the predominant isoform being histone H2B [5]. Recently, two distinct human testis/sperm-specific H2B variants (hTSH2B and H2BFWT) were cloned and characterized [6, 7]. There is evidence to show that these isoform variants might not be uniformly expressed in human spermatozoa, suggesting the presence of different sperm populations in the human ejaculate [6, 8]. Although the exact role of histone H2B variants is unknown, the accumulation of H2B variants during spermatogenesis and the

Correspondence to: Dr Armand Zini, St. Mary's Hospital, 3830 Lacombe Avenue, Montreal, Quebec H3T 1M5, Canada.
Tel: +1-514 345-3511 Fax: +1-514-734-2718
E-mail: ziniarmand@yahoo.com
Received 2007-12-01 Accepted 2008-03-28

association of H2B with telomeres suggest a potential involvement in spermiogenesis and fertilization [7, 9].

Sperm protamine deficiency (partial or complete) is demonstrated in some infertile men [10–13]. Studies suggest that sperm protamine deficiency (or histone retention) is related to sperm DNA damage [14]. As such, we sought to further examine the relationship, if any, between sperm DNA damage and sperm nuclear protein content by examination of sperm chromatin structure (DNA denaturation and DNA stainability) and sperm nuclear somatic histone H2B (more accurately designated as HIST2H2BE [15]) immunostaining in samples from fertile and infertile men. We have focused our studies on H2B as this is the predominant histone isoform in human spermatozoa [5] and we have previously observed that the ratio of H2B to protamine is increased in the spermatozoa of infertile men [13]. However, we cannot exclude the possibility that the levels of other histone species are increased (or decreased) in spermatozoa of infertile men and that these alterations might be associated with chromatin packaging defects.

2 Materials and methods

2.1 Materials

Acridine orange (AO) was purchased from PolySciences (Warrington, PA, USA). Unless specified, all the chemicals in the present study are from Fisher Chemical (Elvet Scientific, Bearpark, Canada).

2.2 Study subjects and semen handling

Semen samples were obtained from consecutive asthenoteratozoospermic men (with < 50% sperm motility, < 15% normal forms and normal sperm concentration [> 20 × 10^6 sperm/mL]) presenting for infertility evaluation (n = 14). All couples presenting for infertility evaluation had primary infertility (no prior pregnancy) and had been unable to conceive naturally for at least one year. The infertile men were selected based on the observation that abnormalities in sperm motility and morphology have been associated with an aberrant histone to protamine ratio in mice with a targeted disruption of the protamine gene [16]. Couples with significant female-factor infertility (tubal obstruction or ovarian failure) were excluded.

Semen samples (n = 6) were also obtained from consecutive fertile controls (men presenting for vasectomy who had previously fathered at least two children with one born in the past 5 years).

Samples were produced by masturbation after 3–5 days of sexual abstinence and allowed to liquefy at room temperature. After liquefaction of semen, standard semen parameters (volume, concentration, motility and morphology) were obtained according to World Health Organization (WHO) guidelines [17]. A small aliquot of semen (approximately 25–100 mL, containing approximately 2 × 10^6 spermatozoa) was frozen at –70°C for later evaluation of sperm DNA damage. All of the semen samples had motile sperm and none had significant numbers of round cells or leukocytospermia, as per World Health Organization guidelines (< 1 000 000 round cells per mL).

All patients signed an informed consent and the information for this study remained confidential and within the institution. This study was approved by the ethics review board at McGill University (Montreal, Canada).

2.3 Immunocytochemistry

Semen was washed with phosphate-buffered saline (PBS, pH = 7.4) and smears were prepared on Fisher Superfrost Plus slides (Elvet Scientific). The smears were fixed in 100% methanol for 2 h, air-dried and then stored at –70ºC. Prior to immunostaining, smears were brought to room temperature, re-hydrated with PBS for 30 min and decondensed in 5 mmol/L DTT and 0.3 μg/mL heparin for 30–60 min (to ensure full decondensation of > 90% of the spermatozoa) at room temperature (22–24ºC).

Immunostaining for H2B was performed using a human-specific rabbit anti-H2B (Upstate, Charlottsville, VA, USA). We and others have demonstrated the specificity of the H2B antibody by Western blot analysis [13, 18]. Briefly, smears were blocked with 5% goat serum in PBS for 30 min, washed with PBS containing 0.1% Triton X-100 (PBS-T), and incubated with the H2B antibody (dilution 1:300) for 1 h at 20ºC. Smears were then washed with PBS-T and incubated with Flurochrome-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). After that, smears were mounted with Prolong Antifade and observed under a Carl Zeiss Axiophot microscope (exciter filter BP450-490, emission filter BP520) at × 1 000 magnification. All immunostaining experiments were carried out on the same run and the data were recorded by two separate and blinded observers (inter-observer variability was 7.7%). At least 200 spermatozoa were assessed per slide. The

mean percentage (± SD) of sperm exhibiting (i) diffuse; and (ii) focal punctate staining was recorded. Negative controls were performed in the absence of primary antibody.

2.4 Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS)

Sperm DNA damage was assessed using the sperm chromatin structure assay and the results were expressed as the percentage of spermatozoa with DNA denaturation (also known as %DFI and %HDS), as previously described [19–21]. Stored semen samples were thawed on ice and 200 μL of TNE (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl and 1 mmol/L EDTA, pH 7.4) was added to the sample. The samples were treated for 30 s with 400 μL of a solution of 0.1% Triton X-100, 0.15 mol/L NaCl and 0.08 mol/L HCl, pH 1.2. After 30 s, 1.2 mL of staining buffer (6 μg/mL AO, 37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L disodium EDTA, 0.15 mol/L NaCl, pH 6.0) was admixed to the test tube and 3 min later the sample was analyzed by flow cytometry.

Following excitation by a 488-nm wavelength light source, AO bound to double-stranded DNA emits green florescence (515–530 nm) and AO bound to single-stranded DNA emits red florescence (≥ 630 nm). The sample is stained with AO, placed into the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the sample flowing to establish excellent sheath/sample flow, and then 3 min after AO staining measurements are taken. A minimum of 5 000 cells from two aliquots of each sample were analyzed by FACS scan interfaced with a data handler (CELLQUEST 3.1, Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA, USA). WinList (Verity Softwarehouse, Topsham, ME, USA) was used to generate the cytogram (red *vs.* green fluorescence) and histogram (total cells *vs.* DFI) plots, as well as, %DFI and %HDS readings. A mean of the two sperm %DFI and %HDS values was reported.

Fresh and frozen-thawed samples have yielded similar results (< 5% variability) [21]. We have shown that the inter-assay variability of sperm %DFI is low (< 5%) by repeat assessments of reference semen samples [21]. Over 300 aliquots of the same semen sample (reference sample) have been stored at –70ºC for ongoing assessment of inter-assay variability. A reference sample is used to set the red and green photomultiplier tube (PMT) voltage gains to give the same means for red and green florescence levels (130/1 000 and 500/1 000 channels + 5). A new reference sample is run every 6–10 samples to avoid drift. We have previously validated our assay by assessing sperm DNA fragmentation in parallel with sperm %DFI and have shown a strong association between these two measures of DNA damage [22].

2.5 Data analysis

Results were expressed as mean ± SD. Inter-group (fertile and infertile men) differences in sperm parameters were assessed by Mann-Whitney rank sum test. The relationships between parameters were examined using linear regression techniques with Pearson's correlation coefficient. All hypothesis testing was two-sided, with $P < 0.05$ deemed as significant. Statistical analysis was performed using Sigma Stat software (SPSS, Chicago, IL, USA).

3 Results

3.1 Sperm parameters, %DFI and %HDS

The mean (± SD) sperm concentration, motility, %DFI and %HDS in samples from fertile and infertile men are shown in Table 1. As expected, sperm concen-

Table 1. Mean (± SD) sperm concentration, motility, the percentage of DNA fragmentation index (%DFI), the percentage of high DNA stainability (%HDS) and the percentage of sperm nuclei with diffuse and punctate histone (H2B) staining in fertile and infertile men. [a]Comparison between fertile and infertile men by Mann-Whitney rank sum test.

	Fertile	Infertile	*P*-value[a]
n	6	14	
Sperm concentration (× 10^6/mL)	116 ± 46	64 ± 46	0.044
Sperm motility (%)	81 ± 7	37 ± 25	0.001
Sperm DFI (%)	10.8 ± 5.6	18.3 ± 7.8	0.042
Sperm HDS (%)	5.0 ± 2.7	6.5 ± 3.1	0.34
Spermatozoa with diffuse H2B staining (%)	1.6 ± 1.2	7.7 ± 4.6	0.005

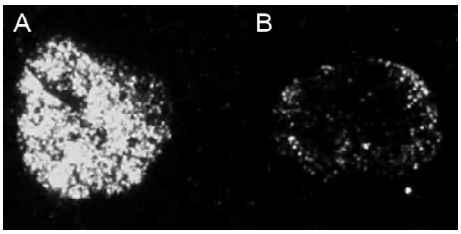

Figure 1. Photomicrographs of decondensed human spermatozoa immunoreacted with H2B antibody. (A): Spermatozoon exhibiting diffuse staining of the nucleus (patient sample). (B): Spermatozoon exhibiting sparse, punctate staining (white spots) of the nucleus (fertile control sample).

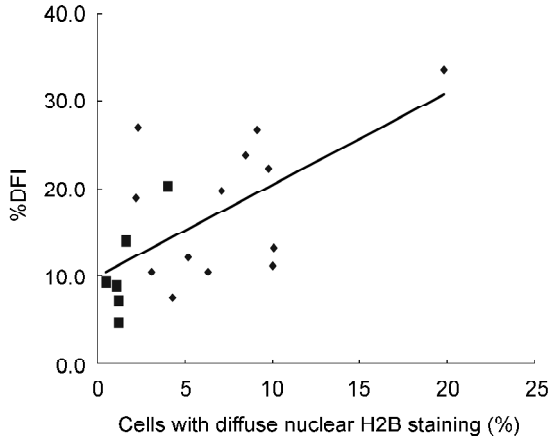

Figure 2. Correlation between the percentage of sperm nuclei with diffuse histone H2B staining and the percentage of DNA fragmentation index (%DFI) in fertile (■) and infertile men (◆).

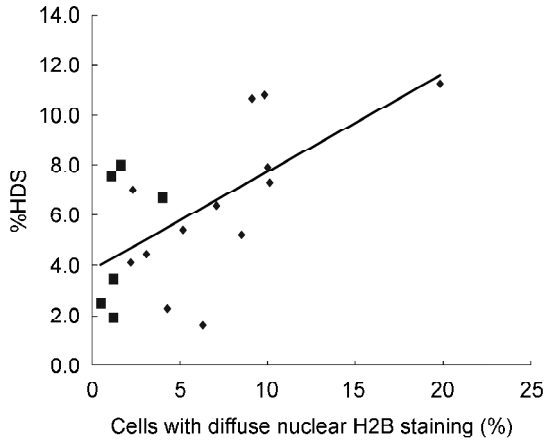

Figure 3. Correlation between the percentage of sperm nuclei with diffuse histone H2B staining and the the percentage of sperm high DNA stainablility (%HDS) in fertile (■) and infertile men (◆).

tration and motility were significantly higher in samples from fertile compared to infertile men ($P < 0.05$). Mean sperm %DFI was significantly higher in samples from infertile compared to fertile men.

3.2 Histone H2B immunostaining

Immunocytochemistry experiments using histone H2B antibody demonstrated one of two sperm nuclear staining patterns: (i) focal, punctate staining (Figure 1A) and (ii) diffuse staining (Figure 1B). Negative controls (absence of primary antibody) demonstrated no detectable sperm nuclear staining (data not shown). Infertile men had significantly higher proportions of spermatozoa exhibiting diffuse nuclear staining patterns than did fertile men (Table 1).

3.3 Relationship between histone H2B immunostaining and sperm motility, %DFI and %HDS

We observed significant inverse relationships between the proportion of spermatozoa with diffuse nuclear histone (H2B) staining and both percentage sperm motility ($r = –0.45$, $P < 0.05$) and sperm concentration ($r = –0.54$, $P < 0.05$). We also observed significant relationships between the proportion of spermatozoa with diffuse nuclear histone (H2B) staining and both sperm %DFI ($r = 0.63$, $P < 0.01$; Figure 2) and sperm %HDS ($r = 0.63$, $P < 0.01$; Figure 3).

4 Discussion

We have found that semen samples from asthenoteratozoospermic infertile men possess a higher percentage of spermatozoa with diffuse sperm nuclear histone H2B staining than sperm from fertile men and that the diffuse nuclear histone H2B staining is inversely related to both sperm motility and sperm concentration. We have previously reported that spermatozoa with diffuse nuclear histone H2B staining have a relative reduction in protamine staining, suggesting that these spermatozoa have an increased histone to protamine ratio [13]. We recognize that the immunocytochemistry data are semi-quantitative and, therefore, have specifically recorded the pattern of nuclear staining (diffuse *vs*. punctate) reflecting different sperm subpopulations, rather than the intensity of staining. Taken together, the data suggest that a diffuse nuclear H2B staining pattern is abnormal and are in keeping with reports indicating that histone reten-

tion is common in infertile men [10–13].

Our data suggest that infertile men with a high percentage of spermatozoa with diffuse nuclear histone H2B have defective spermiogenesis as this is the specific step in spermatogenesis where the final assembly of sperm proteins occurs. Moreover, the finding of two sperm subpopulations suggests that spermiogenesis is also heterogeneous within the testicle (it is during spermiogenesis that sperm nuclear compaction and the exchange of histones to protamines takes place). The focal, punctuate staining observed in the majority of spermatozoa suggests that H2B is principally located at the periphery of the sperm nucleus, as previously demonstrated [7, 9].

Although there is sequence homology between somatic H2B and TSH2B (approximately 95% sequence homology at the C-terminal region [6]) and, to a lesser extent, between somatic H2B and H2BFWT (70% sequence homology at the C-terminal region [7]) that might cause our monoclonal somatic H2B antibody (targeting the C-terminal) to cross-react with other H2B variants, there is good evidence to show that the antibody we have used is specific to somatic H2B. First, Tovich and Oko [18] have used the same antibody and have demonstrated its specificity to somatic H2B. Second, there is good evidence that H2B variants (particularly, TSH2B and somatic H2B) do not co-migrate on acid-urea (AU) gels [5, 6, 23] and we have repeatedly demonstrated a single band on the western immunoblots (from AU gels) with this antibody [13]. Finally, we have further verified the specificity of the antibody by extended separation of nuclear histones on AU gels and again have observed a single band on the western immunoblots [13].

Our results demonstrate that the proportion of spermatozoa with diffuse H2B staining (suggestive of H2B retention) is associated with sperm DNA damage. Other investigators have similarly shown that abnormal sperm nuclear protein composition is associated with sperm DNA damage. Cho *et al.* [16] and Tanaka *et al.* [24] observe that sperm from protamine-deficient mice exhibit reduced chromatin stability (the nuclei possess a lower resistance to chemical disruption when compared to that of wild-type mice), which likely explains the greater susceptibility of protamine-deficient human spermatozoa to DNA fragmentation. Aoki *et al.* [14] demonstrate an inverse relationship between total protamine concentration and DNA fragmentation. Moreover, the same investigators show that spermatozoa with diminished protamine content are more likely to possess DNA damage [25]. Increased sperm DNA damage has also been demonstrated in mice with targeted disruption of the protamine gene and in humans with a single nucleotide polymorphism in the protamine gene [16, 26]. Our data further support the concept that sperm DNA damage might in part be a result of a relative increase in the histone to protamine ratio. The data also suggest that at least some of the DNA damage that is detected in ejaculated spermatozoa originates in the testis, during spermiogenesis (where and when protamines are incorporated into the sperm nucleus).

Our data indicate that increased levels of sperm nuclear histone H2B are associated with an increased proportion of spermatozoa with high DNA stainability. The high DNA stainability is a result of increased accessibility of dyes to the sperm DNA and suggests that increased levels of nuclear H2B might lead to a "looser" or "more porous" sperm chromatin [20, 27]. Indeed, Evenson *et al.* [27] demonstrate that the stainability of histone-complexed DNA in round spermatids (stained with acridine orange after low pH extraction of H1 histones) is fivefold higher than the protamine-complexed DNA in mature spermatozoa. In a case study of a man with a febrile illness, Evenson *et al*. [20] demonstrate that the temporal changes in sperm DNA stainability were linked to changes in the histone to protamine ratio. Singleton *et al*. [28] similarly find that testis-specific H2B levels in human spermatozoa are inversely correlated to chromatin compaction, suggesting that a relative reduction in nuclear protamine levels results in poor sperm nuclear compaction. Our data further support the concept that poor sperm chromatin compaction might also in part be due to a relative increase in the histone to protamine ratio [16, 26].

Sperm DNA damage and chromatin abnormalities are clinically relevant as they have been associated with reduced fertility potential [29, 30]. Couples in whom the husband has a high percentage of spermatozoa with DNA damage have very low potential for natural fertility and a prolonged process of achieving pregnancy [19, 31, 32]. High levels of sperm DNA damage have also been associated with poor pregnancy outcomes after intra-uterine insemination and conventional *in vitro* fertilization [33, 34]. However, the impact of sperm DNA damage on reproductive outcomes after intracytoplasmic sperm injection is less clear [34–36]. Finally, couples with pregnancy resulting in miscarriage demonstrate a trend toward poorer sperm DNA integrity, as compared to that

of highly fertile couples [19, 37].

In summary, we have shown that infertile men possess a higher percentage of spermatozoa with increased levels of sperm nuclear histone H2B staining than do fertile men. We have also found that the increased levels of sperm nuclear histone H2B is associated with poor sperm motility and chromatin compaction and with increased sperm DNA damage. Taken together, the data support the concept that poor sperm chromatin compaction and DNA damage in humans might, in part, be a result of an underlying defect in the sperm histone to protamine ratio.

References

1 Balhorn R. A model for the structure of chromatin in human sperm. J Cell Biol 1982; 93: 298–305.
2 Balhorn R, Brewer L, Corzett M. DNA condensation by protamine and arginine-rich peptides: Analysis of toroid stability using single DNA molecules. Mol Reprod Dev 2000; 56: 230–4.
3 Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW. Sequence-specific packaging of DNA in human sperm chromatin. Science 1987, 236: 962–4.
4 Wykes SM, Krawetz SA. The structural organization of sperm chromatin. J Biol Chem 2003; 278: 29471–7.
5 Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM. Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem 1990; 265: 20662–6.
6 Zalensky AO, Siino JS, Gineitis AA, Zalenskaya IA, Tomilin NV, Yau P, *et al.* Human testis/sperm-specific histone H2B (hTSH2B). Molecular cloning and characterization. J Biol Chem 2002; 277: 43474–80.
7 Churikov D, Siino J, Svetlova M, Zhang K, Gineitis A, Morton Bradbury E, *et al*. Novel human testis-specific histone H2B encoded by the interrupted gene on the X chromosome. Genomics 2004; 84: 745–56.
8 Singleton S, Mudrak O, Morshedi M, Oehninger S, Zalenskaya I, Zalensky A. Characterisation of a human sperm cell subpopulation marked by the presence of the TSH2B histone. Reprod Fertil Dev 2007; 19: 392–7.
9 Gineitis AA, Zalenskaya IA, Yau PM, Bradbury EM, Zalensky AO. Human sperm telomere-binding complex involves histone H2B and secures telomere membrane attachment. J Cell Biol 2000; 151: 1591–8.
10 Bach O, Glander HJ, Scholz G, Schwarz J. Electrophoretic patterns of spermatozoal nucleoproteins (NP) in fertile men and infertility patients and comparison with NP of somatic cells. Andrologia 1990; 22: 217–24.
11 Carrell DT, Liu L. 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 2001; 22: 604–10.
12 de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine P2 in humans. J Biol Chem 1993; 268: 10553–7.
13 Zhang X, San Gabriel M, A Zini A. Sperm nuclear protamine to histone ratio in fertile and infertile men: evidence of heterogeneous sub-populations of spermatozoa in the ejaculate. J Androl 2006; 27: 414–20.
14 Aoki VW, Moskovtsev SI, Willis J, Liu L, Mullen JB, Carrell DT. DNA integrity is compromised in protamine-deficient human sperm. J Androl 2005; 26: 741–8.
15 Marzluff WF, Gongidi P, Woods KR, Jin J, Maltais LJ. The human and mouse replication-dependent histone genes. Genomics 2002; 80: 487–98.
16 Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, *et al.* Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 2001; 28: 82–6.
17 World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 4th edn. Cambridge: Cambridge University Press, 1999.
18 Tovich PR, Oko RJ. Somatic histones are components of the perinuclear theca in bovine spermatozoa. J Biol Chem 2003; 278: 32431–8.
19 Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, *et al*. Utility of the sperm chromatin assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999; 14: 1039–49.
20 Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. J Androl 2000; 21: 739–46.
21 Zini A, Meriano J, Kader K, Jarvi K, Laskin C, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Hum Reprod 2005; 20: 3476–80.
22 Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril 2001; 75: 674–7.
23 Prigent Y, Muller S, Dadoune JP. Immunoelectron microscopical distribution of histones H2B and H3 and protamines during human spermiogenesis. Mol Hum Reprod 1996; 2: 929–35.
24 Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, *et al*. HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. Mol Cell Biol 2005; 25: 7107–19.
25 Aoki VW, Emery BR, Liu L, Carrell DT. Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. J Androl 2006; 27: 890–8.
26 Iguchi N, Yang S, Lamb DJ, Hecht NB. An SNP in protamine 1: a possible genetic cause of male infertility? J Med Genet 2006; 43: 382–4.
27 Evenson DP, Darzynkiewicz Z, Jost L, Janca F, Ballachey B. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry 1986; 7: 45–53.
28 Singleton S, Zalensky A, Doncel GF, Morshedi M, Zalenskaya IA. Testis/sperm-specific histone 2B in the sperm of donors and subfertile patients: variability and relation to chromatin

packaging. Hum Reprod 2007; 22: 743–50.
29 Erenpreiss J, Spano M, Erenpreisa J, Bungum M, Giwercman A. Sperm chromatin structure and male fertility: biological and clinical aspects. Asian J Androl 2006; 8: 11–29.
30 Emery BR, Carrell DT. The effect of epigenetic sperm abnormalities on early embryogenesis. Asian J Androl 2006; 8: 131–42.
31 Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G, *et al*. Sperm chromatin damage impairs human fertility. Fertil Steril 2000, 73: 43–50.
32 Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gyllemborg J, Ernst E, *et al*. Oxidative DNA damage in human sperm influences time to pregnancy. Hum Reprod 2003; 18: 1265–72.
33 Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod 2002; 17: 3122–8.
34 Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, *et al*. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 2007; 22: 174–9.
35 Huang CC, Lin DP, Tsao HM, Cheng TC, Liu CH, Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. Fertil Steril 2005; 84: 130–40.
36 Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B, Francois Guerin J. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertil Steril 2007; 87: 93–100.
37 Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, *et al*. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 2006; 21: 2876–81.

Edited by Dr Ralf Henkel