Testis/sperm-specific histone 2B in the sperm of donors and subfertile patients: variability and relation to chromatin packaging

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BACKGROUND: The compaction of human sperm chromatin is the result of replacement of ~85% of histones with protamines. Germ-line testis/sperm-specific histone 2B (TSH2B) has been detected in only ~30% of mature spermatozoa. Its level in the semen of subfertile patients varies; its function is unknown. We evaluated TSH2B in the sperm samples of 23 donors and 49 subfertile patients and assessed its association with chromatin compaction status.

METHODS: TSH2B level was measured using immunoblotting. Chromatin packaging quality was evaluated by staining with chromomycin A3 (CMA3) which marked spermatozoa with defective packaging. To assess both TSH2B and chromatin status in the same spermatozoon, CMA3 staining and TSH2B immunolocalization were performed sequentially. RESULTS: A significant correlation ($r = 0.55$, $P = 0.0027$) was found between TSH2B level and percentage of CMA3-positive sperm in patient and donor semen samples. When individual spermatozoa were assessed for these parameters, 92% of TSH2B-containing cells were also CMA3 positive. Variation in the total sperm TSH2B level was less in donors than in patients. CONCLUSIONS: CMA3 positive staining of TSH2B-containing individual spermatozoa and a significant correlation between the total TSH2B level and CMA3 percentage in semen samples suggest a structural role for TSH2B in sperm chromatin organization. Low variability of TSH2B level in donors implies a mechanism (however unknown) regulating this parameter.

Key words: chromatin/chromomycin A3/histone TSH2B/male infertility/sperm

Introduction

In the process of spermatogenesis, a germ cell passing through spermatogonial, spermatocyte and spermatid stages finally differentiates into a spermatozoon. Dramatic transformations of the nucleus and chromatin occur during these transitions. Testis-specific variants of histones, followed by transitional proteins and finally protamines replace somatic histones, resulting in the highly condensed state of sperm chromatin (Meistrich, 1989). Importantly, in human sperm, unlike the sperm of other mammals studied, a portion (10-15%) of residual core histones remains associated with DNA (Tanphaichitr et al., 1978; Gusse et al., 1986). The function of these histones in human sperm remains unknown. Suggestions have been made that the retained histones may mark genes for early embryo gene expression (Gatewood et al., 1987; Gardiner-Garden et al., 1998) and/or that they may bind the chromosomal domains required for the initial stages of male pronucleus formation (Zalenskaya et al., 2000).

One of the histones retained in mature human sperm is a homologue of TH2B, a testis-specific variant of histone H2B that is present in rodent male germ line cells (Tanphaichitr et al., 1978; Wattanaseree and Svasti, 1983; van Roijen et al., 1998; Zalensky et al., 2002). Recently, this protein, referred to as testis/sperm-specific histone 2B (TSH2B) has been cloned and characterized (Zalensky et al., 2002; Li et al., 2005).

The main difference of TSH2B from the somatic H2B lies in its N-terminal end, which among other amino acid substitutions contains three new and two repositioned potential phosphorylation sites. It has been proposed that diverse post-translational modifications of histone tails can be recognized by other proteins or protein complexes participating in chromatin remodelling (Strahl and Allis, 2000) and that they may provide a basis for epigenetic marking (Kimmins and Sassone-Corsi, 2005). Along this line are the results of a nucleosome reconstitution study, in which it was discovered that a nucleosomal core containing TSH2B is less stable than the core with a somatic H2B (Li et al., 2005).

Conservation of TSH2B primary structure among mammalian species and occurrence only in male germ line cells suggest a unique, although unknown, function for this histone. Significance of the TSH2B presence within human mature...
sperm cells has not yet been determined. It could be suggested that chromosomal packaging is different in the sperm containing different quantities of TSH2B and that this difference in chromosomal packaging may have an impact on fertilization and early development of the embryo. Interestingly, TSH2B has been immunolocalized in only a fraction (20–30%) of mature sperm cells (van Roijen et al., 1998; Zalensky et al., 2002). In addition, a significant variation in TSH2B levels was found among infertility patients (van Roijen et al., 1998).

However, no data exist on the extent of TSH2B variability in healthy donors. Therefore, it was the purpose of this study to determine variability in TSH2B content among healthy donors as compared with that of subfertile patients, to identify semen samples with altered quantities of TSH2B and to determine whether the TSH2B level in semen, as well as its presence in individual sperm cells, can be related to chromatin packaging. In addition, for all samples studied, the sperm characteristics traditionally employed to evaluate fertility potential have been obtained and compared to TSH2B level.

Materials and methods

Materials

The primary antibody used to detect TSH2B was mouse anti-tyrosine hydroxylase (TyH) antibody (Chemicon International, Temecula, CA, USA) that has been proven to react specifically with histone TSH2B (Unni et al., 1995; van Roijen et al., 1998). The primary antibody used to detect centromeric protein A (CENP-A) was human CREST antibody (The Binding Site, Birmingham, UK). All secondary antibodies were purchased from Zymed, San Francisco, CA, USA. Secondary antibodies used in immunoblotting were horseradish peroxidase (HRP) conjugated goat anti-mouse IgG and goat anti-human IgG. The secondary antibodies used in immunofluorescence were goat anti-mouse IgG and goat anti-human IgG conjugated with fluorescein isothiocyanate (FITC) or Texas Red. The polyvinylidene difluoride (PVDF) transfer membrane was Immobilon-P (Millipore Corporation, Bedford, MA, USA). Enhanced chemiluminescence (ECL) immunodetection was performed using Western Lightning Chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA). Secondary antibodies used in immunoblotting were horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin (IgG) and goat anti-human IgG. The secondary antibodies used in immunofluorescence were goat anti-mouse IgG and goat anti-human IgG conjugated with fluorescein isothiocyanate (FTTC) or Texas Red. The polyvinylidene difluoride (PVDF) transfer membrane was Immobilon-P (Millipore Corporation, Bedford, MA, USA). Enhanced chemiluminescence (ECL) immunodetection was performed using Western Lightning Chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA, USA); chromomycin A3 (CMA3) fluorochrome was purchased from Sigma Chemicals (St Louis, MO, USA). Slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA).

Semen processing

Semen samples were collected from 49 unselected male partners of couples consulting for infertility at the Jones Institute for Reproductive Medicine (subfertile patients) and from 23 normozoospermic healthy donors (not of proven fertility in all cases) after at least 2 days of abstinence. Written informed consent was obtained from all participants. All samples were collected by masturbation and allowed to liquefy for 30 min at room temperature. After liquefaction, the samples were analysed for concentration and motility according to recommendations of World Health Organization (World Health Organization, 1999) using a computer-assisted semen analyser (HTM-IVOS; Hamilton Thorne Research, Beverly, MA, USA). The samples were kept frozen at –80°C until processed for analyses.

Electrophoresis, immunoblotting and quantification of TSH2B

Frozen semen samples were thawed, washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonylflouride (PMSF) by centrifugation at 4°C (800 × g for 10 min). An aliquot of the sperm suspension was diluted 100 times with 1 N NaOH, and the DNA concentration was determined by measuring the optical density at 260 nm (assuming that 1 mg/ml of denatured DNA corresponds to 30 optical units). The samples were made at a DNA concentration of 2 μg/μl, which is equal to ~0.6 × 10^6 cells/μl. An equal volume of 2× Laemmli sample buffer containing 2.5% β-mercaptoethanol was added, and samples were boiled for 5 min. A volume of 5–7.5 μl of the sample (1.5–2.0 × 10^6 cells) was applied on each gel, and the quantity of TSH2B within a sample was expressed as a ratio to this standard. CENP-A, a histone known to be retained consistently in the nucleus of mature human sperm (Haaf et al., 1990; Palmer et al., 1990; Zalensky et al., 1993; Figure 1A), was used as an internal control of the sample load. Linear dose response for the measurements of the band intensities was verified by applying increasing quantities of the standard sample on a gel (Figure 1B and C).

CMA3 staining

CMA3 staining was performed essentially as described (Bianchi et al., 1996). Approximately 15 × 10^6 PBS washed sperm cells were applied into the well of a multi-well glass slide and allowed to air dry at room temperature. The cells were then fixed in methanol at –20°C for 20 min and allowed to air dry again. Twenty microlitres of CMA3 solution (0.25 mg/ml CMA3 in McIlvane buffer, pH 7.0 containing 10 mM MgCl₂) was applied to each well, and the slide was placed in a dark chamber for 20 min at room temperature. The slide was then rinsed in McIlvane buffer and mounted with Vectashield mounting media. The cells were examined using a fluorescent microscope with oil immersion 60× 1.4 NA objective. Images were collected using a MagnaFire digital color camera and MicroFire software (Optronics Inc.). For each sample, at least 500 cells were counted: cells positive for CMA3 showed bright yellow–green fluorescence (presumably defective chromatin packaging), while those negative for CMA3 showed dull yellow staining (normal chromatin packaging).

Immunofluorescence

Immunofluorescent localization of TSH2B and CENP-A in sperm cells was performed as described earlier (Zalensky et al., 1993; Zalensky et al., 2002). Briefly, sperm cells were decondensed using heparin/dithiothreitol (DTT), loaded onto microscope slides and blocked in 3% bovine serum albumin (BSA), 4× standard saline citrate (SSC), 0.1% Tween-20 for 30 min at room temperature. After blocking, the slides were incubated at 37°C for 90 min with a mixture of anti-TyH and CREST antibodies (each at 1:100 dilution) and then washed in PBS. A mixture of anti-mouse antibodies labelled with

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Texas Red and anti-human antibodies labelled with FITC (each at 1:200 dilution) was applied, and the slide was incubated for 45 min at 37°C. The slide was then dehydrated with a series of 70, 80, 90 and 100% ethanol, air-dried and mounted with Vectashield mounting media.

Localization of TSH2B after CMA3 staining was performed as follows. Sperm cells were deposited on a multi-well slide complemented with a grid to ascertain cell positioning on the slide. CMA3 staining was performed and registered as described above. After removing the coverslip, the slide was washed in 2× SSC for 15 min. The sperm cells were decondensed by incubation in 100 mM DTT/40 μg/ml heparin in PBS for 30 min at 37°C. The slide was then rinsed in PBS, and TSH2B localization was performed as described above with the exception that the secondary anti-mouse antibody was labelled with FITC. After washing in PBS, the DNA was counterstained with 1 μg/ml DAPI. The position of sperm cells previously photographed for CMA3 staining was re-established using the slide grid, and localization of TSH2B was registered.

**Statistics**

Statistical analysis (Student’s t-test, Spearman’s rank correlation, Box and whisker plot) was conducted using the GraphPad Prism software (San Diego, CA).

**Results**

**Semen characteristics**

In comparing semen samples from 23 donors and 49 subfertile patients with regard to the parameters traditionally used for the prediction of fertility potential, there was a significant difference in the mean concentration of sperm between the two groups. The donor samples had a mean (±SD) concentration of 304.9 ± 158.4 × 10⁶ compared to a concentration of 51.6 ± 40.3 × 10⁶ for the subfertile patients (P < 0.0001). No significant difference was noted between the donor samples and subfertile patient samples with regard to percentage of motile sperm (61.5 ± 14.0% and 57.5 ± 17.0%, respectively).

**TSH2B level and distribution in sperm.**

To localize TSH2B and CENP-A, spermatozoa were decondensed as described in Materials and methods and immunostained using CENP-A and TSH2B-specific antibodies simultaneously. Figure 1A demonstrates that TSH2B is present only in a portion of the population of spermatozoa and is evenly distributed within the sperm head (red staining).
The high specificity of the antibodies used in this work has been proven earlier (Zalensky et al., 1993; Unni et al., 1995; van Roijen et al., 1998; Zalensky et al., 2002). In addition, preadsorption of TSH2B-specific antibody with a recombinant TSH2B completely eliminated the signal (data not shown). Unlike TSH2B, CENP-A is present in all sperm cells and appears as green or yellow (when CENP-A and TSH2B signals overlap) dots or patches. Earlier, it has been shown that CENP-A co-localizes with centromeric DNA (Zalensky et al., 1993).

The quantity of the histone TSH2B (related to a standard sample) in sperm samples from 23 donors and 49 infertility patients was determined using immunoblotting. Figure 1D presents an example of consecutive immunodetection of TSH2B and CENP-A on the same blot.

Quantification of the TSH2B levels within samples revealed that the mean (±SD) TSH2B level within the sperm samples of donors did not differ significantly from that of subfertile patients (1.0 ± 0.4 versus 0.9 ± 0.6, respectively). We did find, however, that the variation in TSH2B quantity among subfertile patients was significantly greater than among donors.

The TSH2B levels within the samples of donors ranged from 0.53 to 1.83, whereas those of subfertile patients ranged from 0.06 to 2.27 (Figure 2A and B).

No correlation was found between TSH2B level and parameters of the concentration (Spearman $r = -0.149$, $P = 0.9$) and motility (Spearman $r = -0.179$, $P = 0.3$) within the semen samples of donors and patients.

**Chromatin packaging as evaluated by CMA3 staining.**

The chromatin packaging quality of sperm from 6 donors and 23 subfertile patients was assessed using CMA3 staining. Figure 3A illustrates examples of semen with a high and low percentage of CMA3 positively staining cells. The mean percentage of CMA3-positive sperm cells within donor samples in our study was significantly less than that of subfertile patients (25.4 ± 8.0 and 42.5 ± 17.4, respectively ($P < 0.014$), with the latter, showing much greater variability. A significant positive significant correlation (Spearman $r = 0.55$, $P = 0.0027$) was found between the TSH2B level and the CMA3 percentage in a sperm sample (Figure 3B).

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**Figure 2.** Variability in the levels of testis/sperm-specific histone 2B (TSH2B) within samples as determined from immunoblots. (A) scatterplots of TSH2B levels (arbitrary units) in sperm of patients (closed circles) and donors (open circles) (B) Box and whisker plot of TSH2B level distribution among subfertile patients and donors. Whiskers show the range of the data. Median value (horizontal line) is 0.9 for patients and 1.0 for donors.
Individual spermatozoa assessment for chromatin packaging and TSH2B presence.

Each technique, CMA3 staining (Figure 3A) and TSH2B localization by immunofluorescence (Figure 1A), selectively illuminates only a fraction of sperm cells within a given sample based upon their chromatin packaging or presence of the histone. These techniques cannot be applied simultaneously, as TSH2B immunolocalization requires that the sperm be decondensed, which is inappropriate for CMA3 assessment of sperm chromatin packaging quality. To determine whether any consistency exists in the combination of these two parameters at the level of the individual spermatozoa, we performed sequential CMA3 staining and TSH2B immunolocalization. Seven samples from subfertile patients were analysed. The mean percentage of CMA3-positive cells within these samples was 59%. Figure 4 shows an example of this analysis. Following CMA3 staining and registration, the cells were decondensed with heparin/DTT, which was necessary to make hidden epitopes accessible to antibodies. The treatment resulted in nuclear swelling and sometimes in minor position change of the cells on the slide. After application of the TSH2B-recognizing antibodies, the same fields initially analysed with CMA3 were recaptured and assessed for TSH2B presence. CMA3 treatment did not change a proportion of the TSH2B-positive cells. Simultaneously, the sperm were stained with DAPI to ensure that all cells present were analysed and counted. We were able
to clearly identify 177 cells in the randomly chosen recaptured fields. Of those 177 cells, 106 cells (60%) were CMA3 positive and 60 cells (34%) were positive for TSH2B. Of the 60 TSH2B-containing spermatozoa, 55 cells were also CMA3 positive. Therefore, 92% of the spermatozoa that contained TSH2B were characterized by abnormal chromatin packaging as determined by CMA3 staining. At the same time, only 52% of CMA3-positive sperm contained TSH2B (Figure 4B).

**Discussion**

In mature human sperm, the highly compact state of chromatin is developed as a result of replacement of the highly compact state of chromatin with the sperm chromatin by protamines. This state is further strengthened by the formation of covalent (S-S) and non-covalent (SH...Zn...SH) bonds between the cysteine residues of the protamines (Balhorn, 1982; Ward and Coffey, 1991). The condensed state of sperm DNA is thought to be important for protection and transport of the paternal genome to the oocyte.

Many studies have demonstrated that alterations in sperm chromosomal proteins (histones and protamines) might be connected with male infertility. Proteomics approach has revealed several highly conserved (from Caenorhabditis elegans to mammals) spermatogenic and sperm chromatin-associated proteins that might be important for fertility (Chu et al., 2006). Sperm from infertility patients often exhibit anomalies related to the protamine content and composition (Balhorn et al., 1988; Foresta et al., 1992; Belokopytova et al., 1993; de Yebra et al., 1993; Carrell and Liu, 2001; Nasr-Esfahani et al., 2004; Aoki et al., 2005; Oliva, 2006; Torregrosa et al., in press). In addition, an increased ratio between histones and protamines has been reported to exist in the sperm of infertile men (Silvestroni et al., 1976; Terquem and Dadoune, 1983; Juelin et al., 1986; Chevaillier et al., 1987; Auger et al., 1990; Bach et al., 1990; Blanchard et al., 1990; Zhang et al., 2006). Overall, many of these changes in the basic nuclear protein complement, which presumably make the sperm chromatin more vulnerable, have been shown to correlate with the various nuclear and chromatin disturbances that are manifest in the sperm of many infertile men (Agarwal and Said, 2003).

On the other hand, the importance of the sperm genome organization supported by nuclear proteins could lie in its contribution to the proper decondensation required for successful fertilization. Factors responsible for sperm decondensation and the development of the male pronucleus are only partially understood (Perreault 1992; Cagler et al., 2005).

Previous studies have shown that the TSH2B histone quantity within sperm samples varies significantly among subfertile patients (van Roijen et al., 1998). In our study, we analysed, using immunoblotting, the sperm TSH2B in populations of both donors and subfertile patients to determine whether donors would show the same variability in their TSH2B content. We found no significant difference in the mean sperm TSH2B levels between these two groups. We did demonstrate significant inter-individual variability in the TSH2B levels among the samples of subfertile patients, thus confirming the earlier findings of van Roijen et al. (1998). We found that the TSH2B levels in the samples of donors revealed much lower variability, implying that the TSH2B level in semen sperm is subjected to certain, however unknown, regulating mechanisms.

No correlation was found between TSH2B content and conventional semen characteristics (sperm concentration and motility), which not surprisingly indicates an independence of these parameters. Similar results were reported by van Roijen et al. (1998).

On the other hand, variations in nuclear protein composition could be expected to have an impact on chromatin compactness. Therefore, we analysed the relationship between TSH2B level and chromatin packaging.

In many studies, the packaging quality of sperm chromatin in connection with fertility status has been assessed by staining the sperm with CMA3 fluorochrome. CMA3 binds to DNA strand breaks, decreased sperm penetration, the absence of sperm decondensation within the oocyte and IVF and ICSI failure (Bianchi et al., 1993; Gorczyca et al., 1993; Manicardi et al., 1995; Sailer et al., 1995; Esterhuizen et al., 2000; Nasr-Esfahani et al., 2001; Esterhuizen et al., 2002; Razavi et al., 2003).

In this study, we found that CMA3 positive staining was significantly less in the donors (25 ± 8%) than in the patients (42 ± 17%), which was in concordance with the corresponding values obtained in the studies cited above. Upon comparison of the CMA3 percentage with the TSH2B content in the same sample, a significant positive correlation was found between these parameters suggesting involvement of the histone TSH2B in the sperm chromatin compaction. This suggestion was corroborated by results of analysis of individual spermatozoa.

We performed consecutive CMA3 staining and TSH2B detection on the same slide, enabling us to evaluate a given cell with both techniques. We found that of 177 cells analyzed, 106 cells (60%) were CMA3 positive and 60 cells (34%) were TSH2B positive. Significantly, of these 60 TSH2B-positive cells, 55 cells were also CMA3 positive. Therefore, in 92% of the spermatozoa that contained TSH2B, DNA was accessible for the binding of fluorochrome CMA3, thus implying more loosely packaged chromatin. At the same time, TSH2B was immunodetected in only 52% of CMA3-positive cells, suggesting that chromatin packaging is likely to be affected by multiple and not necessarily related factors.

Earlier, it has been proposed that increased binding of CMA3 to DNA may be a result of protamine deficiency in abnormal spermatozoa (Bianchi et al., 1993) or other nuclear protein alterations (Nasr-Esfahani et al., 2004). It has also been suggested that chromatin packaging may be affected by the thiol status of the sperm nuclear proteins (Rufas et al., 1991; Kosower et al., 1992). The contribution of factors other than TSH2B to the enhancement of sperm DNA accessibility to the fluorochrome CMA3 can account for CMA3 positive staining of the TSH2B-negative cells.
Variations in total TSH2B level could be due to a variable content of TSH2B in individual sperm cells or due to variability in the number of TSH2B-containing cells. Correlation found between TSH2B level and a number of CMA3-positive cells indirectly suggests that the TSH2B level could also correlate with a number of TSH2B-containing spermatozoa. However, our preliminary data show that this is not always the case indicating a more complicated scenario. To understand the relationship between these two characteristics, the study is now in progress in which immunofluorescence determination of the percentage of TSH2B-containing sperm in the individual sample is performed in parallel with immunoblot analysis of its TSH2B level.

The higher variability in TSH2B content in the sperm of subfertile patients as compared to that in healthy donors suggests that TSH2B may be involved in the modulation of fertilization potential of spermatozoa, for example, by influencing sperm decondensation and the development of the male pronucleus. Noteworthy, in cell-free extracts of amphibian oocytes, TSH2B-positive sperm cells have been shown to decondense more rapidly and to a greater extent than cells without this histone (Singleton et al., submitted for publication). We suggest, therefore, that activation of the paternal genome in the early stages of male pronuclei formation may be different for sperm with and without TSH2B. As with most sperm nuclear factors, there is still much study needed to determine the true influence of TSH2B on human sperm chromatin status and its effects on the fertilization capacity and early development of the embryo.

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