Incomplete nuclear transformation of human spermatozoa in oligo-astheno-teratospermia: characterization by indirect immunofluorescence of chromatin and thiol status

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BACKGROUND: Sperm heterogeneity in the human, as observed in oligo-astheno-teratozoospermia (OAT), is associated with hypospermatogenesis. METHODS: The chromatin of sperm from OAT and normospermic males was characterized with antibodies specific for nucleosomes, the histone H3.1/H3.2 isoform, histone TH2B, apoptosis-associated H4 acetylation (KM-2) and protamines. Subsequently, sperm samples were stained with the thiol-specific fluorochrome monobromobimane (mBBr) before and after reduction with dithiotreitol (DTT) as most thiol groups reside in the cysteine-rich protamines. We also used fluorescence-activated cell sorter (FACS) for sperm histograms and sorting for high or low free and total thiol levels. These fractions were further analysed for DNA damage with the TdT-UTP nick end-labelling (TUNEL) assay. RESULTS: OAT sperm nuclei stained higher for nucleosomes and KM2-epitopes, and lower for TH2B. For free, and total, thiol groups, OAT sperm were characterized by biphasic distributions, reflecting incomplete thiol oxidation as well as overoxidation, and possibly reduced protamine contents. The TUNEL assay on sperm subfractions, for both control and OAT sperm, revealed that a lower level of free thiol groups is associated with a higher TUNEL incidence, and this relationship was also found for total thiol levels. Hence, both overoxidation and a low total number of thiol groups predestine for sperm apoptosis. CONCLUSIONS: Chromatin characteristics reflecting an incomplete nucleosome to protamine remodelling were found in subfertile males. Sperm apoptosis is related to both incomplete remodelling and protamine overoxidation.

Keywords: spermiogenesis; protamines; histones; chromatin condensation; OAT

Introduction

Mammalian sperm heterogeneity can be defined at many levels such as overall morphology, motility, nuclear differentiation, capacitation and receptor status. Compared with that of farm and experimental animals, human spermatozoa are more variable in overall morphology (Bedford et al., 1973). Sperm concentration in the ejaculate is inversely related to the incidence of abnormal morphology and the variation in motility (Zollner et al., 1996). Oligo-astheno-teratospermia (OAT) largely occurs due to abnormal spermatogenesis (hypospermatogenesis or a combination of this condition with incomplete maturation arrest) (Johnsen, 1970; Levin, 1979; Carrell et al., 2007). The biological significance of the increased sperm heterogeneity observed in OAT has received more attention in recent years due to the use of these sperm samples in classical IVF and ICSI. For the latter application, visual selection of motile and 'normal' looking sperm is the only parameter used worldwide (Ramos and Wetzels, 2001; De Vos et al., 2003; Ramos et al., 2004).

Nuclear elongation halfway during spermiogenesis is accompanied by the transition of chromatin from a nucleosome-based structure to a protamine-based structure (for a review, see Dadoune, 2003), largely reducing the nuclear volume and increasing chromatin compaction. In human and mouse, two types of protamines (PRM1 and PRM2) contribute to the compaction of chromatin. For this purpose, histones are first exchanged for transition proteins 1 and 2 (TNP1 and TNP2), which are later replaced by PRM 1.
and PRM2. During this process, DNA double strand breaks occur (McPherson and Longo, 1993; Laberge and Boissonneault, 2005) and are subsequently repaired. When sperm is passing from the testis to the epididymis, a further stabilization of nuclear structure is achieved by thiol-oxidation of the cysteine-rich protamines. In mouse and man cauda epididymis, ~95% of SH groups are converted into -S-S- bridges (Sawaros and Panyim, 1979; Pellicciani et al., 1983; Seligman et al., 1994). Protamine thiol oxidation has been linked to the stability of the DNA, yielding a shift from red to green acridine orange (AO) fluorescence in rodents and man after acetic acid/ alcohol fixation (Kosower et al., 1992). The replacement of histones by protamines is less complete in the human (85%) (Gatewood et al., 1987) compared with other mammals (Bench et al., 1996). de Yebra and Oliva (1993) were the first to notice with biochemical methods that over a range of infertile patients higher PRM1/PRM2 ratios correlated with higher histone levels. By fluorescence-activated cell sortor (FACS) measurements of total sperm cell thiol levels (after DTT reduction) (Rufas et al., 1991), an indication of the reduced presence of the cysteine-rich PRM1 and PRM 2 proteins in oligospermic patients had already been obtained.

A number of subsequent observations reinforce the conclusion of a nuclear differentiation defect during spermiogenesis in OAT males.

First, CMA3 fluorescence as an indicator for underprotamination (Bianchi et al., 1993) or reduced protamine thiol cross-linking is much more frequently observed in sperm samples from OAT male patients (Iranpour et al., 2000). Furthermore, sperm of infertile men are much more sensitive to DNase I, linking is much more frequently observed in sperm samples from normal and OAT donor. As a follow up study, we characterized SH profiles of human sperm samples by FACS and selected subfractions to measure sensitivity for the TdT-UTP nick end-labelling (TUNEL) reaction and to study chromatin compaction by the use of a double-strand DNA (dsDNA) antibody on whole nuclei.

Evidence for an apoptotic chromatin imprint has been obtained. By the use of an anti-dsDNA antibody, the conclusion is also reached that chromatin compaction is compromised in OAT sperm. For all parameters studied, the increased nuclear heterogeneity in OAT sperm can be related to variability of chromatin remodeling in elongating spermatids.

Our results complement insights generated by other investigators and add to the conclusion that OAT sperm samples quantitatively differ from normospermic samples by an incomplete nucleosome to protamine transition, the basis of which likely is laid during the second half of pachytene meiosis.

**Materials and Methods**

**Sperm samples**

Sperm samples were obtained from 10 normospermic and 13 infertile donors (Table I). Classification was based on World Health Organization (WHO, 1999) criteria. Accordingly, all infertile patients were classified as OAT. Morphology was assessed by the strict criteria (Menkveld et al., 2001). Table I lists the spermogram data. For the histone characterizations (IF experiments) and FACS thiol fluorescence sorting experiments, pools of four normospermic donor samples and four or five oligospermic donor samples were assessed (see Table I for composition of pools). For the determination of dsDNA immunofluorescence in thiol sorted samples, the sperm of one normospermic donor and a pool of two OAT males were used.

All sperm samples were cryopreserved using a dilution 1:1 with cryoprotectant freezing medium (TYB; Irvine Scientific, Santa Ana, CA, USA) in liquid nitrogen vapour. The sperm concentration varied between 5–20 × 10^6/straw in order to allow pilot experiments to be executed on the same samples. Pooled samples were made after thawing aliquots of individual donors and mixed for equivalent numbers of cells per donor. Cryopreserved sperm were thawed at room temperature and washed once with human tubal fluid medium (HTF; Cambrex, Verviers, Belgium) supplemented with 10% human plasma proteins (GPO; CLB, Amsterdam, The Netherlands) in order to eliminate the cryopreservation medium by spinning for 5 min at 500g. The pellet was resuspended in 200 μl phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA), pH 7.4.

**Induction of nuclear expansion for IF**

The sperm suspension (pools normospermic and OAT) was diluted 1:4 in MilliQ-water. Drops of 5 μl were placed on a glass slide and air dried. An amount of 100 μl of freshly prepared decondensing mix [25 mM diithiothreitol (DTT); Roche Biochemicals, Mannheim, Germany]: 0.2% Triton-X-100 (Sigma); 200 IU heparin/ml (Leo Laboratories, The Netherlands) in PBS] was placed over the dried sperm cells that were incubated in a humidified atmosphere for 12–18 min. The speed and degree of nuclear decondensation was followed by phase-contrast microscopy. When the majority of nuclei appeared dull grey with roughly twice the surface area of the undecondensed sperm heads, the slide was placed in a coplin jar with 4% paraformaldehyde (PFA in PBS, Sigma) at pH 7 for 15 min. Subsequently, slides were washed thrice for 5 min in PBS and allowed to dry. A 2 μl cell suspension of mouse spermatogenic cells was placed on one end of the slide for IF control.
Antibodies

Monoclonal antibody PL2-3 recognizes H2A.H2B DNA in a nucleosomal context (dilution 1:2000) (Losman et al., 1992; Dieker et al., 2005); monoclonal antibody KM-2 (1:3000) (Dieker et al., 2007) recognizes H4acK8, 12 and 16 with a preference for apoptotic nuclei and apoptotic bodies; monoclonal antibody #34 (1:1500) recognizes the replication-dependent histone 3 isoform H3.1/H3.2 (van der Heijden et al., 2005; Henikoff and Ahmad, 2005); monoclonal antibody #36 (1:200) (Smeenk et al., 1988) recognizes dsDNA. The tyrosine hydroxylase antibody (1:100) (van Roijen et al., 1998) recognizes hTSH2B (Zalensky et al., 2002). Hup1N and Hup2B monoclonal antibodies (Stanker et al., 1987) specific for, respectively, PRM1 and PRM2 were applied in a 1:3000 dilution.

Mouse monoclonal antibody gH2AX (Upstate #05-636) was used at 1:1000.

Secondary antibodies A11001 fluor 488 goat anti-mouse IgG (H+L) and A11012 fluor 594 goat anti-rabbit IgG (H+L) from Molecular Probes, (Oregon, USA) were, for the detection of primary antibodies, used at a 1:500 dilution. IF was performed as described previously (Baart et al., 2000).

Images were obtained with a Zeiss axioplan fluorescence microscope and captured by a Zeiss AxioCam MR camera with Axiovision 3.1 software (Carl Zeiss). The expanded sperm nuclear grades ++ (+/-) for no decondensation; (+) for minimal decondensation; (++) for open chromatin and (+++) for highly open chromatin. Only sperm nuclei with sufficient decondensation (category ++ and ++++) were subjectively scored.

Figure 1: (A) Decondensation of sperm nuclei after DTT/heparin treatment and IF results

The degree of decondensation was subjectively assessed as (−) for no decondensation; (+) for minimal decondensation; (++) for open chromatin and (+++) for highly open chromatin. Only sperm nuclei with sufficient decondensation (category ++ and ++++) were subjectively evaluated. (B) IF intensity was assessed as: no signal (−); only a few weak foci (+/−); foci all over the nucleus but still of weak intensity (+); foci all over the nucleus with increasing brightness (++) and highly fluorescent nuclei (+++).
7.4) and kept in the dark for 20 min at room temperature (Kosower and Kosower, 1987). The sample was washed once more in PBS and the pellet was resuspended in 500 μl PBS for fluorescence measurements and sorting by FACS.

For the determination of the total amount of \( \text{SH}^- \) groups in the cells, one sperm aliquot was reduced using DTT. First, samples were fixed in 4% PFA in PBS for 10 min at room temperature. The cell suspension was washed once in PBS and the pellet resuspended in 500 μl 1 mM DTT in TBS, pH 9.5 (15 min at room temperature). Reduced sperm samples were washed twice in PBS (5 min, 500g) before staining with mBBr.

Cells were analysed and sorted on an Altra HyperSort flowcytometer (Beckman Coulter, Miami, USA). A 408-nm Vioflame laser running at 25 mW was used for excitation of mBBr and a 525-nm band-pass filter for emission. Spermatozoa were first gated on forward scatter versus side scatter to discriminate sperm from debris and other cells as much as possible (Gate A, Fig. 2). Depending on the final concentration per sample, a minimum of 150 000–500 000

**Figure 2:** (A and B) FACS plot representation of unstained normospermic and OAT pools
Gate ‘A’ represents the sperm population analyzed and sorted for further experiments. (C and D) mBBr stained sperm detecting free thiol groups. (E and F) total thiol content in the DTT reduced mBBr stained sperm samples. Horizontal bars in C–F (R and S populations) give selection criteria for sorting.
particles was sorted for high or low fluorescence in the mBBr and DTT–mBBr stained samples.

The gate A population and stained fractions from the A-gated population were collected in 500 µl HTF medium and spun for 10 min at 1500 g. Sperm cell populations were fractionated (sorted) by high and low free and total SH levels (see Fig. 2). After centrifugation, pellets were resuspended in 30 µl HTF for further processing, such as the determination of DNA breakage by the TUNEL reaction and chromatin compaction by measuring accessibility of the DNA to the dsDNA-specific antibody #36.

**TUNEL staining**
The TUNEL assay (Cell Death Detection kit, Roche Biochemicals) was executed following the manufacturer’s specifications with minor modifications (Ramos et al., 2002). Briefly, air-dried spermatozoa were fixated in 1% PFA in PBS for 10 min at room temperature and rinsed twice with PBS followed by permeabilization with 0.2% Triton X-100 in PBS, for 10 min. Nuclei were exposed to the TdT-labelled nucleotide mix for 60 min at 37°C. Slides were rinsed twice (5 min) in PBS and the sperm nuclei were counterstained with DAPI (0.01 mg/l in PBS). Nuclei were mounted in 25 µl Vectashield. The total number of DAPI blue staining sperm nuclei per field was counted first. A minimum of 200, but mostly between 300 and 400, nuclei per fraction was scored by two observers.

**Nuclear condensation patterns with IF**
Sperm fractions were embedded in a fibrin clot (Hunt et al., 1995) by mixing 1 µl of cell suspension with 3 µl of fibrin (Catalogue number: 341573, Calbiochem) on a precleaned coverslip, after which 1 µl of thrombin (Catalogue number: T-6634, Sigma) was added. A clotting reaction induced by body heat follows within one minute. The clot was washed briefly in PBS. Subsequently, the cells were fixed in 0.5% PFA in PBS for 5 min, treated with 1 mM DTT for 30 min and refixed for 30 min with ice-cold methanol (modified from Zalensky et al., 2002).

**Statistics**
Chi-square analysis was used to test for independency of variables such as observers and staining patterns within samples. The Spearman rank correlation coefficient was used as an estimate for the congruence between observers. In the case of more than two groups, one-way analysis of variance was used. P-values <0.05 were considered statistically different. Statistical analysis was carried out with the SSPS 12.0 software package (SPSS Inc., Chicago, IL, USA).

**Results**

**Histone characterization of expanded sperm nuclei**
Sperm from the OAT pool expanded (decondensed) more slowly than sperm from normal donors. Overall, the results were comparable between expansion classes (+) and (+++) (see Figs. 1 and 3 for examples of expansion classes and IF signals and Table II for results).

Chromatin markers were either expressed in each sperm nucleus (Table II) or only in a fraction, such as for TH2B and KM-2. For TH2B, we observed that a significantly higher percentage of nuclei contained a fine spotted signal in the normospermic pool than in the OAT pool (+/− and +, 38 versus 16%), which is in concordance with other studies (van Roijen et al., 1998; Zalensky et al., 2002). The signal was spread throughout the nucleus. For KM2, an antibody that has recently been characterized to recognize H4 acetylated at K8, 12 and 16 and associates with apoptosis-induced chromatin changes in somatic cells (Dieker et al., 2007), the reverse was found. This marker was present in 44% of nuclei from the OAT pool and in only 21% of nuclei from normospermic males. Heavier labelling patterns (+ + and +++) that were hardly present in normospermics occurred in 3% of OAT nuclei.

The nucleosome-specific PL2-3 antibody, detecting its epitope in each sperm nucleus, clearly showed an increase in signal for the OAT pool (Table II). Also, the variation in signal was larger for OAT sperm. Nucleosomes characterized by the replication-dependent H3.1/H3.2 (mab #34) this were found to a lesser degree (Table II). Mab PL2-3 detects all nucleosomes containing either the replication-dependent H3.1, H3.2 or replication-independent H3.3 isoforms.

For PRM1 (Hup1N), we found some sperm with a low signal (+/−; 10 and 20% for normospermics and OAT, respectively), but the mean intensity for both groups did not differ (see Table II). For PRM2 (Hup2B), only 0.3% of the normospermic pool had a low intensity signal (+/−) compared with 2.7% for the OAT pool (P = 0.05). No difference in mean intensity was observed for this marker as well.

Neither the OAT pool nor the normospermic pool reacted with the monoclonal antibody γH2AX (data not shown).

**Analysis of thiol status by FACS**
The higher intensity of IF signals for nucleosomes theoretically should represent a lower presence of protamines in the OAT pool, but this was not found using IF for PRM1 and 2. Another approach to study underprotamination is the use of mBBr staining in combination with FACS. Therefore, we executed pilot mBBr-FACS runs for free thiol and total thiol measurements. The analysis makes use of the fact that both PRM1 and 2 are rich in cysteines. First pilot experiments used combinations of two OAT donors (see Table I) and (individual) normospermic donor samples. The data of each FACS run were comparable with the results given in Fig. 2. Because of the number of sperm necessary to perform sorting of fractions, a large pool was made (pool normospermic donors: N-2, N-3, N-4, N-5; pool OAT donors: O-2, O-6, O-7, O-8). The FACS run of the pooled samples is represented in Fig. 2.

In the forward and side scatter plots of Fig. 2A and B, each dot represents one sperm cell. Sperm with a specific and regular size were labelled ‘gate A’ in normospermic samples, this gate contained around 80% of the measurements and around 60% for the OAT samples.

In the histograms of Fig. 2C–F, horizontal bars indicate the fluorescence criteria that were used for the sorting of high (R) and low (S) intensity fractions, respectively. Table III gives the frequencies and average fluorescence values of nuclei contained in these windows. Appreciable differences emerged between normospermic and OAT donor pools. For both the free SH groups and total SH groups (after DTT reduction), OAT sperm were characterized by biphasic distributions (Fig. 2D and F), that for the free SH groups was also visible in the normospermic samples, however, not as pronounced (Fig. 2C). For both types of SH measurements, the normospermic samples were much more homogeneous. When comparing
Figure 3: Decondensed human sperm heads were stained by using monoclonal antibodies for different chromatin markers. Only decondensed sperm heads grade (++) or (+++) were used for IF intensity scoring (in this figure, scored cells are given within dotted circles). Depending on the intensity and pattern of the signal, semi-quantitative values were given (−, +/+−, +, ++, +++).
in the normospermic pool, the staining and passage of sperm emerged. The TUNEL scores before or after FACS show that mBBr-sorted sperm. TUNEL was evaluated by two independent observers, between which no statistical differences emerged. The TUNEL scores before or after FACS show that in the normospermic pool, the staining and passage of sperm through the sorter had little effect on the outcome. However, a greater effect of presumably gating was observed in the OAT patients. Both for normospermic and OAT pools, a lower level of free thiol groups was associated with a higher TUNEL score and vice versa (Table IV). Hence, the larger fraction of low free thiol sperm in the OAT sample (Fig. 2D) is responsible for the higher TUNEL level in OAT ejaculates. Likewise, when fractionating for total thiol levels, in both samples the TUNEL score of the low fraction was about twice that of the high fraction.

### Table II. IF intensity values of the different chromatin proteins (protamines, histones types, histone variants and histone modifications for KM-2).

<table>
<thead>
<tr>
<th>Antibody (nucleosomes)</th>
<th>Poola</th>
<th>n</th>
<th>Mean ± SD IF intensity</th>
<th>Ratio O/Na</th>
<th>P-value</th>
<th>IF intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL2-3</td>
<td>N</td>
<td>350</td>
<td>1.71 ± 0.72</td>
<td>1.49</td>
<td>&lt;0.001</td>
<td>High (+ + +)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>300</td>
<td>2.54 ± 1.12</td>
<td></td>
<td></td>
<td>Intermediate (+ + / +)</td>
</tr>
<tr>
<td>#34 (H3.1/H3.2)</td>
<td>N</td>
<td>400</td>
<td>1.51 ± 0.78</td>
<td>1.14</td>
<td>0.001</td>
<td>Low (+ / –)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>350</td>
<td>1.72 ± 0.94</td>
<td></td>
<td></td>
<td>No signal (–)</td>
</tr>
<tr>
<td>TH2B</td>
<td>N</td>
<td>300</td>
<td>0.39 ± 0.50</td>
<td>0.41</td>
<td>&lt;0.001</td>
<td>High (+ + +)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>200</td>
<td>0.16 ± 0.37</td>
<td></td>
<td></td>
<td>Intermediate (+ + / +)</td>
</tr>
<tr>
<td>HuP-1N (protamine 1)</td>
<td>N</td>
<td>500</td>
<td>2.13 ± 0.59</td>
<td>0.97</td>
<td>n.s</td>
<td>Low (+ / –)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>300</td>
<td>2.07 ± 0.71</td>
<td></td>
<td></td>
<td>No signal (–)</td>
</tr>
<tr>
<td>HuP-2B (protamine 2)</td>
<td>N</td>
<td>350</td>
<td>3.11 ± 0.69</td>
<td>1.0</td>
<td>n.s</td>
<td>High (+ + +)</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>300</td>
<td>3.11 ± 0.76</td>
<td></td>
<td></td>
<td>Intermediate (+ + / +)</td>
</tr>
<tr>
<td>KM-2</td>
<td>N</td>
<td>550</td>
<td>0.26 ± 0.57</td>
<td>2.19</td>
<td>&lt;0.001</td>
<td>Low (+ / –)</td>
</tr>
<tr>
<td>(AcH4K8,12,16)</td>
<td>O</td>
<td>600</td>
<td>0.57 ± 0.76</td>
<td></td>
<td></td>
<td>No signal (–)</td>
</tr>
</tbody>
</table>

aN, normospermic pool; O, OAT pool.

### Table III. Mean fluorescence intensity (arbitrary units) and percentage of cells in each fraction (Fig. 2) after FACS sorting.

<table>
<thead>
<tr>
<th>Sorted fractions</th>
<th>Mean intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normo pool</td>
</tr>
<tr>
<td>% cells in Gate Aa</td>
<td>79</td>
</tr>
<tr>
<td>mBBr low (S), n (%)</td>
<td>34 (22)</td>
</tr>
<tr>
<td>mBBr high (R), n (%)</td>
<td>194 (72)</td>
</tr>
<tr>
<td>DTT–mBBr low (S)</td>
<td>695 (7)</td>
</tr>
<tr>
<td>DTT–mBBr high (R), n (%)</td>
<td>3602 (92)</td>
</tr>
</tbody>
</table>

aSee Fig. 2 (FACS plots).

Considered the mean intensity after DTT reduction [normo pool (R): 3602 arbitrary units] as the 100% level of the free SH-groups in a sperm population, the percentage of free thiols per sample was estimated. Using this approach, the normal percentage of free thiols in human sperm cells was ~5%, but was increased in OAT sperm to 13% of the total. Assuming that protamine cysteine thiols are the main contributors to sperm thiols, a lower total SH-content after DTT reduction indicates a lower nuclear protamine content (2790 versus 3602 arbitrary units for the R windows of OAT and normospermics, respectively). Because in the OAT pool DTT–mBBr intensity was overall lower than in the normo pool (Table III), it is assumed that chromatin remodelling in OAT sperm is incomplete or delayed during spermiogenesis.

### TUNEL measurements

Table IV gives the outcomes of the TUNEL measurements of mBBr-sorted sperm. TUNEL was evaluated by two independent observers, between which no statistical differences emerged. The TUNEL scores before or after FACS show that in the normospermic pool, the staining and passage of sperm

### Table IV. Percentage TUNEL positive sperm in the different sorted fractions.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Normo pool</th>
<th>OAT pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% TUNEL</td>
</tr>
<tr>
<td>mBBr (control)a</td>
<td>462</td>
<td>10</td>
</tr>
<tr>
<td>Gate A (control)b</td>
<td>432</td>
<td>7.4</td>
</tr>
<tr>
<td>mBBr low</td>
<td>651</td>
<td>32.7</td>
</tr>
<tr>
<td>mBBr high</td>
<td>697</td>
<td>17.8</td>
</tr>
<tr>
<td>DTT–mBBr low</td>
<td>621</td>
<td>20.9</td>
</tr>
<tr>
<td>DTT–mBBr high</td>
<td>649</td>
<td>11.7</td>
</tr>
</tbody>
</table>

aSperm stained with mBBr before sorting with FACS; bSee Fig. 2 (FACS plot); unstained sperm after a FACS run.

### IF detection of ds DNA in whole mount sperm nuclei

Underprotamination predicts a more open chromatin configuration. In order to establish if we could detect a more open chromatin configuration with IF, we applied to a subset of the samples (see Table I), a monoclonal antibody against dsDNA to the mBBr–FACS sorted spermatozoa. The various patterns and intensities obtained are given in Fig. 4. In both the normospermic and OAT samples, there was considerable variation in antibody penetration-dependent DNA detection. The antibody reacted more intensely at the nuclear periphery, and always accentuated nuclear vacuoles (Fig. 4C). In nuclei with background- or low fluorescence, a ‘negative’ band could be observed (Fig. 4B) situated in the posterior nucleus close to the attachment of the axonema. For the FACS-selected...
samples, this band was most frequently observed in the mBBr+ fraction of the normospermics (82% showed a band contrary to 46% in the corresponding OAT sperm fraction $P = 0.001$), therefore we assume this to represent a normal nuclear differentiation feature. Although less accentuated, a difference in the same direction was preserved after thiol reduction (50 versus 43%, n.s.).

Both samples harboured a fraction of nuclei that did not stain with anti-dsDNA. For this limited set of material (see Table I) the overall level of freely accessible DNA was larger in the OAT sample than in the normospermic samples (Fig. 5). No differences were found between high and low free thiol levels (mBBr) of normospermic samples (see bars in Fig. 5A for ‘control, mBBr+ and mBBr−‘) or OAT samples (compare bars for ‘control, mBBr+ and mBBr−‘). Similarly, for high and low total thiol levels (DTT–mBBr), no difference was found between selected samples (control versus DTT–mBBr+ and DTT–mBBr−) both for normospermic and OAT samples (Fig. 5).

**Discussion**

The purpose of this investigation was to obtain further insight into sperm nuclear differentiation/maturation in oligospermic individuals compared with normospermics. Chromatin remodelling of spermatids is a differentiation characteristic. For the characterization of sperm maturation, SH cross-linking, between and within protamines, is used.
During the nuclear elongation phase of spermiogenesis, chromatin remodelling from a nucleosomal structure to protamine-induced DNA compaction is less complete in the human compared with laboratory rodents and farm animals (Gatewood et al., 1990; Bench et al., 1996). Also, the variation in this process is larger in patients with deficiencies in spermatogenesis compared with normospermic donors (Rufas et al., 1991; de Yebra et al., 1998; Zhang et al., 2006). Besides enrichment of nucleosomes at the telomeres (Mudrak et al., 2005), genes can harbour an alteration of areas with protamines and histones (Wykes and Krawetz, 2003). Centromeric and pericentromeric regions contain nucleosomal chromatin in the bull (Palmer et al., 1990) and human (Zalensky et al., 1993), and this finding has been recently confirmed in the mouse (van der Heijden et al., 2006). Hence, it would appear that DNA repeat areas that are of structural importance to chromosome functioning, such as telomeres and centromeres/centric heterochromatin, are enriched in nucleosomes, providing a memory base for chromatin remodelling upon nuclear expansion after sperm penetration (van der Heijden et al., 2006).

These heterochromatic DNA repeat areas are by definition gene poor. As a consequence, their nucleosomal make-up is derived from S-phase, not influenced by transcription, i.e. associated with chromatin remodelling involving de novo nucleosome formation. Replication-derived nucleosomes contain the H3.1/H3.2 histone variant, whereas transcription-associated nucleosomes contain the H3.3 variant (for a review on functions of histone isoforms and chromatin activity, see Bernstein and Hake, 2006). For nucleosomes containing H3.1/H3.2, we only found a small statistically significant increase in OAT sperm (Table II). For total nucleosomes, the OAT/normal score ratio was 1.5 (Table II). Hence, in the human the transition of nucleosomal chromatin to protamine by preference applies to H3.3 containing nucleosomes that recently have been shown to increase during the second half of the pachytene stage (van der Heijden et al., 2007), a period, i.e. characterized by intense transcription (Geremia et al., 1977). We conclude that a large fraction of OAT sperm seems to be deficient in the remodelling of H3.3 containing nucleosomes, whereas areas where H3.1/H3.2 nucleosomes are present (as assembled at premeiotic and previous S-phases) often may not be exchanged for protamines, either in normal or OAT sperm samples. This analysis would position the origin of the OAT syndrome at the primary spermatocytes stage, for which more indications, such as deficient homologous recombination have been found (Gonsalves et al., 2004). Support for this hypothesis comes from the fact that the histological diagnosis of hypospermatogenesis (Levin, 1979) includes reduced numbers of primary spermatocytes.

Recently, the H4 K8, 12, 16 triacetylation modification, i.e. detected by the monoclonal antibody KM-2, has been shown to be enriched in apoptotic somatic nuclei (Dieker et al., 2007). We found this mark to be about two times more frequently present in OAT sperm which were on average more intensely labelled. Around 40% of nuclei were positive, which is comparable to TUNEL scores in OAT samples as found by us (Table IV) and in the literature (mean 40.9%) (Sergerie et al., 2005). In the normospermic pool, we found around 21% KM-2-positive cells, which is higher than the TUNEL+ frequencies found in our control group (10%), but comparable to the frequencies found in normospermics by other investigators (Chohan et al., 2006).

Indications for an apoptosis-like process in sperm are numerous now, ranging from mitochondrial membrane and caspase determinations (Marchetti et al., 2004) to the discovery of endonuclease activity in the sperm nucleus (Ward and Ward, 2004; Sotolongo et al., 2005). The question of interest here is...
at what stage during spermiogenesis H4 becomes modified, i.e. when does the nucleus receive signals for the apoptotic pathway. Hyperacetylation of H4 is the normal mark at the onset of the histone to protamine change (Sonnack et al., 2002). KM-2 is likely to sense more sites than just the classified epitopes H4K8, 12 and 16, such as the homologous (acyetylated) region in H2A (Dieker et al., 2007).

The frequency of positivity for TH2B in normospermics was comparable with data from the literature (van Roijen et al., 1998; Singleton et al., 2007). Extrapolating from the finding of the latter group that sperm which are positive for TH2B have superior nuclear decondensation after gamete fusion, the lower fraction of TH2B-positive sperm in OAT indicates a lower fraction of optimally differentiated sperm in this syndrome.

We have used FACS measurements of mBBr to substantiate the IF data that were indicative of an increased level of nucleosomes in OAT sperm. Thiol fluorescence of human sperm mainly represents the nucleus, although a low percentage (negligible for the total measurements) of the total mBBr fluorescence is derived from tails (Seligman et al., 1991). When total thiol levels are determined by mBBr fluorescence, the histograms obtained by us agreed with those of Rufas et al., 1991. Under-representation of PRM2 in OAT (for a review, see Carrell et al. 2007) is the main candidate for explaining the higher maximum of free thiol fluorescence in normospermic samples (Fig. 2E). We did not find lower protamine levels by the subjective scoring of IF intensities, pointing to the fact that when epitope levels are high, IF is not a suitable method for quantification. A ratio of around 0.8 (OAT/normo) of total thiol fluorescence in the ‘high windows’ of donor pools (Table III) may well be in line with expectation, although in gel systems such a ratio is not always found (Mengual et al., 2003). The higher maximum of free thiol in the OAT sample (Fig. 2D) is likely due to a delay in, or an interruption to, thiol oxidation during nuclear maturation after spermiation.

The excess of OAT sperm with low free thiol levels could originate from oxidative stress due to ROS production or apoptotic processes, i.e. one aspect of incompletely transformed immature human sperm (Bennetts and Aitken, 2005). The fact that thiol oxidation functions in biological signalling (Moran et al., 2001) and has been implicated in sperm capacitation (de Lamirande and Gagnon, 2003), labels sperm with low free thiol levels as pathogenic. In line with this interpretation, we found TUNEL readings to be increased in fractions with low free SH groups (Table IV), again indicating oxidative stress (Greco et al., 2005; Aitken and Baker, 2006) during epididymal transit as one aspect of the induction of apoptosis.

Protection of the genetic material is always mentioned as one of the prime functions of chromatin remodelling during spermiogenesis and subsequent maturation/compaction of the nucleus by thiol oxidation. Underprolamination by definition entails a lower degree of compaction as does delayed or incomplete nuclear thiol oxidation (Kosower et al., 1992). We have asked ourselves if immunofluorescence with an antibody that detects dsDNA can serve to evaluate the degree of nuclear compaction (hence its accessibility). For this purpose, we adopted a protocol that does not include nuclear expansion and preserves nuclear structures. To achieve this, we avoided settling of sperm cells on glass by encapsulating the spermatozoa in a fibrin clot. Our results show the feasibility of this approach. The nuclear periphery is most reactive, which could partly involve the fact that telomeric histone containing chromatin is adjacent to the nuclear envelope (Zalenskaya et al., 2000). Vacuoles were faithfully recorded, which would argue for proper penetration of the antibody. Overall in this experiment, OAT sperm were by penetration of the antibody less compacted, which is in agreement with the higher nucleosomal content and CMA3 scores (Iranpour et al., 2000). Within pools, no difference was found between oxidized (low free SH) and non-oxidized (high free SH) sperm. When we selected for the sperm fractions with a high total thiol level, hence sperm with higher protamine content, in both pools we did not observe a striking improvement of ‘compaction’. This would argue for another aspect of nuclear structure, likely involving the nuclear matrix, to be not properly developed in OAT sperm, as for instance could be demonstrated by the slower decondensation kinetics in this study.

In conclusion, by the application of various chromatin antibodies, we were able to suggest that H3.1/H3.2 containing nucleosomes may be the most resistant to chromatin remodelling at nucleolar elongation during spermiogenesis. Therefore, H3.3 containing nucleosomes that grow more numerous during the second half of pachytene in the mouse (van der Heijden et al., 2007) may be the major ones that are incompletely exchanged for protamines in OAT sperm.

The free and total thiol status relates to the likelihood of apoptotic development by TUNEL standard, with lower thiol levels relating to a higher likelihood of apoptotic development. Low levels of free thiol groups are indicative of oxidative stress and therefore also predictive for apoptosis. Within normo and OAT pools, free and total thiol levels do not predict the amount of nuclear compaction as measured by the penetrability of a monoclonal antibody for dsDNA. Hence other aspects of nuclear differentiation also differ between normo and OAT sperm.

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