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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 (Saowaros and Panyim, 1979; Pellicciari *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 sorter (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, highlighting the role of uncompacted (higher histone containing) chromatin in infertility (Sakkas *et al*., 2002).

Second, Steger *et al*. (2001) observed that the frequency of *PRM1* and *PRM2* transcribing round spermatids is negatively related to the severity of the spermatogenic defect. In concordance with this finding, the fraction of elongating spermatids, that were immunofluorescence positive for hyperacetylated histone 4 (H4), marking the onset of the nucleosomal transition, was decreased by a factor 0.6–0.75 in azoospermic to oligospermic patients (Sonnack *et al*., 2002).

Third, when the ratio of PRM1 to PRM2, which in normospermic human samples is around 1 (Balhorn *et al*., 1988), is evaluated in oligospermic subjects, often a shortage of PRM2 is found in combination with the presence of PRM2 precursor proteins (de Yebra *et al*., 1998). The same observation is made in mouse models deficient for TNP1 and 2 (Yu *et al*., 2000; Zhao *et al*., 2001). TNP1 and 2 and PRM2 deficiency lead to altered chromatin compaction as demonstrated by electron microscopy (Yu *et al*., 2000; Zhao *et al*., 2001; Cho *et al*., 2003).

In this study, we have used highly specific antibodies against histones in a nucleosomal context, histone subtypes (DNA replication-dependent H3.1/H3.2, TH2B) and histone modifications (acetylated forms of histone 4) in an *in situ* immunofluorescence (IF) investigation of expanded sperm nuclei from normal and OAT donors. 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 spermiogram 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 $5–20 \times 10^6$/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 500*g*. 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 dithiotreitol (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 an IF control.

Table I. Sperm analysis of the samples included in this study and experiments carried out with each sample.

Samples	Code	Volume (ml)	Concentration $\times 10^6$	% motility	% normal morphology	Experiment 1	Experiment 2
Normospermic donors	N-1	2.2	195	80	47	FACS	–
	N-2[a]	2.4	185	60	22	FACS	TUNEL
	N-3[a]	3.8	200	80	33	FACS	IF-dsDNA+TUNEL
	N-4[a]	2.5	100	70	25	FACS	TUNEL
	N-5[a]	3.6	85	55	35	FACS	TUNEL
	N-6	2.3	130	60	17	IF-chromatin	–
	N-7	4.2	150	75	12	IF-chromatin	–
	N-8	1.5	130	75	25	IF-chromatin	–
	N-9	2.4	100	65	12	IF-chromatin	–
	N-10	3.1	100	50	15	IF-chromatin	–
OAT donors	O-1[b]	1.5	30	15	2	FACS	IF-dsDNA
	O-2[b]	1.0	45	20	3		
	O-3[c,e]	7.3	5	25	2	FACS	IF-dsDNA+TUNEL
	O-4[c]	3.6	40	25	7		
	O-5[d]	5.0	7	30	1	FACS	–
	O-6[d,e]	4.2	7	25	5		TUNEL
	O-7[e]	7.9	15	10	1	FACS	TUNEL
	O-8[e]	3.7	20	35	7	FACS	TUNEL
	O-9	8.4	5	50	5	IF-chromatin	–
	O-10	1.1	3.8	10	1	IF-chromatin	–
	O-11	6.3	1.0	15	2	IF-chromatin	–
	O-12	5.3	5.0	40	3	IF-chromatin	–
	O-13	1.7	1.9	20	2	IF-chromatin	–

Experiments 1 and 2: flow cytometry, FACS; immunofluorescence for chromatin markers, IF-chromatin; immunofluorescence with monoclonal antibody against ds-DNA, IF-dsDNA; TdT UTP-nick end labelling, TUNEL. [a]pooled samples for the final FACS sorting experiment (Normo pool); [b,c,d]pooled samples for measurements with FACS; [e]pooled samples for the final FACS sorting experiment (OAT pool).

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 γH2AX (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 ++ and +++ (see Fig. 1A) showed uniform staining when probed wit mab #36 specific for dsDNA. Fluorescence intensities for these expansion classes were subjectively scored in five grades. No signal detected (−); some small specks of low intensity not covering the entire nucleus (+/−); small specks of low intensity covering the entire nucleus (+); overall signal with increasing brightness (++) and overall strong signal up to covering the entire nucleus (+++) (Fig. 1B). For numerical representations, these grades received the values 0–4 [corresponding to (−); (+/−); (+); (++) and (+++), respectively].

Thiol characterizations by FACS and cell separation

For the monobromobimane (mBBr, Calbiochem, CA,USA) staining of free thiol (SH−) groups, sperm cells were fixed for 15 min in 1% PFA in PBS at room temperature and washed twice in PBS (spinning 10 min at 500*g*). The sperm pellet was resuspended in 100 μl mBBr solution [50 μM mBBr in 20 mM Tris-buffered saline (TBS), pH

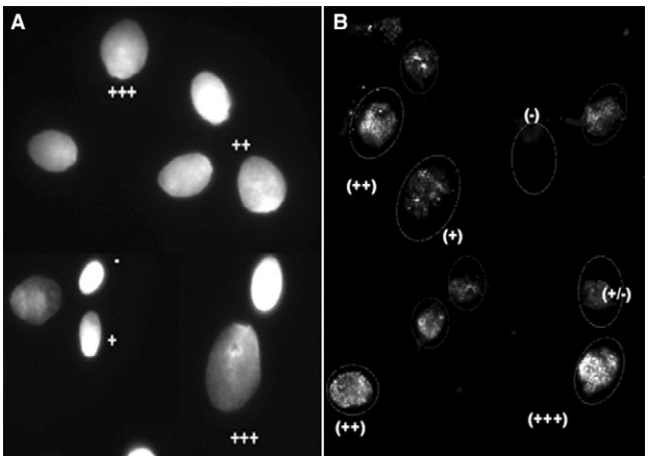

Figure 1: (**A**) Decondensation of sperm nuclei after DTT/heparin treatment and IF results
The degree of decondensation was subjectively assessed as (−) for no decondensation; (+) for minimal decondensation; (++) for open chromatin and (+++) for highly open chromatin. Only sperm nuclei with sufficient 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 strong intensity (++) 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 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, 500*g*) 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

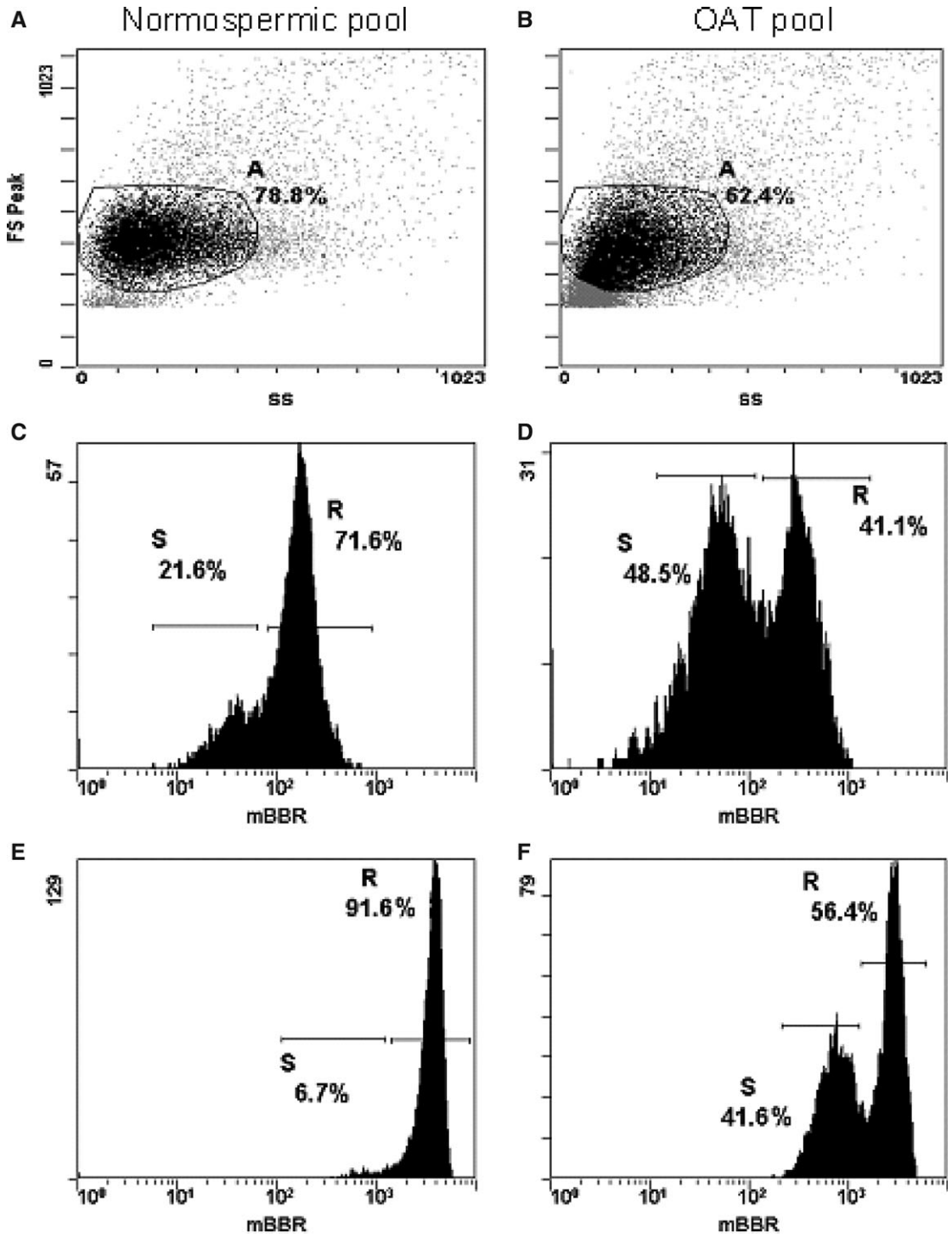

Figure 2: (**A** and **B**) FACS plot representation of unstained normospermic and OAT pools
Gate 'A' represents the sperm population analyzed and sorted for further experiments. (**C** and **D**) mBBr stained sperm detecting free thiol groups. (**E** and **F**) total thiol content in the DTT reduced 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

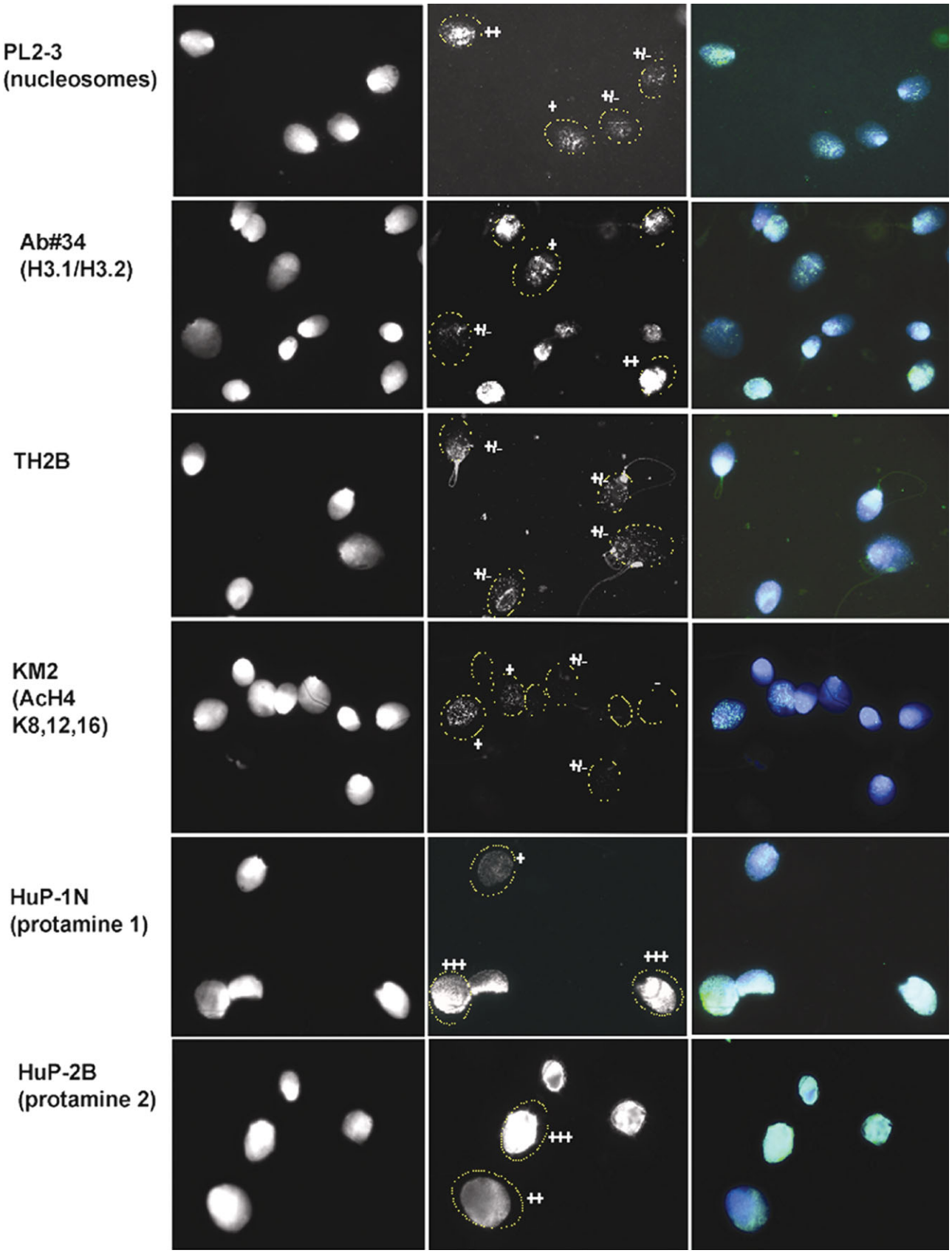

Figure 3: Decondensed human sperm heads were stained by using monoclonal antibodies for different chromatin markers
Only decondensed sperm heads grade (++) or (+++) were used for IF intensity scoring (in this figure, scored cells are given within dotted circles). Depending on the intensity and pattern of the signal, semi-quantitative values were given (−, +/−, +, ++, +++)

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

Antibody	Pool[a]	*n*	Mean ± SD IF intensity	Ratio O/N[a]	*P*-value	IF intensity (%)			
						High (+++)	Intermediate (++/+)	Low (+/−)	No signal (−)
PL2-3 (nucleosomes)	N	350	1.71 ± 0.72	1.49	<0.001	1	57	42	0
	O	300	2.54 ± 1.12			27	50	23	0
#34 (H3.1/H3.2)	N	400	1.51 ± 0.78	1.14	0.001	3	33	64	0
	O	350	1.72 ± 0.94			8	40	50	2
TH2B	N	300	0.39 ± 0.50	0.41	<0.001	0	1	38	61
	O	200	0.16 ± 0.37			0	0	16	84
HuP-1N (protamine 1)	N	500	2.13 ± 0.59	0.97	n.s	1	89	10	0
	O	300	2.07 ± 0.71			3	78	19	0
HuP-2B (protamine 2)	N	350	3.11 ± 0.69	1.0	n.s	30	70	0	0
	O	300	3.11 ± 0.76			32	65	3	0
KM-2	N	550	0.26 ± 0.57	2.19	<0.001	0	4	17	79
(AcH4K8,12,16)	O	600	0.57 ± 0.76			0	10	34	56

[a]N, normospermic pool; O, OAT pool.

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

Sorted fractions	Mean intensity (%)	
	Normo pool	OAT pool
% cells in Gate A[a]	79	62
mBBr low (S), *n* (%)	34 (22)	52 (42)
mBBr high (R), *n* (%)	194 (72)	360 (41)
DTT–mBBr low (S)	695 (7)	730 (42)
DTT–mBBr high (R), *n* (%)	3602 (92)	2790 (56)

[a]See Fig. 2 (FACS plots).

the right borders (upper limits) that give an indication of maximum fluorescence yields, the OAT samples are higher with respect to free thiol groups (Fig. 2D), whereas the normospermics are higher for total SH content (Fig. 2E).

Considering 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 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.

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

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

Fractions	Normo pool			OAT pool		
	n	% TUNEL	*P*-value	*n*	% TUNEL	*P*-value
mBBr (control)[a]	462	10	n.s	477	47	0.008
Gate A (control)[b]	432	7.4		413	33.4	
mBBr low	651	32.7	<0.001	518	59.2	0.002
mBBr high	697	17.8		600	40.8	
DTT–mBBr low	621	20.9	<0.001	148	65.5	<0.001
DTT–mBBr high	649	11.7		615	31.7	

[a]Sperm stained with mBBr before sorting with FACS; [b]See Fig. 2 (FACS plot): unstained sperm after a FACS run.

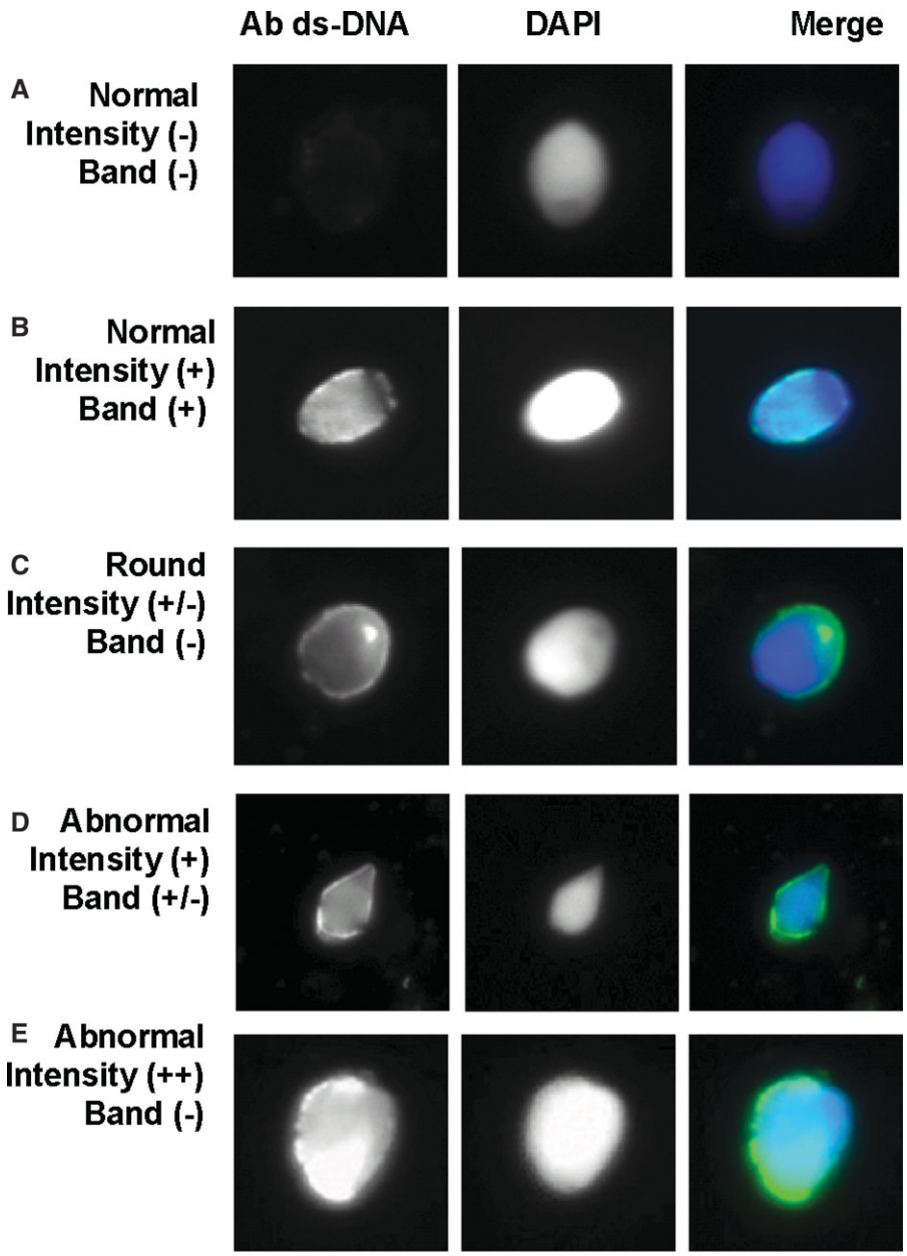

Figure 4: Fluorescence patterns obtained with mab #36 specific for dsDNA
Band describes a fluorescence negative zone, i.e. often found near the nuclear annulus in the posterior nucleus. Intensity refers to the overall level of fluorescence. Only nuclei that showed their biggest circumference were scored

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.

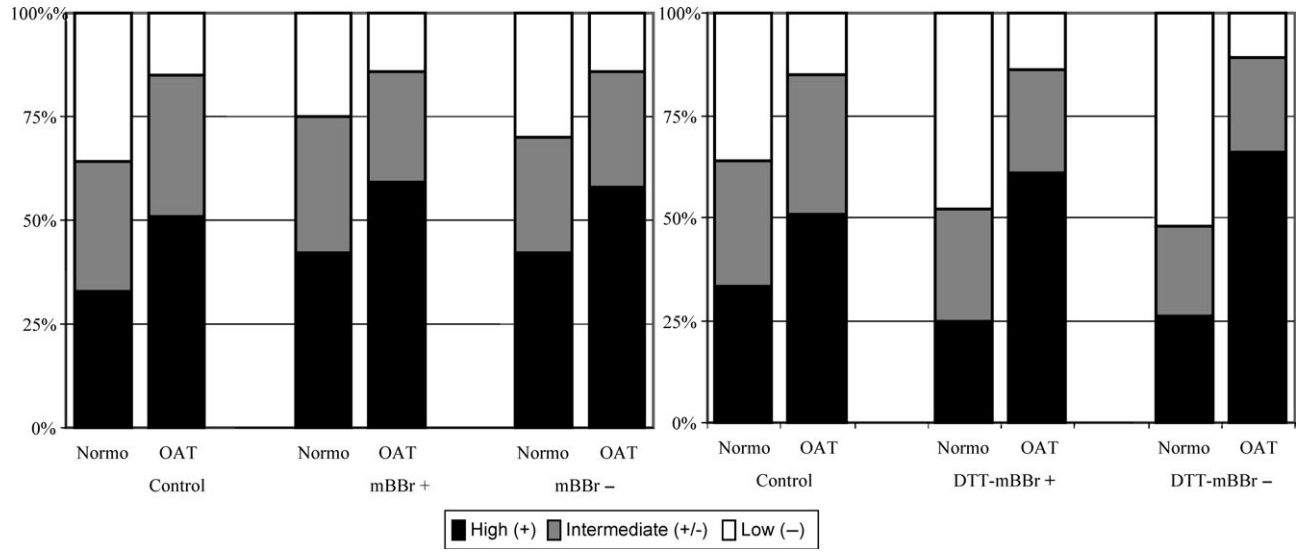

Figure 5: Fluorescence intensity patterns of mAb #36 in the different FACS fractions are represented by bars
Intensity of the signal was characterized as high, intermediate or low. From each sample, an unstained aliquot (control), the mBBr and DTT-mBBr fractions after FACS were stained with mab # 36. No difference in IF staining was found in each sample separately (normospermic or OAT) in the sorted fractions. A marked difference was found between normo and OAT samples but not within donors for the DTT-mBBr+ and DTT-mBBr- sorted fractions

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 appears 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 (acetylated) 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 total 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. Underprotamination 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 nuclear 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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References

Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrinol* 2006;**250**:66–69.

Baart EB, de Rooij DG, Keegan KS, de Boer P. Distribution of Atr protein in primary spermatocytes of a mouse chromosomal mutant: a comparison of preparation techniques. *Chromosoma* 2000;**109**:139–147.

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

Bedford JM, Bent MJ, Calvin H. Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. *J Reprod Fertil* 1973;**33**:19–29.

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

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

Bernstein E, Hake SB. The nucleosome: a little variation goes a long way. *Biochem Cell Biol* 2006;**84**:505–517.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biol Reprod* 1993;**49**:1083–1088.

Carrell DT, Liu L, Christensen G. Polyploidy in mouse embryos derived from in vivo and in vitro fertilization is dependent on the timing of pregnant mare serum gonadotropin (PMSG) injection. *Fertil Steril* 2007;**87**:1470–1472.

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

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 2006;**27**:53–59.

Dadoune JP. Expression of mammalian spermatozoal nucleoproteins. *Microsc Res Tech* 2003;**61**:56–75.

de Lamirande E, Gagnon C. Redox control of changes in protein sulfhydryl levels during human sperm capacitation. *Free Radic Biol Med* 2003;**35**:1271–1285.

De Vos A, Van De Velde H, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. *Fertil Steril* 2003;**79**:42–48.

de Yebra L, Oliva R. Rapid analysis of mammalian sperm nuclear proteins. *Anal Biochem* 1993;**209**:201–203.

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

Dieker JW, Sun YJ, Jacobs CW, Putterman C, Monestier M, Muller S, van der Vlag J, Berden JH. Mimotopes for lupus-derived anti-DNA and nucleosome-specific autoantibodies selected from random peptide phage display libraries: facts and follies. *J Immunol Methods* 2005;**296**:83–93.

Dieker JW, Fransen JH, van Bavel CC, Briand JP, Jacobs CW, Muller S, Berden JH, van der Vlag J. Apoptosis-induced acetylation of histones is pathogenic in systemic lupus erythematosus. *Arthritis Rheum* 2007;**56**:1921–1933.

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

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**:20662–20666.

Geremia R, Boitani C, Conti M, Monesi V. RNA synthesis in spermatocytes and spermatids and preservation of meiotic RNA during spermiogenesis in the mouse. *Cell Differ* 1977;**5**:343–355.

Gonsalves J, Sun F, Schlegel PN, Turek PJ, Hopps CV, Greene C, Martin RH, Pera RA. Defective recombination in infertile men. *Hum Mol Genet* 2004;**13**:2875–2883.

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005;**26**:349–353.

Henikoff S, Ahmad K. Assembly of variant histones into chromatin. *Annu Rev Cell Dev Biol* 2005;**21**:133–153.

Hunt P, LeMaire R, Embury P, Sheean L, Mroz K. Analysis of chromosome behavior in intact mammalian oocytes: monitoring the segregation of a univalent chromosome during female meiosis. *Hum Mol Genet* 1995;**4**:2007–2012.

Iranpour FG, Nasr-Esfahani MH, Valojerdi MR, al Taraihi TM. Chromomycin A3 staining as a useful tool for evaluation of male fertility. *J Assist Reprod Genet* 2000;**17**:60–66.

Johnsen SG. Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* 1970;**1**:2–25.

Kosower NS, Kosower EM. Thiol labeling with bromobimanes. *Methods Enzymol* 1987;**143**:76–84.

Kosower NS, Katayose H, Yanagimachi R. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J Androl* 1992;**13**:342–348.

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

Levin HS. Testicular biopsy in the study of male infertility: its current usefulness, histologic techniques, and prospects for the future. *Hum Pathol* 1979;**10**:569–584.

Losman MJ, Fasy TM, Novick KE, Monestier M. Monoclonal autoantibodies to subnucleosomes from a MRL/Mp(−)+/+ mouse. Oligoclonality of the antibody response and recognition of a determinant composed of histones H2A, H2B, and DNA. *J Immunol* 1992;**148**:1561–1569.

Marchetti C, Gallego MA, Defossez A, Formstecher P, Marchetti P. Staining of human sperm with fluorochrome-labeled inhibitor of caspases to detect activated caspases: correlation with apoptosis and sperm parameters. *Hum Reprod* 2004;**19**:1127–1134.

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

Mengual L, Ballesca JL, Ascaso C, Oliva R. Marked differences in protamine content and P1/P2 ratios in sperm cells from percoll fractions between patients and controls. *J Androl* 2003;**24**:438–447.

Menkveld R, Wong WY, Lombard CJ, Wetzels AM, Thomas CM, Merkus HM, Steegers-Theunissen RP. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. *Hum Reprod* 2001;**16**:1165–1171.

Moran LK, Gutteridge JM, Quinlan GJ. Thiols in cellular redox signalling and control. *Curr Med Chem* 2001;**8**:763–772.

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

Palmer DK, O'Day K, Margolis RL. The centromere specific histone CENP-A is selectively retained in discrete foci in mammalian sperm nuclei. *Chromosoma* 1990;**100**:32–36.

Pellicciari C, Hosokawa Y, Fukuda M, Manfredi-Romanini MG. Cytofluorometric study of nuclear sulphydryl and disulphide groups during sperm maturation in the mouse. *J Reprod Fertil* 1983;**68**:371–376.

Ramos L, Wetzels AMM. Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. *Hum Reprod* 2001;**16**:1703–1707.

Ramos L, Kleingeld P, Meuleman E, van Kooy R, Kremer J, Braat D, Wetzels A. Assessment of DNA fragmentation of spermatozoa that were surgically retrieved from men with obstructive azoospermia. *Fertil Steril* 2002;**77**:233–237.

Ramos L, de Boer P, Meuleman EJ, Braat DD, Wetzels AM. Evaluation of ICSI-selected epididymal sperm samples of obstructive azoospermic males by the CKIA system. *J Androl* 2004;**25**:406–411.

Rufas O, Fisch B, Seligman J, Tadir Y, Ovadia J, Shalgi R. Thiol status in human sperm. *Mol Reprod Dev* 1991;**29**:282–288.

Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 2002;**66**:1061–1067.

Saowaros W, Panyim S. The formation of disulfide bonds in human protamines during sperm maturation. *Experientia* 1979;**35**:191–192.

Seligman J, Shalgi R, Oschry Y, Kosower NS. Sperm analysis by flow cytometry using the fluorescent thiol labeling agent monobromobimane. *Mol Reprod Dev* 1991;**29**:276–281.

Seligman J, Kosower NS, Weissenberg R, Shalgi R. Thiol-disulfide status of human sperm proteins. *J Reprod Fertil* 1994;**101**:435–443.

Sergerie M, Laforest G, Bujan L, Bissonnette F, Bleau G. Sperm DNA fragmentation: threshold value in male fertility. *Hum Reprod* 2005;**20**:3446–3451.

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**:392–397.

Smeenk RJ, Brinkman K, van den Brink HG, Westgeest AA. Reaction patterns of monoclonal antibodies to DNA. *J Immunol* 1988;**140**:3786–3792.

Sonnack V, Failing K, Bergmann M, Steger K. Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis. *Andrologia* 2002;**34**:384–390.

Sotolongo B, Huang TT, Isenberger E, Ward WS. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J Androl* 2005;**26**:272–280.

Stanker LH, Wyrobek A, Balhorn R. Monoclonal antibodies to human protamines. *Hybridoma* 1987;**6**:293–303.

Steger K, Failing K, Klonisch T, Behre HM, Manning M, Weidner W, Hertle L, Bergmann M, Kliesch S. Round spermatids from infertile men exhibit decreased protamine-1 and -2 mRNA. *Hum Reprod* 2001;**16**:709–716.

Van Der Heijden GW, Dieker JW, Derijck AA, Muller S, Berden JH, Braat DD, van der Vlag J, de Boer P. Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. *Mech Dev* 2005;**122**:1008–1022.

van der Heijden GW, Derijck AA, Ramos L, Giele M, van der Vlag J, de Boer P. Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin. *Dev Biol* 2006;**298**:458–469.

Van Der Heijden GW, Derijck AA, Posfai E, Giele M, Pelczar P, Ramos L, Wansink DG, van der Vlag J, Peters AH, de Boer P. Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation. *Nat Genet* 2007;**39**:251–258.

van Roijen HJ, Ooms MP, Spaargaren MC, Baarends WM, Weber RF, Grootegoed JA, Vreeburg JT. Immunoexpression of testis-specific histone 2B in human spermatozoa and testis tissue. *Hum Reprod* 1998;**13**:1559–1566.

Ward MA, Ward WS. A model for the function of sperm DNA degradation. *Reprod Fertil Dev* 2004;**16**:547–554.

WHO. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 34th ed. edn. Cambridge University Press, 1999.

Wykes SM, Krawetz SA. The structural organization of sperm chromatin. *J Biol Chem* 2003;**278**:29471–29477.

Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proc Natl Acad Sci USA* 2000;**97**:4683–4688.

Zalenskaya IA, Bradbury EM, Zalensky AO. Chromatin structure of telomere domain in human sperm. *Biochem Biophys Res Commun* 2000;**279**:213–218.

Zalensky AO, Breneman JW, Zalenskaya IA, Brinkley BR, Bradbury EM. Organization of centromeres in the decondensed nuclei of mature human sperm. *Chromosoma* 1993;**102**:509–518.

Zalensky AO, Siino JS, Gineitis AA, Zalenskaya IA, Tomilin NV, Yau P, Bradbury EM. Human testis/sperm-specific histone H2B (hTSH2B). Molecular cloning and characterization. *J Biol Chem* 2002;**277**:43474–43480.

Zhang X, San Gabriel M, Zini A. Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. *J Androl* 2006;**27**:414–420.

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM *et al.* Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Mol Cell Biol* 2001;**21**:7243–7255.

Zollner U, Schleyer M, Steck T. Evaluation of a cut-off value for normal sperm morphology using strict criteria to predict fertilization after conventional in-vitro fertilization and embryo transfer in asthenozoospermia. *Hum Reprod* 1996;**11**:2155–2161.

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