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Influence of incubation on the chromatin condensation and nuclear stability of human spermatozoa by flow cytometry

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Flow cytometry analysis was used for the acurate and objective evaluation of sperm chromatin condensation and chromatin stability of sperm nuclei. It was also possible to determine the influence of incubation on sperm chromatin. Different types of spermatozoa were studied: unprocessed spermatozoa at 1 and 45 min after ejaculation, after swim-up (migrated), spermatozoa incubated for 6 h in non-capacitating conditions (aged), or in B2 medium (capacitated) or B2 medium followed 1 h later with A23187 (reacted). All types of spermatozoa were analysed before and after treatment with various decondensation agents: sodium dodecyl sulphate (SDS), SDS plus EDTA and SDS plus disulphide-reducing agent [dithiotreitol (DTT)]. Sperm nuclei were enzymatically isolated and stained with propidium iodide. Three flow cytometric parameters were then measured: forward light scatter (cellular size), side light scatter (cellular complexity) and fluorescence (uptake of propidium iodide). Fluorescence was the most suitable parameter to study the degree of condensation and resistance to decondensation of DNA in the spermatozoa. Unprocessed spermatozoa 1 min after ejaculation underwent decondensation by all assessed treatments (anionic detergent, chelating or disulphide-reducing agents). Unprocessed spermatozoa 45 min after ejaculation and migrated spermatozoa did not undergo decondensation with SDS treatment, but decondensation occurred after treatment with SDS + EDTA or SDS + DTT. Spermatozoa incubated for 6 h under both non-capacitating (aged spermatozoa) and capacitating conditions (capacitated spermatozoa) and reacted spermatozoa were decondensed only after treatment with SDS + DTT. In conclusion, the post-ejaculation and incubation time have to be taken into account when clinical interpretation of the effect of different treatments on sperm chromatin condensation is made.

Key words: chromatin/flow cytometry/spermatozoa

Introduction

During spermiogenesis, somatic histones are replaced by protamines (Poccia, 1986). Protamines contain a large number of cysteine residues, which are initially present as thiols. These thiols are oxidized to disulphides during sperm maturation (Huang *et al.*, 1984). As a consequence, the nuclei of mature spermatozoa have a degree of condensation which is reflected in the fact that the quantity of fluorochromes (e.g. acridine orange, ethidium bromide and propidium iodide) bound by the sperm nuclei is below the haploid level (Clausen *et al.*, 1982; Fossa *et al.*, 1989; Gledhill *et al.*, 1990) and is very resistant to mechanical and chemical disruption.

Three phases have been described in the evolution of chromatin stability after ejaculation. Immediately after ejaculation, not all the thiol groups are oxidized to form S-S bonds and a large number of bridges between nuclear proteins are non-covalent bound. Secondly, sperm chromatin is stabilized by seminal plasma: zinc from the prostatic fluid is probably the main cause of this stabilization (Arver and Eliasson, 1982; Kvist and Björndahl, 1985; Björndahl and Kvist, 1990). Zinc enters the chromatin and binds to the free thiol groups, thus stabilizing its quarternary structure. Thirdly, when spermatozoa are incubated in seminal plasma (Kvist, 1980; Kvist and Eliasson, 1980; Björndhal and Kvist, 1985; Lipitz *et al.*, 1992; or under capacitating conditions (Huret and Courtot, 1984; Rosenborg *et al.*, 1990), chromatin stability is increased. This state of hyperstability is attributed to a gradual depletion of the chromatin zinc store caused by the seminal or culture medium ligand (citrate, protein), which exposes the free thiol groups and permits the formation of additional interchromatin fibre S-S bridges.

Several assays have been developed to quantify the state of the sperm chromatin; with these methods, differences in the degree of sperm chromatin condensation and chromatin stability can be revealed. The degree of sperm chromatin

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condensation can be evaluated by Toluidine or Aniline Blue staining (Krzanowska, 1982) or by the uptake of an intercalating compound (acridine orange, ethidium bromide and propidium iodide) and by posterior analysis using either fluorescence microscopy (Gledhill, 1983) or flow cytometry (Spano *et al.*, 1984; Evenson *et al.*, 1989; Engh *et al.*, 1992;). This latter technique is probably the most objective and recommended (Gledhill *et al.*, 1990). Chromatin stability can be evaluated by treating the spermatozoa with different chelating, e.g. EDTA, or disulphide-reducing agents, e.g. dithiotreitol (DTT) (Calvin and Bedford, 1971; Beil and Graves, 1977) or anionic detergents, e.g. sodium dodecyl sulphate (SDS), which disrupt non-covalent bonds. After these treatments, the degree of chromatin decondensation can be evaluated by phase-contrast microscopy (Bedford *et al.*, 1973; Kvist, 1980), electron microscopy (Lipitz *et al.*, 1992) or by evaluating the uptake of intercalating compounds (Evenson *et al.*, 1980; Kosower *et al.*, 1992).

It has been observed that ageing of the spermatozoa *in vivo* (de Leon and Boice, 1985) or *in vitro* (Munné and Estop, 1991) produces an increase in chromosome abnormalities and in susceptibility to DNA denaturation (Estop *et al.*, 1993). The influence of capacitation on this damage is controversial. Munné and Estop (1993) have observed a higher percentage of chromosome structural abnormalities in human spermatozoa stored *in vitro* 24 h prior to capacitation than in previously non-incubated capacitated spermatozoa.

A better knowledge of the phenomena occurring in sperm nuclei during in-vitro incubation is important for an understanding of both the mechanisms controlling normal human fertilization and the causes of its defects. The aim of this study is to describe a method to evaluate sperm chromatin condensation and chromatin stability by isolation of sperm nuclei and analysis by flow cytometry, and to investigate the influence of incubation on sperm chromatin.

Materials and methods

Sperm source and preparation

Fertile semen samples were obtained from 16 healthy male volunteers all of whom had fathered a child within the past 2 years and had a normal semen analysis according to World Health Organization criteria (WHO, 1992). All men were asked to abstain from sexual activity for 72 h before the sample was collected. The sample was produced by masturbation into a dry wide-mouthed sterile plastic container. Sperm concentration, percentage of motility, and percentage of normal forms were determined by light microscopy. All samples tested negative for the presence of antisperm antibodies by means of the mixed antiglobulin reaction test (WHO, 1992).

Aliquots of unprocessed semen were analysed 1 and 45 min after ejaculation to determine the sperm chromatin condensation and chromatin stability. The remainder of the ejaculate was divided into 1 ml aliquots in 10 ml centrifuge tubes (ICN Flow Laboratories, High Wycombe, Bucks, UK) and diluted in B2 medium (Bio Mérieux, Marcy l'Etoile, France) followed by two cycles of centrifugation and resuspension of the resulting pellet in B2 medium. The final sperm suspension was centrifuged again, and the pellet was overlayered with 1 ml of B2 medium balanced with 5% CO_2 in air and incubated at 37°C for 30 min to allow motile spermatozoa to swim up. The migrated spermatozoa were then recovered from the tubes with the upper 0.5 ml of the medium. Aliquots of these sperm suspensions were separated immediately and used in futher experiments under the designation of 'migrated spermatozoa'. In order to capacitate the spermatozoa, aliquots of supernatant adjusted to 1×10^6 motile spermatozoa/ml by adding B2 medium were incubated for 6 h at 37°C and 5% CO_2. Spermatozoa treated in this way were referred to as 'capacitated'. Another aliquot of migrated spermatozoa was centrifuged again, and resuspended in Biggers-Whitten-Whittingham (BWW) medium (Biggers *et al.*, 1971) and then incubated for 4 h at room temperature; these spermatozoa were used in further experiments and termed 'aged spermatozoa' (Munné and Estop, 1993). The acrosome reaction was induced in capacitated spermatozoa by calcium ionophore A23187 (Sigma, St. Louis, MO, USA), and these spermatozoa were henceforward termed 'reacted spermatozoa'. Briefly, a stock solution of 200 mM ionophore A23187 in dimethyl sulphoxide (Sigma, St. Louis, MO, USA) was prepared and added to capacitated sperm suspensions up to a final concentration of 10 μM. Spermatozoa were then incubated with the ionophore at 37°C and 5% CO_2 in air for 60 min. After this, spermatozoa were washed twice in Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) to remove the culture medium. A sample of these spermatozoa was analysed to determine the effect of the acrosome reaction on sperm chromatin condensation and chromatin stability. The remaining spermatozoa were used to verify that the acrosome reaction had taken place. Spermatozoa were smeared on microscope slides and air-dried. The smears were then treated for 30 s with methanol to permeabilize the sperm membranes, followed by incubation with fluorescein isothiocyanate-labelled *Pisum sativum* lectin (Sigma, St. Louis, MO, USA). The incubations were carried out in moisture chambers at room temperature for 30 min. The smears were washed for 10 min in abundant water, allowed to air-dry again and examined in an epifluorescence microscope as described elsewhere (Ramirez *et al.*, 1994). All semen samples showed >25% reacted spermatozoa.

Nuclear sperm chromatin condensation

Sperm chromatin condensation was studied by staining the sperm nuclei with propidium iodide. In order to permeate the sperm membrane, enzymatic digestion was performed using trypsin (0.5%) in a spermine-tetrahydrochloride buffer for 10 min at room temperature. To stop trypsin action and to remove double-stranded RNA, trypsin inhibitor and ribonuclease A (12 mg/ml) were added for a further 10 min. Subsequently, ice-cold propidium iodide (50 mg/ml) and spermine-tetrahydrochloride in citrate buffer were added. Samples were incubated for 10 min in the dark before analysis. All reagents used for preparation of semen samples were purchased as the Cycle Test DNA reagent kit from Becton Dickinson (Mountain View, CA, USA).

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Table I. Flow cytometer parameters of unprocessed spermatozoa at 45 min from ejaculation before and after treatment with EDTA + SDS

	FLC			SLC			FL		
	PC	MC	CV	PC	MC	CV	PC	MC	CV
Untreated	29 ± 3	31 ± 1	39 ± 9	12 ± 1	33 ±5	97 ± 5	26 ± 3	27 ± 3	30 ± 7
(n = 10)	(9)	(14)	(11)	(4)	(5)	(1)	(2)	(1)	(6)
EDTA + SDS	27 ± 4	59 ± 10[a]	90 ± 25[a]	8 ± 2[b]	28 ± 4[b]	128 ± 19	24 ± 1	35 ± 3[b]	39 ± 9
(n = 10)	(4)	(25)	(18)	(18)	(20)	(6)	(1)	(5)	(8)

EDTA = ethylenediaminetetracetic acid; SDS = sodium dodecyl sulphate; FLC = forward light scatter; SLC = side light scatter; FL = fluorescence; PC = peak channel; MC = median channel; CV = coefficient of variation of signals within the peak.
Numbers in parentheses are coefficients of intra-assay variation.
[a] P <0.01 versus untreated.
[b] P <0.05 versus untreated.

Chromatin stability and decondensation treatment

Chromatin stability was studied by analysing the sperm chromatin condensation, as described above, after applying different decondensation treatments to the spermatozoa. In the SDS treatment, spermatozoa were suspended in a solution of 1% SDS in borate buffer (0.05 M; pH 9.0) for 60 min (Kvist *et al.*, 1980). For EDTA + SDS treatment, spermatozoa were suspended in a solution of 6 mM EDTA and 1% SDS in borate buffer for 5 min according to the method of Huret (1983). In DTT + SDS treatment, spermatozoa were suspended in sodium borate buffer containing 2 mM DTT and 1% SDS for 5 min to reduce S-S bonds (Rodriguez *et al.*, 1985). To study how time from ejaculation, incubation, capacitation and acrosome reaction influenced the chromatin structure, six fertile semen samples were subjected to the following treatment: SDS, EDTA + SDS, DTT + SDS at 1 min, 45 min, 90 min and 6 h after ejaculation. Spermatozoa studied at 1 and 45 min were from unprocessed semen, the samples studied at 90 min were those previously categorized as migrated spermatozoa, and those incubated for 6 h were those previously categorized as aged spermatozoa in BWW, capacitated in B2 medium, and reacted in B2 medium and then 1 h more with calcium ionophore A23187. Acrosomal status was evaluated in this latter group.

Flow cytometry analysis

All the samples were analysed with a Facscan flow cytometer (Becton Dickinson). To eliminate cellular debris and reduce the orientation-based variability, sperm nuclei were analysed by an interactively adjusted gating using the combination of forward and side light scattering, as suggested by Zucker *et al.* (1992). For each determination 10 000 sperm nuclei were measured. The instrument set-up was checked before each analysis using chicken erythrocyte nuclei, human peripheral blood mononuclear cells and DNA standards (Figure 1). The data were analysed using Consort 30 software from Becton Dickinson. We analysed sperm chromatin condensation by three flow cytometry parameters: forward light scatter (FLC), side light scatter (SLC) and fluorescence (FL). Channel number was directly proportional to cellular size in FLC, cellular complexity in SLC or intensity of cellular fluorescence in FL. From each flow cytometry parameter, three variables were used for the statistical analysis according to Engh *et al.* (1992): (i) peak channel (PC), the channel associated with the largest number of cells; (ii) median channel (MC), the median of the distribution of signals; (iii) coefficient of variation (CV) of signals within the peak, which provides a measure of the peak width. Fluorescence parameters were analysed at the mean peak (sperm nuclei peak).

To test the reproducibility of the technique, two complete sperm chromatin condensation and chromatin stability studies were performed on each unprocessed semen sample (n = 10) before and after treatment with EDTA + SDS at 45 min after ejaculation. The two sets of measurements of sperm chromatin condensation did not vary by >10%. However, after SDS + EDTA treatment the two sets of measurements of FLC and SLC varied by >20%. Fluorescence of sperm nuclei after SDS + EDTA treatment did not vary by >5%. Thus FL is the most suitable flow cytometer parameter to study the degree of condensation and resistance to decondensation of DNA in spermatozoa. Only this parameter was analysed in the following experiments on chromatin state (Table I).

Five fertile donors donated a second semen sample 1 month after the first. These additional samples underwent the same study as the previous batch. Repeated measurements of sperm chromatin condensation and chromatin stability on two different samples from each of the same five fertile donors gave within-subject coefficients of variation for mean channel of fluorescence of 5.3% for sperm chromatin condensation and 8.2% for chromatin stability after SDS + EDTA treatment at 45 min after ejaculation.

Statistical analysis

Data are presented as the mean ± SD. Shapiro–Wilk's test was used to check normal distribution. The data in Table I were analysed by the multivariate Hotteling's T2 and matched t test. Analysis of variance of repeated measurements with two grouping factors (treatment and time) were used to analyse data from Table II, and differences between the means were examined by orthogonal comparisons.

Results

The sperm nuclei analysed by FLC and SLC exhibited a dispersed distribution. However, the fluorescence signal had the narrowest distribution. In all untreated samples, the sperm peak appeared ~0.5 of the fluorescence intensity of the haploid cells (MCα55) (Figure 1). After decondensing treatment with SDS + EDTA at 45 min from ejaculation we observed first

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Table II. Effects of different conditions of incubations and treatment on the uptake of propidium iodide by sperm nuclei

Treatment	Time post-ejaculation		Incubation for 6 h			
	Fresh 1 min	Fresh 45 min	Migrated 90 min	Aged (BWW)	Capacitated Reacted (B2 medium)	(B2 medium + A23187)
Untreated	29.0 ± 1.9*	28.9 ± 1.8	27.0 ± 1.3	27.1 ± 1.1	26.9 ± 1.8	27.3 ± 1.2
SDS	36.2 ± 2.0[a]	27.9 ± 2.1[b]	28.4 ± 2.0[b]	28.2 ± 1.5[b]	27.9 ± 2.0[b]	27.0 ± 1.1[b]
SDS + EDTA	37.8 ± 1.7[a]	37.5 ± 2.8[a,c]	33.5 ± 1.[1a,c,d,e]	28.0 ± 1.2[d,e,g]	28.2 ± 1.2[d,e,g]	27.3 ± 1.9[d,e,g]
DTT + SDS	39.2 ± 2.1[a]	38.3 ± 1.6[a,c]	34.1 ± 1.8[a,c,h]	33.7 ± 1.8[a,c,f,h]	34.1 ± 1.6[a,c,f,h]	34.0 ± 1.2[a,c,f,h]

EDTA = ethylenediaminetetracetic acid; SDS = sodium dodecyl sulphate; DTT = dithiothreitol; BWW = Biggars–Whitten–Whittingham medium.
*Represents mean of mean channel ± SD.
[a]$P < 0.05$ versus fresh spermatozoa untreated at the same time.
[b]$P < 0.02$ versus fresh spermatozoa treated with SDS at 1 min.
[c]$P < 0.01$ versus fresh spermatozoa treated with SDS at the same time.
[d]$P < 0.01$ versus fresh spermatozoa treated with SDS+EDTA at 1 min.
[e]$P < 0.01$ versus fresh spermatozoa treated with SDS+EDTA at 45 min.
[f]$P < 0.01$ versus fresh spermatozoa treated with SDS+EDTA at the same time.
[g]$P < 0.05$ versus migrated spermatozoa treated with SDS+EDTA
[h]$P < 0.05$ versus fresh spermatozoa treated with DTT+SDS at 1 and 45 min.

an increase in the flow cytometry parameter of FLC, which reflected an increase in the sperm nuclei size, then a decrease in the SLC parameter, which reflected a reduction in nuclei density, and finally an increase in the fluorescence of sperm nuclei, concomitant with an increase in the uptake of propidium iodide (Figure 2, Table I). As previously noted, FL is the most suitable flow cytometer parameter to study the degree of condensation and resistance to decondensation of DNA in spermatozoa. Only this parameter was analysed in the following experiments on chromatin state.

The sperm chromatin condensation was not related to time after ejaculation. A lesser, but not significant, stainability of the selected spematozoa (migrated, aged, capacitated and reacted) than in whole semen (1 and 45 min from ejaculation) was observed. However, the nuclear stability showed a strong relationship with time. Unprocessed spermatozoa at 1 min from ejaculation underwent decondensation by all assessed treatments (anionic detergent, chelating or disulphide-reducing agents). Unprocessed spermatozoa at 45 min after ejaculation and migrated spermatozoa did not undergo decondensation with SDS treatment, but decondensation occurred after treatment with chelating or disulfide-reducing agents. Migrated spermatozoa after SDS + EDTA or DTT + SDS treatment underwent significantly less decondensation than whole semen, suggesting a greater chromatin stability. Aged spermatozoa were decondensed only after treatment with disulphide-reducing agents (Table II). No difference was found in sperm chromatin condensation and chromatin stability between spermatozoa incubated under non-capacitating conditions (aged), capacitating conditions (capacitated) and reacted spermatozoa (Figure 3, Table II).

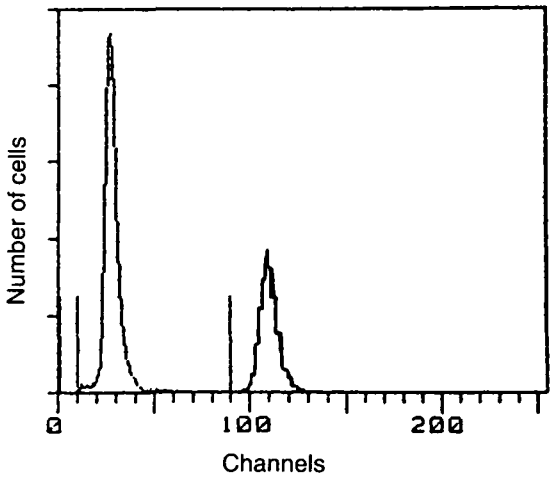

Figure 1. Flow cytometric analysis of a mixture of migrated spermatozoa (1N) and peripheral blood mononuclear cells (2N) by fluorescence after propidium iodide staining. The first peak (channels 20–40) represents sperm nuclei. The second peak (channels 100–120) represents nuclei from peripheral blood mononuclear cells. The vertical lines at channels 10 and 90 show the total 1N and 2N peaks respectively. All cells were processed as indicated in the text.

Discussion

Sperm nuclei size (FLC) and density (SLC) distributions determined by flow cytometry are characterized by an asymmetric peak with an extension toward greater size and density values respectively. These peaks are wider than those observed when we analyse the fluorescence parameter, probably because the variable presence of debris after removing sperm cytoplasm and membranes affects only size and density parameters but not the uptake of intercalating DNA compounds, such aspropidium iodide. This could be the main reason why FLC and SLC show a high coefficient of intra-assay variation, and why fluorescence exhibits a low coefficient of variation. Another possibility, suggested by Zucker *et al.* (1992), is related to the idea that the particle morphology may be altered by the staining process with propidium iodide. Therefore we recommend only the use of the flow cytometry parameter of fluorescence for the study of the sperm chromatin state.

Differences in the presentation of results (x-axis scale) (Clausen *et al.*, 1982), and in preparation and staining techniques (Steen and Hanson, 1981; Clausen *et al.*, 1982; Evenson *et al.*, 1986) are the main technical reasons why comparison between studies on DNA flow cytometry in human ejaculates is so difficult (Fossa *et al.*, 1989).

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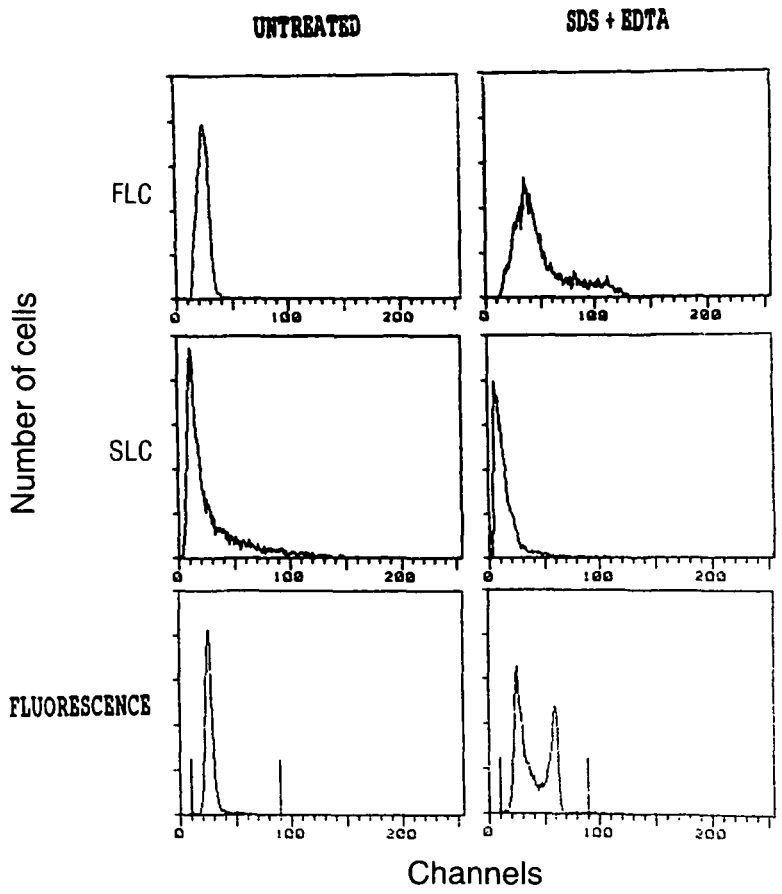

Figure 2. Flow cytometry analysis of a migrated sperm sample by forward light scatter (FLC, relating to cell size), side light scatter (SLC, relating to cell density) and fluorescence (relating to DNA uptake of propidum iodide). Spermatozoa were analysed at 45 min from ejaculation before (untreated) and after treatment (treated) with sodium dodecyl sulphate (SDS) + EDTA (see text).

Comparison of the coefficients of variation of fluorescence, 5.3% for sperm chromatin condensation and 8.2% for chromatin stability after SDS + EDTA treatment at 45 min after ejaculation, with coefficients of variation for sperm concentration, total sperm number, motility, progression and morphology reported respectively as being 46, 58–80, 27–36 (range 6–49), 19–20 (range 2–49) and 15 (Overstreet, 1984; Working, 1988) indicates that chromatin structure displays less variation over time than other semen parameters previously studied. These data are in agreement with the results previously reported by Evenson *et al.* (1991). Such a comparison of biological variation emphasizes the utility of quantitative measurements of chromatin structure as markers for reproductive potential. The high variability in the sperm chromatin decondensation observed by other authors (Huret, 1986) between subjects and different ejaculates might be due to the indirect method (variations of the head size by optical microscopy) used by these authors as compared with the direct method (flow cytometer) we used.

Sperm preparation techniques for the selection of highly mobile spermatozoa produce spermatozoa which have improved nuclear maturity, as shown by our observations on the reduced uptake of stain by migrated spermatozoa after decondensation treatment compared with spermatozoa in whole semen. Pasteur *et al.* (1992), using discontinuous Percoll gradient centrifugation, and Huret and Courtot (1984) using swim-up, showed that spermatozoa selected in this way belong to a population with a more compact and denser chromatin compared to the residual spermatozoa.

Condensation of the chromatin during spermatogenesis, and epididymal transport and its decondensation at the time of fertilization are essential for successful fertilization. Alterations in chromatin structure are associated with infertility. Although interstudy comparisons are difficult because of the different methods used, in general, there is agreement in associating infertility with chromatin decondensation after SDS treatment or with an excess of decondensation after SDS + EDTA at 45 min post-ejaculation (Bedford *et al.*, 1973; Kvist, 1980; Kvist and Eliasson, 1980; Huret, 1986; Kosower *et al.*, 1992). However, hyperstability of human sperm chromatin is interpreted variously as detrimental to delivery of the male genome in the oocyte (Rosenborg *et al.*, 1990; Huret and Miquereau, 1984), or as essential to avoid chromosomal damage, and thus produce successful fertilization (Lipitz *et al.*, 1992). These discrepancies may be due to the fact that these studies were carried out by subjective methods (analysing variations in sperm morphology).

The mechanisms involved in the changes in chromatin stability in spermatozoa are unknown. Jager *et al.* (1983) observed that decondensation depends on time, temperature and pH, suggesting that human sperm decondensation is more a physico-chemical process than an enzymatic one. Human

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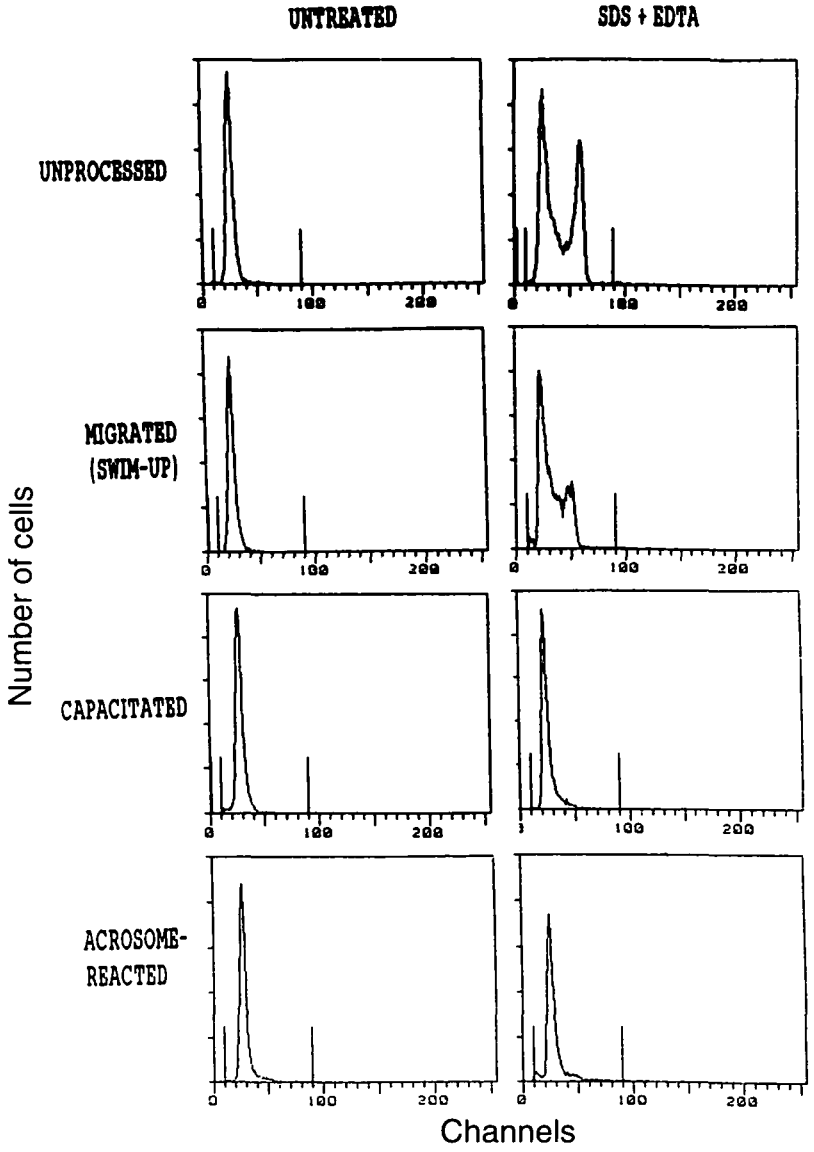

Figure 3. Flow cytometric analysis before (untreated) and after (treated) treatment of spermatozoa with sodium dodecyl sulphate (SDS) + EDTA under different conditions: fresh (unprocessed), after swim-up (migrated), incubated 6 h in B2 medium (capacitated) and incubated 6 h in B2 medium plus 1 h with A23187 (reacted).

spermatozoa generate reactive oxygen radicals which induce the conversion of sulphydryl groups to disulphide bridges (Marushige and Marushige, 1975; Huang *et al.*, 1984). We have shown that intracellular Ca^{2+} ions, which are very important in the physiology of spermatozoa (capacitation, hyperactivation and acrosome reaction), are not related to chromatin states because the incubation of capacitated spermatozoa with calcium ionophore A23187 did not have any influence on the chromatin stability. However, they are related to acrosome reaction. Nevertheless, findings such as those of Delgado *et al.* (1980) that trysin inhibitor inhibits decondensation induced by DTT, or those of Jager *et al.* (1983) that phenylsulphonil fluoride inhibits decondensation, suggest that endogenous enzymes are involved in human sperm decondensation.

In summary, the assessment of nuclear maturity by the uptake of propidium iodide after different decondensation treatments as determined by flow cytometry appears to be a direct technique to use in the andrology laboratory.

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