A novel in vitro sperm head decondensation protocol for rapid flow cytometric measurement of deoxyribonucleic acid content

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Objective: To set up a novel protocol of sperm head in vitro decondensation that obviates the problematic effect of the variable degree of sperm chromatin packaging on DNA staining needed for flow cytometric analysis.
Design: Development of a new cytofluorimetric assay.
Setting: University laboratory.
Patient(s): Semen specimens were obtained from normospermic healthy volunteers at the Department of Life and Environmental Sciences, Università Politecnica delle Marche.
Intervention(s): Setup of the novel in vitro sperm head decondensation protocol; sperm were then stained and analyzed by flow cytometry to measure DNA content.
Main Outcome Measure(s): Mean fluorescent channel, DNA content, percentage diploid sperm.
Result(s): Native nondecondensed fluorochrome-labeled sperm show significant under-staining, resulting in an underestimated C-value (approximately 1.4 pg). This protocol ensures stoichiometric staining of sperm DNA, which becomes fully reachable by fluorescent probes and makes the diploid (7.12 pg) over haploid (3.56 pg) sperm frequency quantification easier.
Conclusion(s): This study establishes a simple method for in vitro sperm head decondensation, which allows accurate detection of the real sperm DNA content. (Fertil Steril® 2013;99:1857–61. ©2013 by American Society for Reproductive Medicine.)
Key Words: Sperm decondensation protocol, flow cytometry, DNA content, sperm chromatin packaging

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Flow cytometry (FCM) has evolved into a greatly dependable technology, and new fluorochromes and techniques having potential application to sperm investigation by FCM are continuously being planned (1, 2). The accurate analysis of sperm nuclear status is made difficult by the unique compacted nature of human spermatozoa, thus hindering fluorochromes and other probes' accessibility (3, 4). During spermiogenesis, positively charged protamines interact with the negatively charged DNA, allowing the DNA strands to be brought into close proximity with each other to facilitate compaction. The chromatin stability is further reinforced by the creation of intra- and intermolecular protamine disulphide bonds (5). Current decondensation assays of sperm DNA are based on the use of different chemicals that allow the loosening of chromatin structure (6, 7). An effective decondensation of sperm nuclei destroys the spatial integrity of sperm cells, impairing further analysis if samples are not previously fixed on a solid substrate (7). In this study we propose a procedure of sperm head

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TABLE 1

DNA content (pg per nucleus) of human sperm obtained after testing different intrinsic variables (i.e., PFA, heparin, DTT, and incubation time).

		Decondensation			
Parameter	**Stabilization (% PFA)**	**DTT (mM)**	**Heparin (U/mL)**	**Time (min)**	**DNA content (pg/nucleus)**
Native sample	–	–	–	–	1.43 ± 0.09
Effects of stabilization solution	–	5	100	30	0
	0.2	5	100	30	3.53 ± 0.03
	0.5	5	100	30	3.52 ± 0.04
	1	5	100	30	3.55 ± 0.05
	2	5	100	30	3.14 ± 0.06
	4	5	100	30	2.10 ± 0.03
Effects of decondensation solution	0.5	–	–	30	1.40 ± 0.06
	0.5	5	–	30	1.88 ± 0.03
	0.5	–	100	30	1.54 ± 0.03
	0.5	5	100	30	3.55 ± 0.04
Effects of time of decondensation	0.5	5	100	0	1.38 ± 0.04
	0.5	5	100	15	3.09 ± 0.14
	0.5	5	100	30	3.54 ± 0.08
	0.5	5	100	45	3.6 ± 0.09
	0.5	5	100	60	3.6 ± 0.09

Note: Results are expressed as mean ± SD. Every variable was tested on 10 aliquots from different samples and in total approximately 2,000,000 gated sperm were analyzed.

Antonucci. In vitro sperm decondensation protocol. Fertil Steril 2013.

in vitro decondensation that obviates the troublesome effect of the variable degree of sperm chromatin packaging on DNA staining. This protocol ensures stoichiometric staining of sperm DNA, which becomes fully reachable by propidium iodide (PI), allowing accurate quantification of DNA content (C-value) and to clearly distinguish haploid from diploid sperm.

MATERIALS AND METHODS

Semen Specimens and Samples Processing

Normospermic semen specimens were obtained with informed consent from healthy volunteers at the Department of Life and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy. Institutional review board approval was obtained.

Semen samples were provided after sexual abstinence of 3–5 days by 10 volunteers, analyzed according to World Health Organization guidelines (8), aliquoted (2×10^6 cells per aliquot), and stored at −80°C. For setting up of the protocol, an aliquot of each patient was analyzed for every intrinsic variable taken into consideration (i.e., paraformaldehyde [PFA], heparin, dithiothreitol [DTT], and the incubation time) (Table 1).

In Vitro Sperm Head Decondensation

The in vitro sperm head decondensation procedure is based on two steps: [1] chromatin stabilization with a mild chromatin cross-linking agent (PFA); and [2] a controlled decondensation by combining DTT (reducing agent for protamine disulphide bonds) (9), heparin (as a protamine remover by electrostatic interactions) (10), and Triton X-100.

After thawing, 2×10^6 spermatozoa were washed in 4 mL of phosphate-buffered saline (PBS) and 0.5% bovine serum albumin. Cells were centrifuged at 400 × *g* for 10 minutes, resuspended with 500 μL of chromatin stabilization solution composed of 0.5% PFA in filtered PBS, and then kept shaking on ice in the dark for 10 minutes. Cell suspensions were then washed, centrifuged at 400 × *g* for 10 minutes, and resuspended in 600 μL of decondensation solution (5 mM 1,4- DTT, 100 U/mL heparin, and 0.1% Triton X100 in PBS). Suspension was incubated at a controlled temperature of 25°C for 30 minutes, in the dark.

To set up the protocol several intrinsic variables were taken into consideration: the effects of the chemicals used (PFA, heparin, and DTT) and the incubation time (0–60 minutes) required for effective decondensation (Table 1).

Preparation of Chicken Red Blood Cells and Sperm for DNA Content Analysis

Chicken red blood cells (CRBCs) were used as internal DNA content standard (2.5 pg per nucleus). Spermatozoa, both decondensed and native, and CRBCs were postfixed in 70% ethanol for approximately 2 hours and resuspended after centrifugation in 1 mL DNA staining solution, (40 μg/mL PI, 0.1 mg/mL RNase-DNase free, and 0.1% Triton X-100) for 30 minutes in the dark at room temperature until FCM analysis was performed.

FCM Analysis

Flow cytometry was performed using an Epics XL flow cytometer (Beckman Coulter). A histogram of sperm DNA content distribution (FL3 linear scale) was used to quantify the degree of sperm DNA accessibility to PI. A minimum of 10,000 gated cells was analyzed. All offline analysis was performed on FCS files using Weasel software developed by The Walter and Eliza Hall Institute (WEHI), The Cytometry Laboratory. Fluorescence intensity (FI) of histograms was expressed as mean fluorescence channel (MFC). The DNA content (picograms of DNA per nucleus) was calculated by the formula $(X/S) \times 2.5$, where 2.5 represents picograms of