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# Cryopreservation and sperm DNA integrity

L. Gandini[1], F. Lombardo[1], A. Lenzi[1], M. Spanò[1,2] and F. Dondero[1,*]
[1]*Laboratory of Seminology and Reproductive Immunology, Department of Medical Pathophysiology, University of Rome 'La Sapienza', 00100 Rome, Italy;* [2]*Section of Toxicology and Biomedical Sciences, ENEA CR Casaccia, Via Anguillarese 301, 00060 Rome, Italy; *Author for correspondence (e-mail: franco.dondero@uniroma1.it)*

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**Abstract**

Cryopreservation of sperm is an extremely important issue in the field of male infertility as freezing can have detrimental effects on a variety of sperm functions, some of them not accessible to the traditional semen quality analysis. In this study, chromatin structure variations in human spermatozoa in semen were studied with the sperm chromatin structure assay (SCSA), both before and after cryopreservation. Samples were divided into two aliquots: the first was analysed without further treatment, while the second was stored in liquid nitrogen at −196 °C using standard cryopreservation techniques. The fresh and thawed aliquots were also assessed by light and fluorescence microscopy (after Acridine Orange staining, AO), and computer-assisted semen analysis (CASA) of motility. Overall sperm quality was found to deteriorate after cryopreservation. When thawed spermatozoa were subjected to an extra swim-up round, a general improvement in nuclear maturity was seen in post-rise spermatozoa.

*Abbreviations:* ALH – Amplitude of Lateral Head displacement; AO – Acridine Orange; ART – Assisted Reproductive Technology; BCF – Beat Cross Frequency; CASA – Computer-Assisted Semen Analysis; DFI – Dna Fragmentation Index; FCM – Flow Cytometric; LIN – Linearity Index; SCSA – sperm chromatin structure assay; VEL – Curvilinear Velocity; WHO – World Health Organization

## Introduction

Sperm DNA chromatin integrity appears to be important for correct spermatozoa functioning (Agarwal and Said 2003; Sakkas et al. 2003) and methods focusing on characterisation of sperm chromatin condensation and stability have received increasing attention (Perreault et al. 2003). One of the most interesting techniques today available is the flow cytometric (FCM) sperm chromatin structure assay (SCSA) (Evenson et al. 1980, 2002), which has demonstrated to be an independent predictor of fertility either *in vivo* (Evenson et al. 1999; Spanò et al. 2000) or *in vitro* (Larson-Cook et al. 2003; Saleh et al. 2003). The assessment of sperm DNA/chromatin quality in human semen samples, carried out using a variety of techniques, is becoming a relevant end-point as sperm DNA and chromatin abnormalities have been associated with failures in *in vitro* assisted

reproductive techniques (ART) (Sun et al. 1997; Lopes et al. 1998; Host et al. 2000; Duran et al. 2002; Morris et al. 2002; Tomsu et al. 2002; Benchaib et al. 2003; Tesarik et al. 2004).

Sperm cryopreservation may be of help to any assisted reproduction program, mainly, for example in the case of semen cryopreserved prior to chemo- or radiotherapy, which may induce azoospermia or teratogenecity, together with sperm aneuploidies and DNA fragmentation (Martin et al. 1997; Robbins et al. 1997; Morris 2002; Meistrich and Byrne 2002; Ståhl et al. 2004) It is well known that temperature variations (e.g. during cooling, freezing and thawing) can cause detrimental changes in sperm functions (i.e. motility) and structure (nucleus, membrane and mitochondria) that can impair its fertility capabilities (Spanò et al. 1999; Donnelly et al. 2001a, 2001b; Duru et al. 2001; Duty et al. 2002; Thompson-Cree et al. 2003; Isachenko et al. 2004), but the results are somehow conflicting as far as the extent of the induced cell damage and the influence of the sperm processing procedure employed.

Therefore, it would be interesting to assess nuclear chromatin damage induced by extreme temperature changes and express sperm quality in terms of cell-to-cell integrity using a variety of endpoints, thus helping the physician increase the chance of achieving successful fertilisation. In this study the FCM SCSA was used to evaluate the chromatin structure in fresh semen of normozoospermic subjects and to measure sperm nuclear integrity changes after cryopreservation. Results were compared with those of Acridine Orange (AO) staining on slide, of standard microscopy assessment and of sperm motility by computer-assisted semen analysis (CASA). The same endpoints have also been applied to assess sperm quality of the post-rise thawed sperm undergoing the swim-up technique.

## Materials and methods

### *Subjects*

We studied 19 healthy subjects, aged 30–38 years, attending our Laboratory of Seminology and Immunology of Reproduction, Rome, Italy for pre-marriage checks. All samples were considered normal under World Health Organization guidelines (WHO 1999). None of the subjects had received medical treatment in the 3 months prior to the study. They were all advised on the study's nature and purpose and signed a detailed consent form.

### *Semen quality analysis*

Semen was collected by masturbation after 3–5 days of abstinence. Ejaculates were left to liquefy at 37 °C and examined within 1 h of collection. The parameters considered were ejaculate volume (ml), sperm concentration ($n \times 10^6$/ml), forward motility (%), percentage of atypical forms (sperm and head morphology). The evaluation was carried out under a light microscope under WHO criteria (WHO 1999). The same biologist performed all seminal fluid examinations in order to minimize variability. CASA of sperm motility was also performed. The CASA system (Cell Soft; Cryo Resources, New York, NY, USA) was equipped with a heated (35 °C) stage. The parameters assessed were curvilinear velocity (VEL, $\mu$rn/s), linearity index (LIN), amplitude of lateral head displacement (ALH, $\mu$m), and beat cross frequency (BCF, Hz). The CASA analysis was performed under the following parameter settings: 25 frames to be analysed, 25 frames/s, 2 track points for calculation of motility, 8 track points for calculation of velocity, velocity range 10–150 $\mu$/s, cell size range 4–20, 8 track points for ALH calculation, minimum velocity for ALH calculation 20 $\mu$m/s, minimum linearity for ALH calculation 3.5 $\mu$m. At least 300 cells were examined in each sample. Each sample was divided into two aliquots: (A) the first was evaluated without further processing; (B) the second was stored under standard cryopreservation techniques at −196 °C in liquid nitrogen. Finally, (C) an aliquot of the cryopreserved sample was subjected to a swim-up procedure after thawing. Each aliquot was analysed under the light microscope, by fluorescence microscopy after AO staining and by FCM SCSA.

### *Semen cryopreservation*

After liquefaction, an aliquot from each sample was diluted (1:1) with freezing medium (test yolk buffer; Irvine Scientific, Santa Ana, CA, USA).

After 15 min at 37 °C for equilibration, the mixture was aspirated into 0.25 ml straws, and powder-sealed. The straws were frozen in liquid nitrogen vapour for 8 min at −80 °C and then plunged into liquid nitrogen (−196 °C) for storage. Finally, samples were removed from the liquid nitrogen, thawed at room temperature for 15 min and examined by light microscopy and CASA system.

*Swim-up after cryopreservation*

After thawing, semen aliquots were diluted with Earle's solution (1:2) and centrifuged for 10 min at $300 \times g$. The supernatant was discarded and 0.5 ml Earle's solution was layered on the pellet. Spermatozoa were then allowed to migrate in the solution in 5% $CO_2$ for 30 min at 37 °C. The solution was then carefully collected and examined by light microscopy and CASA after migration.

*Acridine orange test*

The spermatozoa were washed twice in a salt solution, smeared on a slide, air-dried, fixed overnight in Carnoy's solution (3:1, methanol:glacial acetic acid) and air-dried once more. Slides were dipped in a 0.1 M citric acid solution (pH 2.5) for 5 min at room temperature and then rinsed several times with distilled water. They were then stained with an AO solution (0.2 mg/ml in water). After 5 min, each smear was washed with distilled water and protected with a coverslip. They were examined with a fluorescence microscope (Dialux 22, Leica, Darrmstadt, Germany) with a 490 nm excitation light and 530 nm barrier filter. Nuclei from 500 spermatozoa were examined and scored as green- or red-fluorescing. To minimize photobleaching effects, each microscopic field was evaluated for no more than 40 s. Spermatazoa may show green fluorescence at the head (bicatenary DNA, normal) or red-fluorescence at the head (monocatenary DNA, denatured).

*FCM SCSA*

The SCSA procedure described by Evenson et al. (2002) was used with minor modifications. Samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA, pH 7.4) containing 10% glycerol and transferred to 2 ml Eppendorf snap cap tubes, with a final sperm concentration of $2 \times 10^6$/ml. Tubes were stored on dry ice until transfer to an ultra-cold freezer (−80 °C) for storage until FCM analysis. All samples were coded and FCM measurements were carried out blindly. After thawing on crushed ice, sperm cells were subjected to *in situ* partial DNA denaturation and then stained with AO. 0.2 ml aliquots were mixed with 0.4 ml of a low pH detergent solution (0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.2). After 30 s, cells were stained by adding 1.2 ml of a solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) containing 6 mg/l of chromatographically-purified AO (Molecular Probes, Eugene, OR, USA). Cells were analysed by a FACScan flow cytometer (Becton Dickinson, San Josè, CA, USA), equipped with 15 mW air-cooled Arrgo laser. The SCSA analysis is based on the phenomenon that chromatin with abundant double DNA breaks has a tendency to denaturate when exposed to acid–detergent, whereas normal chromatin remains stable. Acridin orange stains the native double-stranded DNA and the single-stranded nucleic acids and in excitation of blue-light the intact DNA emits green (530 ± 30 nm) fluorescence, whereas the denaturated DNA emits red fluorescence (>630 nm) (Evenson and Jost 2000).

About 5000 cells were measured for each sample. All measurements began 3 min after AO staining, at a flow rate of 200 cells/s. The extent of DNA denaturability is expressed as the DNA Fragmentation Index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity. DFI hereby expresses the percentage of cells containing denaturated DNA, mostly because of the presence of DNA breaks (Evenson et al. 2002). DFI was calculated using the ListView software (Phoenix Flow systems, San Diego, CA, USA).

*Statistical analysis*

This was carried out using the SPSS software (SPSS Inc., Chicago, IL, USA). Analysis of variance for repeated measures was used to assess the overall significance.

## Results

### *Microscopy*

All the results are summarized in Table 1. Mean values (± SD) for fresh semen samples were: volume 3.8 ± 1.1 ml (range 3.0–7.0 ml), sperm concentration/ml 76.4 ± 43.7 × $10^6$ (range 28–180 × $10^6$), forward motility 54.7 ± 8.6% (range 35–65%), total atypical forms 44.4 ± 8.6% (range 36–68%), head atypical forms 32.9 ± 8.7% (range 20–52%). Kinetic parameters values evaluated by CASA were: VEL 58.5 ± 3.6 μm/s (range 52.8–65.3 μm/s), LIN 6.1 ± 0.3 (range 5.4–6.6), ALH 3.9 ± 0.4 μm (range 3.0–4.4 μm), BCF 13.9 ± 0.3 Hz (range 13.2–14.3 Hz). Green fluorescent head percentage after AO staining was 71.7 ± 7.7% (range 59–89%). The sperm parameters for microscopically evaluated raw semen, cryopreserved, and post-rise spermatozoa after cryopreservation are shown in Table 1. A significant difference was seen for all parameters between fresh and cryopreserved samples. Post-rise spermatozoa from cryopreserved samples showed a clear improvement in associated variables over the cryopreserved samples. Taken together, the results indicate a general deterioration in semen quality caused by cryopreservation, whereas post-rise spermatozoa represent a subpopulation of cells characterized by an improvement in kinetic parameters, an enhanced percentage of green fluorescent heads and a reduction in morphologically abnormal forms.

*Table 1.* Mean and SD of sperm parameters from (A) raw semen, (B) after cryopreservation and (C) post-rise spermatozoa from the cryopreserved samples.

| Methods | A | B | C |
|---|---|---|---|
| Light microscopy | | | |
| Forward motility (%) | 54.7 ± 8.6 | 35.0 ± 8.2b | 84.5 ± 6.4a |
| Atypical forms (%) | 44.4 ± 8.6 | 51.5 ± 8.7b | 28.4 ± 4.5a |
| Abnormal heads (%) | 32.9 ± 8.7 | 36.9 ± 7.4b | 24.4 ± 4.3a |
| Fluorescence microscopy | | | |
| Green fluorescent heads (%) | 71.7 ± 7.7 | 67.4 ± 5.9b | 76.9 ± 3.1a |
| CASA | | | |
| VEL (μm/s) | 58.5 ± 3.6 | 51.9 ± 3.5b | 76.9 ± 3.1a |
| ALH (μm) | 3.9 ± 0.4 | 3.7 ± 0.2 | 4.9 ± 0.2a |
| LIN | 6.1 ± 0.3 | 5.6 ± 0.5b | 7.6 ± 0.2a |
| BCF (Hz) | 13.9 ± 0.3 | 13.1 ± 0.4b | 15.0 ± 0.2a |
| FCM SCSA | | | |
| DFI (%) | 12.1 ± 7.9 | 15.2 ± 8.7b | 10.8 ± 6.9a |

CASA = computer-assisted semen analysis; VEL = curvilinear velocity; ALH = amplitude of lateral head displacement; LIN = linearity; BCF = beat cross frequency; FCM = flow cytometric method; SCSA = sperm chromatin structure assay; DFI = DNA Fragmentation Index (ratio of red to total (red plus green) fluorescence intensity). Statistically significant differences ($p < 0.05$) after analysis of variance: a B vs. A; b B vs. C.

### *SCSA*

The DFI numerical results of the SCSA assessment are also given in Table 1. DFI mean values (± SD) calculated on fresh samples was 12.1 ± 7.9% (range 4.5–40.1%). This value significantly rised to 15.2 ± 8.7% for sperm thawed after cryopreservation. Frozen sperm undergoing the swim-up selection are characterized by a significantly lower value (10.8 ± 6.9%), better than that observed in sperm cells from fresh ejaculates. The increase in abnormal sperm cell frequency may be indicative of the physical stresses experienced by the cryopreserved samples, leading to chromatin deterioration in some of the spermatozoa present in the native sample. However, the migrated spermatozoa (post-rise) population showed a general improvement in all SCSA-related parameters in comparison with the unselected populations.

## Discussion

Cryopreservation is widely used in ART to preserve male fertility before cytotoxic chemotherapy, radiotherapy, or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction. Freezing of sperm before initiation of treatment gives patients a sort of fertility insurance that may allow them to father their own children through the use of IVF or intracytoplasmic sperm injection (ICSI). Despite many refinements in cryopreservation and separation methodology, the salvage of post-thaw sperm remains poor even though it seems not to hamper fertilization or pregnancy rate in ART (Hammadeh et al. 2001a; Kuczynski et al. 2001). The most commonly reported detrimental effect of cryopreservation on human sperm is a marked reduction in motility (Kramer et al. 1993), but also DNA integrity has recently started to be extensively checked.

Chromatin in its final form is packed into the sperm nucleus as the result of a long, complex

process that begins in the earliest stages of spermiogenesis, where transition proteins replace the original histones finally substituted by protamines (Boissonneault 2002; Brewer et al. 2002). Progressive packing of sperm chromatin seems to be associated with a complex DNA cutting and ligating process (Marcon and Boissonneault 2003). During the transit through the epididymis, epididymal fluid also participates in the evolution of chromatin stability. This pathway leads to a smaller, mature sperm cell, which needs less energy for its motility and is ready for fertilisation.

DFI is a SCSA parameter which closely mirrors sperm chromatin integrity defined as susceptibility of DNA to acid induced denaturation in situ; it has been shown to correlate with the results obtained from other more direct tests, such as the TUNEL assay (Gorczyka et al. 1993; Sailer et al. 1995; Zini et al. 2001) and the COMET assay (Anderson et al. 1997; Aravindan et al. 1997; Cordelli et al. 2003). The DFI values obtained in this study therefore seem to be linked to chromatin alterations associated with DNA nicks. This corroborates previous results obtained by using the SCSA approach (Spanò et al. 1999) where some deterioration of sperm nuclear integrity after cryoconservation has been reported. Using the Comet assay, other Authors have noted a differential effect depending if the donor was normozoospermic or not (Donnelly et al. 2001b). Other studies attributed the sperm damage after cryopreservation more to the membrane damage induced by thawing than to sperm integrity evaluated by the Tunel assay (Duru et al. 2001). Noteworthy, SCSA, being an FCM technique takes advantage of the peculiar features of this computer-interfaced technology, such as automation, standardization, reproducibility, objectivity, speed, precision, and statistical robustness.

Notwithstanding the diverse methods available for evaluation of the maturity of sperm chromatin, most evidence highlights the key role of chromatin integrity during spermatogenesis and fertilisation. A lower packing quality in morphologically normal and motile spermatozoa may be one of the most limiting factors for fertilisation capability. The tertiary and quaternary chromatin structure is thus of prime importance in protecting genetic information, and possibly in early post-fertilization events. The definition of normal values for fresh semen may be very useful for any clinical application. However, there have been proposed SCSA-DFI values where above which human fertility becomes problematic, regardless the sperm number, concentration, morphology and motility. Several studies have observed that when DFI values are higher than 30%, the chance to father a child is greatly decreased, either *in vivo* and *in vitro* (Evenson et al. 1999; Spanò et al. 2000; Larson-Cook et al. 2003; Saleh et al. 2003). When examining the cell fraction with abnormal chromatin in our group of normozoospermic subjects, we observed a mean DFI value of 12.1 ± 9.8%. It is worth noting that the highest value of 40.4% was from semen of the donor who had the lowest sperm concentration ($28 \times 10^6$/ml) and one of the highest numbers of sperm cell abnormalities (57%), although this was still in the normal range for normozoospermia. SCSA DFI values in this range are in close agreement with the figures from cohorts of healthy fertile men when compared to the higher values obtained in groups of dyspermic men or with fertility problems (Evenson et al. 1980, 1999; Zini et al. 2001, 2002: Saleh et al. 2002, 2003). This is a quite general finding as the same difference has been noted in several other studies measuring sperm DNA damage with other techniques (Lopes et al. 1998; Host et al. 1999; Gandini et al. 2000; Irvine et al. 2000; Carrell and Liu 2001; Hammadeh et al. 2001b; Erenpreisa et al. 2003; Muratori et al. 2003). However, SCSA parameters are generally weakly correlated with WHO parameters and therefore they represent an independent parameter to be incorporated in semen quality assessment (Evenson et al. 1991, 1999, 2002; Spanò et al. 1998, 2000).

Changes in nuclear maturity are therefore used as a measure of sperm quality in fresh semen and to check gamete quality following sperm manipulation, after cryopreservation (Donnelly et al. 2001a, b; Duty et al. 2002; Isachenko et al. 2004) but also after sperm selection procedures (Larson et al. 1999). In this study, we have considered the swim-up technique, which was used to select spermatozoa from cryopreserved samples. We have observed the that swim-up techniques, which sorts a subpopulation of highly motile cells, select also cells with better performances assessed by light and fluorescent microscopy, together with a remarkable improvement of the sperm chromatin features as evaluated by the SCSA. In particular, the

fraction of DFI markedly decreased in all samples and the mean value was almost one-third of the fraction observed in native semen samples. Therefore, post-rise spermatozoa are characterized by superior and more homogeneous chromatin structure characteristics than that of unselected fresh (and, in the vast majority of cases, also cryopreserved) semen samples. There are several reports confirming the results from this study on the superior performances of the swim-up procedure to improve the sperm nuclear integrity and the overall semen quality of the fresh unselected semen, basing the analysis of defective sperm either by using the SCSA (Golan et al. 1997; Spanò et al. 1999; Zini et al. 2000) or other techniques (Molina et al. 1995; Younglai et al. 2001; Lachaud et al. 2004). However, other studies reported different findings with other separation techniques being more efficient (Hammadeh et al. 2001a; Sakkas et al. 2000). Noteworthy, among the WHO parameter, motility exhibits the strongest correlation with the SCSA DFI (Giwercman et al. 2003): this can explained on the basis that sperm acquire motility and in parallel complete the nuclear packaging during the epididymal transit. This could be attributable to the variety of physical stresses the sample experiences during the cryopreservation also leading to a reduction of sperm motility and an increase of atypical sperm forms. A possible hypothesis is that cryopreservation, correctly carried out, is not able to damage spermatozoa per se, but can enhance defects already present in the sperm population. This has already been partially demonstrated using sperm function tests and CASA (Dondero et al. 1995).

In conclusion, our study demonstrated a generaI improvement over the subpopulation obtained by a physiological selection procedure, e.g. swim-up, after cryopreservation. This result is indicative of a strong correlation between 'good' spermatozoa with a high probability of fertilizing and sperm chromatin integrity. It is suggested that both parameters, being strong and independent predictors of the fertility status when evaluated by operator-independent, objective methods such as FCM and CASA, can complement each other, which provides additional diagnostic and prognostic criteria for male factor infertility evaluations and in the ART setting.

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