Journal of Andrology, Vol. 25, No. 3, May/June 2004
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Evaluation of ICSI-Selected Epididymal Sperm Samples of Obstructive Azoospermic Males by the CKIA System

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ABSTRACT: The objective of this study was to evaluate nuclear normality in intracytoplasmic sperm injection (ICSI)-selected epididymal sperm from obstructive azoospermic (OA) patients. We evaluated whether the selection criteria used in routine ICSI (morphology and motility at a magnification of 400×) is adequate for selecting ''normal'' sperm from epididymal samples. Surgically retrieved spermatozoa from the caput epididymis of 15 OA patients and ejaculated sperm samples from 9 normospermic donors were evaluated with a DNA-specific stain (Feulgen) and in combination with the computerized karyometric image analysis (CKIA) system. Original (unselected) samples and ICSI-selected sperm were compared in donor and patient samples. In the original fraction, a larger variation in almost all measured parameters was found in epididymal sperm than in ejaculated sperm. After sperm selection, the morphometry was comparable between epididymal and ejaculated sperm. However, for those parameters related to the DNA stainability and chromatin texture (nuclear condensation), significant differences between patients and donors were observed. This result suggests that the size and form of the sperm do not necessarily hold similar internal structures. Thus, the frequency of ''normal'' sperm significantly increased after ICSI selection, but the improvement was more marked in donor than in OA patients' samples. In conclusion, at least a twofold increase in the number of normal spermatozoa was achieved after ICSI selection. The heterogeneity in the stainability and chromatin condensation of epididymal samples from OA patients indicates that some of the selected spermatozoa have a hypocondensed or hypercondensed chromatin. Even in the best of donor cases, no more than 55% of the selected sperm scored normal with CKIA, indicating that the present routine ICSI selection criteria are not sufficient for selecting normal condensed nuclei.

Key words: Sperm selection, sperm normality, chromatin, condensation, DNA stainability.

J Androl 2004;25:406–411

The integrity of spermatozoa is of fundamental importance for fertilization and embryo development (Aytoz et al, 1998; Van Dyk et al, 2000; Bartoov et al, 2001; Morris et al, 2002; Tesarik et al, 2002). During in vivo conception and in vitro fertilization (IVF), a natural process is involved in the selection of the fertilizing gametes. With the introduction of intracytoplasmic sperm injection (ICSI), however, the selection depends on the technician's eye and experience and the optical resolution of the microscope. Sperm selection by ICSI criteria is mainly based on motility and morphologic characteristics at a maximum magnification of 400×. By this magnification, only gross morphologic abnormalities can be identified. Still, high fertilization and pregnancy rates can be achieved with ICSI (Palermo et al, 1992), indicating its competence for the treatment of male infertility. Whether these selection criteria are adequate for all sperm samples is of concern, especially from extremely poor sperm samples and in nonejaculated sperm.

One approach to obtaining information about the adequacy of the ICSI criteria in selecting ''normal sperm'' from (non)ejaculated samples is to assess the sperm normality and DNA integrity of the sperm fraction selected for injection. To obtain further insight into the quality of spermatozoa, we adapted a computerized karyometric image analysis (CKIA) system to evaluate the sperm nucleus (Ramos et al, 2002a). This method is based on the stainability of the cells with a DNA-specific stain (Feulgen) in combination with a computer image analyzer system. The advantage of the CKIA system is the integral and objective assessment of the head features of human spermatozoa. CKIA analyzes a series of parameters that are related to different aspects of the nucleus: morphometry (eg, form, size), DNA stainability, and chromatin texture characteristics.

In this study, we focused on the evaluation of epididymal sperm from obstructive azoospermic (OA) patients. In these samples, there was a mixture of sperm with different maturational stages. In addition to immature and mature sperm, with incomplete and complete DNA con-

Supported by the Ministry of Health, Welfare, and Sport (VWS) of the Netherlands.

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Received for publication September 24, 2003; accepted for publication December 18, 2003.

densation, respectively (Saowaros and Panyim, 1979; Evenson et al, 1986), postmature and damaged cells may be present in epididymal samples. Whether the maturational differences can be recognized using the ICSI selection criteria (morphology and motility) is unknown.

In a previous report concerning the evaluation of sperm markers and DNA integrity, we found that motility is a good marker for the selection of sperm with low damage evaluated with the terminal deoxynucleotidyl transferase–mediated deoxyuridine-5′-triphosphate nick end labeling (TUNEL) assay (Ramos and Wetzels, 2001). This finding was also valid for motile epididymal sperm from OA patients (Ramos et al, 2002b). The goal of the present study was 1) to examine the nuclear characteristics of epididymal spermatozoa from OA patients; and 2) to evaluate whether the selection criteria routinely used with ICSI (normal morphology and motility) are adequate markers for the selection of ‘‘normal’’ spermatozoa in ejaculated and epididymal samples according to the CKIA criteria.

Materials and Methods

Patients and Controls

Surgically retrieved spermatozoa from the caput epididymis of 15 azoospermic patients undergoing a microsurgical sperm aspiration or a percutaneous epididymal sperm aspiration were examined. Cryopreserved semen samples of 9 normospermic donors from the sperm bank of the University Medical Centre Utrecht (UMC Utrecht) were used as controls.

All patients signed an informed consent for participation in the study. The project was approved by the Ethical Committee of the University Medical Centre Nijmegen (UMCN, CMO) and the Dutch Central Committee for Research Involving Human Subjects (CCMO, The Hague).

Sperm Samples

Epididymal and donor sperm samples were processed as described previously (Ramos et al, 2002b). Sperm samples were washed in human tubal fluid medium (Cambrex; Bio Whittaker Europe, Vervier, Belgium) supplemented with 10% human plasma proteins (CLB, Amsterdam, the Netherlands) and were concentrated by centrifugation at $500 \times g$ for 5 minutes.

From patients and donors, a droplet of the washed sperm samples (total/unselected population) was allowed to air dry on a glass slide. The rest of the sperm samples were used for the selection of spermatozoa using the ICSI criteria. For this purpose, we mimicked the routine ICSI procedure, except that selected sperm were placed on a glass slide for further evaluation. Briefly, 1 μL of sperm suspension was placed in 5 μL of a 5% polyvinylpyrrolidone solution (Medicult, Jyllige, Denmark) and left to swim out for 2 to 5 minutes. Using a magnification of 400×, morphologic normal motile spermatozoa were aspirated using micromanipulators connected to an ICSI injection pipette (Humagen, Charlottesville, Va). This procedure was repeated until enough sperm (50–100) were collected for analysis. The selected sperm fraction was allowed to dry before the fixation and staining procedure was continued.

Feulgen Stain

All chemicals were provided by Merck, Darmstadt, Germany, unless indicated differently. Air-dried sperm samples were prefixed in a freshly prepared Carbowax/NaCl 0.9% solution (1:1 [vol/vol]) (100% Carbowax: 2% Polyethyleneglycol MW 1500 in 50% ethanol) for at least 24 hours (at 4°C). Subsequently, the samples were fixed for 1 additional day with 100% Carbowax (at 4°C) and then immersed in Böhm solution (10% formalin, 5% acetic acid glacial, and 85% methanol) for 2 hours. After fixation, nuclei were stained according to the Feulgen-Schiff reaction (hydrolysis in 5 N HCl for 60 minutes and stain in Schiff reagent for 30 minutes at room temperature). The slides were mounted in Permount (Fischer Scientific, Fairlawn, NJ).

The CKIA Method

The measurement specifications of the CKIA method have been extensively described previously (van der Poel et al, 1992; Ramos et al, 2002a). Cytomorphologic measurements were made using a microscope connected to a CCD-video camera (Vision Technology, Eindhoven, the Netherlands). The system consists of a frame grabber board (VFG frame grabber; Image Technology, Woburn, Mass) connected to a personal computer. Using 1000× magnification, images of 512×512 pixels were captured, digitized, and stored in the computer before analysis. The images were corrected for background and shading, and they were filtered before applying local segmentation. Each cell image was then processed independently from the images of other cells. The nuclear boundary was delineated and separated from the background. The nuclei were automatically analyzed and numbered, enabling ‘‘postanalysis’’ verification of the objects. After computer analysis, each detected nucleus was visually screened, and artifacts or faulty segmented nuclei were eliminated. The karyometric parameters recorded for each cell were grouped into the following 3 categories:

1) Morphometry: describe size and shape of the nucleus. Size is measured by counting the number of pixels occupied by the object (area, μm^2). Shape is described by elongation/elliptic factor (fell: minor axis/major axis, a value of 1 is for a circle and is smaller than 1 for an ellipse); the nuclear roundness (ben: decreases when the object deviates from a circle, a measurement of an irregularly shaped object. This value is expressed in arbitrary units); and nominal mean curvature (nmac: combines the head contour with smooth Freeman difference chain code of the cell perimeter and is expressed in arbitrary units).

2) Densitometry: related to DNA staining intensity (according to Feulgen; it correlates with the DNA content or the accessibility of the stain to the DNA). These parameters are the optical density (OD: mean optical density of pixels in the nucleus: −log transmission), the integrated OD (IOD: OD × area), and the variation of the OD (varOD).

3) Chromatin texture: quantify stain distribution patterns (the ‘‘stickiness’’ of the pixels with the same grey value; it uses grey-value classes for comparison to neighboring pixels). The parameters describing the chromatin patterns are as follows: the ‘‘meanhis’’ (average OD along the maximal diameter), the

Table 1. *Summarized description of the parameters, codes, and normal ranges determined for sperm analysis with the CKIA method (normal ranges are adapted from Ramos et al, 2002a)**

Category	Code	Description of the Parameter	Reference Range
Morphometry (describes size and shape of cells)	area	Nuclear area in μm2	5.5–8.0
	fell	Elliptic factor (minimum/maximum diameter)	0.52–0.70
	ben	Bending energy (difference between the highest and lowest value in the smooth Freeman difference chain code [SFDC])	1100–1800
	nmac	Nominal mean curvature; factor derived from the SFDC	22–38
Densitometry (related to the stain intensity)	OD	Optical density (mean optical density of the pixels or strainability of the cell)	0.75–0.86
	IOD	Integrated optical density (area × OD)	4.29–5.27
	varOD	Mean variation of the OD of the nucleus	0.18–0.30
Chromatin texture (quantification of the pattern distribution)	meanhis	Mean grey value of the line of maximal diameter	85–135
	sdhis	Mean standard deviation of the grey values of the line of maximal diameter	38–54
	cvhis	Mean coefficient of variation of sdhis	0.37–0.57

* CKIA indicates computerized karyometric image analysis.

''sdhis'' (standard deviation of the meanhis), and the ''cvhis'' (coefficient of variation of the sdhis; larger values indicate differences in staining within the nucleus, but all of them are expressed in arbitrary units).

The codes and description of all karyometric parameters measured are summarized in Table 1. The normal ranges are based on the mean ± 1 standard deviation obtained from previous assessments from normospermic donors (Ramos et al, 2002a). Cryopreservation of the sperm samples did not influence the outcome of CKIA (data not shown), but special attention is required to achieve stabilization of the light source during the CKIA assessment.

In this study, we defined the *qualitative* analysis of a sperm as the value assessed for each parameter per cell (evaluation of each parameter in a spermatozoon). A cell was considered ''normal'' when the values assessed for all parameters analyzed were within the predefined normal ranges. The *quantitative* analysis was defined as the frequency of ''normal'' sperm in each category (morphometry, DNA stain, and chromatin texture) and in the summed categories. This result is expressed as the percentage of normal sperm per sample.

Statistical Analysis

Differences between the samples were analyzed by the 1-way analysis of variance and the Mann-Whitney *U* test when corresponding. *P*-values $<.05$ were considered statistically significant. Data were analyzed using the SPSS 11.1 software package (SPSS Inc, Chicago, Ill).

Results

Qualitative Analysis of the CKIA

In Table 2, the mean and standard deviation values of each parameter in the unselected and selected sperm fractions are presented. Unselected sperm samples showed statistically significant differences between patients and donors in those parameters related to the cell morphometry (area, fell, ben, and IOD). In contrast, in the selected sperm fractions, the means of all parameters were statistically significantly different between patients and donors, except for the area and the area-related parameter, IOD. In other words, the selection of sperm by morphology at a magnification of 400× results in sperm heads of similar size (area), but the chromatin texture and nuclear stainability differ between epididymal and ejaculated samples. Note that the variation (expressed in the SD) of the individual parameters in epididymal samples indicates a larger heterogeneity in these samples than in the ejaculated sperm samples from fertile donors.

Quantitative Analysis of the CKIA

The biologic interpretation of the assessed parameters is complex and difficult to apply in the clinical evaluation of male infertility. Therefore, sperm ''normality'' per category or sample was also calculated. The quantitative

Table 2. *Qualitative analysis of the CKIA parameters (mean ± SD) for patients and fertile donors in the original sample (unselected sample) and after selection of cells using ICSI criteria**

Parameter†	Unselected Spermatoza			Selected Spermatoza		
	Patients	Donors	*P*	Patients	Donors	*P*
area	7.09 ± 0.59	6.35 ± 0.53	.004	6.76 ± 0.54	6.71 ± 0.39	NS
fell	0.60 ± 0.05	0.65 ± 0.03	.01	0.61 ± 0.04	0.65 ± 0.03	.018
ben	1589 ± 148	1454 ± 102	.02	1547 ± 155	1400 ± 69	.01
nmac	32.6 ± 4.0	30.3 ± 2.0	NS	32.1 ± 2.9	29.1 ± 2.5	.012
OD	0.80 ± 0.04	0.82 ± 0.04	NS	0.77 ± 0.04	0.81 ± 0.02	.003
IOD	5.69 ± 0.47	5.19 ± 0.45	.015	5.21 ± 0.53	5.45 ± 0.25	NS
varOD	0.25 ± 0.03	0.23 ± 0.03	NS	0.27 ± 0.03	0.23 ± 0.02	.002
meanhis	108 ± 10	107 ± 13	NS	117 ± 14	105 ± 6	.015
cvhis	0.44 ± 0.05	0.43 ± 0.08	NS	0.41 ± 0.06	0.48 ± 0.04	.005
sdhis	46.3 ± 3.3	44.7 ± 3.2	NS	46.0 ± 3.4	48.7 ± 3.4	.05

* NS indicates not significant; CKIA, compterized karyometric image analysis; and ICSI, intracytoplasmic sperm injection.
† The codes of each parameter are described in Table 1.

CKIA analysis involves the combination of normal values for all individual parameters, and it is expressed as the percentage of normal spermatozoa per category (or per sample). In Table 3 and the Figure, the results of the quantitative analysis (grouped into the categories of morphometry, DNA stainability, and chromatin texture and the combination of these categories) are presented. The Figure helps to visualize these results and also shows the median and extreme values. No statistical differences were found in the unselected (original) samples between patients and donors in any category (Figure). The ICSI selection of epididymal and ejaculated sperm resulted in an increase in the frequency of normal spermatozoa in each category (Table 3), yielding comparable morphologic features in both patients and donors. However, the improvement in the frequency of ''normal'' sperm after selection was significantly higher in donor samples than in patient samples in chromatin texture and DNA stainability and, consequently, in the total sample (Figure).

Discussion

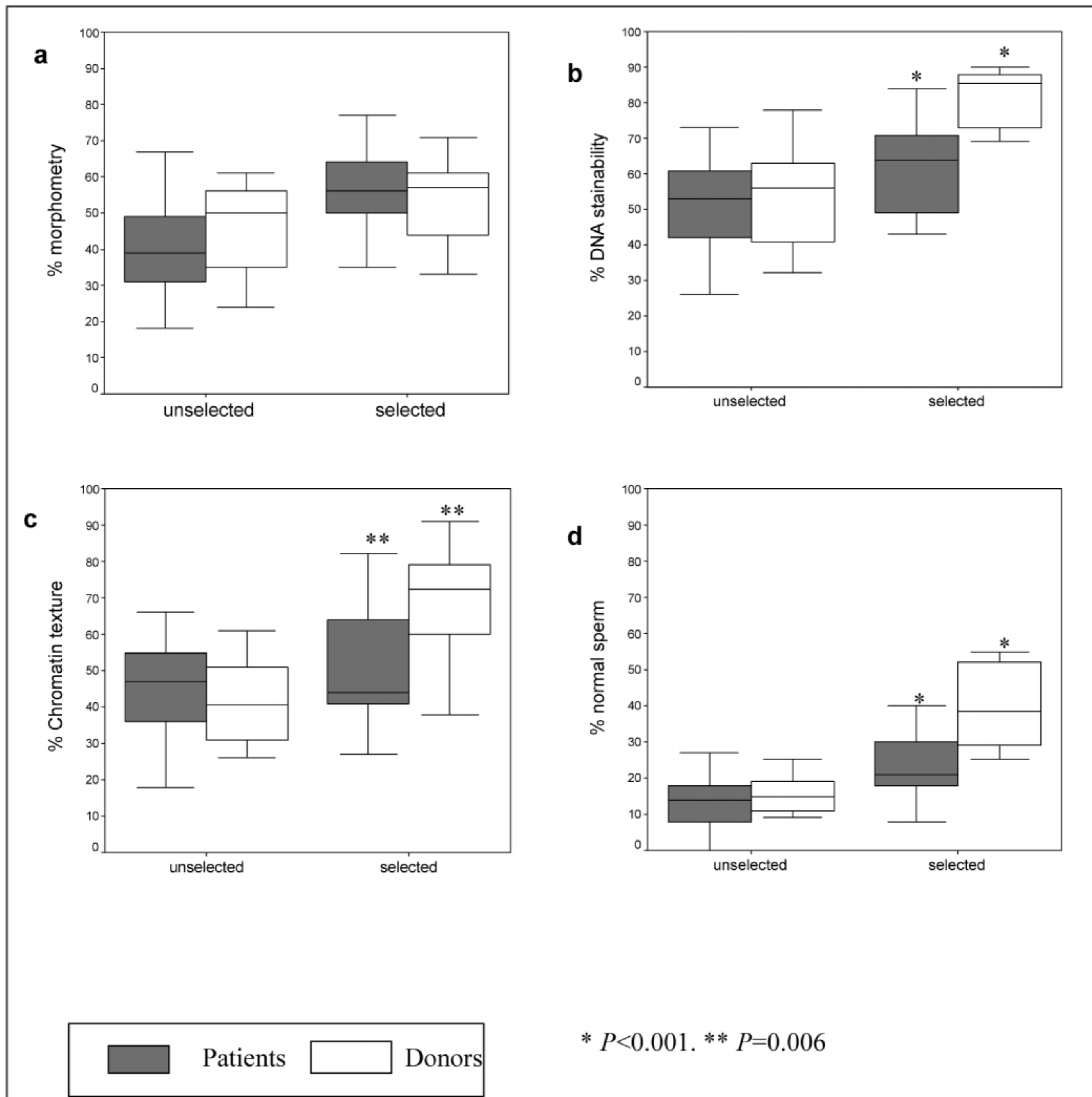
In this study, unselected and ICSI-selected epididymal and ejaculated sperm samples were analyzed by the CKIA system. The original sperm samples (unselected sperm) were compared with a sperm fraction that was selected for motility and normal morphology, mimicking the ICSI procedure. Using these markers for selection (morphology and motility), at least a twofold increase in the frequency of ''normal'' sperm was achieved in epididymal and ejaculated sperm samples. Nevertheless, even in the best samples, only 55% of the selected spermatozoa scored normal with CKIA.

One possible explanation for the relatively low numbers of sperm scoring ''normal'' with CKIA relies on the normal ranges used. The normal ranges described in our previous study (Ramos et al, 2002a) were derived from the mean ± 1 standard deviation obtained from sperm samples that were previously classified as normospermic by World Health Organization criteria; however, the CKIA assessment is based on a principle that is different from that of the standard morphology. Moreover, normospermia is not necessarily equivalent to fertility. Validation and, eventually, adjustment of these normal ranges need to be performed in a much larger fertile population. On the other hand, in the perspective of the present study, emphasis is placed on the outcome of nuclear differences of the same samples previous to or after selection and not the accuracy of the normal values.

Table 3. *Quantitative analysis of the unselected and selected sperm fractions of patients and fertile donors presented as the percentages of normal sperm nuclei in each category and in all categories (% total normal)**†

% Normal Sperm per Category	Patients			Donors		
	Unselected	Selected	*P*	Unselected	Selected	*P*
% Morphometry	40.5 ± 12.5	54.6 ± 12.8	.005	46.2 ± 11.9	53.9 ± 12.1	NS
% DNA stainability	48.0 ± 17.8	61.5 ± 14.1	.037	52.8 ± 15.0	81.3 ± 8.5	<.001
% Chromatin texture	46.1 ± 15.1	49.7 ± 16.2	NS	42.3 ± 12.1	69.3 ± 16.2	.001
% Total	12.3 ± 8.2	23.7 ± 9.4	.001	17.2 ± 8.4	40.5 ± 11.8	<.001

* *P* values were calculated by the Mann-Whitney *U* test.
† NS indicates not significant.



Box plot representation of the improvement of nuclear normality after selection by computerized karyometric image analysis (CKIA) analysis: percentage normal for each category separately **(a, b, c)** and for all categories integrated **(d)**.

A remarkable finding of the qualitative measurements is that only the parameters related to the morphometry were significantly different between donors and patients in unselected sperm samples. After selection, however, the inverse was found: epididymal and ejaculated sperm samples differed in all parameters, except for the area and the IOD (morphometric-related parameters). This finding suggests that morphology selection at a magnification of 400× is adequate for the selection of similar-sized sperm but that it does not discriminate between other nuclear features. We conclude that spermatozoa with similar external characteristics do not necessarily hold the same internal structures. This points to a shortage in the routine sperm selection and needs further investigation.

Contrary to our expectation, a lower OD and IOD mean value was found for epididymal sperm. For human sperm, the OD of the Feulgen stain is related to the accessibility of the stain in the nuclear chromatin (Bito et al, 1999), which is higher in immature sperm (Saowaros and Panyim, 1979). Thus, an increase in the OD of the sample should be found. This was not always the case in caput spermatozoa from OA patients. A larger variation in the staining within samples and between OA patients was found, indicating a large heterogeneity in chromatin packaging in these samples. We speculate that in OA men, a fraction of the epididymal samples are ''hypercondensed'' sperm (aged). These postmature or aged spermatozoa may have continued thiol crosslinking, presenting some degree of chromatin hypercondensation. Hypercondensed nuclei are less accessible for Feulgen stain, leading to our present results.

Taking into account the results of the quantitative analysis for each CKIA category (see Table 3; Figure), patient and donor samples did not differ before selection. After selection, a larger frequency of ''normal'' spermatozoa was found in both patients and donors, but the proportion of normal sperm in the donor samples was significantly higher than in the latter group. ICSI selection increases the frequency of ''normal'' sperm as assessed by the CKIA system, but the improvement differs per sperm sample and origin. We should also take into account that the freeze-thaw procedure may act as an extra selection for the donor sperm samples, increasing the frequency of sperm with normal chromatin compaction.

In conclusion, an increase in the frequency of morphologic normal spermatozoa as evaluated by the CKIA method was observed after selection by morphology and motility under ICSI conditions. This finding joins the results of our previous work, where motile-selected spermatozoa showed an increase in the nuclear integrity assessed by TUNEL (Ramos et al, 2002b). The present results give rise to further research on sperm selection procedures for ICSI, effective chromatin packaging, and DNA integrity in spermatozoa from different origins.

Acknowledgment

The authors thank Mr P. Peelen from the Department of Experimental Urology for the Feulgen staining of the samples.

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