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Use of Computerized Karyometric Image Analysis for Evaluation of Human Spermatozoa

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ABSTRACT: The objective of this study was to evaluate a computer image system for its ability to determine morphological and nuclear semen characteristics in an integral and reproducible way. Semen samples from 19 normospermic fertile donors were used to estimate preliminary cutoff values for spermatozoa and to test the reproducibility of the system. Ten aliquots of 1 sample were used to investigate the sensitivity of the system for experimental conditions by exposure to different laboratory variables. Human spermatozoa were stained with Feulgen dye and analyzed with a magnification of 1000×. A panel of 21 parameters was measured for each sperm nucleus using the computerized karyometric image analysis (CKIA) system. Eight parameters were found to be sensitive for differentiating normal or abnormal human spermatozoa, and cutoff values for each parameter were defined for quantitative analysis. These 8 parameters were grouped into 3 categories depending on their descriptive value: morphometry, DNA condensation (stainability), and chromatin texture. Intrapatient and interpatient variabilities were tested by calculating the reliability coefficient for each of the 8 parameters as well as for each category. Reliability coefficients were all >70% (indicative of the suitability of the system to identify differences between spermatozoa). Interpatient variability (SD) was 5%. Although it was not statistically significant, a variation of 10.9% in measurements was found when the effects of experimental conditions were tested. We conclude that an objective description of the human sperm nucleus can be achieved with CKIA, yielding high interpatient and intrapatient reliability coefficients (reproducibility), thereby adding a new tool for the quantification of normal sperm.

Key words: Chromatin, condensation, Feulgen, morphometry, DNA stainability.

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Determination of male fertility status in the laboratory is mainly based on routine semen analysis (ie, sperm count, motility, and morphology). Except for morphology, this historic heritage has a poor predictive value for male fertility status and fertilization outcome in in vitro fertilization (IVF) programs (Liu and Baker 1992; Barratt et al, 1995; Oehninger and Kruger,1995; Menkveld et al, 2001). Determination of sperm morphology requires high standardization to reduce interlaboratory and intralaboratory results (Cooper et al, 1992, 1999). However, great variability in morphology assessment occurs despite the many quality control programs in use around the world (Neuwinger et al, 1990). The development of a system that can eliminate bias and subjectivity and increase reproducibility between observers has been a matter of interest in human andrology as well as in animal breeding programs for a long time (Moruzzi et al, 1988; Sailer et al, 1996). In an attempt to reduce the subjectivity in assessing human sperm head morphology, a number of semiautomated computer analysis systems have been developed in the last decades (Moruzzi et al, 1988; Garrett and Baker, 1995; Kruger et al, 1995; Sailer et al, 1996). These systems have not gained great use in routine analysis of semen samples in fertility centers. This lack of success is probably due to the high degree of standardization and investment of labor needed.

In our center, computerized karyometric image analysis (CKIA) has been successfully applied in pathology for many years, especially in the field of urological oncology (van der Poel et al, 1990, 1991, 1992). We have adapted this system for characterizing (qualitative evaluation) and quantifying human spermatozoa. For this purpose, we first defined the normal karyometric values, then evaluated the reproducibility of CKIA and, finally, we determined the influence of laboratory variables (experimental factors) on the results.

Materials and Methods

Semen Samples and Controls

A total of 19 cryopreserved samples from normospermic fertile donors evaluated by World Health Organization (1999) criteria

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were used in this study, except for one fresh sample, which was used to test experimental conditions. Semen samples were washed twice in human tubal fluid medium (Bio Whittaker Europe, Belgium) supplemented with 10% (v/v) human plasma solution (CLB, Amsterdam, The Netherlands; Quinn et al, 1985) in order to remove seminal plasma and reduce background staining. Cryopreserved bull spermatozoa (from a fertile specimen) were used as controls and to calibrate the system (see below). Fixation and staining of human samples and bull controls were carried out simultaneously.

Fixation and Feulgen Stain

All chemicals were provided by Merck (Darmstadt, Germany) unless otherwise indicated. One droplet of washed semen sample was placed on a glass slide and left to dry. The cells were prefixed with standard methods in one part of 100% Carbowax (2% polyethylene glycol, molecular weight 1500 in 50% ethanol) and one part of physiological saline solution (0.9% NaCl) at 4°C for 3 days. Thereafter, the samples were fixed one additional day with 100% Carbowax at 4°C. Before staining, slides were immersed in Böhm solution (10% formaldehyde [37%], 5% glacial acetic acid, and 85% absolute ethanol). The fixed slides were stained with Feulgen-Schiff stain (hydrolysis in 5 N HCl for 60 minutes and 30 minutes in Schiff-reagent at room temperature). Cells were mounted in Permount (Fischer Scientific, Fairlawn, NJ). At least 100 spermatozoa per sample were recorded for analysis.

Study Design

To study the reproducibility and accuracy of CKIA for determining semen characteristics, duplicates of 19 samples were stained and evaluated at different moments. CKIA was performed on 100 to 120 cells per slide.

To study the influence of experimental conditions (laboratory variables) on the measurements, different aliquots of 1 sample (fresh and cryopreserved) were used. Four experimental conditions were analyzed against a standard condition. We defined the standard condition for fresh semen as prefixation in freshly made fixative at 4°C for 3 days. While keeping the rest of the variables unchanged, we tested the effect of the following conditions in CKIA: 1) cryopreserved semen, 2) prefixation at room temperature, 3) prefixation in 6-month-old fixative, or 4) extended prefixation for 10 days. As a result, 5 × 2 (= duplicates) = 10 aliquots were used for the analysis.

Calibration of the System

Control of the stain intensity and calibration of the microscope light is crucial for CKIA. Therefore, light calibration of the system with a nucleus with constant staining intensity before measurements is required. For this purpose, bull spermatozoa were used and stained together with the rest of the samples. The advantage of bull spermatozoa is the low rate of variation in the DNA condensation in fertile animals (more than 90% shows normal condensation; Dobrinski et al, 1994).

Computerized Karyometric Image Analysis

This technique, which is routinely used at the Nijmegen Urology Department for karyometric analysis of bladder tumors (van der Poel et al, 1990) has been applied to sperm head morphology. Cytomorphological measurements were made using a microscope connected to a CCD-video camera (Vision Technology, Eindenhoven, The Netherlands). The system consists of a framegrabber board (VFG frame grabber, Image Technology, Woburn, Mass) connected to a personal computer (van der Poel et al, 1992). 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 filtered before applying local segmentation. Each cell image is then processed independently from the images of other cells. The nuclear boundary is 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 time required to perform the analysis is about 1 minute per field; the complete procedure takes no more than 15 to 30 minutes per sample, depending on sperm concentration.

The karyometric parameters recorded for each cell were grouped into three categories: 1) morphometric parameters that describe size and shape of the nucleus, 2) densitometric parameters that are related to staining intensity (DNA condensation), and 3) chromatin texture parameters that quantify stain distribution patterns. The codes and description of all karyometric parameters measured are presented in Table 1.

Determination of Cutoff Values and Definition of Normal Karyometry

In the first phase of the study, preliminary cutoff ranges for each nuclear parameter were calculated. These were based on means ± 2 SD values from a total of 483 randomly selected sperm cells from 10 fertile normospermic donors. Comparing these values with those of other cell types (data not shown), we found that 14 of 21 parameters were appropriate for identification of human spermatozoa (the + and ++ categories in Table 1). We chose only 8 parameters (++) grouped in the aforementioned 3 categories for further CKIA evaluation.

In a second phase of the study, determination of the cutoff ranges for ''normal'' head morphology was carried out by analyzing 160 spermatozoa that were visually selected according to their conformation to WHO (1999) criteria. The mean ± SD values derived from this population were used as cutoff values to define the normal human sperm nucleus. Also, 430 visually selected abnormal sperm were analyzed and evaluated for differences compared to the normal or unselected sperm population. For quantification of normal forms, we applied the following criterion: whenever the measured value of a parameter suits the defined normal range of that parameter, the cell is classified as normal for that specific parameter. Thus, classification of normal sperm heads (for each category) is based on the combination of normal ranges for all parameters in that category. The combination of normal values for the 3 categories results in a quantitative value for that sample (total normal karyometry).

Statistical Methods

Reproducibility of each CKIA parameter was evaluated using one-way analysis of variance (ANOVA) with patients considered

Table 1. *Suitability of nuclear measurements of CKIA for sperm head analysis, grouped by category*

Category	Code	Description of the Parameter	Suitable
Morphometric parameters (Size and shape of cells)	area*	Nuclear area in μm²	++†
	perimeter	Nuclear perimeter (in μm)	+
	fpe	Nuclear roundness factor = (4π × area)/perimeter²	++
	maxd	Maximal diameter of the nucleus	+/−
	fell*	Elliptic factor (minimum/maximum diameter)	++
	posX/Y	Position of the nucleus in the screen	−
	ben*	Bending energy (difference between highest and lowest value in the smooth Freeman difference chain code [SFDC])	++
	nmac*	Nominal mean curvature; factor derived from SFDC	++
	thre	Threshold value from the form factors, derived from SFDC	+
	diss	Distance between values of threshold limits	−
	pass	Number of times threshold values have been passed	+/−
	form1	Total bending energy of SFDC	−
Densitometric parameters (DNA condensation)	od*	Optical density (stainability of the cell)	++
	iod	Integrated optical density (area × od)	+
	varod*	Mean variation of the od of the nucleus	++
	cvod	Mean coefficient of variation of the od	+
Chromatic texture parameters (Quantification of DNA distribution patterns)	meanhis*	Mean grey value of the line of maximal diameter	++
	sdhis	Mean standard deviation of the grey values of the line of maximal diameter	+
	cvhis*	Mean coefficient of variation of sdhis	++
	scathis	Mean scatter of grey values in the line of diameter	+/−
	edghis	Mean grey value of the nuclear edge	+/−

* Parameters used later for quantitative analysis.
† − indicates not suitable; +/−, poorly suitable; +, suitable; ++, very suitable.

as a random effect. The validity of the parameters was measured by the reliability coefficient and computed for interpatient and intrapatient variability. Reliability coefficients ≥70% are considered suitable for characterization of human spermatozoa and discrimination between samples. Also, differences between experimental factors in measurements were tested for statistical significance using one-way ANOVA. The experimental conditions were analyzed as independent variables and the mean of each parameter were analyzed separately as a dependent variable. The estimated mean square error (MSE) between slides (samples) and standard errors are presented. $P < .05$ was considered statistically significant. Calculations were performed using the Statistical Analysis System computer program (SAS Institute Inc, Cary, NC).

Results

Selection of Parameters, Definition of Normal Values for Human Spermatozoa, and Evaluation of Suitability for Differentiation Between Samples

The total panel of karyometric parameters and its suitability for analysis of human sperm is presented in Table 1. The mean and SD values of the selected parameters (++, except nuclear roundness factor, or fpe) were evaluated by comparing unselected, and visually selected normal and abnormal sperm heads (Table 2). Also, two examples of other cell types (bull sperm and human cu-

Table 2. *CKIA measurements for different cell populations*

		Categories/Mean (± SD)							
		Morphometry				DNA Condensation		Chromatin Texture	
Cell Type*	N	area	fell	ben	nmac	od	varod	meanhis	cvhis
Unselected	483	6.43 (0.77)	0.61 (0.09)	1522 (314)	32.4 (7.8)	0.78 (0.07)	0.24 (0.04)	115 (19)	0.37 (0.10)
Selected normal	160	6.87 (0.65)	0.63 (0.05)	1394 (183)	28.9 (5.2)	0.83 (0.04)	0.22 (0.04)	111 (11)	0.39 (0.06)
Selected abnormal	430	6.82 (1.65)	0.53 (0.13)	1914 (499)	39.3 (8.9)	0.79 (0.08)	0.25 (0.05)	113 (19)	0.40 (0.11)
Bull sperm	487	18.0 (1.7)	0.44 (0.03)	1978 (200)	28.9 (2.2)	0.26 (0.15)	0.052 (0.014)	190 (8)	0.051 (0.01)
Human cumulus cells	140	25.6 (7.1)	0.75 (0.09)	1463 (346)	14.1 (3.8)	0.79 (0.06)	0.21 (0.04)	101 (26)	0.37 (0.12)

* Human spermatozoa unless mentioned otherwise.

Table 3. *Percentage of normal cells per category and total normal spermatozoa analyzed with CKIA (based on defined cutoff ranges for normal human spermatozoa)*

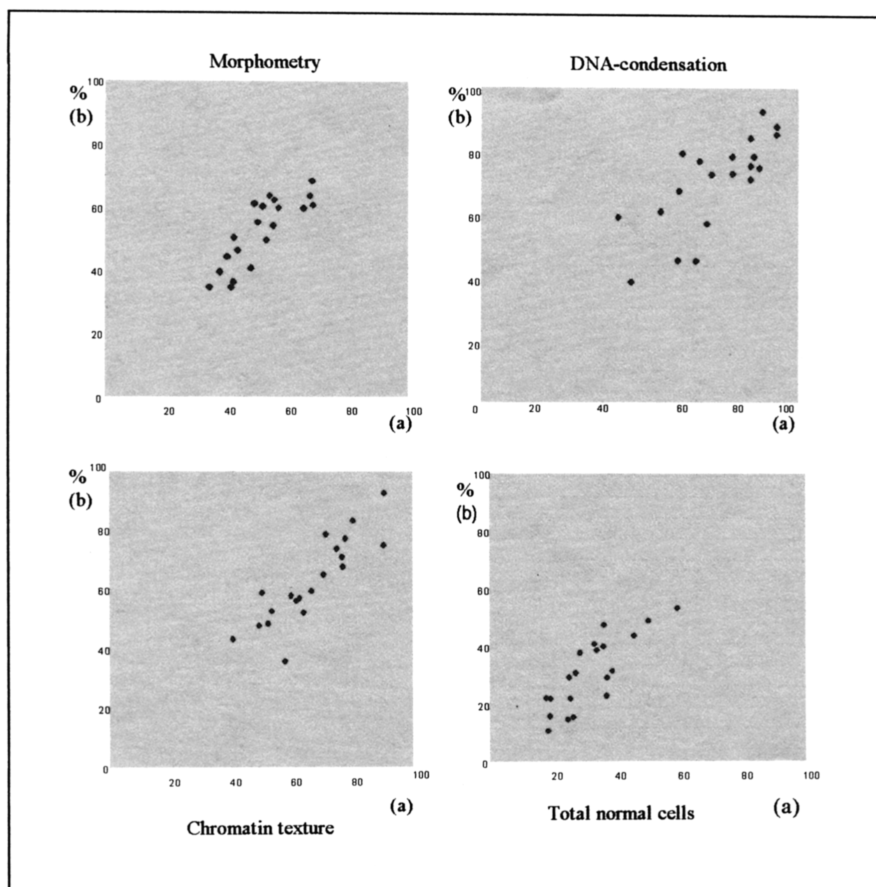
Category/ Type of Sperm	Morphometry	DNA Condensation	Chromatin Texture	Total Normal Karyometry*
Unselected (original sample)	57%	60%	53%	26%
Selected normal	91%	91%	93%	76%
Selected abnormal	17%	57%	52%	5%

* To consider a cell "normal," the same cell has to score normal after a combination of all three categories. All groups are significantly different ($P < .01$).

mulus cells) are presented. When quantitative analysis (percentage of normal cells in each sample) was carried out, statistically significant differences were found for all groups ($P < .01$; Table 3). Differences between unselected sperm cells (phase 1, see "Materials and Methods") and abnormal sperm cells (phase 2) were found for morphometry (57% vs. 17%, $P < .01$). Also in these cell populations, the percentage of cells with total normal karyometry was statistically different (26% vs. 5% $P < .01$). From the visually selected normal sperm heads, 76% were classified as normal by karyometry with CKIA ($P < .01$ compared to unselected and abnormal sperm populations).

Reproducibility of Karyometric Measurements (Quantitative Analysis)

An average of 127 randomly selected spermatozoa were analyzed in duplicates for each of the 19 semen samples. The relationship between the duplicates is presented in the Figure. The interpatient variability was calculated in order to validate the discrimination capacity of CKIA between normal and abnormal cells. The SD within donors was 5% for the percentage of sperm with normal karyometry as determined by CKIA (see Table 4), whereas the intrapatient variability (SD between donors) was 10.7%. The reliability coefficients for each parameter are also presented in Table 4. Reliability coefficients >70% are by definition considered good (suitable) for differentiation between spermatozoa (normal/abnormal) and between samples (patients).



Repeatability of CKIA measurements per category obtained from 19 samples analyzed in duplicate (a vs. b). Repeatability of duplicates for each category were $r = .84, .76, .87$, and .82 for morphometry, DNA condensation, chromatin texture, and total normal karyometry, respectively.

Table 4. *Intra- and interpatient variability and reliability coefficients for 19 donors calculated for each individual parameter and per category*

Parameter/Category	Mean	SD Within Donors	SD Between Donors	Reliability Coefficient
area	7.01	0.299	0.502	73.7%
fell	0.62	0.007	0.043	97.6%
ben	1512	25.1	135.9	96.7%
nmac	30.6	0.86	2.35	88.3%
od	0.84	0.012	0.020	72.4%
varod	0.21	0.011	0.018	68.5%
meanhis	103.4	3.95	6.24	71.4%
cvhis	0.42	0.024	0.030	60.1%
Normal morphometry (%)	51.9	4.2	9.8	84.1
Normal DNA condensation (%)	72.6	7.2	13.0	76.5
Normal chromatin texture (%)	64.4	5.6	12.9	84.2
Total normal karyometry (%)	31.4	5.0	10.7	82.2

Reliability coefficients ≥ 70% are considered good for discrimination between normal and abnormal sperm cells and discrimination between samples.

Assessment of the Influence of Experimental Conditions on CKIA

Evaluation of the influence of some laboratory variables in CKIA measurements is shown in Table 5. MSE values indicate that differences in qualitative analysis are within the range of clinical relevance. The differences found cannot be related to one specific experimental condition. Although not significant for the quantitative analysis, an MSE =10.9% for influence of experimental factors suggests that some conditions are likely to influence the determination.

Discussion

The present study describes the application of CKIA for objective characterization of human sperm heads and quantification of normal forms. After staining semen samples with Feulgen stain, cells were digitized and each image was analyzed individually. For each nucleus a panel of morphometric, densitometric, and chromatin texture parameters was determined. An important advantage of CKIA relies on recording imperceptible differences by the human eye in distribution and intensity of the stain, and by identifying other differences in spermatozoa rather than morphology alone. Therefore, the present assumption that morphological normal spermatozoa are fertile may be not always be correct because other characteristics in the sperm nucleus may contribute to the fertility potential of human spermatozoa. Another benefit of CKIA is that objectivity and high reproducibility can be achieved.

The need for assessing the fertility potential of spermatozoa has been a long-term issue for andrologists. Until now, the percentage of sperm with normal morphology and the percentage of spermatozoa with a normal reaction to acid-induced denaturation of DNA (using green/red acridine orange fluorescence) seem to be the most important predictive factors for fertilization rates in vitro (Liu and Baker, 1992; Oehninger and Kruger, 1995; Evenson et al, 1999). However, the accuracy of morphology determination in predicting fertilization failures in IVF programs is still poor. The deficiencies of morphology determination can be summarized as follows: 1) the subjectivity of the determination of normal forms, 2) the reproducibility of the results, and 3) little information on DNA/chromatin condensation.

Table 5. *Mean CKIA values and percentage of normal forms (quantitative CKIA) after exposing cells to different experimental factors*

Categories/ Experimental factors*	Morphometry			DNA Condensation			Chromatin Texture		
	area	fell	ben	nmac	od	varod	meanhis	cvhis	CKIA§
Standard†	7.50	0.612	1481	29.01	0.823	0.23	110.0	0.400	49.2%
Cryopreserved sperm	7.60	0.600	1512	29.92	0.838	0.22	106.8	0.402	42.0%
Old fixative	6.89	0.600	1506	31.19	0.836	0.22	105.4	0.406	61.0%
Room temperature	6.86	0.615	1439	30.38	0.814	0.23	116.8	0.358	41.3%
Long fixing	7.42	0.620	1446	28.69	0.821	0.23	112.0	0.379	52.0%
MSE‡	0.30	0.007	20	0.96	0.015	0.01	4.8	0.028	10.9%

* All other conditions were kept standard.
† Standard conditions: fresh washed semen, 3 days at 4°C in 50% freshly made Carbowax.
‡ MSE: mean square error (SD between slides).
§ Total normal karyometry per sample.

Subjectivity by technicians in determining normal forms is one of the most important biases in morphology determination in routine analysis; quality control programs still show large differences in results (Neuwinger et al, 1990; Kruger et al, 1995; Cooper et al, 1999). In this respect, CKIA leads to an objective determination, although some items have to be kept in mind for its interpretation and use. Selection of sperm cells for determination of normal cutoff ranges may carry some subjectivity. Selection of normal cells is mainly based on shape (morphology), whereas differences in DNA stain or chromatin condensation are mostly imperceptible by the human eye, but can be detected by a computer. These features may explain that, when manually selected normal spermatozoa are analyzed with CKIA, only 76% rather than 100% of sperm heads were classified as ''normal.'' This difference gives evidence that CKIA measurements are an integral analysis of the cell nucleus. Evaluation and verification of the defined normal karyometry for fertility must be performed in a larger population of fertile and infertile men (in study).

We found that the reproducibility of the results within donors has a correlation of $r = .82$, explaining $r^2 = 68\%$ of the variance, probably because intradonor repeatability was evaluated by selecting independent samples and not the reproducibility of each individual cell. Still, high reliability coefficients were found for almost all parameters, indicating that the system can effectively discriminate between patients (SD between donors = 10.7%) and is constant for each patient (interpatient SD = 5%). Only the parameter ''cvhis'' (Table 1) was found to be not relevant for our system and will probably be replaced in the future by ''sdhis'' (reliability coefficient 73%, data not shown).

Although only 10 aliquots of one sample were used to study the influence of some experimental factors in CKIA, we found that no factor by itself was critical for determining differences in measurements. In particular, the use of fresh or cryopreserved samples for karyometric measurements did not show significant differences. This result was observed previously in a larger group of patients (data not shown). Although quantitative analysis for evaluation of experimental conditions showed differences in results (10.9% individual variation among measurements), it was found not to be statistically significant. This finding underlines the importance of the standardization of every step in the fixation and staining procedure in order to reduce the random effects in the measurements.

Besides morphology, increasing evidence indicates that the condensation status of spermatozoa may play an important role in fertilization outcome in human and animal programs (Bito et al, 1999). Therefore, it is necessary to assess those sperm defects that may explain low fertilization rates in some patients (Dobrinski et al, 1994; Gravance et al, 1996; Evenson et al, 1999). Abnormal DNA condensation in spermatozoa can hardly be detected by the human eye with routine morphology stains. DNA-specific dyes such as Feulgen were found to correlate well with the chromatin structure assay and have been used to assess chromatin condensation (Dobrinski et al, 1994; Dadoune 1995; Sailer et al, 1996). Feulgen stain (a stoichiometric dye) selectively binds to the aldehyde groups of the purines, allowing anomalies such as coarse or fine clumping of the nuclear material to be determined (Peluso et al, 1992). Also, Feulgen staining has revealed a higher percentage of heterogeneous DNA distribution in semen from infertile compared with fertile donors (Moruzzi et al, 1988; Sailer et al, 1996).

One disadvantage of CKIA is overestimation of some sperm populations by rejection of aberrant forms. The program automatically rejects incorrect images (which mostly do not correspond to that of spermatozoa). Therefore, very abnormal forms or spermatozoa that are too large (probably diploid or multiploid cells) are not always recognized as such and are automatically eliminated in analysis. This can lead to underrepresentation of some types of aberrant cells. Also, postcontrol of the captured cells before data analysis must be done because the computer does not always discriminate unfocused cells or debris. Elimination of these images is necessary, although accounting for no more than 2% of the images. Another item to consider with CKIA is that when extra condensed, diploid, or multiploid sperm cells are present in the sample, the optical density does not increase linearly with cellular DNA content. Small increases in optical density may lead to incorrect interpretation of DNA content if the area is not taken into account. Therefore, monitoring the variability of the stain can be better achieved using cells with a constant morphology and stainability characteristics (in our case, bull spermatozoa) in order to eliminate stain variances.

In conclusion, CKIA offers an objective and integral method for sperm head characterization with high reproducibility. Image analysis not only describes morphometric parameters of the cell but can also detect small differences in stain intensity and distribution, which are related to changes in DNA/chromatin condensation. Differences in DNA condensation may explain differences in fertilization potential of some spermatozoa. Still, validation for the diagnostic value of CKIA must be carried out before it can be used for clinical proposes.

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