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Male Fertility

Morphometric comparison by the ISAS® CASA-DNAf system of two techniques for the evaluation of DNA fragmentation in human spermatozoa

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DNA fragmentation has been shown to be one of the causes of male infertility, particularly related to repeated abortions, and different methods have been developed to analyze it. In the present study, two commercial kits based on the SCD technique (Halosperm® and SDFA) were evaluated by the use of the DNA fragmentation module of the ISAS® v1 CASA system. Seven semen samples from volunteers were analyzed. To compare the results between techniques, the Kruskal–Wallis test was used. Data were used for calculation of Principal Components (two PCs were obtained), and subsequent subpopulations were identified using the Halo, Halo/Core Ratio, and PC data. Results from both kits were significantly different (*P* < 0.001). In each case, four subpopulations were obtained, independently of the classification method used. The distribution of subpopulations differed depending on the kit used. From the PC data, a discriminant analysis matrix was obtained and a good *a posteriori* classification was obtained (97.1% for Halosperm and 96.6% for SDFA). The present results are the first approach on morphometric evaluation of DNA fragmentation from the SCD technique. This approach could be used for the future definition of a classification matrix surpassing the current subjective evaluation of this important sperm factor.

INTRODUCTION

In humans, approximately 15% of patients with male factor infertility have normal semen analysis results, and so a definitive diagnosis of male infertility often cannot be made solely from results of routine semen analysis.[1] This implies that new seminal parameters must be included in the routine analysis for discriminating other causes of male infertility.

The possible significance of DNA fragmentation on fertility was indicated some years ago.[2–5] To evaluate this semen trait, different techniques have been developed,[6,7] including the TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling) assay,[8–10] the Comet assay,[10–12] the chromomycin A3 test,[13,14] Acridine Orange metachromatic staining,[15–17] DNA Breakage Detection-Fluorescence *In Situ* Hybridization,[18] the SCSA (Sperm Chromatin Structure Assay) test,[19] and the SCD (Sperm Chromatin Decondensation) test.[20]

DNA fragmentation in human sperm samples after evaluation by the Comet technique is higher in infertile males than fertile males, and spermatozoa with abnormal morphology and low levels of motility have more DNA damage than normal cells.[5] By using the TUNEL technique, it has been demonstrated that specific abnormal sperm morphology can be correlated with chromosomal abnormalities and the level of DNA

fragmentation in human spermatozoa.[21] Development of simple kits for the diagnosis of DNA fragmentation has increased the number of studies on the significance of DNA fragmentation in several species,[22] but there is some controversy over the diagnostic significance of the differential tests, making it difficult to decide which is the best to use.[23,24]

Two commercial kids have been developed around the SCD technique: Halosperm® (Halotech, Madrid, Spain) and SDFA (ACECR, Tehran, Iran). The purpose of the present study was to compare the results from these commercial kits, by performing a morphometric analysis with the ISAS® v1 DNA fragmentation module (Proiser, Valencia, Spain). These morphometric data were used, for the first time to our knowledge, to define mathematical clusters that provide a classification matrix of different subpopulations of sperm head DNA-reacted cells.

MATERIALS AND METHODS

Study population

Seven volunteers signed informed consent form to participate and have their semen used in the study. Semen samples were collected by masturbation after sexual abstinence for 3–5 days. Each sample was collected in a clean 60-ml wide-mouthed universal container and stored at 37°C in an incubator for 30 min to allow liquefaction.

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Assessment of DNA fragmentation

Two commercial kits were used to assess the level of sperm head DNA fragmentation by the SCD approach: the Halosperm® test (Halotech DNA, S.L., Madrid, Spain) and the Sperm DNA Fragmentation SDFA test (ACECR, Tehran, Iran). For both tests, semen samples were diluted with Sydney IVF Sperm Medium (Cook® Medical, Bloomington, IN, USA) to a sperm concentration of $5–10 × 10^6$ cells ml^{-1}. Agarose gel from the kit (500 µl for Halosperm or 100 µl for SDFA) was incubated in an Eppendorf tube for 5 min at 90–100°C to melt the agarose and then 5 min at 37°C in temperature-controlled water bath after which 25 µl (Halosperm test) or 50 µl (SDFA test) of the semen sample was added into an Eppendorf tube and mixed carefully. For both tests, 15 µl of the mixture was placed onto a kit-provided super-coated slide, placed on a cold surface, and covered with a 22 mm × 22 mm coverslip. Slides were kept for 5 min at 4°C in a refrigerator to create a microgel with the contained spermatozoa.

For the Halosperm test, coverslips were then carefully removed, and the slides immersed into acid denaturation solution for 7 min, transferred to a tray of the kit's lysing solution for 25 min incubation, rinsed with distilled water and dehydrated for 2 min in each of 70%, 90%, and 100% (v/v) ethanol. After being dried, the slides were stained with Diff-Quik (Medion Diagnostics, Düdingen, Switzerland) in a horizontal position, first in Eosin (red color) for 7 min, then in Azur B (blue color) for 7 min, and finally rinsed in distilled water and allowed to dry at room temperature.

For the SDFA test, coverslips were carefully removed, and a few drops of solution A were added to the slide, which was incubated for 7 min. Slides were transferred to solution B and incubated for 15 min, rinsed with distilled water and dehydrated for 2 min in increasing concentrations of ethanol (70%, 90%, and 100%). After being air-dried, the slides in a horizontal position were stained sequentially with the kit's staining solutions: solution C for 75 s, solution D for 3 min, and solution E for 2 min, then rinsed in distilled water and allowed to dry at room temperature.

Morphometric analysis

Analyses were conducted by using the DNA fragmentation module of the ISAS® v1 (Proiser R+D S.L., Paterna, Valencia, Spain) CASA-DNAf system. The camera used was Proiser 782 m (Proiser R+D S.L.) attached to a microscope UB203 (UOP/Proiser, Paterna, Valencia, Spain). Images were captured through a 40 × bright field objective (AN 0.7) with resolution of the analyzed images of 0.21 µm/pixel for both axes. The software renders three morphometric parameters: the total Halo and central Core areas (µm^2), distinguished by the intensity of staining (**Figure 1**), and the Ratio between them.

Statistical analysis

Clustering procedures were performed on the datasets to identify sperm subpopulations from the Halo parameter values and the Ratio criteria. In both cases, the parameter values were examined using a nonhierarchical clustering procedure (k-means model and Euclidean distance), to classify the spermatozoa of the dataset.[25] The first step was to perform a principal component analysis (PCA) of the DNA fragmentation data. The morphometric database comprised a total number of 1775 spermatozoa. To select the number of principal components that should be used in the next step of analysis, the criterion of selecting only those components with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion) was chosen. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA to determine the subpopulation structure.
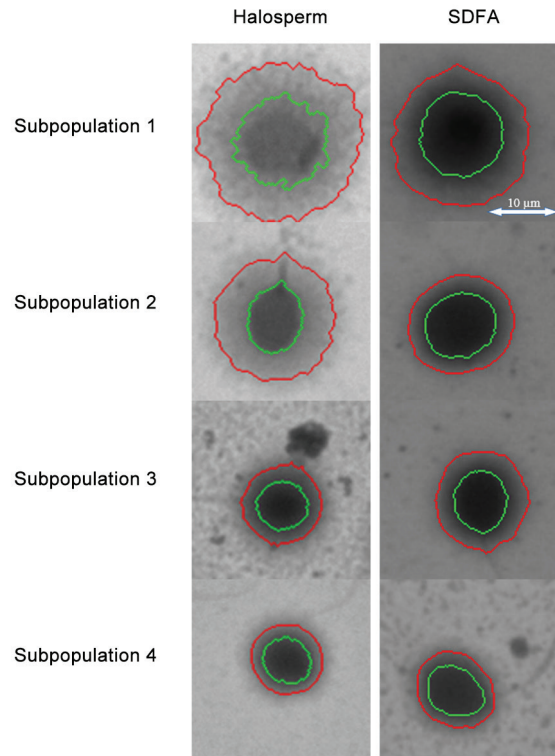

Figure 1: Images of DNA-reacted cells after treatment with both kits (Left column Halosperm, Right column SFDA). One representative cell from each subpopulation is shown (Top-to-bottom – SP1 large, SP2 large-medium, SP3 medium-small, SP4 small). Scale bar = 10 µm applicable to each figure.

The relative distribution frequency of spermatozoa belonging to each subpopulation for each patient was analyzed by the Chi-squared and Mantel–Haenszel Chi-squared tests. The morphometric data on the DNA fragmentation and the multivariate method were first tested for normality and homoscedasticity by using Shapiro–Wilks and Kolmogorov–Smirnov tests, respectively. Because no parameters satisfied either criterion, nonparametric analyses were performed with the Kruskal–Wallis test. The results were presented as mean ± standard deviation (s.d.). Statistical significance was considered at $P < 0.05$.

Discriminant analysis[26] was performed from the principal components and the classification by subpopulations of the DNA fragmentation to obtain a classification matrix. The analysis was done considering the morphometric data independently, with a linear stepwise procedure to identify those parameters that were most useful for classifying individual cells into one of the four subpopulations. In all cases, principal component vectors were added to the discriminant function variables to obtain significantly better discrimination. It was found in all cases that all the variables were useful for discrimination. The classification matrix obtained after this discriminant analysis was applied to the whole population to establish the proportion of cases in each category by each DNA fragmentation kit.

All data were analyzed using InfoStat Software v2008 (University of Córdoba, Córdoba, Argentina) for Windows.[27]

RESULTS

Correlation of morphometric parameter values between kits

Comparison of the total number of cells analyzed from both kits produced a significant correlation between considered morphometric data ($P < 0.001$) although the correlation values between both kits were not enough to

permit to extrapolation of one set of data from the other. The value of *r* for Halo was of 0.54, for Core of 0.44, and for the Halo/Core Ratio of −0.35.

Principal component analysis

For the two kits considered, principal component analysis of the three parameters analyzed (Halo, Core, and Halo/Core Ratio) rendered two PCs. In the case of Halotech, PC1 was related to the three parameters and explained 80% of the variance. PC2 was essentially related to Ratio, explaining the remaining 20% (**Table 1**). In the case of SDFA, PC1 was positively related to the Halo and Core areas, and negatively to the Ratio, explaining 79% of the variance, and PC2 was related to Ratio and Halo, explaining 20% (**Table 1**).

Subpopulation structure

The distribution of DNA-reacted cells in the subpopulations was made on the basis of the Halo area, the Halo/Core Ratio, and from the PC data. Independently of the classification criteria and the kit used, four subpopulations were found. In all cases, the subpopulations comprised four size classes: SP1, large; SP2, large-medium; SP3, medium-small; and SP4, small (**Table 2** and **Figure 1**). Only the values of Ratio showed no differences between SP1 and SP2 from Halosperm samples with the Halo classification method. No differences in Core values were found between SP2, SP3, and SP4 for the SDFA samples for Core values examined by the Ratio classification method or between SP3 and SP4 for Ratio with the PC classification method (**Table 2**).

Table 1: Principal component analysis of morphometric data from DNA-reacted cells for each DNA fragmentation kit application

Parameter	Halosperm		SDFA	
	PC1	PC2	PC1	PC2
Halo	0.6		0.6	0.6
Core	0.6	−0.5	0.6	
Ratio	0.5	0.8	−0.5	0.8
Explained variation (%)	80	20	79	20

Only eigenvalues >0.3 are shown. PC: principal component; SDFA: sperm DNA fragmentation assay

For both kits and methods, SP1 was the less frequent with the exception of the SDFA kit for the Ratio method. The distribution of the other SPs depended on the kit and method (**Table 2**). With the Halo area as the classification criterion, the larger reacted cells (SP1) with both kits comprised similar and low percentage of cells, but SP2 was <10% for Halosperm and >20% for SDFA. For the Halosperm kit, SP4 was more abundant than SP3, but for SDFA, SP3 was more frequent than SP4 (**Table 2**). The distribution of subpopulations in patients varied both between classification techniques and kits used (**Table 3**).

Discriminant analysis

For this study, we only used the data from PCA. During the previous subpopulation analysis, each cell was assigned to one of the four subpopulations, and this assignment was used to define the canonical cells for the discriminant analysis performance. Fisher discriminant linear coefficients for both kits were obtained (**Table 4**). After reclassification of canonical cells following the Fisher matrix, the percentage of well-classified cells was 97.2 for Halosperm and 96.9 for SDFA. In the Halosperm samples, all the cells of SP1 were well classified while 5.2% of the cells from SP4 were classified as belonging to SP3. In the SDFA samples, both SP1 and SP2 showed 100% correct classification while 9.7% of the SP3 were classified as SP2 (**Table 5**).

DISCUSSION

Different studies have shown that DNA fragmentation evaluated by the SCD technique is a good parameter for predicting fertility in humans,[28–30] even better than "standard parameters" when combined with mitochondrial membrane potential.[31] In addition to studies on fertility, this technique has been applied to different clinical and toxicological situations such as varicocele,[32] ejaculatory abstinence,[33] cigarette smoking and alcohol consumption,[34] and genitourinary infection.[35] From SCD criteria, independently of the test kit used, sperm cells with very small halos or without halos, as well as degraded sperm cells, are classified as containing fragmented DNA, and cells with intermediate or large halos are not considered fragmented.[20,22,29,36] In some papers, the description is somewhat more accurate, including

Table 2: Morphometric values (mean±s.d.) by subpopulation for each parameter presented by the kit used and the classification method

CM/P	Halosperm				SDFA			
	%	Halo	Core	Ratio	%	Halo	Core	Ratio
Halo (µm²)								
SP1	2.9	378.2±55.7a,m,x	125.7±29.1a,m,x	3.1±0.5a,m,x	6.3	318.2±54.4a,n,x	299.0±64.8a,n,x	1.1±0.3a,n,x
SP2	7.8	228.7±33.6b,m,x	75.5±13.9b,m,x	3.1±0.5a,m,x	23.7	199.8±25.7b,n,x	158.9±51.2b,n,x	1.4±0.6b,n,x
SP3	31.2	117.7±20.9c,m,x	50.7±9.4c,m,x	2.3±0.3b,m,x	36.4	126.5±19.2c,n,x	85.4±33.4c,n,x	1.7±0.5c,n,x
SP4	58.1	65.6±16.8d,m,x	31.8±7.6d,m,x	2.1±0.3c,m,x	33.6	58.0±19.6d,n,x	31.8±14.9d,n,x	1.9±0.4d,n,x
Ratio								
SP1	5.8	244.6±88.4a,m,y	70.2±26.3a,m,y	3.5±0.3a,m,y	38.4	188.4±73.3a,n,y	178.6±71.3a,n,y	1.1±0.03a,n,x
SP2	18.4	150.2±91.3b,m,y	55.9±31.8b,m,y	2.6±0.2b,m,y	9.1	125.0±70.3b,n,y	46.9±27.0b,n,y	2.7±0.4b,m,y
SP3	46.8	92.6±35.7c,m,y	41.0±15.3c,m,y	2.2±0.1c,m,y	23.1	103.0±56.2c,m,y	48.0±26.0b,n,y	2.1±0.1c,m,y
SP4	29.0	64.1±23.3d,m,y	35.5±12.7d,m,y	1.8±0.1d,m,y	29.4	86.7±40.8d,n,y	49.0±21.9b,n,y	1.8±0.1d,m,x
PC								
SP1	3.2	343.8±88.3a,m,x	126.4±25.5a,m,x	2.7±0.4a,m,z	8.7	297.3±58.1a,n,x	284.3±56.8a,n,x	2.4±0.4a,n,y
SP2	7.3	223.5±71.2b,m,x	66.4±20.1b,m,x	3.4±0.4b,m,z	29.5	157.3±38.4b,n,z	148.6±37.3b,n,x	1.9±0.2b,n,z
SP3	49.4	89.6±31.3c,m,y	37.5±11.3c,m,z	2.4±0.2c,m,x	18.9	148.6±57.2b,n,z	64.2±27.5c,n,z	1.1±0.01c,n,z
SP4	40.1	79.8±33.7d,m,z	40.8±14.0d,m,z	1.9±0.2d,m,z	42.9	76.3±33.0c,n,z	41.4±18.9d,n,z	1.1±0.01c,n,y

a–dDifferent superscripts indicate significant differences between subpopulations within each CM for each parameter and kit; m–nDifferent superscripts indicate significant differences between kits for each subpopulation within each CM and parameter; x–zDifferent superscripts indicate significant differences between CM for each subpopulation for each parameter and kit. Significant differences were considered at *P*<0.05 after Kruskal–Wallis test. CM: classification method; P: parameter; PC: principal component; Ratio: halo/Core; SP: subpopulation; %: percentage of total sperm number comprising each SP; s.d.: standard deviation; SDFA: sperm DNA fragmentation assay

Table 3: Distribution of subpopulations (%) for each volunteer in each kit from different classification methods

	Halosperm				SDFA			
	SP1	SP2	SP3	SP4	SP1	SP2	SP3	SP4
Halo CM								
1*,x	0.0	0.0	34.4	65.6	1.1	38.6	50.0	10.2
2x	24.8	54.5	13.9	6.9	31.5	40.7	17.6	10.2
3*,x	0.0	0.0	0.0	100.0	0.0	0.0	52.1	47.9
4	0.0	3.5	29.9	66.7	0.5	9.2	32.4	57.8
5*,x	0.0	0.6	57.1	42.2	3.2	33.8	40.9	22.1
6*,x	0.7	5.6	47.6	46.2	10.4	18.7	29.9	41.0
7*,x	0.0	0.0	10.7	89.3	0.0	22.0	43.3	34.7
Ratio CM								
1*,y	0.0	33.3	61.5	5.2	54.5	0.0	4.5	40.9
2*,y	49.5	34.7	9.9	5.9	53.7	12.0	21.3	13.0
3*,y	1.8	5.3	77.2	15.8	4.2	0.0	37.5	58.3
4*	0.0	4.0	40.8	55.2	8.6	14.6	33.0	43.8
5*,y	0.0	20.5	47.8	31.7	59.7	4.5	12.3	23.4
6*,y	0.7	24.5	36.4	38.5	42.5	10.4	21.6	25.4
7*,y	0.7	14.1	67.8	17.4	40.0	12.0	30.7	17.3
PC CM								
1*,z	0.0	1.0	86.5	12.5	6.8	47.7	5.7	39.8
2*,z	22.8	55.4	8.9	12.9	39.8	13.9	32.4	13.9
3*,z	0.0	1.8	84.2	14.0	0.0	4.2	10.4	85.4
4*	1.0	0.0	26.9	72.1	1.1	7.6	27.0	64.3
5*,z	0.6	0.0	52.8	46.6	5.2	53.2	11.7	29.9
6*,z	2.1	4.9	42.0	51.0	10.4	32.1	17.9	39.6
7*,z	0.0	0.7	73.8	25.5	1.3	38.7	18.0	42.0

*For each volunteer significant differences in subpopulation distribution between kits for each CM, x–zFor each volunteer significant differences in subpopulation distribution between CM for each kit. Significant differences by Chi-squared test were considered at $P<0.05$. CM: classification method; PC: principal component; Ratio: halo/Core; SP: subpopulation; SDFA: sperm DNA fragmentation assay

Table 4: Discriminant linear coefficients classification matrix (Fisher) from principal component data

	SP1	SP2	SP3	SP4
Halosperm				
PC1	4.9546562	2.9556255	−0.1947948	−0.6903666
PC2	−1.7570925	1.2368074	0.3601852	−0.5285625
SDFA				
PC1	3.3050860	1.1265700	−0.8204570	−1.0799150
PC2	0.6298914	−0.6116444	1.1426632	−0.2098324

SP: subpopulation; SDFA: sperm DNA fragmentation assay; PC: principal component

Table 5: Percentage of cells of the reference population assigned to each class after discriminant analysis of the principal component data

	SP1	SP2	SP3	SP4	Total
Halosperm					
SP1	100	0.0	0.0	0.0	100
SP2	0.0	97.0	3.0	0.0	100
SP3	0.0	0.2	96.7	3.1	100
SP4	0.0	0.0	5.2	94.8	100
SDFA					
SP1	100	0.0	0.0	0.0	100
SP2	0.0	100.0	0.0	0.0	100
SP3	0.0	9.7	90.3	0.0	100
SP4	0.0	0.0	4.1	95.9	100

Overall 97.1% and 96.6% of the reference sperm population by Halosperm and SDFA, respectively, were classified correctly. SDFA: sperm DNA fragmentation assay; SP: subpopulation

references to relatives sizes, but proper morphometric measures have not been done.[37]

As with other seminal parameters, when subjective evaluation is done, relative criteria are used. How is a small halo to be defined? Just because it is not medium or big, but how is it defined? The actual expanse of the Halo is a continuous variable that cannot be reduced to one discrete value just by choice. In the present work, it was demonstrated that with appropriate statistics, the classification can be based on the real nature of the variables. The four subpopulations we have found in all cases paralleled the subjective evaluations of "no halo," "small halo," "medium," and "large halo," respectively, but on the basis of mathematical data, they can be applied to future classifications. Correlation studies between both approaches must be done. The differences observed in classification criteria between kits used indicate that even with results from the same principle (SCD), differences could originate from the composition of the solutions. For future work, it is thus necessary to mention specifically the technique used when results are presented.

Instead of using just one of the morphometric parameters offered by CASA-DNAf systems for classification, it is better to integrate them through the use of principal component analysis, and for this reason, we have used this approach for the definition of the classification matrix that can be used for the future work. We acknowledge that the number of cases and spermatozoa presented here is insufficient to establish a definitive classification matrix, but this paper represents a start in the rigorous objective sorting of spermatozoa processed to provide assessment of their nuclear DNA fragmentation. More work will be done in the way described here.

CONCLUSION

The present data are the first demonstration of a new evaluation of DNA fragmentation from morphometric criteria, but we have not aimed to compare the former definition of fragmentation with the new purpose. Future work on more data following this method will provide a classification matrix to be used in future evaluation, by the use of automatic CASA-DNAf systems, of the impact of DNA fragmentation on male fertility in both clinical and research work.

AUTHOR CONTRIBUTIONS

SS and CS conceived and designed the experiments; AGM, FC, and SF performed the experiments; AV and CS analyzed the data; CS wrote the paper.

COMPETING INTERESTS

CS is Professor at Valencia University and acts as Scientific Director of Proiser R+D S.L Research and Development Laboratory. Neither he nor the other authors have interests that influenced the results presented in this paper.

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