Asian Journal of Andrology (2016) 18, 819–823

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INVITED ORIGINAL ARTICLE

Semen Analysis

Comparison of different statistical approaches to evaluate morphometric sperm subpopulations in men

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This study was designed to characterize morphometric sperm subpopulations in normozoospermic men by using different statistical methods and examining their suitability to classify correctly different sperm nuclear morphologies present in human ejaculates. Ejaculates from 21 normozoospermic men were collected for the study. After semen collection and analysis, samples were prepared for morphometric determination. At least 200 spermatozoa per sample were assessed for sperm morphometry by computer-assisted sperm morphometry analysis (CASA-Morph) using fluorescence. Clustering and discriminant procedures were performed to identify sperm subpopulations from the morphometric data obtained. Clustering procedures resulted in the classification of spermatozoa into three morphometric subpopulations (large-round 30.4%, small-round 46.6%, and large-elongated 22.9%). In the second analysis, using discriminant methods, the classification was made independently of size and shape. Three morphological categories according to nuclear size (small <10.90 μm², intermediate 10.91–13.07 μm², and large >13.07 μm²) and four categories were defined on 400 canonical cells (100 × 4) from 10 men according to sperm nuclear shape (oval, pyriform, round, and elongated). Thereafter, the resulting classification functions were used to categorize 4200 spermatozoa from 21 men. Differences in the class distribution were observed among men from both clustering and discriminant procedures. It was concluded that the combination of CASA-Morph fluorescence-based technology with multivariate cluster or discriminant analyses provides new information on the description of different morphometric sperm subpopulations in normal individuals, and that important variations in the distribution of morphometric sperm subpopulations may exist between men, with possible functional implications.

Asian Journal of Andrology (2016) **18**, 819–823; doi: 10.4103/1008-682X.186872; published online: 13 September 2016

Keywords: computer-assisted sperm morphometry analysis; man; sperm subpopulations

INTRODUCTION

Spermatozoa are highly specialized cells that have to function in the complex environment of the female genital tract. Spermatozoa present in ejaculates are heterogeneous, and the existence of sperm subpopulations in mammalian ejaculates is now widely accepted.[1] These subpopulations may be an adaptive mechanism to increase the chance of a fertilization.

Sperm subpopulations in semen have been identified in different species on the basis of biochemistry (humans[2,3]), function (boars[4,5]), motility (stallions;[6] red deer;[7,8] dogs;[9] bulls;[10] rams;[11–13] and blue foxes[14]), and morphometry (stallions;[15] boars;[16,17] red deer;[7,18] bulls;[17,19] brown bears;[20] rams;[12,13,17,21,22] Goeldi's monkey;[23] and marmosets[24]). There is increasing evidence that the heterogeneity of these subpopulations has functional relevance. For example, relationships have been found between the sperm subpopulations and fertility,[8,25] and the ability to survive cryopreservation.[9,26,27] Theoretically, heterogeneity of spermatozoa ensures a greater potential to fertilize an oocyte at some unpredictable interval after ejaculation.[28]

The introduction of computer-assisted sperm morphometry analysis (CASA-Morph) systems has increased the objectivity and sensitivity of sperm morphological evaluation. The use of morphometric data obtained with this technique has changed the classical approach of considering the whole ejaculate as a single homogeneous population with a normal distribution, by showing the existence of sperm subpopulations.[24] Thus, there is a substantial loss of information when traditional statistical procedures are applied to the results, because the real distribution of sperm morphometric forms is not uniform and normal, but rather structured in separate subpopulations.[24,29] An association between computerized and statistical techniques allows classifying the overall sperm population of semen samples into homogeneous, separated subpopulations, grouping spermatozoa with similar morphometry characteristics.[12] Two different

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statistical methods, cluster and discriminant analyses, have been used to disclose sperm morphometric subpopulations in different species.[29] The aim of this study was to characterize sperm morphometric subpopulations in normozoospermic men by using different statistical methods and examining their ability to classify correctly different sperm nuclear morphologies present in ejaculates. This study may constitute the basis for future analyses of the relationships of sperm quality, freezing capacity, and human male fertility.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich Chemical Company (Alcobendas, Madrid, Spain) and were of the highest grade available.

Donors and sample selection

The study was approved by the Institutional Ethics Committee and written informed consent was given by all patients. Semen samples from 21 volunteers, with mean age of 24.2 years (range 21–32 years), were obtained by masturbation after 3–5 days of sexual abstinence. Only men with clinically normal semen parameters, judged from the World Health Organization[30] reference values, were included in the study.

After collection, the semen was allowed to liquefy at 37°C for at least 30 min and then was examined within 1 h. Each ejaculate was thoroughly mixed, and aliquots were prepared for sperm morphometric assessment as previously described.[31,32] In brief, semen smears were allowed to air dry for a minimum of 2 h, fixed with 2% (v/v) glutaraldehyde in PBS for 3 min, washed thoroughly in distilled water and labeled with Hoechst 33342 as detailed below.

Sperm morphometric determination by computer-assisted sperm morphometry analysis (CASA-Morph)

Semen smears were stained by placing 20 µl of a Hoechst 33342 suspension (20 µg ml^{-1} in a TRIS-based solution) between the slide and a coverslip, which was then incubated for 20 min in the dark at room temperature.[31] The coverslip was then removed and the slide was washed thoroughly with distilled water and allowed to dry. Digital images of the fluorescent sperm nuclei were recorded by means of a setup composed of an epifluorescence microscope (DM4500B, Leica, Wetzlar, Germany; A-UV filter cube, BP340–380 excitation filter, LP425 suppressor filter, dichromatic mirror: DM400) with a 63× plan apochromatic objective, and photographed with a Canon Eos 400D digital camera (Canon Inc., Tokyo, Japan). The camera was controlled by a computer using DSLR Remote Pro software (Breeze Systems, Camberley, UK).

At least 200 sperm cells per sample were randomly captured at least two slides per sample. From each captured image, sperm nuclei morphometry was automatically analyzed by the ImageJ open software (available on-line at http://rsbweb.nih.gov/ij/download.html), with a plug-in module created for this purpose.[31] Each sperm nucleus was measured for four primary parameters and four derived parameters for nuclear shape. Primary parameters were Area (A, µm^2, as the sum of all pixel areas contained within the boundary), Perimeter (P, µm, as the sum of external boundaries), and Length (L) and Width (W) (µm, the highest and lowest values, respectively, of the Feret diameters, i.e., the projection of the sperm nucleus on the horizontal axis measured at angles of rotation of 0°, 30°, 60°, 90°, 120°, and 150°; Length and Width are not necessarily orthogonal). Derived nuclear shape parameters were Ellipticity (L/W), Rugosity ($4\pi A/P^2$), Elongation ([L – W]/[L + W]), and Regularity ($\pi LW/4A$).

Statistical analysis

Statistical analyses were performed with the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). Two methods were used to obtain sperm subpopulations based on the morphometric data. The first method was based on two-step cluster procedures.[12,17] The first step was to perform a principal component analysis (PCA) of the morphometric data. The purpose of PCA is to derive a small number of linear combinations (principal components) from a set of variables that retain as much of the information in the original variables as possible. This allows the summarizing of many variables in few, jointly uncorrelated, principal components. A preferred result is when there are few principal components accounting for a large proportion of the total variance. To select the number of principal components that should be used in the next step of the analysis, the criterion was used of selecting only those with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion). The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA. This analysis allows the identification of sperm subpopulations and the detection of the outliers.

The second method was based on a two-step discriminant analysis.[33,34] The first step consisted of defining the nuclear size into three categories: small, intermediate, and large. Each threshold was established on the basis of the area values, considering the 25th centile and below for small, 26th–74th centile for intermediate, and 75th centile and above for large. The second step focused on nuclear shape, initially comprising four subjective forms: round, elongated, oval, and pyriform (**Figure 1**). Oval nuclei were the most frequently represented and may be considered analogous to the normal cells described by the WHO.[30] Round, elongated, and pyriform spermatozoa also have a correspondence to the round, narrow-tapered, and pyriform cells described by the WHO.[30] For each class, a number of 100 canonical cells/category obtained from 10 men were selected and used for the subsequent calculation of the classification matrix. Different donor samples (10 donors) were used to define canonical cells from those used for the global discriminant analysis (21 donors). Discriminant analysis was performed by the linear stepwise procedure to identify the most useful parameters for the shape classification of these cells. Variables were added one by one to the discriminating functions until it was found that the addition of a new variable did not give a better discrimination. Wilk's lambda

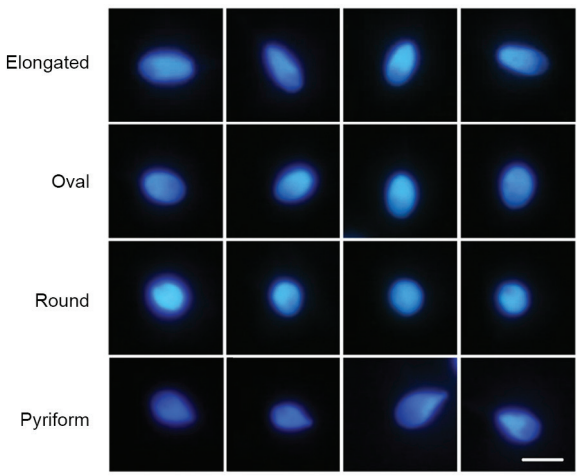

Figure 1: Four examples of each of the sperm categories defined according to sperm nuclear shape. Scale bar for all images, 5 µm.

was used to test discrimination. Then, the classification matrix was applied to the whole population to establish the proportion of each class (subpopulation) per animal.

To study the distributions of subpopulations between men, the Chi-squared test was used. The values obtained were expressed as mean ± standard error of the mean (s.e.m.). The statistical level of significance was set at $P < 0.05$.

The variability of each parameter at different grouping levels was determined from the coefficients of variation (CV).[31] In all the donors, within-man and between-man CVs were calculated for all the morphometric parameters. Within-man CVs were expressed as the mean of individual values.

RESULTS

Spermatozoa in humans are characterized by substantial between-man variability. The highest variability in sperm nuclear morphometry was identified for elongation and area (CV 38.98% and 20.34%, respectively). All morphometric parameters showed a higher degree of variation between individuals than within individuals.

From the two-step cluster procedure, PCA analysis revealed three components with eigenvalues over 1, representing more than 97.4% of the cumulative variance (**Table 1**). The first factor (PC1) was defined positively by primary (P and L) and secondary (Ellipticity and Elongation) parameters, the second (PC2) by positive primary (A and W) and negative secondary (Ellipticity and Elongation) factors, and the third (PC3) by Regularity.

The second step of clustering analysis revealed the existence of three sperm subpopulations (**Table 2**). Subpopulation 1 (SP1) had positive values for PC2, so this cluster includes large and round spermatozoa. Subpopulation 2 (SP2) had negative values for PC1, so it comprises small and round spermatozoa. Subpopulation 3 (SP3) had positive values for PC1 and negative for PC2, thus comprising large and elongated spermatozoa. Of the total spermatozoa, 29.8%, 47.5%, and 22.7% were included in Subpopulations 1, 2, and 3, respectively. The distribution of sperm subpopulations was completely different among men ($P < 0.001$, **Table 3**).

In the second analysis, with discriminant methods, the classification was made independently of size and shape. For shape, the matrix of classification obtained gave the Fisher's discriminant linear functions for each class as listed in **Table 4**. This matrix was applied to the reference population with a globally correct assignment of 86.4% of cells (**Table 5**). Round and oval sperm nuclei were more accurately classified (more than 94%) than elongated and pyriform forms. When the allocation matrix for shape was applied to the whole population, oval cells were the most frequently represented (54.1%), while the most infrequent were round and elongated spermatozoa (11.5% and 13.0%, respectively).

For size, the sperm nuclei were divided into three classes from the area data, considering the small ones as those equal to and below the 25th centile, the large ones as those equal to and above the 75th centile, and the intermediate ones between these limits. The values observed were: small, area <10.90 μm^2; intermediate, 10.90 μm^2 ≥ area ≤13.07 μm^2; and large, area >13.07 μm^2. Differences in the class distribution were observed among men ($P < 0.001$), with some donors having more than 90% of the cells with a small area, while others had 0%. This extent of difference was also present for the other two size classes (**Table 6**). Moreover, differences in the distribution of the shape classes were found between men (**Table 6**).

Table 1: Results of the PCA performed on the CASA Morph data obtained from 21 normozoospermic men

Morphometric parameters	*PC1*	*PC2*	*PC3*
Area	0.689	0.717	–0.077
Perimeter	0.846	0.528	–0.037
Length	0.969	0.213	–0.039
Width	0.245	0.962	0.090
Ellipticity	0.722	–0.668	–0.130
Rugosity	–0.758	0.531	–0.140
Elongation	0.710	–0.671	–0.140
Regularity	0.193	–0.113	0.970

PCA: principal component analysis; PC: principal component

Table 2: Results of the two-step cluster procedure in men with the morphometric indices (PC) as variables

Cluster	*PC1*		*PC2*		*PC3*	
	Mean	*s.e.m.*	*Mean*	*s.e.m.*	*Mean*	*s.e.m.*
1	0.2802	0.0178	1.1263	0.0120	0.0336	0.0218
2	–0.6700	0.0174	–0.2602	0.0124	–0.0458	0.0234
3	1.0325	0.0252	–0.9311	0.0169	0.0518	0.0495

s.e.m: standard error of mean; PC: principal component

Table 3: Percentage distribution of sperm subpopulations in the different men

Donor	*Sperm subpopulations*		
	1	*2*	*3*
1	0.5	86.5	13
2	5.5	72.0	22.5
3	0.5	73.5	26.0
4	0.0	84.5	15.5
5	15.0	39.0	46.0
6	84.5	6.0	9.5
7	56.0	13.0	31.0
8	27.5	30.0	42.5
9	62.5	24.0	13.5
10	26.5	37.5	36.0
11	24.1	71.9	4.0
12	3.0	39.0	58.0
13	35.0	44.0	21.0
14	35.0	52.5	12.5
15	14.0	36.5	49.5
16	33.5	57.1	9.4
17	38.0	43.0	19.0
18	16	56.5	27.5
19	79.5	18.0	2.5
20	68.0	21.0	11.0
21	14.5	73.5	12.0
Mean	30.4	46.6	22.9

Significant statistical differences were found among individuals (Pearson's χ^2, $P<0.001$)

DISCUSSION

The subjective evaluation of sperm morphology lacks precise replication, and the coefficients of variation associated with this analysis are very high.[35,36] This fact points to the need to establish quantitative criteria for the definition of sperm cell morphology, which has been improved by the introduction of CASA-Morph systems. Morphometry

Table 4: Discriminant classification matrix showing Fisher's linear discriminant functions for shape of human sperm cells

	Coefficient of function of classification			
	Round	*Elongated*	*Oval*	*Pyriform*
Area	−628.227	−627.311	−630.724	−629.547
Perimeter	1141.248	1133.718	1143.111	1135.543
Length	−653.350	−669.300	−660.204	−649.375
Width	833.203	878.004	855.534	862.678
Ellipticity	1743.192	1555.550	1559.625	1511.497
Rugosity	12246.360	12174.289	12264.073	12159.884
Elongation	1280.710	2361.626	2136.304	2297.684

Values were obtained from a reference population of 400 canonical sperm cells (100×4) by linear stepwise discriminant analysis

Table 5: Percentage of sperm heads in each class of the reference population assigned to each class after discriminant analysis

Canonical forms	*Percentage of spermatozoa allocated to group*			
	Round	*Elongated*	*Oval*	*Pyriform*
Round	98.0	0.0	2.0	0.0
Elongated	0.0	83.0	5.0	12.0
Oval	0.9	0.9	94.3	3.8
Pyriform	0.0	11.4	18.1	70.5

86.4% of the reference population was classified correctly

Table 6: Percentage distribution of sperm subpopulations in different men

Donor	*Size*			*Shape*			
	Small	*Intermediate*	*Large*	*Round*	*Elongated*	*Oval*	*Pyriform*
1	68.0	32.0	0.0	12.5	14.0	62.5	11.0
2	60.0	37.5	2.5	14.0	26.0	49.5	10.5
3	81.0	18.5	2.5	6.0	40.0	45.5	8.5
4	92.0	8.0	0.0	9.0	23.0	59.0	9.0
5	8.5	74.0	17.5	1.5	9.0	47.0	42.5
6	0.0	29.5	70.5	16.0		66.0	18.0
7	0.0	35.0	65.0	1.5	4.0	52.0	42.5
8	11.0	53.5	35.5	10.0	13.5	34.0	42.5
9	4.0	50.0	46.0	14.0	1.5	61.0	23.5
10	1.0	73.0	26.0	4.5	6.0	52.5	37.0
11	15.1	75.9	9.0	6.0	3.0	84.9	6.0
12	44.5	53.0	2.5	7.5	53.5	22.5	16.5
13	5.0	62.0	33.0	3.0	5.0	69.5	22.5
14	13.5	69.0	17.5	5.0	3.5	74.0	17.5
15	6.5	67.0	26.5	1.0	12.5	44.0	42.5
16	12.3	72.4	15.3	18.7	2.5	63.5	15.3
17	8.5	68.5	23.0	13.0	6.5	56.0	24.5
18	20.0	73.5	6.5	6.0	17.0	53.0	24.0
19	2.0	29.0	69.0	19.0	0.5	75.5	5.0
20	1.5	45.0	53.5	20.0	0.5	60.0	19.5
21	43.5	50.5	6.0	26.5	9.5	53.5	10.5
Mean	23.7	51.3	25.1	10.2	12.6	56.4	21.4

Significant statistical differences were found among individuals (Pearson's χ^2, $P<0.001$)

analyzed by these systems has been considered a powerful tool for the selection of human patients for ART.[37]

A wide range of values for sperm nuclear morphometry parameters was evident here, both within samples and among men. Differences in values both within samples and among individuals are also present in other animal species.[31,32] Despite this variability among men, the mean values for the sperm nuclear dimensions are higher than those reported by some,[38–42] and lower than those described by others[43,44] for the whole sperm head. These results are not surprising because previous work has shown that variation in fixation, staining, or the software used can cause important differences in sperm head morphometr.[29]

Human spermatozoa are highly heteromorphous, with morphological differences both in the same ejaculate and in different individuals.[40,45] The different sperm subpopulations may be considered to work synergistically to increase fertilization success.[26,46,47] Morphometric data provided by CASA-Morph systems may be analyzed by traditional statistical procedures although, given the heterogeneity of spermatozoa in the human ejaculates, the study of sperm subpopulations may be more informative.[29] A combination of computerized and statistical techniques has allowed classification of the overall sperm population of semen samples into homogeneous, separate subpopulations in different species, by grouping spermatozoa with similar morphometry characteristics.[29] However, research into morphometric sperm subpopulations has received little attention in the human species.

In the present study, two alternative statistical procedures were compared to disclose sperm morphometric subpopulations: two-step cluster and two-step discriminant analyses. These methods have been successfully used in different species.[7,15–21,29,33,34,48] From the two-step cluster procedure, different sperm subpopulations were obtained and their distribution varied significantly among men, providing more information than classical analysis of sperm morphometric data that are based on mean values. However, this method provides a classification of spermatozoa mainly based on head size and elongation.[17] The two-step discriminant analysis, however, allows a separate and more precise classification of spermatozoa according to their head size and shape, and its use may be more adequate for heteromorphous species,[33,34] particularly in those species, as man, in which a previous standard has been defined.[30] While measuring sperm head size can be considered an easy task, shape evaluation is commonly evaluated subjectively and expressed in descriptive terms: round, elongated, oval, and pyriform. Here, oval nuclei were the most frequently represented and may be considered analogous to the normal cells described by the WHO.[30] However, there was no concordance between our results and those indicated in the WHO manual. Certainly, we are considering only the nuclear shape and size, even separately, and other cell components can contribute to the normal/abnormal definition, but we observed a higher proportion of oval cells with our technique than that indicated by the WHO. This implies that both the technique and the statistical approach used here can be a new model for human sperm morphological evaluation.

It is concluded that the combination of our defined CASA-Morph fluorescence-based technology with multivariate cluster or discriminant analyses provides new information on the description of different morphometric sperm subpopulations in normozoospermic individuals. Important variations in the distribution of morphometric sperm subpopulations may exist among men, with possible functional implications.

AUTHOR CONTRIBUTIONS

JLY and PS conceived and designed the experiments; SVF, CS, PR, TC, AB, and JMB performed the experiments; PS analyzed the data; and JY 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.

ACKNOWLEDGMENTS

The authors would like to thank Neil Macowan for assistance with the English translation. We are especially grateful for the support from the healthy semen donor volunteers that enabled us to perform the present study. This work was supported by the Spanish MINECO (grant AGL2014-52775-P), and the DGA-FSE (grant A40).

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