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INVITED ORIGINAL ARTICLE

Semen Analysis

Morphometric and kinematic sperm subpopulations in split ejaculates of normozoospermic men

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This study was designed to analyze the sperm kinematic and morphometric subpopulations in the different fractions of the ejaculate in normozoospermic men. Ejaculates from eight normozoospermic men were collected by masturbation in three fractions after 3–5 days of sexual abstinence. Analyses of sperm motility by computer-assisted sperm analysis (CASA-Mot), and of sperm morphometry by computer-assisted sperm morphometry analysis (CASA-Morph) using fluorescence were performed. Clustering and discriminant procedures were performed to identify sperm subpopulations in the kinematic and morphometric data obtained. Clustering procedures resulted in the classification of spermatozoa into three kinematic subpopulations (slow with low ALH [35.6% of all motile spermatozoa], with circular trajectories [32.0%], and rapid with high ALH [32.4%]), and three morphometric subpopulations (large-round [33.9% of all spermatozoa], elongated [32.0%], and small [34.10%]). The distribution of kinematic sperm subpopulations was different among ejaculate fractions ($P < 0.001$), with higher percentages of spermatozoa exhibiting slow movements with low ALH in the second and third portions, and with a more homogeneous distribution of kinematic sperm subpopulations in the first portion. The distribution of morphometric sperm subpopulations was also different among ejaculate fractions ($P < 0.001$), with more elongated spermatozoa in the first, and of small spermatozoa in the third, portion. It is concluded that important variations in the distribution of kinematic and morphometric sperm subpopulations exist between ejaculate fractions, with possible functional implications.

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INTRODUCTION

Ejaculate fractioning is typical for species with a high ejaculate volume, such as the boar[1] and stallion.[2] In spite of its comparatively small volume, a clear fractioning of the ejaculate has also been described in men,[3,4] with more total and motile spermatozoa in the first fraction of a split ejaculate. In different studies, the human ejaculate has been fractionated into two to six portions although many researchers have opted for the study of three-portion ejaculate.[5,6]

Spermatozoa present in the ejaculates are heterogeneous, and the existence of sperm subpopulations in mammalian ejaculates is now widely accepted.[7] There is increasing evidence that the heterogeneity among these subpopulations has functional relevance. For example, associations have been found between the sperm subpopulations and fertility[8–10] and their ability to survive cryopreservation.[11–13]

To the best of our knowledge, the possible contribution of ejaculate fractions to the heterogeneity of the ejaculates has not been considered in previous studies. Although the morphology of sperm cells has been described as normal in all split fractions in different studies,[14,15] little is known about the distribution of sperm subpopulations in the different split portions of the human ejaculate. The aim of this study was to analyze possible differences in sperm kinematic and morphometric subpopulations in the different fractions of the ejaculate from normozoospermic men.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich Chemical Company (Alcobendas, Madrid, Spain) and were of the best 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 eight volunteers aged 23 to 36 years were obtained by masturbation after 3–5 days of sexual abstinence. The volunteers received a box with three independent containers and were instructed to collect the complete semen sample into them in three successive portions (F1 to F3). The first and second spurts were collected in the first and second containers, respectively. The remainder of the ejaculate was collected in the third container. Only men with clinically normal sperm parameter values (judged from the World Health Organization[16]

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reference values on previous complete semen samples) were included in the study.

After collection, the semen aliquots were allowed to liquefy at 37°C for at least 30 min and were then examined within 1 h after recovery. When liquefaction was complete, sperm concentration and sperm motility were determined using a computer-assisted sperm analyzer (ISAS® v1, PROISER R+D, S.L., Paterna, Valencia, Spain) after placing a semen sample in a Makler® counting chamber (10 μm depth; Makler®; Sefi Medical Instruments, Haifa, Israel) in duplicate. The semen of each split ejaculate portion was then carefully mixed, and sample aliquots were prepared for sperm morphometry assessment as previously described.[17,18] Briefly, 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 motility determination by computer-assisted sperm analysis (CASA-Mot)

The ISAS® v1 computer-assisted sperm analyzer was used to assess sperm motility. Sample aliquots (5 μl) were placed in a prewarmed Makler chamber and examined in an Olympus BX 40 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a heated stage set at 37°C, a 20× phase-contrast objective lens and a Basler A310F digital video camera (Basler Vision Technologies, Ahrensburg, Germany). Two consecutive drops and at least 200 sperm cells were analyzed by CASA-Mot for each sample. Established setup parameters were 25 frames s^{-1}, with 2–40 μm^2 for head area and minimum curvilinear velocity (VCL) >10 $\mu m\ s^{-1}$ to classify a spermatozoon as motile. Reported parameters were curvilinear velocity (VCL, $\mu m\ s^{-1}$), straight line velocity (VSL, $\mu m\ s^{-1}$), average path velocity (VAP, $\mu m\ s^{-1}$), sperm linearity (LIN [VSL/VCL]) as a measure of a curvilinear path, straightness (STR [VSL/VAP]) as the linearity of the average path, wobble (WOB [VAP/VCL] as a measure of the oscillation of the actual path about the average path), amplitude of lateral sperm head displacement (ALH, μm), and beat cross frequency (BCF, Hz).[19,20] Only actively moving cells were included in the analysis of movement patterns.

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 $\mu g\ ml^{-1}$ in a TRIS-based solution) between the slide and a coverslip, and incubating for 20 min in the dark at room temperature.[17] The coverslip was then removed and the slide washed thoroughly with distilled water and allowed to dry. Digital images of the fluorescent sperm nuclei were recorded using 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 apo-chromatic 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).

From each captured image, the sperm nuclear morphometry was automatically analyzed by ImageJ open software (available on-line at http://rsbweb.nih.gov/ij/download.html), with a plug-in created for this purpose.[17] At least 100 spermatozoa per sample were assessed for sperm morphometry by CASA-Morph, making a total of 2400 cells. Each sperm nucleus was assessed by measuring four primary parameters and calculating 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), 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°). 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). Clustering procedures were performed to identify sperm subpopulations from the CASA-Mot and CASMA-Morph data.[10,21] The first step was to perform a principal component analysis (PCA) of the motility and morphometry 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 indexes obtained after the PCA. This analysis allowed the identification of sperm subpopulations and the detection of the outliers.

Differences in sperm motility or morphometric parameter values among the subpopulations of the split ejaculate fractions were examined through analysis of variance (ANOVA) by using generalized linear models. To study the distributions of subpopulations between ejaculate splits, the Chi-squared test was used. The values obtained were expressed as mean ± standard deviation (s.d.). The statistical level of significance was set at $P < 0.05$.

RESULTS

Significantly higher sperm concentrations and motility parameter values were observed in F1. This fraction, which on average represented 20.0% of the volume of ejaculate, contained 63.1% of the total ejaculate spermatozoa and 78.3% of the motile spermatozoa. In relation to morphometric attributes, the first fraction contained spermatozoa with a more elongated nucleus (higher Elongation and Ellipticity; $P < 0.001$) whereas the sperm nuclei in F3 were smaller and shorter (lower A, P, and L; $P < 0.01$). From the two-step cluster procedure, PCA analysis revealed three components with eigenvalues > 1, representing more than 87.9% of the cumulative variance (**Table 1**). The first factor (PC1) was defined mainly by primary (A, P, W, and L) parameters and secondary (Ellipticity and Elongation) parameters, the second (PC2) by primary (low W) and secondary (Ellipticity and Elongation) factors, and the third (PC3) by Rugosity.

The second clustering analysis revealed the existence of three sperm subpopulations (**Table 2**). Subpopulation 1 ($SP1_{morpho}$) had positive values for PC1 and negative for PC2, so this cluster includes large and round spermatozoa; subpopulation 2 ($SP2_{morpho}$) had positive values for PC1, so this comprises elongated spermatozoa; and subpopulation 3 ($SP3_{morpho}$) had negative values for PC1, so comprises small spermatozoa. Of the total spermatozoa, 33.9%, 32.0%, and 34.1% were included in subpopulations 1, 2, and 3, respectively. The distribution of sperm subpopulations was different among the ejaculate fractions ($P < 0.001$, **Table 3**), with more spermatozoa of the $SP2_{morpho}$ (elongated) in F1 and of the $SP3_{morpho}$ (small) in F3.

In the analysis of kinematic variables of motile spermatozoa, significantly lower values of VCL, VAP, and ALH were observed in F3, together with an increase in LIN and WOB from F1 to F3 ($P < 0.01$). From the two-step cluster analysis, PCA rendered two principal components with eigenvalues >1 (PC1 and PC2; **Table 4**), which accounted for more than 80% of the cumulative variance. The first principal component was related to rapid movement, whereas the second principal component was related to slow curvilinear movement (VCL), including narrow head lateral displacement (ALH). Both PC1 and PC2 were related to high LIN and STR.

The second clustering analysis, with the two principal components as variables, revealed the presence of three sperm subpopulations in men (**Table 5**). Subpopulation 1 ($SP1_{mot}$) had positive values for PRIN2, so this cluster includes spermatozoa with low VCL and ALH. Subpopulation 2 ($SP2_{mot}$) had negative values for PC1 and PC2, so this comprises spermatozoa with circular trajectories (low LIN, STR, and VSL). Subpopulation 3 ($SP3_{mot}$) had positive values for PC1 and negative for PC2, thus including rapid spermatozoa with high ALH. Of the total spermatozoa, 35.6%, 32.0%, and 32.4% were included in subpopulations 1, 2, and 3, respectively. The distribution of kinematic sperm subpopulations was different among ejaculate fractions ($P < 0.001$, **Table 6**), with more spermatozoa of the $SP1_{mot}$ (slow) in F2 and F3 and with a more equal distribution of sperm subpopulations in F1.

DISCUSSION

Humans produce semen in clear fractions during the ejaculation process.[22] A greater abundance of spermatozoa has been confirmed in the first portion of the ejaculate by different authors. This first sperm-rich split ejaculate fraction has been considered more fertile[16] and is widely used for artificial insemination.[4,5,14] Our results are in agreement with those of investigators who have reported that spermatozoa in the first fraction have higher motility than those in the other fractions and the whole ejaculate[4,5,14] although these findings have not always been confirmed.[3,23]

The study of sperm motility and morphology has been markedly improved by the introduction of computer-assisted sperm analysis (CASA) systems. Measurements of sperm motility by CASA-Mot and morphometry by CASMA-Morph have been considered powerful tools for the selection of human patients for ART.[24] The development of these systems has also enabled the use of morphometric[25] and motility[9,10,12,26–29] parameters to be used to identify sperm subpopulations in different species. The use of computerized and statistical techniques allows the classification of the overall sperm populations of semen samples into clearly separate, homogeneous subpopulations, by grouping spermatozoa with similar motility or morphometric characteristics. The application of these techniques in the present study allowed us to describe for the first time differences between the portions of the human split ejaculate, related to CASA-Mot- and CASA-Morph-measurable sperm parameters and to the distribution of sperm subpopulations.

Seminal plasma is composed of a mixture of the contents of the cauda epididymidis and the secretions of the accessory sexual glands, which are emptied to the urethral lumen in a fractionated, concerted way. It is generally accepted that during ejaculation in man, the accessory sex glands secrete in the following order: Cowper's glands, prostate, and seminal vesicles.[22,30] Epididymal fluid, which contains the spermatozoa, is liberated after release of prostatic fluid. The ejaculate is, therefore, composed of a series of fractions, the first contains secretions of Cowper's gland, the prostate and epididymis; the second of the prostate and seminal vesicles; and the third of the seminal vesicles.[5] In fact, split ejaculation was established as a technique for understanding the origin of the different components present in semen, as well as to evaluate the pathological condition of different glands.[5]

In the present study, clear differences were found between split ejaculate fractions, not only in total sperm motility but also in the morphometric and kinematic sperm parameters, and in the distribution

Table 1: Results of the principal component analysis (PC1, PC2, and PC3) from morphometric parameters, performed on the CASA-Morph data from eight normozoospermic men

Morphometric parameters	*PC1*	*PC2*	*PC3*
Area	0.932	−0.274	−0.073
Perimeter	0.985	−0.062	−0.008
Length	0.944	0.316	−0.018
Width	0.688	−0.722	0.009
Ellipticity	0.250	0.963	−0.010
Rugosity	0.014	−0.028	0.967
Elongation	0.245	0.965	−0.012
Regularity	0.361	0.106	0.253

PC: principal component; CASA: computer-assisted sperm analysis

Table 2: Results of the two-step cluster procedure in eight men from the morphometric indices (PC1, PC2, and PC3) as variables

Cluster	*PC1*		*PC2*		*PC3*	
	Mean	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*
$SP1_{morpho}$	0.439	0.849	−0.950	0.610	−0.090	0.151
$SP2_{morpho}$	0.388	0.774	1.009	0.637	−0.034	0.223
$SP3_{morpho}$	−0.768	0.527	−0.054	0.502	0.0396	0.150

s.d: standard deviation; PC: principal component; SP: subpopulation

Table 3: Percentage distribution of morphometric sperm SPs in the different ejaculate fractions (*n*=8)

Ejaculate fraction	*Sperm SPs*		
	$SP1_{morpho}$	$SP2_{morpho}$	$SP3_{morpho}$
F1	37.42	34.89	27.69
F2	40.42	30.79	28.79
F3	23.83	30.34	45.82
Mean	33.89	32.01	34.10

Significant statistical differences were found among ejaculate fractions (Pearson's Chi-squared test, $P<0.001$). SP: subpopulation

Table 4: Results of the principal component analysis (PC1, PC2) of kinematic parameters, performed on the CASA data from eight normozoospermic men

CASA parameters	*PC1*	*PC2*
VCL	0.644	−0.729
VSL	0.970	0.018
VAP	0.849	−0.436
LIN	0.667	0.717
STR	0.648	0.580
WOB	0.457	−0.642
ALH	0.435	−0.856
BCF	0.553	0.125

CASA: computer-assisted sperm analysis; PC: principal component; BCF: beat cross frequency; WOB: wobble; LIN: linearity; VCL: curvilinear velocity; VSL: straight line velocity; VAP: average path velocity; STR: straightness; ALH: amplitude of lateral sperm head displacement

Table 5: Results of the two-step cluster procedure in eight men with the kinematic indexes (PC1, PC2) as variables

Cluster	*PC1*		*PC2*	
	Mean	*s.d.*	*Mean*	*s.d.*
$SP1_{mot}$	0.098	0.860	1.028	0.533
$SP2_{mot}$	–0.954	0.516	–0.387	0.628
$SP3_{mot}$	0.833	0.637	–0.743	0.718

s.d: standard deviation; PC: principal component; SP: subpopulation

Table 6: Percentage distribution of kinematic sperm SPs in the different ejaculate fractions (*n*=8)

Ejaculate fraction	*Sperm SPs*		
	$SP1_{mot}$	$SP2_{mot}$	$SP3_{mot}$
F1	32.16	35.06	32.75
F2	39.65	25.97	34.38
F3	54.25	22.16	23.48
Mean	35.55	31.99	32.44

Significant statistical differences were found among ejaculate fractions (Pearson's Chi-squared test, $P<0.001$). SP: subpopulation

of sperm subpopulations. Differences in sperm morphometry between ejaculate portions have also been described in boars[1] and llamas.[31] The causes of the different characteristics of the spermatozoa in these ejaculate splits are still obscure, but the changing physicochemical differences in the composition of the seminal plasma fractions may explain them. Other possible explanations include the retention of spermatozoa in the seminal vesicles,[32] and the variations in the assembly and degree of maturation of individual spermatozoa within the epididymis.[1]

In this paper, it is concluded that the combination of CASA-Mot and CASA-Morph technologies with multivariate cluster analyses provides new descriptive information on the sperm subpopulations in the split ejaculate portion of normozoospermic individuals. These variations in the morphometric and kinematic sperm parameter values, and in the distribution of sperm subpopulations that exist among split ejaculate portions, may be important with functional implications.

AUTHOR CONTRIBUTIONS

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