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LETTER TO THE EDITOR

Semen Analysis

Updating semen analysis: a subpopulation approach

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Dear Editor,

Reproductive problems affect 8%–12% of human populations worldwide; 40%–50% of all infertility cases are due to the male, and up to 2% of men have suboptimal sperm quality.[1] Semen analysis is the first test in male infertility clinics, but interpretation of the results is not straightforward, with the significance of results for predicting fertility after intrauterine insemination (IUI), intracytoplasmic sperm injection (ICSI), and *in vitro* fertilization (IVF) differing between studies.[2] Classical semen evaluation treats seminal variables individually, so that whereas poor semen quality is an indicator of subfertility,[3] good semen quality is no guarantee of fertility.[4]

Considering that all the data and their distribution provide more information, computing variables by multivariate statistics offers a new understanding of the relationship of semen quality to fertility.[5] Different datasets corresponding to several variables were standardized from human semen samples and combined here to examine subpopulations of seminal variables with the eventual aim of providing a conceptual mathematical approach to fertility studies.

Thirteen volunteers (aged 25–59 years) signed informed consent forms to participate in the study and provide samples for the study. Semen samples were collected by masturbation after 3–5 days of abstinence. The mean of this age range was 36.9 years. Seminal volume and pH using strips (Macherey-Nagel, Düren, Germany) were assessed within 30 s of collection. The resting parameters were evaluated after 30 min for liquefaction at 37°C, counting almost 250 cells per sample. Sperm motility and concentration (total sperm count) were determined in a reusable 10-μm deep Spermtrack® counting chamber (Proiser R+D S.L., Paterna, Valencia, Spain), following the WHO (2010) classification.[6] Analyses were done subjectively on the monitor of the ISAS®v1 CASA-Mot system (Proiser R+D S.L.), equipped with an ISAS® CM13-ON video camera attached to a UB203 microscope (UOP, Proiser R+D S.L.), with a ×10 negative phase contrast objective and an integrated self-heated stage maintained at a constant temperature of 37°C.

Sperm vitality was assessed by eosin–nigrosin (Merck KGaA, Darmstadt, Germany) staining. Cells were assessed subjectively at ×20 as nonvital (pink) or vital (unstained).

Sperm DNA fragmentation (F) was assessed by the Halosperm® G2 kit (Halotech DNA, S.L., Madrid, Spain) from the extent of chromatin dispersion. Semen was diluted with FertiCult™ Flushing medium (FertiPro N.V., Beernem, Belgium) to 20×10^6 sperm cells per ml and processed as per the kit. The stained slides were analyzed with the ISAS®v1 CASA-DNAf, with a ×10 bright-field objective. The software automatically classified the sperm heads according to the kit criteria as having no DNA fragmentation (large- or medium-sized halo) or fragmented DNA (small or no halo).

Sperm DNA maturation (AB) level was assessed on well-mixed semen smears. Formaldehyde-fixed air-dried slides were stained for 5 min with 5% (*w*/*v*) aniline blue dye (Merck KGaA) and mounted on Neo-Mount® medium (Merck KGaA) under a large coverslip. The sperm heads were assessed subjectively with a ×40 bright-field objective as having mature DNA (unstained) or immature DNA (totally or partially stained).

Sperm chromatin stability (TB) was evaluated from toluidine blue-stained cells by the Sperm Chromatin Assay kit (Avicenna Research Institute, Evin, Tehran, Iran). The sperm heads were assessed subjectively with a ×40 bright-field objective as having stable chromatin (red or purple) or unstable chromatin (blue).

Clustering procedures were used to identify sperm subpopulations from the entire dataset. Principal component analysis (PCA) included the feasibility of factorial analysis, verified by the Bartlett's test of sphericity, to confirm that the correlation matrix was an identity matrix, and the Kaiser–Meyer–Olkin index, which determines the correlations between two variables once the influence of other variables is eliminated. Only components with an eigenvalue >1 were used for the next, two-step cluster procedure with the sperm-derived indices obtained by the PCA. All the data were assessed in a nonhierarchical clustering procedure (*k*-means model and Euclidean distance), which classifies the spermatozoa into subpopulations according to joint characteristics and allows the detection of outliers. The effects of clusters within and between the semen measurements were analyzed by a generalized linear model. Statistical significance was considered as $P < 0.05$ with data analyzed by InfoStat Software (version 2017; InfoStat, Córdoba, Argentina) for Windows.

PCA rendered three components, explaining 77% of the variation. The first component was related positively to seminal pH, progressive sperm motility, AB, and TB and negatively to sperm immobility and F. The second was related positively to total count and nonprogressive motility and negatively to TB. The third was positively related to vitality, nonprogressive motility, and TB. Although F, AB, and TB each indicated an aspect of DNA status, there was a greater correlation between AB and TB ($r = 0.712$) than that between F and AB ($r = 0.242$) or TB ($r = 0.126$). Cluster analysis revealed two subpopulations, SP1, characterized by progressive motility and high levels of TB and AB,

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Table 1: Subpopulations analysis of the seminogram data

Variables	*SP1 (n=8, 61.5%)*			*SP2 (n=5, 38.5%)*		
	MD	*Q1*	*Q3*	*MD*	*Q1*	*Q3*
Semen volume (ml)	3.15	2.50	5.30	4.00	3.80	4.50
Semen pH	7.65	7.30	8.00	7.50	7.30	7.50
Total count (10^6)	415	188	543	801	659	947
Sperm motility (%)						
c	1.30	0.90	1.40	3.00*	2.70	3.20
d	38.00	25.30	46.90	49.10	39.30	54.00
a + b	60.65	46.20	69.30	48.40	42.20	57.5
Sperm vitality (%)	60.55	49.36	64.85	64.19	62.27	77.78
Sperm DNA fragmentation (%)	8.30	4.75	11.20	14.10	12.82	16.61
Sperm chromatin stability (%)	85.58	82.30	87.20	73.95*	64.50	80.68
Sperm DNA stability (%)	77.63	52.81	81.02	62.50	30.00	69.00

*P<0.05. SP1 and SP2 are represented with eigenvalues. a: fast and progressive; b: rapid nonprogressive and means; c: slow; MD: median; Q1: first quartile; Q3: third quartile

and SP2 with total count and vitality, poor motility, and F (**Table 1**). Eight (61.5%) samples were included in SP1.

For decades, clinicians have sought a subjectively evaluated semen parameter that defines the fertility of a sample.[7] This approach implies a great limitation in the value of the results, particularly for that parameters not completely standardized, as DNA fragmentation. Objectively, computer-assisted sperm analysis (CASA)-generated data were initially considered independently, although this approach has limited power.[8] There are studies on concomitant evaluation, by logistic regression, of multiple parameters evaluated subjectively[3] and by subpopulation studies on human semen variables.[9] The holistic work on the subpopulations of ejaculated spermatozoa has changed the vision of a race among more or less equivalent cells, to that of competition among different groups of spermatozoa with similar characteristics.[10] This first such study on adult human semen samples is promising for improving the diagnostic potential of semen analysis. For developing a mathematical model integrating all semen variables, future studies must include more men, and the concept must be extended to subjects of variable age, fertility, residence, socioeconomic status, ethnicity, and with pharmacological and toxicological conditions. This will make possible definition of universal criteria that improve the prediction of fertility and optimize the diagnosis for assisted reproduction techniques.

AUTHOR CONTRIBUTIONS

AGM conceived and designed the study, and drafted and revised the manuscript. A Valverde conducted statistical design of the study and data analysis. DB, A Vendrell, and CC participated in data acquisition. CS participated in designing the study and critical review of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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