Relationship Between Standard Semen Parameters, Calcium, Cholesterol Contents, and Mitochondrial Activity in Ejaculated Spermatozoa From Fertile and Infertile Males

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Purpose: To correlate levels of cholesterol (CH), calcium (Ca^{2+}), and mitochondrial activity (MA) with the standard semen parameters and to compare them between fertile and infertile men.

Methods: We studied 151 semen samples from infertile ($n = 60$) or fertile ($n = 91$) males. Basic sperm parameters were analyzed. Ca^{2+} and CH concentrations on seminal plasma were determined by enzyme immunoassay. Intracellular Ca^{2+} and CH concentrations in the sperm plasma membrane and mitochondrial activity by fluorometry.

Results: There was a significant positive correlation between sperm membrane CH and sperm morphology. Intracellular Ca^{2+} was lower in infertile patients compared to fertile. No differences were found regarding Ca^{2+} and CH concentrations in seminal plasma. MA is directly and strongly related with sperm motility.

Conclusions: Intracellular concentrations of Ca^{2+} and the proportion of CH in the sperm membrane are two important markers of the sperm quality due to its direct relationship with sperm morphology and fertility potential.

KEY WORDS: Ca^{2+} ; cholesterol; fertility; mitochondrial activity; human spermatozoa.

INTRODUCTION

To date we lack data regarding molecular aspects of male infertility, useful to be employed as a diagnostic test, apart from those results obtained by classical WHO (1) analysis and the recent sperm chromatin structure assay (SCSA) (2,3). Sperm DNA fragmentation is increasingly being recognized

as an important cause of infertility. Clinical studies indicate that DNA fragmentation levels above 30% measured by the SCSA, are not compatible with the initiation and maintenance of a term pregnancy (2).

Nevertheless, a significant proportion of the couples attending our clinic suffer from unknown origin infertility, because semen parameters evaluated following WHO criteria are normal and the study of their partners revealed no gynecological problem. This suggests that male infertility could be caused by different deficiencies not described yet, distinct from those related with basic sperm analysis.

A key feature in the function of the spermatozoa is the lipid composition of seminal plasma and sperm membrane. Within all the lipids present in the sperm and seminal plasma, CH has a special relevance, since

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is the most present lipid in the spermatozoa among all the mammalian species (4).

The relationship of lipids with male fertility has also been studied, and knowing the role of lipids in the phenomena of maturation and capacitation of spermatozoa, subtle modifications on their composition within the plasma membrane will lead to functional defects. CH/phospholipids ratios in sperm from patients with unexplained infertility are twice than fertile donors (5,6).

In patients with infection in the urogenital tract a significant reduction in total seminal plasma CH is noted, and this is potentially affecting sperm function (7).

Also, seminal plasma lipid composition is also important because of the ability of sperm cells to take up lipid components or fatty acids from the surrounding environment under determined circumstances (8).

Another relevant factor is the Ca^{2+} ion. The change of the lipid composition in the capacitation progress provokes an increase in the Ca^{2+} influxes as a first event (9). It has been described that high concentrations of Ca^{2+} in the external medium may decrease the motility and metabolism of sperm. Nevertheless, hyperactivation, a process that assists sperm cells in penetrating the cumulus matrix directly depends on it. Moreover, after the acrosome reaction, the zona pellucida is trespassed by raising intracellular Ca^{2+} concentrations in sperm (10).

Undoubtedly considering Ca^{2+} as an important factor because of its direct effect on sperm metabolism, a direct method to assess Ca^{2+} could yield important information regarding the fertility potential of the spermatozoa and its relationship with basic sperm parameters. However, previous studies indicate that free intracellular Ca^{2+} parameters are only weakly correlated with the penetrating ability of sperm from infertile men (11).

Sperm motility is also a relevant feature in the sperm function, directly dependent on mitochondrial action, and the classic method to assess it on spermatozoa is the determination of the percentage of progressive motile cells under a light-microscope. The results obtained by means of this analysis show an inherent variability, given that samples are evaluated by different individuals, and that only few sperm numbers are analyzed.

Some laboratories employ the combination of mitochondria-specific fluorescent staining coupled with fluorometry to provide the initial estimate of semen quality. It allows a rapid and precise analysis of thousands of spermatozoa in each sperm sample

and this strongly correlates with the motility determination. In addition, this motility evaluation serves as an estimate of the proportion of viable spermatozoa (12), but studies regarding these parameters with male fertility are lacking.

The aim of the current study was to correlate the levels of significant sperm function markers as CH and Ca^{2+} , together with mitochondrial activity on semen with basic sperm parameters and fertility potential. To this end, we determined Ca^{2+} and CH concentrations on both seminal plasma by enzymeimmunoanalysis techniques and Ca^{+} intracellular concentrations, CH concentrations in the sperm plasma membrane and mitochondrial activity by fluorometry.

MATERIALS AND METHODS

Semen samples for the infertile group were obtained from males attending our centre for assisted conception ($n = 91$) and fertile sperm samples were selected from semen donors who get pregnancies within the donor artificial insemination program ($n = 60$) (13). Inclusion criteria to consider a male infertile were duration of infertility of at least 12 months, nonsevere seminal pathology (more than 15 mill./mL, 25% sperm with progressive motility, and total number of motile sperm higher than 40 million), and no other demonstrable cause of infertility in the female partner. All samples were collected by masturbation into a sterile recipient after 3–5 days of sexual abstinence. All participants gave their informed consent for the utilization of a fraction of their samples for research purposes. The study was approved by the Institutional Review Board of the Instituto Valenciano de Infertilidad.

After allowing the liquefaction of the semen at 37° , 5% CO_2 , for 10 min, the samples were examined for concentration and motility according to the WHO guidelines (8) on a Mackler[®] chamber. Then, the total sperm number taken was standardized to 50 million to provide enough spermatozoa for all tests by centrifugation 10 min at 400 g and storage at -20° of seminal plasma for different quantifications. These cells were differentially treated according to their final use for plasma membrane CH quantification, intracellular Ca^{2+} or mitochondrial activity quantification. No ejaculate showed significant (>1 mill/mL) levels of leukocytes, as demonstrated by Quick Panoptic (QCA, Barcelona, Spain) staining of semen smears.

All the determinations were done blindly by two different observers (M.M. and N.G.), and the final

results are the mean of the different observations when they presented less than 5% of discordance.

CH and Ca²⁺ Determinations in Seminal Plasma

The CH measurements were performed in seminal plasma ($n = 52$) by using an ALCYON[®] analyzer 300/300i with the B2D273 kit (Abbot, Madrid, Spain) following manufacturer's instructions. The method is based in a controlled enzymatic reaction where the final product is measured at 500 nm (14). Detection limit was 1 mg/dL.

Ca²⁺ determinations were performed from seminal plasma ($n = 53$) by using an ALCYON[®] analyzer 300/300i with B8D263 kit (Abbot, Madrid, Spain) also following the instructions provided by the manufacturer. The method is based in the reaction between Arsenazo III colorant and Ca²⁺ which complex is measured at 600 nm. Detection limit was 0.9 mg/dL.

Plasma Membrane CH, Intracellular Ca²⁺, and Mitochondrial Activity Measurements

Forty eight sperm samples were destined to plasma membrane CH measurements. Sperm cells were pelleted by centrifugation (400 g, 10 min) and then a wash step with PBS was done (400 g, 10 min). Cells were treated as described by Cerolini *et al.* (8) for boar sperm. Briefly, total lipids were extracted in excess chloroform:methanol (2:1) (v:v) from the washed spermatozoa. A total of 50 million cells were used for CH analysis. CH was measured by using the Amplex Red CH Assay Kit (Molecular Probes, Leiden, The Netherlands). The assay is based on an enzyme-coupled reaction that detects both free CH and cholesteryl esters in each sample. Quantification was performed by using a fluorometer (Fluoroskan, Labsystems) (Ex: 530 nm, Em: 520 nm). Assay's detection limit is 200 nM (80 ng/mL).

Forty-seven sperm samples were destined to intracellular Ca²⁺ measurements. Twenty five million of sperm cells were pelleted by centrifugation (400 g, 10 min) and a following wash with PBS were performed (400 g, 10 min). Cells were resuspended in 2.5 μ M of Fluo-4 (Molecular Probes, Leiden, The Netherlands) in PBS and incubated 30 min at 37°C. Fluo-4 has a very large fluorescence intensity increase in response to Ca²⁺ binding, being suitable for detecting intracellular Ca²⁺ levels in the 1 μ M–1 mM range. Finally, samples were pelleted and resuspended in PBS and fluorescence was immediately measured

with a fluorometer (Fluoroskan, Labsystems) (Ex: 485 nm Em: 535).

Thirty-four sperm samples were destined to mitochondrial activity measurements. Twenty-five million of sperm cells were pelleted by centrifugation (400 g, 10 min) and a following wash with PBS was performed (400 g, 10 min). Sperm cells were resuspended in 100 nM MitoTracker Green in PBS (Molecular Probes, Leiden, The Netherlands) in PBS and incubated 30 min at 37°C. MitoTracker is a mitochondrion-selective stain that is concentrated by active mitochondria and retained during fixation. Finally, samples were pelleted by centrifugation and resuspended in PBS. Fluorescence was immediately measured with a fluorometer (Fluoroskan, Labsystems) (Ex: 485nm Em: 535).

Statistical Analysis

Seminal plasma CH and Ca²⁺, together with intracellular Ca²⁺, plasmatic membrane CH concentrations and mitochondrial activity were correlated with basic sperm parameters by using linear regression analysis followed by analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

Nonparametric tests were used to compare study parameters in fertile and infertile males when the data did not follow a normal distribution. Subsequently, in these cases, Mann–Whitney's *U*-tests were carried out. Significance was defined as $p < 0.05$. *T*-Tests were employed for comparisons between groups when the data followed a normal distribution.

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL) and MedCalc Software (Ghent, Belgium).

RESULTS

Seminal Characteristics

Table I shows the semen quality of all the subjects included in this study; infertile males and fertile donors.

Only patients with unexplained infertility, subsequently with elevated spermatozoa counts have been included, this allowed to reach the sperm cells needed for the experiments.

Nevertheless, there were not significant differences between groups in any of the sperm parameters studied. Subsequently, their cause of infertility is not apparently related with any macro or microscopical

Table I. Seminal Characteristics of Fertile and Infertile Males Involved on this Study

	Fertile	Infertile	<i>p</i>
Volume (mL)	3.9 ± 0.2	3.9 ± 0.2	ns
Concentration (Mill./mL)	78.7 ± 5.3	84.3 ± 5.1	ns
A+B (%)	55.2 ± 1.6	52.4 ± 1.5	ns
A+B+C (%)	63.0 ± 2.0	62.7 ± 1.4	ns
Normal forms (%)	13.1 ± 0.7	12.6 ± 0.6	ns
Head defects (% of abnormal)	86.5 ± 2.5	88.7 ± 2.1	ns
Midpiece def. (% of abnormal)	36.3 ± 3.7	43.5 ± 2.6	ns
Tail defects (% of abnormal)	5.3 ± 0.5	5.3 ± 0.5	ns

Note. Values expressed as mean ± standard error of the mean (SEM).

sperm alteration, as stated by the WHO semen analysis and Kruger's strict criteria, thus suggesting other alterations are responsible of their condition.

Correlation Between Male Fertility and Ca^{2+} , CH, and Mitochondrial Activity Contents

With regard to CH concentration in seminal plasma and CH contents in the sperm membrane, no correlation was initially found to be statistically significant. For seminal plasma CH quantification, significance was $p = 0.956$, and for the amount of CH in the sperm membrane resulted $p = 0.688$ (Table II).

Regarding seminal plasma and intracellular sperm Ca^{2+} concentrations, a statistically significant difference between intracellular Ca^{2+} in infertile versus fertile males ($p = 0.020$) was found, while no differences were observed for seminal plasma Ca^{2+} concentrations ($p = 0.113$) (Table II). Then, intracellular Ca^{2+} seems to be a marker of sperm fertility, since males with unexplained infertility have lower intracellular concentrations of this divalent cation.

There was no relationship between male fertility status and mitochondrial activity (Table II).

Table II. Comparison of Ca^{2+} , CH, and Mitochondrial Activity in Human Semen Between Fertile and Infertile Males

	Fertile	Infertile	<i>p</i> value
Seminal cholesterol (mg/dL)	40.30 ± 4.71	39.91 ± 4.46	ns
Membrane chol. ($\mu\text{g}/50$ million)	5.79 ± 1.47	6.59 ± 1.14	ns
Seminal Ca^{2+} (mg/dL)	26.16 ± 1.57	25.7 ± 0.77	ns
Intracellular Ca^{2+} (fau/50 million)	0.81 ± 0.13	0.53 ± 0.05	0.020
Mitochondrial act. (fau/50 million)	1.66 ± 0.18	1.59 ± 0.25	ns

Note. Values expressed as median ± SEM.

WHO Semen Parameters and Ca^{2+} , CH, and Mitochondrial Activity

First we studied the relationship between basic sperm parameters and Ca^{2+} , CH and mitochondrial activity by linear regression analysis. The summary of the parameters compared are presented in Table III. As expected the fluorometric measurement of mitochondrial function were highly correlated with microscopic estimation of motility, this information has been also corroborated by other groups (12). Neither volume, concentration nor motility (except mitochondrial activity) were directly related with all the molecular markers studied. But, when sperm morphology were analyzed we observed a linear positive relation between sperm membrane CH contents and sperm morphology ($r = 0.395$, $p = 0.011$). Subsequently, the higher the percentage of normal forms is, the higher the amount of CH (Fig. 1). No relation was observed between the other molecular markers studied and sperm morphology.

Furthermore, we aimed to compare the expression of these factors as a function of conventional WHO parameters of motility and morphology.

To this end, we first grouped all the samples depending on their characteristics and divided in asthenozoospermic patients (less than 50% spermatozoa), and patients with normal motility parameters (more than 50% type A + B), as represented on Table IV. After statistical analysis only mitochondrial activity was higher in patients with more than 50% of progressive motile sperm as expected by previous publications (12).

Also, we divided the samples according to their morphology parameters, following strict criteria. We divided the samples into three groups with severe, moderate, or mild-normal morphology. As expected by linear regression results, the proportion of sperm membrane CH were higher in sperm samples with 11 or higher percentage of normal forms compare with samples with less than 5% of normal forms. Subsequently CH contents are significantly lower in the most severe situations (Table IV).

DISCUSSION

The analysis of male fertility and sperm function has been classically limited to the microscopic study of semen samples, together with the measurement of other macroscopic parameters such as volume. From the results obtained with these methods, we aim to present

Table III. Correlation Between Semen Parameters and CH, Ca²⁺ and Mitochondrial Activity

	Seminal Cholesterol (mg/dL)		Membrane cholesterol		Seminal Ca ²⁺		Intracellular Ca ⁺		Mitochondrial activity	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Volume	-0.044	0.383	-0.132	0.233	-0.040	0.391	-0.091	0.292	0.264	0.083
Concentration	0.011	0.470	0.265	0.068	0.171	0.118	-0.042	0.401	-0.175	0.182
%A+B	0.157	0.143	0.123	0.243	-0.180	0.105	-0.051	0.482	0.785	0.001
TMP	0.011	0.471	0.107	0.277	0.021	0.443	-0.059	0.361	-0.104	0.296
Normal forms	-0.006	0.483	0.395	0.011	0.217	0.065	0.077	0.323	-0.189	0.163
Head defects	0.157	0.138	-0.121	0.251	0.070	0.311	-0.142	0.205	0.045	0.408
Midp. Defects	-0.127	0.190	0.060	0.371	0.059	0.340	-0.210	0.110	0.131	0.249
Tail defects	0.093	0.260	-0.320	0.035	0.091	0.259	-0.010	0.476	0.077	0.345
TMNS	-0.024	0.437	0.064	0.361	0.178	0.108	0.151	0.182	-0.261	0.086

Note. TMNS: total normal motile sperm.

a concrete diagnose and even recommend (always under the Andrology Laboratory point of view) the best fitting assisted reproduction technique.

New tools in the study of male factors related to infertility are provided by molecular studies about sperm physiology. To this end, first we must comprehend all the molecular events leading spermatozoa to fail fertilization and/or initiation of an inadequate embryo development.

Then, new research on specific biochemical markers must be conducted, in order to design concrete and adequate therapeutic tools to improve male fertile potential.

From the results obtained in this research work we can conclude that intracellular concentration of Ca²⁺

and the proportion of CH in the sperm membrane are two important indicators of the sperm quality.

Concerning Ca²⁺ concentrations undoubtedly this cation is directly linked with sperm metabolism, concretely with hyperactivation process taking place in the oocyte fertilization (10).

Ca²⁺ variations between fertile and infertile males are predictable in a value that is not directly related with any of basic sperm parameters studied, because no differences among these two populations were observed. Possibly, this higher concentration is associated with an elevated metabolic activity, taking these cells to a better predisposition to the process of fertilization: to escape from mucosal pockets, to move through oviductal mucus, to reach the ampulla, or to penetrate inside the cumulus matrix.

No association was initially found between CH concentrations and male infertility status. However CH proportions in the fresh ejaculate are increased in samples with high percentage of normal forms suggesting that, adequate morphology of non-capacitated sperm is also related with higher proportion of CH content in the sperm membrane. Possibly this phenomenon is caused by the relation between the proportion of this main component of the membrane with the fluidity of this structure, thus maintaining an adequate shape.

Several works confirmed that, in the preparation of semen samples, there is a progressive loss of sperm CH, without a significant acrosomal loss until several hours later (4). This suggests that an adequate timing of the process and starting with adequate CH levels are mandatory for a successful fertilization, and that the physical features of the sperm membrane are critical.

The inverse relation between sperm tail defects with CH contents is also pointing in the same way.

Fig. 1. Scatter plot of sperm membrane cholesterol contents vs. percentage of normal forms.

Table IV. WHO Semen Parameters on Semen Samples and the Relation with Ca^{2+} , CH, and Mitochondrial Activity in Human Semen

	Morphology			<i>p</i>	Motility		<i>p</i>
	<5% normal forms	5–10% normal forms	≥11% normal forms		<50% A+B	>50% A+B	
Seminal cholesterol	26.6 ± 1.3 (<i>n</i> = 10)	26.2 ± 1.3 (<i>n</i> = 12)	25.8 ± 1.2 (<i>n</i> = 30)	ns	26.3 ± 1.3 (<i>n</i> = 18)	25.7 ± 0.9 (<i>n</i> = 34)	ns
Membrane cholesterol	2.8 ± 0.6* (<i>n</i> = 11)	6.0 ± 1.6 (<i>n</i> = 16)	7.8 ± 1.5* (<i>n</i> = 21)	0.048	5.8 ± 1.4 (<i>n</i> = 20)	6.7 ± 1.2 (<i>n</i> = 28)	ns
Mitochondrial activity	1.1 ± 0.2 (<i>n</i> = 4)	1.7 ± 0.2 (<i>n</i> = 4)	1.7 ± 0.2 (<i>n</i> = 26)	ns	1.0 ± 0.2 (<i>n</i> = 14)	1.9 ± 0.2 (<i>n</i> = 20)	0.001
Seminal Ca^{2+}	31.1 ± 4.2 (<i>n</i> = 9)	39.3 ± 9.8 (<i>n</i> = 11)	43.6 ± 4.6 (<i>n</i> = 33)	ns	45.6 ± 5.1 (<i>n</i> = 18)	37.1 ± 4.2 (<i>n</i> = 35)	ns
Intracellular Ca^{2+}	0.89 ± 0.14 (<i>n</i> = 7)	0.67 ± 0.06 (<i>n</i> = 11)	3.07 ± 1.76 (<i>n</i> = 29)	* <i>p</i> < 0.05	2.5 ± 1.8 (<i>n</i> = 16)	4.0 ± 2.5 (<i>n</i> = 31)	ns

Note. Data are expressed as median and SEM, with the number of observations in parantheses.

Considering that sperm morphology is comparable in both groups (fertile vs. infertile), no differences should be expected in the CH contents between fertile and infertile males. Then, CH should be especially important on male infertility with aberrant morphology.

In this research work we have also supported the effectiveness of the mitochondrial activity measurement in the sperm motility quantification (12), although its task in sperm fertility is not crucial.

To this end, new research on specific biochemical markers must be conducted, in order to design concrete and adequate therapeutic tools to improve male fertile potential.

First we should study the regulation mechanism of the Ca^{2+} flux into the sperm cells, the possible intermediaries of this process and its direct relation with fertilization ability. Second, we could revise external factors that are affecting CH concentrations in the sperm membrane (i.e., dietary CH support) and potential solutions to avoid these variations.

In conclusion, knowing the ability of sperm cells to exchange lipids with external medium an exogenous addition of CH to culture media could improve sperm quality.

To finally demonstrate the usefulness of controlling Ca^{2+} and CH, further investigations are needed.

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REFERENCES

1. WHO laboratory Manual for the examination of human semen and sperm-cervical mucus interaction. World Health Organization, United Kingdom, Cambridge, 1999
2. Larson-Cook KL, Brannian JD, Hansen KA, Kasperperson KM, Aamold ET, Evenson DP: Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003;80(4):895–902
3. Mesequer M, Garrido N, Martínez-Conejero JA, Simón C, Pellicer A, Remohí J: The role of cholesterol, Ca^{2+} and mitochondrial activity in the susceptibility for cryodamage after a cycle of freezing and thawing. *Fertil Steril* 2004; 81(3):588–594
4. Cross L: Role of cholesterol in sperm capacitation. *Biol Reprod* 1998;59:7–11
5. Davis BK: Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vesicles. In *Symposium on the Pharmacological Effects of Lipids*, JJ Kabara (ed), Champaign, IL, The American Oil Chemists Society, 1978; pp. 145–157
6. Vignon F, Clavert A, Cranz C, Koll-Back MH, Reville P: Alterations in the lipid composition of seminal plasma patients with a chronic infection of the urogenital tract. *Urol Int* 1993;50(1):36–38
7. Sugkraroak P, Kates M, Leader A, Tanphaichitr N: Levels of cholesterol and phospholipids in freshly ejaculated sperm and Percoll-gradient pelleted sperm from fertile and unexplained infertile men. *Fertil Steril* 1991;55(4):820–827
8. Cerolini S, Maldjian A, Pizzi, F, Gliozzi TM: Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reprod.* 2001;121:395–401
9. Breitbart H: Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Mol Cell Endocrinol* 2002;187(1–2):139–44. Review
10. Suarez SS: Hyperactivated motility in sperm. *J Androl* 1996;17(4):331–335

11. Shimizu Y, Nord EP, Bronson RA: Progesterone-evoked increases in sperm Ca²⁺ correlate with the egg penetrating ability of sperm from fertile but not infertile men. *Fertil Steril* 1993;60(3):526–532
12. Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE: Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biol Reprod*. 1997;57:1401–1406
13. Garrido N, Zuzuarregui JL, Meseguer M, Simon C, Remohi J, Pellicer A: Sperm and oocyte selection and management: experience of a 10 year follow-up of more than 2100 candidates. *Hum Reprod* 2002;17(12):3142–314
14. Roeschlau P, Bernt E, Gruber W: Enzymatic determination of total cholesterol in serum. *Z Klin Chem Klin Biochem* 1974;12: 226