

High Cholesterol Content and Decreased Membrane Fluidity in Human Spermatozoa are Associated with Protein Tyrosine Phosphorylation and Functional Deficiencies

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Abstract

Poor-quality sperm show reduced capacity to undergo capacitation-induced protein tyrosine phosphorylation and hyperactivation. Given that these deficiencies can be overcome by membrane-permeant stimulators of the cAMP-dependent kinase system, we hypothesize that the main defect underlying these deficiencies resides on the sperm plasma membrane. Spermatozoa from semen samples obtained from fifteen consenting healthy donors were separated in two subpopulations, L45 (first interface) and L90 (pellet), using a 45:65:90% ISolate gradient-centrifugation method. These sperm fractions were studied before and after a 6h-capacitating incubation for sperm motion parameters (computer-assisted analysis), including hyperactivation, protein tyrosine phosphorylation (immunofluorescence), membrane fluidity (Laurdan fluorescence) and sterol and phospholipid content (high performance thin layer chromatography). In summary, data indicate that L45 (poor motility) spermatozoa present an excess of cholesterol and desmosterol, which impairs the normal increase in membrane fluidity during capacitation and its consequent activation of protein tyrosine phosphorylation and hypermotility. Therefore, a defect in membrane composition and dynamics is underlying human sperm biochemical and functional deficiencies related to inadequate capacitation.

Key words: sperm; phosphorylation; capacitation; hyperactivation; plasma membrane; cholesterol.

55 **Introduction**

Human spermatozoa must undergo capacitation before becoming fully competent to fertilize an egg. This process encompasses structural, biochemical and functional changes, which, in vivo, occur during the sperm transit through the female genital tract (Yanagimachi, 1994). Capacitation can also be achieved in vitro provided the seminal
60 plasma is removed, and sperm are incubated in medium containing protein, calcium and bicarbonate (Visconti and Kopf, 1998). Although capacitation may be triggered by these post-testicular changes, the molecules and structures that enable the process are established during spermatogenesis, spermiogenesis and epididymal maturation. The output of these processes in humans is typically varied, and so is the quality of the sperm
65 produced.

From a functional point of view, human spermatozoa are notoriously heterogeneous. Using Percoll/ISolate® gradient centrifugation, they can be separated in distinct subpopulations showing different functional quality (Buffone et al, 2004; Calamera et al,
70 2003; Ollero et al, 2000). The upper portion, for instance, the one isolated from the interface of 45 and 90% Percoll layers, has been demonstrated to produce more reactive oxygen species (ROS) and have less motility and more DNA damage than the one recovered from under the 90% layer. This subpopulation, so called pellet sperm, on the contrary, displays the best functional quality and is the one used for assisted reproductive
75 technologies (Mortimer and Mortimer, 1992).

We have previously demonstrated that sperm isolated from the 45/65 interface (poor quality sperm) show an inability to improve motion parameters and develop hyperactivation under capacitating conditions, which is associated with an incapacity to increase protein tyrosine phosphorylation. This deficiency, however, does not appear to be at the kinase level since stimulation with permeable cAMP overcomes the impairment (Buffone et al, 2004). We therefore postulate that the main defect resides on the plasma membrane, a structure that undergoes multiple changes during capacitation, and it is critically involved in the acquisition of sperm fertilizing ability.

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In this investigation, we examine the sterol and phospholipid contents of human sperm subpopulations and their impact on membrane fluidity, hyperactivation and protein tyrosine phosphorylation before and after a capacitating incubation.

90 **Methods**

Preparation of spermatozoa

Semen samples were obtained from 15 consented healthy donors, by masturbation, after 3-5 days of sexual abstinence. The protocol was approved by the LER Institutional Review Board. Spermatozoa from individual ejaculates were not pooled. All samples had normal semen parameters, as evaluated from a sperm concentration $> 40 \times 10^6$ spermatozoa/ml, a percentage of progressive cells $\geq 50\%$, a percentage of viable spermatozoa $> 80\%$, and a percentage of normal forms $\geq 14\%$ as assessed by Kruger's strict criteria (Kruger et al, 1986).

Samples were allowed to liquefy for one hour at room temperature and sperm
100 concentration and motility were assessed using a computer-assisted semen analysis
(Hamilton Thorne IVOS V10.8s, Hamilton Thorne Research, Danvers, MA). Sperm
viability was assessed in the original semen samples as well as in the isolated fractions at
all subsequent experimental conditions by light microscopy using the Eosin Y assay
(WHO, 1999).

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Sperm fractionation and incubation

Aliquots of semen (1 ml) were loaded onto a 45, 65 and 90% discontinuous ISolate®
(Irvine Scientific, Santa Ana, CA) gradient. Density gradients were performed by
layering 1 ml of each ISsolate® concentration into a 15-ml conical tube, and centrifuged
110 for 20 min at 400 x g. The resulting interfaces between the layers of 45% and 65% (L45)
and the pellet of the 90% layer (L90) were aspirated and transferred to separate tubes.
Sperm suspensions were then diluted with Ham's F10 medium containing 3 mg/ml
bovine serum albumin (Ham/BSA) and centrifuged twice for 10 min at 400 x g. An
aliquot of each interface was used to assay sperm concentration, motility and
115 morphology. Washed spermatozoa were resuspended in 1 ml of Ham/BSA at a
concentration adjusted to 1×10^7 spermatozoa/ml. Cells were used either immediately after
washing (T0), or after incubation for 6 hours (T6) at 37°C, in a 5% CO₂ atmosphere.
Some experiments were performed incubating spermatozoa in the presence of cholesterol
sulfate (CHO-SO₄)-saturated BSA (20 μM) (Sigma, St. Louis, MO). In this case, the
120 BSA was preincubated with the CHO-SO₄ for 30 min prior to its addition to the sperm
suspension. Due to the need to perform the functional assays with as many cells as

possible, we did not measure CHO content after incubation with CHO-SO₄-saturated BSA. Instead, we assumed effective loading of the sterol based on previously reported results by Cross (2003). As control, we evaluated the addition of CHO-saturated BSA to the cells T0. We did not find any difference in the values with or without cholesterol (125 data not shown). At each time point, cells were assessed for motility, hyperactivation, sterol content, protein tyrosine phosphorylation and membrane fluidity. Due to the number of spermatozoa of each sample, not all parameters were assessed in all samples. The sample size used for each study is stated in its corresponding table or figure.

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Measurement of sterol and phospholipid content

The cholesterol and desmosterol content of sperm fractions was analyzed by micro high-performance thin-layer chromatography (HPTLC) as described before (Alvarez and Storey, 1995). Following extraction, the lower phases were evaporated to dryness and 135 resuspended in 50 µl of chloroform-methanol (1:1, v/v). Aliquots of 4 µl of the different samples and of cholesterol and desmosterol standards at a concentration of 0.1 mg/ml were applied to AgNO₃-impregnated HP-K plates, developed in chloroform-acetone (95:5 v/v), and stained with the CuSO₄ reagent. The resulting bands were scanned at 400 nm in the reflectance mode using a Shimadzu CS-9000U spectrodensitometer (Shimadzu 140 Scientific, Inc., Columbia, MD).

Sperm motility parameters and hyperactivation

Aliquots of each sperm suspension were loaded into a 37°C prewarmed, 20 µm-deep disposable chambers (Microcell, Conception Technologies, San Diego, CA). Computer-

145 assisted sperm motion analysis was performed using a Hamilton-Thorne digital image analyzer (HTR-IVOS v 10.8s, Hamilton Thorne Research, Danvers, MA). At least 300 spermatozoa and 5 fields were assessed.

Eight motion parameters were assessed in this study: 1) motility (%); 2) average path velocity (VAP, $\mu\text{m}\cdot\text{s}^{-1}$); 3) track speed or curvilinear velocity (VCL, $\mu\text{m}\cdot\text{s}^{-1}$); 4) 150 progressive or straight-line velocity (VSL, $\mu\text{m}\cdot\text{s}^{-1}$); 5) straightness (STR, %); 6) beat cross frequency (BCF, Hz); 7) linearity (LIN, %) and 8) lateral head amplitude (ALH, μm). The following settings were used during the analysis: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 85; minimum cell size, 4 pixels; straightness threshold, 80%; low VAP cut off, $5 \mu\text{m}\cdot\text{s}^{-1}$; medium VAP cut off, $25 \mu\text{m}\cdot\text{s}^{-1}$; head size-nonmotile, 155 12 pixels; head intensity – nonmotile, 130 AU; static head size, 0.68-2.57 pixels; static head intensity, 0.31-1.21 AU; static elongation, 23-100%. The playback function was used to accurately identify motile cells. Hyperactivated motility (HA, %) was defined as motility with starspin or high-amplitude thrashing patterns and short trajectory distances (Burkman, 1984). This percentage represents the portion of motile spermatozoa 160 displaying hyperactivated movement. The criterion for detecting hyperactivated spermatozoa was: $\text{VCL} > 150 \mu\text{m}\cdot\text{s}^{-1}$, $\text{ALH} > 7.0 \mu\text{m}$, $\text{LIN} < 50\%$ (Mortimer and Mortimer, 1992).

Protein tyrosine phosphorylation: Indirect immunofluorescence

165 Immunofluorescence (IF) was used to examine the subcellular localization of proteins phosphorylated in tyrosine residues, as well as the incidence of this process in the sperm subpopulation. Immunolabeling was performed in the absence of phosphatase inhibitors

which could preclude biologically relevant differences between the assessed sperm subsets. Spermatozoa from the different ISolate® fractions were incubated under
170 capacitating conditions 6 h and washed twice with PBS. Sperm concentration was adjusted to 5×10^6 cell/ml. An aliquot of 15 μ l of the sperm suspension was spotted onto 8-well glass slides, air-dried, fixed and permeabilized with methanol for 30 min at room temperature. Slides were next incubated for 1.5 h at room temperature in a humidified chamber in the presence of anti-phosphotyrosine antibody PY20 (50 μ g/ml in PBS-0.1%
175 BSA) (ICN Biomedicals Inc, Aurora, OH). After washing twice with PBS, slides were incubated for 30 min at room temperature in a humidified chamber with 50 μ g/ml of goat anti-mouse IgG fluorescein isothiocyanate conjugated (FITC) (ICN Biomedicals Inc, Aurora, OH). After incubation, slides were washed with PBS three times, air-dried and mounted with Antifade® (Molecular Probes, Eugene, OR). Spermatozoa were examined
180 using a fluorescence microscope Olympus BX40F (Olympus America, Mellville, NY). At least 200 cells were counted in different fields and the percentage of spermatozoa showing fluorescence in their tails was calculated. Negative controls were performed by blocking PY20 with *ortho*-D, L phosphotyrosine (Sigma, St. Louis, MO).

185 ***Membrane fluidity***

Sperm membrane fluidity was evaluated using the fluorescent probe 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan, Molecular Probes Inc., Eugene, OR). Laurdan is a probe that spontaneously incorporates into membranes at the glycerol backbone of phospholipids, distributing itself evenly among the different lipid domains (Parasassi and
190 Gratton, 1995). Laurdan responds to variations in the number of water molecules

accessible to the probe, which in turn depends on both lipid packing and cholesterol content. This alteration in the polarity of Laurdan's microenvironment is visualized as an alteration in its fluorescence excitation and emission spectra. Spermatozoa were mixed with 0.2 μ M Laurdan and incubated for 15 min at 37°C to allow the incorporation of the probe into the plasma membrane. After incubation, membrane fluidity was evaluated by changes in Laurdan generalized polarization (GP) calculated as:

$$GP = \frac{I_{430} - I_{480}}{I_{430} + I_{480}}$$

where I_{430} and I_{480} are the fluorescence intensities at 430 nm and 480 nm, respectively, ($\lambda_{excitation}$: 350 nm) (Parasassi et al, 1995), measured at 37°C in a Kontron SFM-25 spectrofluorometer with temperature control (Kontron Instruments SpA, Milan, Italy). The lower the GP ratio, the higher the fluidity of the membrane.

205 *Statistical analysis*

Results are expressed as mean \pm standard error of the mean (SEM). Statistical differences between two groups were evaluated by unpaired student's *t*-tests. Comparisons of more than two groups of sperm fractions were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. All tests were two-tailed with a statistical significance assessed at $P < 0.05$ level. Statistical analyses were performed using the GraphPad Prism 4.0 program (GraphPad software, San Diego, CA).

210 **Results**

As previously reported, spermatozoa recovered from the 45:65 ISolate® interface (L45) showed significantly less motility, poorer sperm movement than those collected from the pellet (L90) (Table 1).

Testing our hypothesis that sperm phosphorylation and functional deficiencies were linked to membrane lipid alterations, we first analysed the membrane lipid profile of spermatozoa in both subsets (L45 and L90). Prior to incubation under capacitating conditions (T0), L90 cells contained significantly lower amounts of cholesterol (CHO) and desmosterol than L45 cells, displaying a lower CHO to phospholipid ratio (Table 2). Upon incubation under capacitating conditions (T6), both L45 and L90 subsets decreased their content of CHO and desmosterol. However, the decrease in CHO in L90 sperm was higher in magnitude (42%) than that of L45 spermatozoa (25%). On the other hand, the decrease in desmosterol content was similar in both cell fractions, and no changes were observed in phospholipid (PL) level (Table 2). As a result, although not statistically significant, after 6 h under capacitating conditions, L90 cells decreased their CHO to PL ratio by 31 %, while this change was only 14 % in L45 cells (Table 2).

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To assess if the membrane CHO excess found in L45 spermatozoa translated into a membrane dynamic impairment, we compared the fluidity of the sperm membrane in both subpopulations, utilizing changes in Laurdan fluorescence emission. L45 sperm had less fluid membranes than L90 sperm from the beginning of the incubation and regardless of the time point of study (Figure 1). Unlike their L90 counterpart, L45 sperm were unable to increase membrane fluidity with capacitation. In the presence of CHO-SO₄ in

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the medium, L45 sperm membrane fluidity also remained unchanged. Conversely, the presence of CHO in the incubation medium abolished the capacitation-associated increase in membrane fluidity in spermatozoa from the L90 subset (Figure 1).

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To verify if this CHO excess and membrane fluidity alteration in L45 sperm caused an impairment in capacitation-associated parameters, we evaluated the effect of addition of CHO-saturated BSA (20 $\mu\text{mol/ml}$) to the incubation medium on the percentage of hyperactivated and tyrosine phosphorylated spermatozoa. After a 6 h incubation (T6), L45 and L90 fractions significantly increased the amount of hyperactivated sperm in comparison to baseline (T0) values (Figure 2). The addition of CHO to the incubation medium did not affect sperm hyperactivation in the L45 subset, significantly reducing, however, the ability of L90 sperm to hyperactivate.

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In a similar fashion, addition of CHO-saturated BSA to the medium significantly reduced the number of L90 spermatozoa undergoing tyrosine phosphorylation (Figure 3). Excess CHO in the medium, did not change the percentage of tyrosine phosphorylated sperm in the L45 subset in a statistically significant manner.

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Discussion

Human spermatozoa recovered from the upper layers of a Percoll gradient have been shown to be of poorer quality than those recovered from the bottom of the gradient (pellet) (Calamera et al, 2003; Gil-Guzman et al, 2001; Ollero et al, 2000). These

260 spermatozoa show a variety of functional deficiencies. The cause(s) for such deficiencies,
however, has not been fully established.

We previously reported impairment in tyrosine phosphorylation as a potential mechanism
underlying the functional deficiencies observed in Percoll-separated human sperm
265 subpopulations (Buffone et al, 2004). Treating poor-quality sperm recovered from the
upper layers of a Percoll gradient (L45) with activators of the cAMP-dependent kinase
(PKA) pathway (dbcAMP + Pentoxifylline), we were able to overcome both their
functional and protein tyrosine phosphorylation deficiencies. Given that the membrane-
permeant activators bypassed the sperm plasma membrane and directly acted on
270 intracellular molecules, these findings indicated that the main kinase systems implicated
in capacitation-associated sperm protein tyrosine phosphorylation were intact. Similar
results were obtained when asthenozoospermic samples showing deficient tyrosine
phosphorylation were treated with PKA activators (Buffone et al, 2005).

275 The defect, therefore, should be upstream from the kinases. In a hypothetical model for
regulation of sperm protein tyrosine phosphorylation proposed by Visconti and Kopf
(1998), the earliest capacitation-associated events occur at the level of the sperm plasma
membrane. Efflux of cholesterol increases membrane fluidity, allowing for
conformational changes and relocation of receptors and channels whose activation
280 stimulates the intracellular kinase system. Cholesterol acceptors like serum albumin, β -
cyclodextrins, and high density lipoproteins have been implicated in these changes in
several species (Flesch and Gadella, 2000; Osheroff et al, 1999, Visconti et al, 1999).

Considering our previous data on human sperm subpopulations and the information
285 existing in the literature, we hypothesized that the defect leading to tyrosine
phosphorylation and functional deficiency in poor-quality human sperm resided on the
sperm plasma membrane. We further speculated that such inability to increase membrane
fluidity was due to an excess of membrane cholesterol. In this work, measurement of
sterols by HPTLC showed 2.4- and 6.6-fold more cholesterol and desmosterol,
290 respectively, in L45 sperm than in L90 spermatozoa. The CHO/PL ratio was also higher
(0.69 vs. 0.35) for L45 sperm, a fact that remained unchanged after a 6 h-capacitating
incubation (0.59 vs. 0.24).

Using the variation in the generalized polarization of the fluorescent hydrophobic probe
295 Laurdan, we then determined the lipid order of sperm membranes in both L45 and L90
subpopulations. Results clearly showed that the membranes of L45 (poor-quality) sperm
were less fluid (i.e., higher lipid packing and rigidity) than those of L90 (good-quality)
spermatozoa. Furthermore, unlike L90 sperm, spermatozoa from L45 subpopulations
could not increase membrane fluidity during the 6h-capacitating incubation. This
300 deficiency was associated with an impairment in protein tyrosine phosphorylation and an
incapacity to develop hyperactivation. An association between inability to increase
membrane fluidity during capacitation and tyrosine phosphorylation and functional
deficiencies was also observed in pathological semen samples of patients with
asthenozoospermia and varicocele (Buffone et al, 2006; Buffone et al, 2005). Thus, a

305 common theme connecting defects in membrane dynamics and sperm functional quality
is evident.

Having an intrinsically high membrane CHO content, addition of CHO-SO₄ to the
incubation medium, which prevents the efflux of sterols from the plasma membrane
310 (Cross, 2003), did not change the membrane fluidity or the functionality of L45 sperm.
Conversely, incubated in the presence of CHO-saturated BSA, L90 sperm displayed
decreased ability to modulate their membrane fluidity and a concomitant reduction in
protein tyrosine phosphorylation and hyperactivation.

315 Loss of sperm sterols, cholesterol and desmosterol, is an obligatory step in human sperm
capacitation. Removal of these sterols likely accounts for the change in membrane
fluidity observed during capacitation and the consequent decrease in the membrane
CHO/PL ratio (Travis and Kopf, 2002). Steady-state fluorescence anisotropy of the
membrane probe diphenyl hexatriene (DPH) decreases during capacitation in a sterol-loss
320 dependent manner, suggesting a diminution of sterol-mediated phospholipid ordering
(Cross, 2003). Using another fluorescent probe, merocyanine 540, increased membrane
lipid disorder in macaque spermatozoa was associated with caffeine and cAMP-
stimulated capacitation (Baumber and Meyers, 2006).

325 Cholesterol efflux during capacitation has been linked to both increased membrane
fluidity and signal transduction. Sperm incubated with β -cyclodextrins, potent CHO-
binding heptasaccharides, initiate transmembrane signalling, leading to increased tyrosine

phosphorylation, capacitation and acquisition of fertilizing ability (Osheroff et al, 1999; Visconti et al, 1999). It has been proposed that CHO efflux from membrane subdomains
330 called lipid rafts might initiate transmembrane signalling by allowing previously
partitioned integral membrane proteins to interact with one another (Travis and Kopf,
2002). Another group, however, suggested that the preferential loss of cholesterol from
the non-raft pool may be the stimulus that promotes raft clustering and activates signal
transduction (Shadan et al, 2004). Regardless of the precise place in the membrane
335 where CHO is coming from, it is clear that high concentrations of CHO can inhibit
capacitation by indirectly diminishing the conformational freedom and hence the
biological activity of sperm surface proteins. Alternatively, CHO might directly affect
specific membrane proteins, e.g., adenylyl cyclase, that function in transmembrane
signalling (Travis and Kopf, 2002).

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Flesch and coworkers demonstrated that bicarbonate-stimulated phospholipid scrambling
induces CHO redistribution and enables CHO depletion from the plasma membrane of
porcine sperm (Flesch et al, 2001). Interestingly, the authors note that only one
subpopulation of spermatozoa responded to bicarbonate-induced capacitation with
345 changes in lipid architecture and CHO loss. They conclude that the subpopulation
differences were caused by variable efficiencies in epididymal maturation as judged by
cell morphology. Studying ejaculates from ten different boars, they found a straight-line
relationship between the percentage of cells that showed a bicarbonate-induced
phospholipid scrambling and CHO loss and the percentage of cells with no cytoplasmic
350 droplet.

Studying human sperm separated by a discontinuous density gradient in four fractions, as well as mouse testicular and epididymal sperm, Ollero and coworkers demonstrated a net loss of docosahexaenoic acid (DHA), the major polyunsaturated fatty acid in human spermatozoa, saturated fatty acids, CHO and desmosterol during the process of sperm maturation (Ollero et al, 2000). Fraction one, containing the highest proportion of immature and defective spermatozoa showed the highest content of CHO and saturated fatty acid mass. Similar results were observed when sperm from pathological semen samples were fractionated using an ISolate® gradient (Ollero et al, 2001).

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It is plausible, therefore, that the observed low membrane fluidity as well as the high CHO/desmosterol content of L45 sperm may be due to insufficient loss of sterols during the process of epididymal maturation. Since the length of sperm capacitation in different species has been directly related to their membrane CHO content (Yanagimachi, 1994), it would be possible to hypothesize that L45 spermatozoa might suffer only a temporary, time-dependent inability to undergo capacitation-induced changes. If true, it would be in agreement with observations by Benoff (Benoff, 1993) who showed that prolonged incubation and further loss of CHO of sperm from infertile patients who had failed in vitro fertilization correlated with increased expression of sperm surface receptors and successful fertilization. However, as previously demonstrated by our group (Buffone et al, 2004), L45 spermatozoa are unable to achieve normal levels of protein tyrosine phosphorylation and hyperactivation even after 18 h of incubation under capacitating conditions. It is highly likely, therefore, that poor-quality sperm such as those isolated

from the lower-density Percoll layers (L45) may possess more than one defect which
375 impairs their ability to capacitate adequately. At the membrane level, for instance, these
spermatozoa are known to suffer more lipid peroxidation (Ollero et al, 2000; Aitken et al,
2007). This susceptibility could be explained, in part, by their membrane composition,
which is responsible for the reported higher oxidation coefficient of these cells (Calamera
et al, 2003). Since the generation of membrane lipid hydroperoxides has been associated
380 with membrane fluidity reduction, peroxidative damage could be another cause for the
membrane-associated functional deficiencies in this population of spermatozoa (Aitken et
al, 1993, 1994; Windsor et al, 1993). Furthermore, increased oxidative damage may also
be responsible for altered ion fluxes and membrane hyperpolarization by affecting
membrane transporters, co-transporters and enzymes linked to signal transduction and
385 capacitation (Wertheimer et al, 2008). In this manuscript, however, we focused on the
impact of excess sterols in the membranes of poor-quality spermatozoa isolated from
normozoospermic samples and the biochemical and functional consequences of such
defect.

390 Our data, together with previous findings reported in the literature, suggest that functional
deficiencies in capacitation-induced protein tyrosine phosphorylation and hyperactivation
may be linked to defects in membrane lipid composition and dynamics due to defective
sperm maturation. Membrane defects entailing excess sterols and decreased fluidity
would therefore be a causative factor underlying poor functional quality in human
395 spermatozoa.

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490 **Figure legends**

Figure 1

Membrane fluidity assessed by changes in Laurdan generalized polarization (GP) in L45 (▨) and L90 (▩) subsets of human spermatozoa before (T0) and after (T6) incubation under capacitating conditions either in the absence (T6 – CHO) or the presence (T6 + CHO) of CHO-SO₄-saturated BSA (20 μmol /ml). Results are shown as mean ± SEM (n=5). * statistically different in comparison with values obtained from L45 at T0 (P < 0.05); ** statistically different from values obtained in the same cell subset at T0 (P<0.005).

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Figure 2

Percentage of hyperactivation (HA %) in L45 (▨) and L90 (▩) subsets of human spermatozoa before (T0) and after (T6) incubation under capacitating conditions either in the absence (T6 – CHO) or the presence (T6 + CHO) of CHO-SO₄-saturated BSA (20 μmol /ml). Results are shown as mean ± SEM (n=12). * statistically different from the values obtained at T0 in the same cell subset (P < 0.005); ** statistically significant compared to cells from the same fraction incubated in the absence of CHO (P < 0.05).

Figure 3

Incidence and intensity of protein tyrosine phosphorylation (PTY %) of L45 (▨) and L90 (▩) subsets of human spermatozoa before (T0) and after (T6) incubation under capacitating conditions either in the absence (T6 – CHO) or the presence (T6 + CHO) of CHO-SO₄-saturated BSA (20 μmol /ml), and evaluated by immunofluorescence. Results are shown as mean ± SEM (n=12). * statistically different from values of cells from the

515 same subset at T0 ($P < 0.005$); ** statistically significant compared to cells from the same subfraction incubated in the absence of CHO ($P < 0.05$).

TABLE 1. Motion parameters, morphology and viability of ISolate-separated human sperm subpopulations

	L45	L90
Concentration (x 10⁶/ml)	22.3 ± 3.3	28.2 ± 7.0
Motility (%)	32.9 ± 3.7	86.6 ± 5.0 *
VAP (µm.s-1)	38.0 ± 9.1	87.4 ± 13.0 *
VSL (µm.s-1)	28.6 ± 7.7	69.0 ± 17.2 *
VCL (µm.s-1)	73.5 ± 17.2	166.9 ± 13.2 *
ALH (µm)	3.9 ± 1.3	6.7 ± 0.8
LIN (%)	40.9 ± 5.8	42.8 ± 4.8
Viability (%)	83.6 ± 3.9	90.9 ± 5.3
Morphologically normal cells (%)	8.6 ± 6.1	22.4 ± 10.0

Basic parameters of L45 and L90 subsets of human spermatozoa. Results are expressed as mean ± SEM (n=12). * statistical significance (P < 0.05) compared to values obtained with L45 cells.

TABLE 2. Sterol and phospholipid content of ISolate-separated human sperm subpopulations.

	T0		T6	
	L45	L90	L45	L90
CHO (nmol/10⁶ sperm)	0.90 ± 0.08	0.38 ± 0.07 *	0.68 ± 0.20	0.22 ± 0.09
Desmosterol (nmol/10⁶ sperm)	0.80 ± 0.24	0.12 ± 0.04 *	0.51 ± 0.20	0.09 ± 0.08
PL (nmol/10⁶ sperm)	1.30 ± 0.16	1.06 ± 0.24	1.15 ± 0.16	1.11 ± 0.28
CHO/PL	0.69 ± 0.12	0.35 ± 0.16	0.59 ± 0.16	0.24 ± 0.16

Cholesterol (CHO), demosterol and phospholipid (PL) contents in L45 and L90 subsets of human spermatozoa. Results are shown as mean ± SEM (n=6). * statistically significant compared to values of L45 cells (P < 0.05).

Figure 1

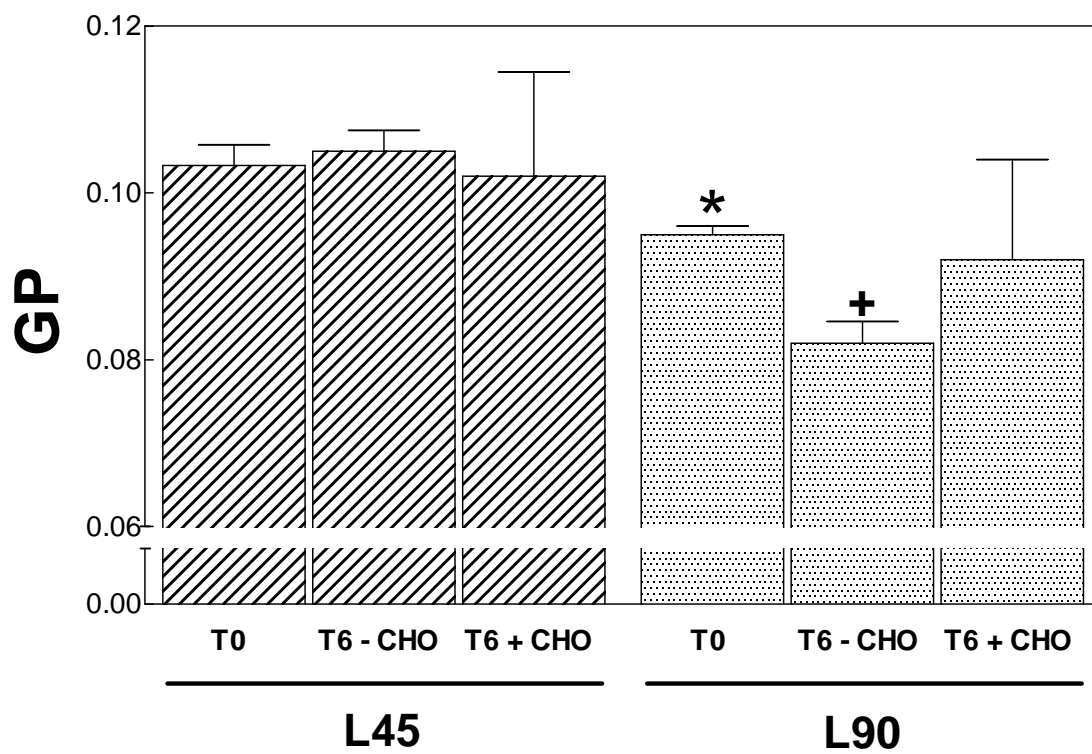


Figure 2

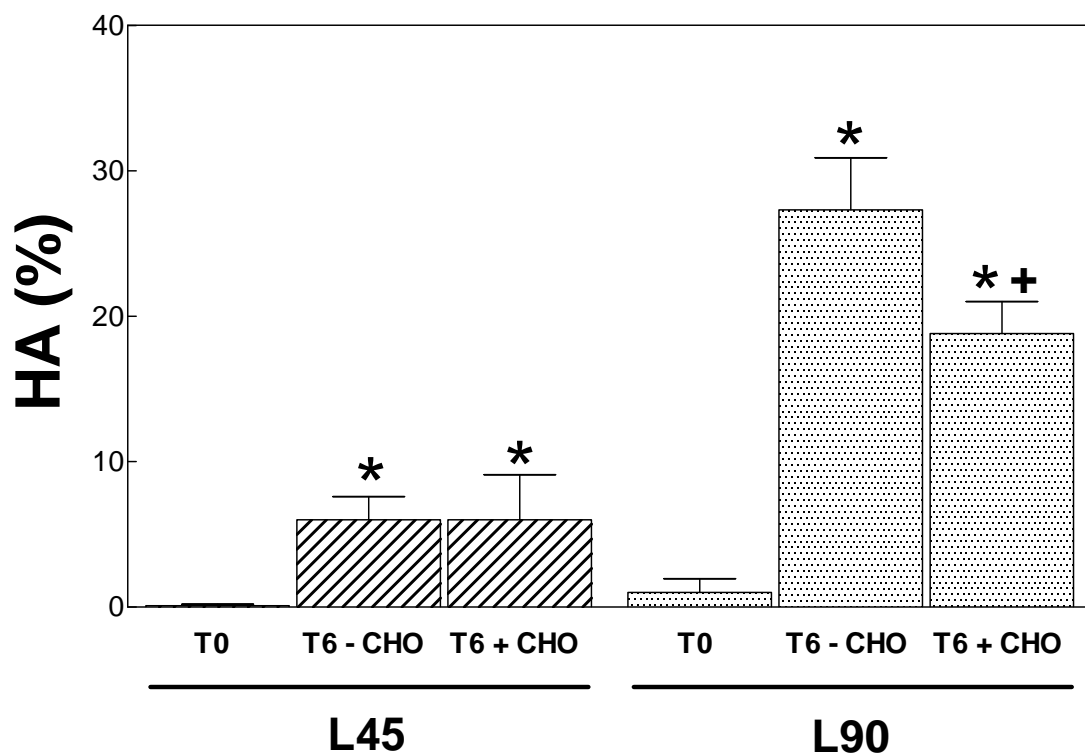


Figure 3

