Human Reproduction Vol.19, No.1 pp. 139–146, 2004 DOI: 10.1093/humrep/deh040

Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation

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BACKGROUND: Human semen is composed of a heterogeneous population of sperm with varying degrees of structural and functional differentiation and normality, which result in subpopulations of different quality. METHODS: Using a discontinuous Percoll gradient, we separated three subsets of sperm [(45%; L45), (65%; L65) and (90%; L90) fractions] from normozoospermic human semen samples from healthy donors and proceeded to characterize their morphology, motility and hyperactivation, as well as their ability to undergo tyrosine phosphorylation under capacitating conditions. RESULTS: As expected, sperm isolated from the lowest density layer (L45) showed the poorest quality, displaying the smallest percentage of morphologically normal and motile sperm. During a capacitating incubation, this subset of cells also showed deficient capacity to undergo hyperactivation and protein tyrosine phosphorylation. Conversely, sperm isolated from the other layers (L65 and L90) showed a time-dependent progressive increment in tyrosine phosphorylation, establishing statistically significant differences with sperm from L45. The tyrosine phosphorylation deficiency of L45 sperm could be overcome when sperm from that fraction were stimulated with activators of the cAMP-dependent kinase (PKA) pathway (dbcAMP + pentoxifylline), pointing to the sperm's plasma membrane as the main site of such deficiency. CONCLUSIONS: Poor quality sperm isolated from a Percoll gradient display an intrinsic tyrosine phosphorylation deficiency, possibly caused by a plasma membrane defect, which is associated with their inability to undergo normal capacitation and, ultimately, acquire optimal fertilizing potential.

Key words: capacitation/human sperm/hyperactivation/Percoll gradient/protein tyrosine phosphorylation

Introduction

Human semen is comprised of a heterogeneous cell population with different degrees of maturation, varying in functional quality and fertilizing ability (Huszar et al., 1993, 1998). Fractionation of sperm by a density gradient centrifugation can separate these subpopulations, resulting in a considerable improvement in the quality of sperm recovered in the pellet. Several reports indicate that higher percentages of motile and morphologically normal sperm can be recovered from the pellet, in comparison with lower density fractions (Menkveld *et al*., 1990; Mortimer and Mortimer, 1992; Chen and Bongso, 1999; Ollero *et al*., 2000). Furthermore, sperm collected from the pellet possess less DNA damage (Larson *et al*., 1999; Sakkas *et al*., 1999) and produce less reactive oxygen species (ROS) than those recovered from the lower density layers, where abnormal sperm are predominantly found (Ollero *et al*., 2001). Applying an ISolate® gradient to semen samples from normal donors and infertile patients, Ollero *et al*. (2000) were able to separate four sperm subpopulations, which revealed different membrane lipid composition and varying degrees of sperm maturation and normality.

Protein tyrosine phosphorylation is an important post-translation event involved at multiple levels of cellular regulation (Hunter, 1991). This type of protein modification has been associated with certain aspects of sperm physiology such as capacitation, acrosome reaction, and hyperactivation (Visconti and Kopf, 1998).

In the mouse, protein tyrosine phosphorylation has been associated with sperm capacitation and fertilizing ability (Visconti *et al*., 1995a). This association appears to be a phenomenon common to many mammalian species, including humans (Carrera *et al*., 1996; Vijayayraghavan *et al*., 1997; Pukazhenthi *et al*., 1998; Si and Okuno, 1999; Yeung *et al*., 1999; Beverley and Aitken, 2001). The increase in protein tyrosine phosphorylation in humans is regulated by a cAMP-dependent pathway that involves protein kinase A (PKA) activation (Leclerc *et al*., 1996). Furthermore, the *in vitro* dependence of sperm tyrosine phosphorylation on the albumin concentration of the incubation medium suggests a correlation

between membrane cholesterol efflux and cAMP-induced protein tyrosine phosphorylation (Visconti *et al*., 2002).

Protein tyrosine phosphorylation has been associated with the ability of normal sperm to capacitate and fertilize an egg. Its deficiency, on the other hand, has been postulated as a possible cause of sperm dysfunction, in particular asthenozoospermia (Yunes *et al*., 2003). Although sperm with varying degrees of quality possess different fertilizing potential, the relationship between the degree and incidence of tyrosine phosphorylation and the functional quality of human sperm subpopulations remains a matter of speculation.

The goal of the present work was to examine the relationship between sperm quality and level of protein tyrosine phosphorylation in different sperm subpopulations in an attempt to determine whether human sperm showing the poorest quality have an intrinsic deficiency in protein tyrosine phosphorylation. In the present study, human sperm were subjected to Percoll gradient centrifugation to separate different cell populations. Several parameters of sperm quality were measured (e.g. morphology, motility and hyperactivation) and compared with the degree and incidence of capacitation-related protein tyrosine phosphorylation in those cells. Additionally, sperm from each subpopulation were stimulated with activators of the PKA pathway, and their protein tyrosine phosphorylation response was assessed.

Materials and methods

Preparation of sperm

Semen samples were obtained by masturbation from 15 healthy donors after 3–5 days of abstinence. Sperm from individual ejaculates were not pooled. All samples had a sperm concentration $>40\times10^6$ sperm/ml (range 40.9–177.7), a percentage of progressive cells $\geq$50% (range 50–75), a percentage of viable sperm >80% (range 85–95), and a percentage of normal forms $\geq$14% (range 14–25) (assessed by Kruger's strict criteria).

Samples were allowed to liquefy for 1 h at room temperature and then sperm concentration and motility were assessed using computer-assisted semen analysis (CASA; Hamilton Thorne IVOS V10.8s, Hamilton Thorne Research, USA). Sperm viability was assessed by light microscopy in the original semen samples as well as the isolated fractions at all incubation times using the Eosin Y assay (World Health Organization, 1999).

Percoll fractionation and incubation of sperm

Aliquots of semen (1 ml) were loaded onto a 45, 65 and 90% discontinuous Percoll (Sigma Chemical Co., USA) gradient. Density gradients were performed by layering 1 ml of each Percoll concentration into a 15 ml conical tube. The tube was then centrifuged at 400 *g* for 20 min. The resulting interfaces between the layers of 45 and 65% (L45), 65 and 90% (L65) and the 90% pellet (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 at 400 *g* for 10 min. An aliquot of each interface was used to assay sperm concentration, motility and morphology (Kruger *et al*., 1987).

Washed sperm were resuspended in 1 ml of Ham/BSA at a concentration adjusted to ~10×10^6 sperm/ml. Other sperm parameters (motility and protein tyrosine phosphorylation) were determined immediately after washing (T0), or after incubation at 37°C, 5% CO_2 for five (T5) and 18 h (T18).

In one set of experiments, 1 mmol/l dibutyryl cyclic adenosine monophosphate (dbcAMP) and 1 mmol/l pentoxifylline (PTX) (Sigma) were added to the incubation medium.

Motility parameters and sperm hyperactivation

Aliquots of each sperm suspension were loaded into a 20 μm deep disposable chamber (Microcell; Conception Technologies, USA), pre-warmed at 37°C. Computer-assisted sperm motion analysis was performed using a Hamilton Thorne digital image analyzer (HTR-IVOS v 10.8s). At least 300 sperm and five fields were assessed.

Six motion parameters were assessed in this study: motility (%); track speed (VCL, μm/s); progressive velocity (VSL, μm/s); straightness (STR, %); beat cross frequency (BCF, Hz); and lateral head amplitude (ALH, μm). The settings used during the analysis were: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 85; minimum cell size, 4 pixels; straightness threshold, 80%; low VAP threshold, 5 μm/s; medium VAP threshold, 25 μm/s; head size—non-motile, 12 pixels; head intensity—non-motile, 130; static head size, 0.68–2.57; static head intensity, 0.31–1.21; static elongation, 23–100. The playback function was used to accurately identify motile cells. Hyperactivated motility (HA, %) was defined as a motility with starspin or high-amplitude thrashing patterns and short trajectory distances (Burkman, 1984). The criterion for detecting hyperactivated sperm was: VCL >150 μm/s, ALH >7.0 μm, LIN <50% (Mortimer *et al*., 1998).

Indirect immunofluorescence of sperm

Immunofluorescence was employed to examine the subcellular localization of proteins phosphorylated in tyrosine residues. Sperm from the different Percoll fractions were capacitated during various periods of time and washed twice with phosphate-buffered saline (PBS). Sperm concentration was adjusted to 5×10^6 cell/ml and 15 μl of the sperm suspension were spotted onto 8-well glass slides. Cells were air-dried on the slides, fixed and permeabilized with methanol for 30 min at room temperature. The slides were incubated with anti-phosphotyrosine antibody PY20 (ICN Biomedicals Inc., USA), diluted 1:20 (50 μg/ml) in PBS–0.1% BSA, for 1.5 h at room temperature in a humidified chamber. After washing twice with PBS, slides were incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; ICN Biomedicals Inc.), diluted 1:20 (50 μg/ml) in PBS–0.1% BSA for 30 min at room temperature in a humidified chamber. Following the incubation, slides were washed with PBS three times, air-dried and mounted with Antifade® (Molecular Probes, USA). Sperm were examined using a fluorescence microscope Olympus BX40F (USA). At least 200 cells were counted in different fields and the percentage of sperm showing fluorescence in their tails was calculated. Negative controls were performed by blocking PY20 with *o*-D,L-phosphotyrosine (Sigma).

Western blot analysis of sperm proteins

Proteins from sperm were analysed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and western immunoblot analysis. Cells were washed twice with PBS and resuspended in Laemmli sample buffer (0.025 mol/l Tris, 0.5% SDS, 5% glycerol, pH 6.8) (Laemmli, 1970). Samples were centrifuged at 6000 *g* for 5 min. The supernatants were recovered and heated at 100°C for 5 min in the presence of 70 mmol/l 2-β mercaptoethanol and stored at –20°C until use. Solubilized proteins [obtained from 2×10^6 sperm per lane (~5 μg of protein)] were separated on 7% polyacrylamide gels under denaturing conditions. Prestained molecular weight markers (Amershan Life Science Inc., Canada) were run in parallel. For

western blot analysis, proteins were electroblotted and transferred onto nitrocellulose (Bio Rad, USA) at 100 V, 4°C for 2 h. To block non-specific binding sites, the membrane was first incubated with 2% dry skimmed milk in PBS–0.1% Tween 20 (blocking solution). After that it was incubated for 1 h with a monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, USA) diluted 1:5000 in blocking solution. After 4 washes with PBS–0.1% Tween 20, an antimouse peroxidase-conjugated IgG (Jackson Immuno-Research Laboratories Inc., USA) diluted 1:5000 in blocking solution was added. Following 1 h of incubation, the membrane was washed four times with PBS–0.1% Tween 20, and reactive bands were detected by enhanced chemiluminescence using the ECL kit (Amershan Life Science Inc., Canada) according to the manufacturer's instructions. All incubations were performed at room temperature.

To quantify changes in protein tyrosine phosphorylation, rectangular boxes were drawn around bands on scanned digital images of ECL contact photographs of western blots, and adjusted optical densities for each lane were obtained using ImageJ software 1.30 V (National Institute of Health, USA).

Statistical analysis

Results are expressed as mean ± SD. Statistical differences between two groups were evaluated by Student's *t*-test. Results obtained on different sperm fractions at the same incubation time were compared by two-way analysis of variance (ANOVA) and Student–Newman–Keuls test. All tests were two-tailed with a statistical significance assessed at the $P < 0.05$ level. Statistical analysis was performed using the Graphpad InStat program (GraphPad software, USA).

Results

Motility, morphology and motion parameters of sperm separated by a discontinuous Percoll gradient and incubated under capacitating conditions

Using a three-layer (45, 65 and 90%) Percoll gradient, semen samples ($n = 14$) were separated in three subpopulations, the resulting interfaces between 45 and 65% (L45), and 65 and 90% (L65), and the 90% pellet (L90). Aliquots of these subpopulations were studied at baseline (T0), and 5 (T5) and 18 (T18) h in a capacitating incubation. The subpopulations contained significantly different percentages of morphologically normal and motile sperm. The percentage of normal forms was significantly lower in L45 than in L65 ($P < 0.05$) and L90 ($P < 0.01$) sperm fractions (Figure 1). The percentage of motile cells was also significantly lower ($P < 0.001$) in L45 than in L65 and L90 (Figure 2). These differences were maintained throughout the incubation times. Sperm viability was conserved, with differences ⩽10% between L45 and L90 at any time-point (Figure 3).

The subpopulations also showed differences in the quality of sperm movement, judged by motion analysis of the sperm tracks. Curvilinear velocity (VCL) values of sperm from L45 fraction were significantly lower than those of L65 ($P < 0.01$ at T0 and $P < 0.05$ at T5 and T18) and L90 ($P < 0.01$ at all times) sperm (Figure 4A). Mean values of amplitude of lateral head displacement (ALH) were significantly different when comparing L45 with L65 ($P < 0.05$) and L90 ($P < 0.01$) at all times (Figure 4B). Contrasting with these results, no statistical differences were observed for the values of flagellar beat-cross frequency (BCF) at any incubation time (Figure 4C).

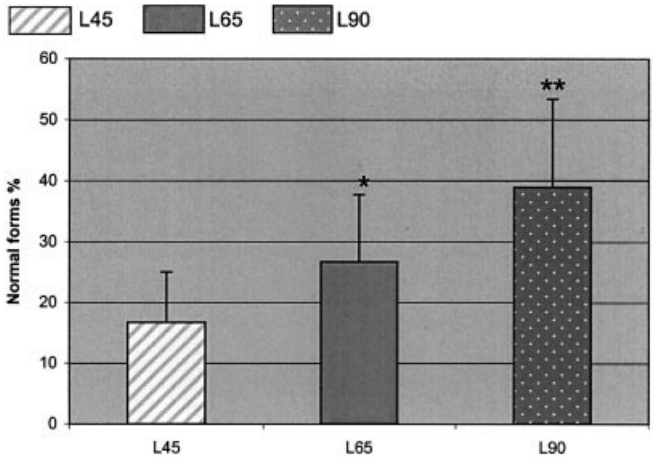

Figure 1. Percentages of morphologically normal sperm recovered from different Percoll layers. Results are expressed as mean ± SD. Statistical significance compared with L45: $*P < 0.05$; $**P < 0.01$ ($n = 14$). L45 = 45/65% Percoll interface; L65 = 65/90% interface; L90 = 90% pellet.

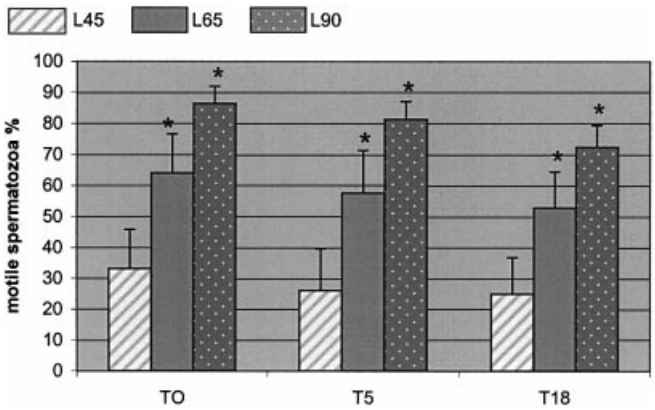

Figure 2. Percentage of motile sperm recovered from different Percoll layers (L45, L65, L90) immediately after centrifugation (T0), and after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Results are expressed as mean ± SD. Sperm from L45 showed a significantly lower proportion of motile cells throughout all the incubation times in comparison with L65 and L90 ($*P < 0.001$) ($n = 14$).

Percoll-separated sperm subsets showed different abilities to develop hyperactivated motility when they were incubated under capacitating conditions for 5 and 18 h (Figure 5). At T5, the percentage of hyperactivated cells in L45 was significantly lower in comparison with the percentages observed in the L65 and L90 fractions ($P < 0.01$). Statistical differences in hyperactivation percentages were also observed at T18, but only between sperm from L45 and L90 ($P < 0.01$).

Protein tyrosine phosphorylation in Percoll-separated sperm subpopulations

The incidence of tyrosine phosphorylation in the described sperm subpopulations ($n = 14$) was determined by immunofluorescence with a specific anti-phosphotyrosine antibody,

while the intensity of tyrosine phosphorylation was evaluated on western blots of total sperm protein extracts.

Although sperm showed phosphotyrosine immunolabelling on both head and tail, the signal associated with tail proteins was stronger and more consistent than that of the head; therefore, tail labelling was used to consider a spermatozoon as 'positive' (Figure 6).

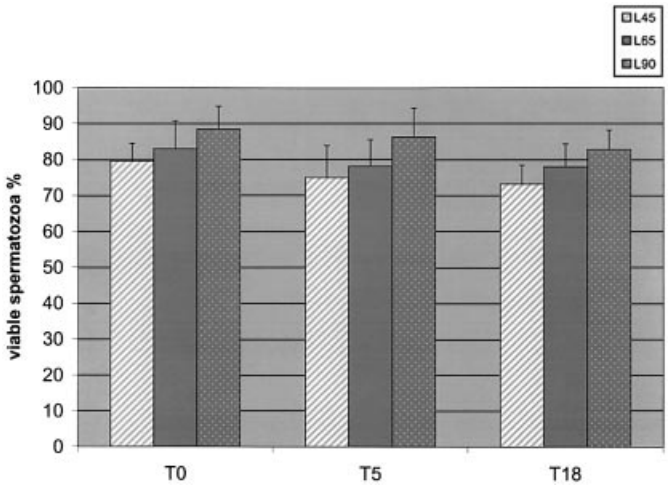

Figure 3. Percentage of viable sperm recovered from different Percoll layers (L45, L65, L90) immediately after centrifugation (T0), and after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Results are expressed as mean ± SD (n = 14).

At T0, the incidence of sperm with phosphotyrosine-immunoreactive tails (Figure 7) and the degree of tyrosine phosphorylation (Figure 8) were different only when sperm of the poorest functional quality, isolated from the L45 fraction, were compared with those of the best functional quality, isolated from the pellet of the gradient (L90) ($P < 0.05$). This difference, however, increased and became statistically significant between all three subpopulations (analysed with ANOVA) when sperm had been incubated under capacitating conditions for 5 and 18 h (Figure 7). After 5 h of incubation under capacitating conditions, the percentage of phosphotyrosine-immunoreactive sperm was significantly different between all three subpopulations ($P < 0.0002$). A comparison by pairs (Tukey post-tests) revealed that the percentage of labelled sperm found in L90 was significantly higher than the percentages found in L45 and L65 ($P < 0.01$ and 0.05 respectively). Differences were more pronounced in sperm incubated for 18 h ($P < 0.0001$; L45 versus L65: $P < 0.05$; L45 versus L90: $P < 0.01$; L65 versus L90: $P < 0.01$).

In accordance with these findings, western blot analysis revealed major differences in the level of tyrosine-phosphorylated proteins recovered from the three sperm subsets after 5 and 18 h of incubation under capacitating conditions. The lowest signal was detected in proteins extracted from sperm of the poorest quality (i.e. from L45) (Figure 8). Analysing the major protein bands densitometrically, at T5, L65 revealed a 1.4-fold increment in the overall signal compared with L45. L90 showed a 2-fold increase in signal intensity compared with

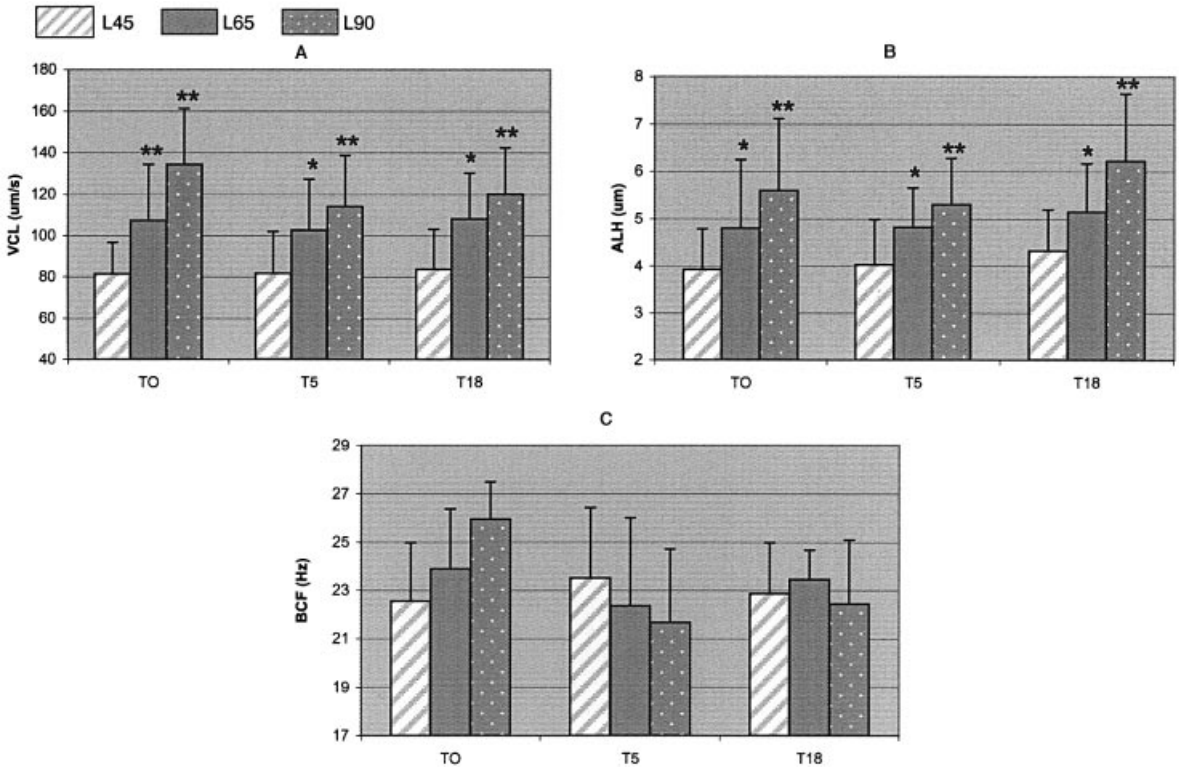

Figure 4. Motion parameters obtained from sperm recovered from different Percoll layers (L45, L65, L90) immediately after centrifugation (T0), and after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Results are expressed as mean ± SD. (**A**) Curvilinear velocity (VCL). (**B**) Lateral head displacement (ALH). (**C**) Beat cross frequency (BCF). Statistical significance compared with L45 at the same incubation time: *$P < 0.05$; **$P < 0.01$ (n = 14).

L45. At T18, the average signal increase was 1.5-fold and 2.5-fold respectively, comparing L65 and L90 to L45.

Effect of cAMP and pentoxifylline on protein tyrosine phosphorylation in Percoll-separated sperm subsets

Sperm tyrosine phosphorylation has been shown to be stimulated by cAMP analogues and/or phosphodiesterase inhibitors via direct protein kinase A activation which occurs downstream from action at the plasma membrane (Visconti *et al*., 1995b; Leclerc *et al*., 1996). To test whether sperm from the L45 subpopulation could be forced to overcome their protein tyrosine phosphorylation deficiency, we designed an experiment where each subpopulation of sperm was incubated with pentoxifylline and dbcAMP.

Sperm recovered from the three layers were incubated for 6 h with pentoxifylline in combination with dbcAMP to increase their endogenous levels of cAMP and induce tyrosine phosphorylation. Untreated control samples showed higher percentages of tyrosine-phosphorylated sperm than those previously observed. This difference may be due to a longer incubation time as well as the smaller sample size employed in this study. The pentoxifylline/dbcAMP experiments (n = 4) showed an increase in the incidence of phosphotyrosine-immunoreactive sperm in all three fractions ($P < 0.05$) (Figure 9). Although the incidence of phosphotyrosine-immunoreactive sperm was lower in L45 than in L90 ($P < 0.05$), compared to the untreated sample, protein kinase A stimulation induced a statistically significant increase in the number of tyrosine-phosphorylated sperm from the L45 fraction, which was equal to or even greater in relative magnitude than that of sperm from L65 or L90 fractions.

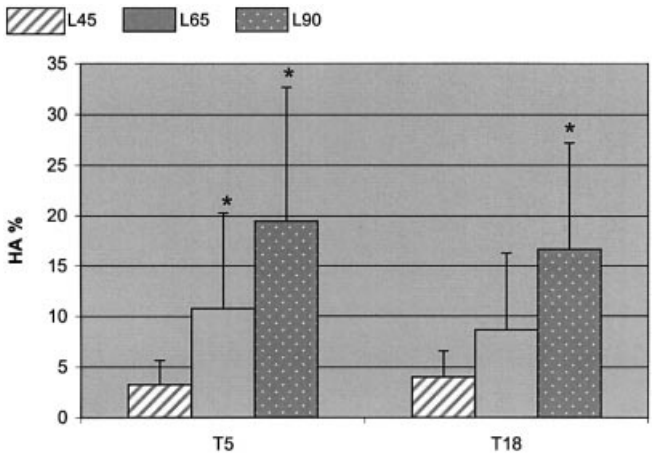

Figure 5. Percentage of hyperactivated sperm (HA%) recovered from different Percoll fractions (L45, L65 and L90) after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Results are expressed as mean ± SD. Statistical significance compared to L45 at the same incubation time: *$P < 0.01$ (n = 14).

Discussion

Human semen is composed of a heterogeneous population of sperm with varying degrees of structural and functional differentiation and normality, resulting in sperm subpopulations of different quality. These subsets may be separated by centrifugation using a discontinuous Percoll gradient. Data presented in this report indicate that the subpopulation of sperm isolated at the interface between the 45 and 65% layers of a

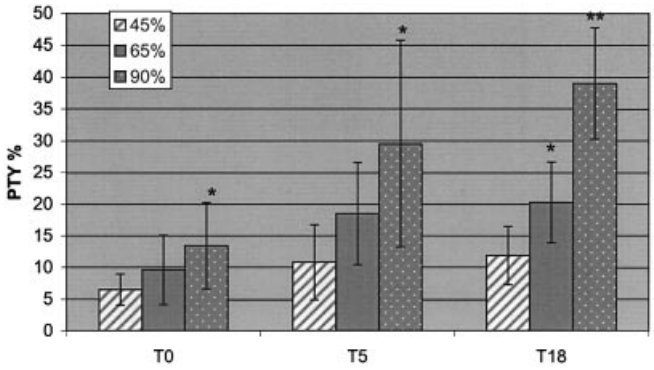

Figure 7. Percentage of sperm with phosphotyrosine-immunoreactive tails (PTY%) isolated from the three Percoll layers at different incubation times, immediately after separation (T0) and after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Results are expressed as mean ± SD. There was a statistically significant increase in the percentage of labelled sperm from L45 to L90 at all three times studied (T0, $P < 0.005$; T5, $P < 0.0002$; T18, $P < 0.0001$). Statistical significance (by pairs, Tukey post-tests) compared with L45 at the same incubation time: *$P < 0.05$; **$P < 0.01$ (n = 14).

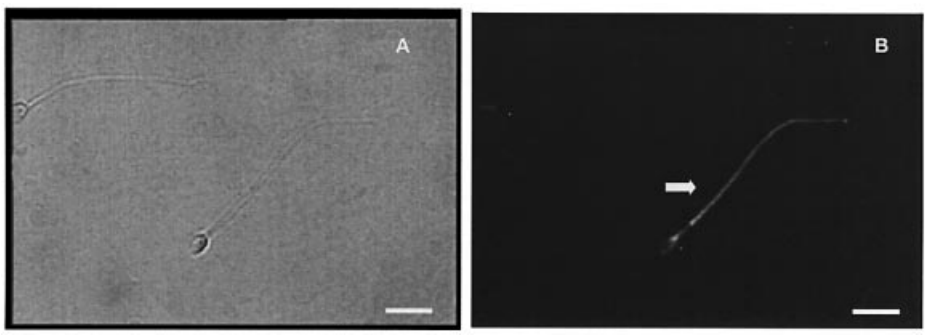

Figure 6. Immunofluorescent localization of phosphotyrosine residues on human sperm incubated under capacitating conditions as detected by immunoreactivity with the anti-phosphotyrosine monoclonal antibody PY20. Phase contrast (**A**) and corresponding immunofluorescence (**B**) photographs. Arrow shows the immunolocalization of phosphotyrosine along the principal piece of the sperm tail. Scale bar = 12.5 μm.

45:65:90% Percoll gradient showed a clear impairment in their capacity to undergo protein tyrosine phosphorylation. This was particularly evident when sperm were challenged with a capacitating incubation (5–18 h at 37°C, 5% CO_2 in Ham/BSA). This subpopulation also showed the lowest percentage of motile and morphologically normal sperm and the poorest quality of movement, including a relative incapacity to develop hyperactivated motility. The opposite was observed for the subpopulation isolated at the 90% layer (pellet), which displayed the best functional quality in association with the highest incidence and magnitude of phosphotyrosine-immunoreactive sperm. The differences among subpopulations in terms of tyrosine-phosphorylated and hyperactivated sperm cannot be explained by their differences in percentage viability, since these differences were small and the incidence of viable sperm in all three subsets at all time-points was >73%, far exceeding the percentages of sperm showing tyrosine phosphorylation or hyperactivation.

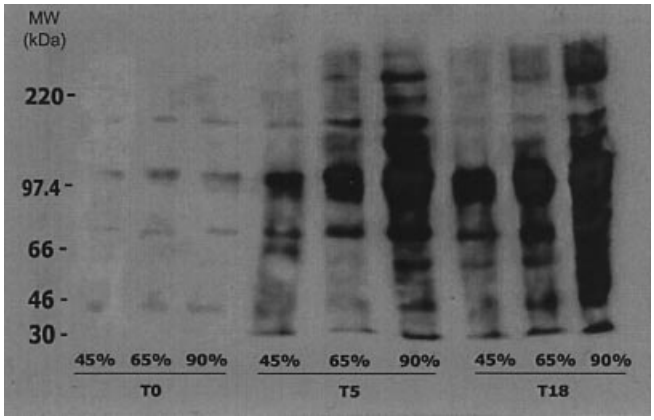

Figure 8. Western blot analysis of sperm tyrosine-phosphorylated proteins from different subsets of sperm (45% = L45; 65% = L65; 90% = L90) immediately after centrifugation (T0) and after 5 h (T5) or 18 h (T18) of incubation under capacitating conditions. Representative blot from $n = 14$ experiments.

Studying enriched populations of highly and poorly motile human sperm separated by the swim-up technique, Turner *et al*. (1999) found that the percentages of tyrosine-phosphorylated cells were not statistically significantly different between these two subpopulations. Furthermore, they found no association between the tyrosine phosphorylation status of the major fibrous sheath protein of human sperm, hAKAP82, and significant differences in motility. Although these results appear to be in disagreement with those presented in our report, differences in the composition of the sperm subpopulations studied and in the ingredients of the medium used for incubation, as well as the length of the capacitating challenge may explain, at least in part, such apparent discrepancy. Turner and co-workers used a swim-up protocol to separate sperm, incubated the cells for 90 min in HTF + 3% BSA, and focused their analysis on the phosphorylation and processing of two tail proteins, pro-hAKAP82 and hAKAP82. In contrast, we used a discontinuous three-layer Percoll gradient which separated sperm subpopulations more precisely, incubated sperm under capacitating conditions for up to 18 h, and based our assessment on the incidence and intensity of all phosphotyrosine proteins. These conditions may have widened the differences between the sperm subsets, making the tyrosine phosphorylation deficiency of poor quality sperm more evident.

The observed differences in the functional quality and tyrosine phosphorylation of the sperm subpopulations evaluated in our study could result from defects at the sperm plasma membrane originated during spermatogenesis or epididymal maturation. Sperm subsets isolated by a similar gradient have been reported to present differences in their sperm membrane lipid composition, particularly in their content of docosahexaenoic acid and cholesterol (Ollero *et al*., 2000). Since cholesterol efflux has been implicated in triggering capacitation (Cross, 1998) and tyrosine phosphorylation (Osheroff

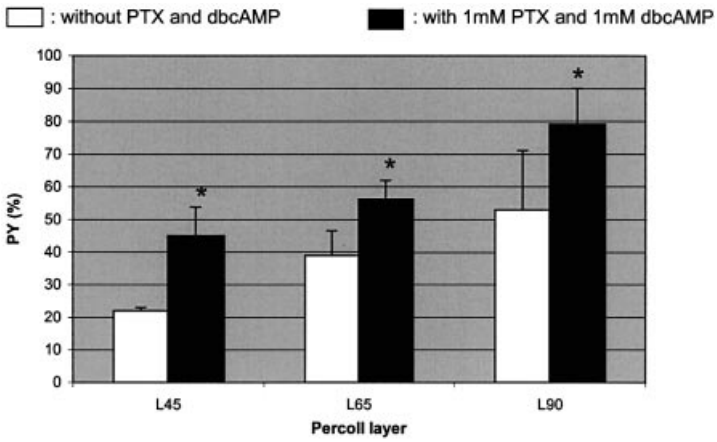

Figure 9. Percentage of sperm with phosphotyrosine-immunoreactive tails (PY%) isolated from the three Percoll layers (L45, L65, L90) after 6 h of incubation under capacitating conditions with or without pentoxifylline (PTX) and dbcAMP. Results are expressed as mean ± SD. The experiments showed an increase in the incidence of phosphotyrosine-immunoreactive sperm in all three fractions. Sperm from L45 incubated with PTX and dbcAMP achieved a percentage of tyrosine-phosphorylated cells similar to that of untreated sperm from L90, overcoming their original deficiency. *Significantly different from sperm incubated without PTX and dbcAMP ($P < 0.05$) ($n = 4$).

et al., 1999), an incompletely or abnormally differentiated sperm membrane with high content of docosahexaenoic acid and cholesterol could be the cause for inadequate tyrosine phosphorylation of sperm proteins. The phosphorylation deficiency might also be due to incomplete or abnormal differentiation of the signal transduction systems, which could be part of a more general sperm abnormality, including the membrane and other sperm structures (Huszar and Vigue, 1993; Aitken *et al*., 1998; Gil-Guzman *et al*., 2001).

Sperm tyrosine phosphorylation has been shown to be stimulated by cAMP analogues and/or phosphodiesterase inhibitors via a direct protein kinase A activation which occurs downstream of the plasma membrane (Visconti *et al*., 1995b; Leclerc *et al*., 1996). In the present study, the effect of such compounds upon protein tyrosine phosphorylation was analysed on sperm subpopulations of different quality isolated through Percoll-gradient centrifugation. Results showed that the defective tyrosine phosphorylation of L45 sperm could be overcome when those stimulators were added to the incubation medium, indicating that signal transduction mechanisms downstream of PKA were not significantly affected in these cells. This stimulation recruited a number of newly phosphorylated sperm in L45, which represented a 100% increase over the basal (untreated) values. Moreover, the percentage of tyrosine-phosphorylated sperm after stimulation reached the level of untreated L90 cells. These findings suggest that the deficiency in tyrosine phosphorylation observed in the poor quality sperm from L45 would be associated with defects in the composition and/or dynamics of the plasma membrane. Alternatively, such deficiency could also be associated with alterations of other enzymes located upstream of PKA, e.g. soluble adenyl cyclase.

The role of adenyl cyclase, bicarbonate and cholesterol acceptors in capacitation–induction has been well demonstrated in sperm from several mammalian species (Harrison, 1996; Visconti and Kopf, 1998; Gadella and Harrison, 2000). In porcine sperm, bicarbonate induces a change in membrane architecture consisting of phospholipid scrambling and lateral re-distribution and apical concentration of cholesterol, phenomena that facilitate cholesterol removal by acceptor molecules such as albumin (Flesch *et al*., 2001). Only a subpopulation of ejaculated sperm responds to bicarbonate, and such a property appears to be related to the degree of epididymal maturation.

We propose that the low level of protein tyrosine phosphorylation observed in poor quality sperm recovered from the 45% layer may be related to their membrane lipid composition, particularly to high cholesterol content, which would impair the ability of these sperm to respond to capacitation-inducing stimuli with adequate membrane architectural changes and related signal transduction. The biological and clinical significance of the above-described findings lies in the association between structural and functional parameters of human sperm quality and a relatively new biochemical marker of capacitation, an indispensable process in human fertilization. This association represents a step toward the elucidation of defective molecular mechanisms that may be the real cause of the reduced fertilizing capacity of pathological sperm.

In conclusion, data presented above show that human sperm subpopulations isolated from normal semen samples through Percoll gradient centrifugation display clear differences in their ability to undergo capacitation-associated protein tyrosine phosphorylation. The subpopulation bearing the poorest quality shows an intrinsic impairment of tyrosine phosphorylation; a defect that may be related to incomplete or abnormal differentiation of these cells, and in particular, to a specific defect of their plasma membrane which contains high levels of cholesterol. This deficiency would impair a normal development of the capacitation process, ultimately compromising the acquisition of optimal fertilizing capacity by the sperm.

Acknowledgement

The authors wish to thank Ms Christine J.Farrigan for her excellent editorial contribution to this manuscript.

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Submitted on May 23, 2003; resubmitted on September 25, 2003; accepted on September 25, 2003