Capacitation-associated protein tyrosine phosphorylation and membrane fluidity changes are impaired in the spermatozoa of asthenozoospermic patients

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Abstract

Sperm protein tyrosine phosphorylation has been associated with capacitation, motility changes, zona binding, and fertilizing ability. We previously demonstrated that gradient-isolated human sperm subpopulations differ in their plasma membrane composition, their ability to phosphorylate proteins in tyrosine residues, and their capacity to undergo hyperactivation. In this study, we have characterized capacitation-associated changes in protein tyrosine phosphorylation and membrane fluidity in spermatozoa of asthenozoospermic and normozoospermic patients consulting for infertility. Semen samples were studied at baseline and after a capacitating incubation with or without the addition of a permeable cAMP analog and a phosphodiesterase inhibitor. Basic sperm and computer-assisted motion parameters, hyperactivation, protein tyrosine phosphorylation (immunofluorescence and Western blot), and membrane fluidity (fluorescent Laurdan probe) were the main study parameters. In comparison with normozoospermic and proven-fertile donor semen, asthenozoospermic samples showed lower motility, velocity, and amplitude of lateral head displacement, both originally and after a 6-h capacitating incubation. Unlike those in normal samples, asthenozoospermic spermatozoa were unable to increase protein tyrosine phosphorylation during capacitation. Such impairment, however, was overcome when they were incubated with a membrane-permeable cAMP analog and a phosphodiesterase inhibitor, indicating a possible membrane defect. Confirming this hypothesis, plasma membranes of asthenozoospermic sperm showed decreased fluidity (increased Laurdan polarization), even after a capacitating incubation. In conclusion, spermatozoa from functional asthenozoospermic samples may owe their poor motility, and their inability to properly capacitate and develop hyperactivation, to an impairment in the tyrosine phosphorylation of critical proteins caused by decreased membrane fluidity. These findings suggest a molecular pathogenetic mechanism for a common seminal pathology associated with male infertility.

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Introduction

Capacitation-associated protein tyrosine phosphorylation has been described in the spermatozoa of numerous species, including humans (Visconti *et al.* 1995a, Carrera *et al.* 1996, Pukazhenthi *et al.* 1998, Si & Okuno 1999, Yeung *et al.* 1999). Furthermore, it has been associated with acquisition of the fertilizing ability of sperm (Visconti *et al.* 1995a, Sakkas *et al.* 2003) and development of sperm hyperactivation (Nassar *et al.* 1999, Si & Okuno 1999, Yunes *et al.* 2003).

Tyrosine phosphorylation in spermatozoa is regulated by a complex series of molecular events involving membrane cholesterol efflux and hyperpolarization, increase in intracellular NaHCO₃, Ca²⁺ and cAMP, and activation of

phosphokinases (Visconti & Kopf 1998, Visconti *et al.* 2002). Following capacitation-associated cholesterol efflux and its consequent increase in membrane fluidity, sperm show a rise in cAMP produced by a NaHCO₃-dependent soluble adenyl cyclase bound to the cytoskeletal fraction in mature sperm, which precedes an upregulation of sperm motion parameters, hypermotility, and tyrosine phosphorylation (Visconti *et al.* 1995b, Leclerc *et al.* 1996, Bajpai & Doncel 2003, Luconi *et al.* 2005). Tyrosine (protein tyrosine kinases; PTK) and cAMP-dependent (protein kinase A; PKA) kinases are involved in these processes as their inhibition in human spermatozoa decreases tyrosine phosphorylation and motility concomitantly (Bajpai & Doncel 2003, Bajpai *et al.* 2003).

Studying sperm subpopulations from normozoospermic human semen samples, we also demonstrated that Percoll-isolated low-quality spermatozoa showed an impairment in the development of tyrosine phosphorylation during capacitation (Buffone *et al.* 2004). Such deficiency, however, was overcome with permeable activators of PKA, which led us to postulate that the main defect in those spermatozoa was localized upstream of PKA, possibly at the plasma membrane. Changes in plasma membrane composition have been associated with both capacitation (Davis 1981, Cross 1998) and sperm pathology (Calamera *et al.* 2003). Considering these findings as well as preliminary data gathered from samples with abnormal motility, we hypothesized that such combined alteration may be the underlying cause of certain sperm pathologies, especially those displaying low motility (Yunes *et al.* 2003).

In order to verify this hypothesis and to expand the characterization of such molecular defects in pathological sperm, we have studied the capacitation-associated changes of protein tyrosine phosphorylation and membrane fluidity in spermatozoa of asthenozoospermic, normozoospermic, and proven-fertile human subjects. Herein, we describe the results of this investigation.

Materials and Methods

Semen collection and analysis

Semen samples were obtained by masturbation from patients consulting our center for infertility. After a basic semen analysis, the unused parts of these samples were included in the study with the patients' consent. Subjects ($n = 34$) were 25–40 years of age and collected semen after 3–5 days of sexual abstinence. Ejaculates were allowed to liquify for 1 h at room temperature and sperm concentration and motility were assessed using a computer-assisted semen analysis (Hamilton Thorne IVOS V10.8; Hamilton Thorne Research, Danvers, MA, 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). Sperm morphology was assessed by Kruger's strict criteria (Kruger *et al.* 1986).

Sperm samples were classified as normozoospermic (sperm concentration $> 20 \times 10^6$ spermatozoa/ml, percentage of motile cells $> 50\%$, percentage of viable spermatozoa $\geq 80\%$, and percentage of normal forms $\geq 14\%$) or asthenozoospermic (sperm concentration $> 20 \times 10^6$ spermatozoa/ml, percentage of motile cells $< 40\%$, percentage of viable spermatozoa $\geq 80\%$, and percentage of normal forms $\geq 14\%$). We also studied samples from seven sperm donors of proven fertility.

Motility parameters and sperm hyperactivation

Aliquots of each sperm suspension were loaded into a 20 μm deep disposable chamber (Microcell; Conception

Technologies, San Diego, CA, USA) pre-warmed at 37 °C. Computer-assisted sperm motion analysis was performed using a Hamilton Thorne digital image analyzer (HTR-IVOS v 10.8; Hamilton Thorne Research) before and after a 6 h capacitating incubation. At least 300 spermatozoa and five fields were assessed.

Eight motion parameters were assessed in this study: (1) motility (%); (2) average path velocity (VAP, $\mu\text{m/s}$); (3) track speed or curvilinear velocity (VCL, $\mu\text{m/s}$); (4) progressive or straight-line velocity (VSL, $\mu\text{m/s}$); (5) straightness (STR, %); (6) beat cross frequency (BCF, Hz); (7) linearity (LIN, %); (8) 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 cut off, 5 $\mu\text{m/s}$; medium VAP cut off, 25 $\mu\text{m/s}$; head size – non-motile, 12 pixels; head intensity – non-motile, 130 Units(U); static head size, 0.68–2.57 pixels; static head intensity, 0.31–1.21 U; static elongation, 23–100%. The playback function was used to accurately identify motile cells. Hyperactivated motility (%) was defined as motility with starspin or high-amplitude trash-ing patterns and short trajectory distances (Burkman 1984). This percentage represents the portion of motile spermatozoa displaying hyperactivated movement. The criteria for detecting hyperactivated spermatozoa were: VCL $> 150 \mu\text{m/s}$; ALH $> 7.0 \mu\text{m}$; LIN $< 50\%$ (Mortimer *et al.* 1998).

Preparation of spermatozoa

Sperm cells were separated from seminal plasma by dilution with Ham's F10 medium (Gibco BRL, Grand Island, NY, USA) containing 3 mg/ml bovine serum albumin (BSA; Sigma, St Louis, MO, USA) (Ham/BSA) and centrifugation at 400 g for 5 min (three times). The pellet was resuspended in 1 ml Ham/BSA and an aliquot of it was used to assay sperm concentration and motility. This was considered the first incubation time (T0). Sperm concentration was adjusted to approximately 10×10^6 spermatozoa/ml and incubated for 6 h (T6) at 37 °C in 5% CO_2 . In some instances, sperm were separated from seminal plasma using Percoll-gradient centrifugation (500 g for 20 min) and subsequent washing with Ham/BSA. To increase intracellular levels of cyclic AMP (cAMP), sperm were incubated for 1 h with dibutyryl cAMP (dbcAMP; 1 mM) and pentoxifylline (PTX; 1 mM).

Indirect immunofluorescence of spermatozoa

Immunofluorescence was employed to examine the sub-cellular localization of proteins phosphorylated in tyrosine residues as well as the incidence of tyrosine-phosphorylated sperm in a given population. Spermatozoa from the different groups were capacitated during various periods of time and washed twice with phosphate-buffered saline (PBS). Sperm concentration was adjusted to 5×10^6 cells/ml and 15 μl of the sperm suspension was spotted

onto eight-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., Aurora, OH, 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 fluorescein isothiocyanate-conjugated goat anti-mouse IgG (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, Eugene, OR, USA). Spermatozoa were examined using a fluorescence microscope (Olympus BX40F, Melville, NY, USA). 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).

Western blot analysis of sperm proteins

Proteins from spermatozoa were analyzed by SDS-PAGE and Western immunoblotting. Cells were washed twice with PBS and resuspended in Laemmli sample buffer (25 mM Tris, 0.5% SDS and 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 mM 2β-mercaptoethanol and stored at –20°C until use. Solubilized proteins (obtained from 2 × 10⁶ spermatozoa per lane (~5 µg protein)) were separated on 7% polyacrylamide gels under denaturing conditions. Prestained molecular weight markers (Amersham Life Science Inc., Oakville, Ontario, Canada) were run in parallel. For Western blot analysis, proteins were electroblotted and transferred onto nitrocellulose (BioRad, Hercules, CA, USA) at 100 V at 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). Then it was incubated for 1 h with the monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:5000 in blocking solution. After four washes with PBS–0.1% Tween 20, an anti-mouse peroxidase-conjugated IgG (Jackson Immuno-Research Laboratories Inc., West Grove, PA, 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 (Amersham Life Science Inc.) 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 Institutes of Health, Bethesda, MD, USA).

Membrane fluidity

Sperm membrane fluidity was evaluated using the fluorescent probe 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan; Molecular Probes Inc., Eugene, OR, USA). Normozoospermic and asthenozoospermic spermatozoa were mixed with Laurdan at 1:1000 (probe:lipid ratio) and incubated for 15 min at 37°C to allow the incorporation of the probe into the membrane. After incubation, membrane fluidity was evaluated by changes in Laurdan generalized polarization (GP) calculated as: $GP = (I_{430} - I_{480}) / (I_{430} + I_{480})$ where I_{430} and I_{480} are the fluorescence intensities at 430 nm and 480 nm respectively (λ excitation: 350 nm) (Harris *et al.* 2002) measured in a Kontron SFM-25 spectrofluorometer with temperature control (Kontron Instruments SPA, Milan, Italy).

Statistical analysis

Results are expressed as means ± S.D. or S.E.M. Statistical differences between two groups were evaluated by Student's *t*-test. Results obtained from different sperm groups at the same incubation time were compared by two-way 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, San Diego, CA, USA).

Results

Motion parameters and hyperactivated motility of spermatozoa incubated under capacitating conditions

By definition, asthenozoospermic samples showed significantly lower original motility than normozoospermic and fertile samples ($P < 0.01$ in both cases) (Table 1). After 6 h of capacitating incubation, asthenozoospermic samples showed a proportionally greater decrease in motility (31.9% drop) than the other two groups (normozoospermic, 15.8% and fertile donors, 13.2%). Viability of spermatozoa, on the other hand, was not significantly different among the study groups at any time of incubation.

Judged by motion analysis of the sperm tracks, asthenozoospermic samples also revealed differences in the quality of sperm movement. At T0, they displayed significantly ($P < 0.01$) lower velocity (VAP, VSL, and VCL) compared with normozoospermic and fertile samples. At T6, the difference between normozoospermic and asthenozoospermic samples increased in magnitude and statistical significance ($P < 0.001$). No significant differences were observed between normozoospermic and fertile samples.

Mean values of ALH displacement were significantly ($P < 0.05$) lower for asthenozoospermic when compared with normozoospermic and fertile samples at T0. This

Table 1 Motion parameters obtained from spermatozoa after 0 (T0) and 6 (T6) h of incubation under capacitating conditions. The results are expressed as means $\pm$ s.d.

Variable	Normozoospermic		Asthenozoospermic		Fertile	
	T0	T6	T0	T6	T0	T6
Motility (%)	65.9 $\pm$ 7.7	55.5 $\pm$ 9.2	26.6 $\pm$ 7.0 ^a	18.1 $\pm$ 5.7 ^a	64.5 $\pm$ 7.0	56.0 $\pm$ 2.4
VAP (μ m/s)	61.0 $\pm$ 9.9	71.8 $\pm$ 10.8	45.4 $\pm$ 7.7 ^a	48.7 $\pm$ 14.9 ^a	67.7 $\pm$ 9.5	59.5 $\pm$ 16.5
VSL (μ m/s)	50.6 $\pm$ 8.1	62.5 $\pm$ 9.8	36.0 $\pm$ 6.6 ^a	41.6 $\pm$ 14.4 ^a	57.7 $\pm$ 10.6	54.0 $\pm$ 17.5
VCL (μ m/s)	89.5 $\pm$ 19.8	119.9 $\pm$ 23.0	66.8 $\pm$ 16.8 ^a	84.3 $\pm$ 13.0 ^a	102.7 $\pm$ 16.6	82.1 $\pm$ 22.1
ALH (μ m)	3.9 $\pm$ 0.7	4.9 $\pm$ 1.1	2.9 $\pm$ 0.7 ^b	3.9 $\pm$ 0.6	4.5 $\pm$ 0.6	4.6 $\pm$ 0.6
BCF (Hz)	25.0 $\pm$ 9.1	26.4 $\pm$ 4.5	23.9 $\pm$ 5.2	33.0 $\pm$ 11.7	23.8 $\pm$ 2.5	25.0 $\pm$ 3.1
STR (%)	75.0 $\pm$ 19.6	85.7 $\pm$ 2.8	80.2 $\pm$ 6.8	75.7 $\pm$ 23.2	80.0 $\pm$ 5.8	87.0 $\pm$ 5.5
LIN (%)	57.9 $\pm$ 5.3	53.6 $\pm$ 7.1	55.1 $\pm$ 7.8	50.8 $\pm$ 9.6	53.8 $\pm$ 4.4	57.0 $\pm$ 3.3
Viability (%)	93.6 $\pm$ 3.0	89.4 $\pm$ 2.7	89.9 $\pm$ 5.3	80.5 $\pm$ 7.8	95.0 $\pm$ 0.5	90.3 $\pm$ 3.3

Superscripts a and b represent statistical differences between asthenozoospermic and normozoospermic samples at the same incubation time ($P < 0.01$ and $P < 0.05$ respectively). No significant differences ($P > 0.05$) were observed between normozoospermic and proven-fertile samples.

difference was no longer statistically significant at T6. Furthermore, no statistical differences were observed for values of flagellar BCF, STR, and LIN of trajectory at any incubation time.

The studied groups revealed different abilities to develop hyperactivated motility when they were incubated under capacitating conditions for 6 h (Fig. 1A). Out of the total number of motile spermatozoa, the percentage of hyperactivated cells in asthenozoospermic samples was

significantly lower than that observed in normozoospermic ($P < 0.01$) and fertile samples ($P < 0.05$).

Protein tyrosine phosphorylation under conditions conducive to capacitation

The incidence of tyrosine phosphorylation in the described sperm populations 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 spermatozoa showed phosphotyrosine immunolabeling on both head and tail, the signal associated with tail proteins was stronger and more consistent than that of the head; tail labeling was therefore used to consider a spermatozoon as 'positive' in the immunofluorescence assessment (Fig. 2).

After 6 h of incubation under capacitating conditions, the incidence of spermatozoa with phosphotyrosine-immunoreactive tails (Fig. 1B) was significantly lower in the asthenozoospermic group than in the normozoospermic and fertile donor groups ($P < 0.05$). These latter groups were not statistically different from one another. The difference between asthenozoospermic and normozoospermic samples was observed regardless of the method used to separate sperm from seminal plasma (data not shown).

Similar results were observed following the Western blot analysis of spermatozoa (Fig. 3). Major differences were observed in the level of tyrosine-phosphorylated proteins recovered from the three groups studied. The lowest signal was detected in proteins extracted from spermatozoa of asthenozoospermic patients (Fig. 3). Densitometric analysis of the major protein bands (range: 77–170 kDa) showed that at T6 normozoospermic samples revealed a 1.8-fold increase in the overall signal compared with T0. The average increase in signal for fertile samples was 1.9-fold. Asthenozoospermic samples, however, showed no increase in the signal intensity of most of their protein bands at T6, revealing an inability to respond to capacitating conditions with the enhanced tyrosine phosphorylation observed in normozoospermic and fertile samples.

Figure 1 Percentage of (A) hyperactivated spermatozoa and (B) phosphotyrosine (pY) immunoreactive sperm after 6 h of incubation under capacitating conditions. Results are means $\pm$ S.E.M. Values with the same superscript differ: a, $P < 0.01$ and b, $P < 0.05$. * $P < 0.05$ in comparison with normozoospermic and fertile donors.

Figure 2 Immunofluorescent localization of phosphotyrosine proteins on human spermatozoa incubated under capacitating conditions as detected by immunoreactivity with the anti-phosphotyrosine monoclonal antibody PY20. (Left) Phase contrast and (right) corresponding immunofluorescence photographs. The arrow shows the immunolocalization of phosphotyrosine along the principal piece of the sperm tail.

Effect of cAMP analogs and pentoxifylline on protein tyrosine phosphorylation

To evaluate whether spermatozoa from asthenozoospermic samples could overcome their tyrosine phosphorylation deficiency when the kinase system was directly stimulated bypassing the plasma membrane, we designed an experiment where sperm were incubated with PTX and dbcAMP. Spermatozoa from the normozoospermic and asthenozoospermic groups were incubated for 6 h in capacitating conditions, adding PTX (1 mM) and dbcAMP (1 mM) during the last hour to increase their endogenous levels of cAMP and induce tyrosine phosphorylation.

Baseline incidence of phosphotyrosine-immunoreactive spermatozoa after a capacitating incubation (T6) was significantly lower ($P < 0.05$) in the asthenozoospermic than in the normozoospermic group (Fig. 4). cAMP stimulation induced a statistically significant ($P < 0.05$) increase in the number of tyrosine-phosphorylated spermatozoa in the asthenozoospermic group, which was equal to or even greater in relative magnitude to that of spermatozoa from normozoospermic samples, suggesting that the main block to increasing tyrosine phosphorylation during capacitation resides upstream of the cAMP-dependent kinase(s).

Assessment of membrane fluidity

Since sperm membrane changes have been associated with capacitation-triggered tyrosine phosphorylation and precede cAMP production, we decided to assess sperm membrane fluidity in normo- and asthenozoospermic spermatozoa. Sperm membrane fluidity was evaluated following the variation in the GP of the fluorescent probe Laurdan (Fig. 5). This molecule spontaneously incorporates into membranes at the glycerol backbone level, distributing itself evenly among the different lipid domains (Parasassi & 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. Results are expressed as ratios of the probe's polarization. The lower the ratio, the higher the fluidity of the membrane. Asthenozoospermic samples showed a 21% higher GP ratio ($P < 0.0001$) prior to their capacitation (T0) in comparison with normozoospermic samples (0.097 ± 0.002 and 0.117 ± 0.004 for normozoospermic and asthenozoospermic spermatozoa respectively), indicating lower membrane fluidity.

When incubated for 6 h in a capacitating medium, both normozoospermic and asthenozoospermic cells significantly decreased their GP values ($P < 0.01$) in comparison with those found before capacitation, reflecting an increase

Figure 3 Western blot analysis of sperm tyrosine-phosphorylated proteins extracted immediately after washing (T0) and after 6 h (T6) of incubation under capacitating conditions. Representative blot from 24 experiments. A, normozoospermic; B, asthenozoospermic; C, fertile.

Figure 4 Percentage of phosphotyrosine (pY) immunoreactive sperm (upper, immunofluorescence) and intensity of pY proteins (lower, Western blot) in normozoospermic and asthenozoospermic samples after 6 h of incubation under capacitating conditions with (+) or without (-) PTX and dbcAMP. Results are expressed as means $\pm$ S.E.M. The experiments showed an increase in the incidence of phosphotyrosine-immunoreactive spermatozoa, as well as in the intensity of pY phosphorylation, in normozoospermic and asthenozoospermic samples. Spermatozoa from asthenozoospermic samples incubated with PTX and dbcAMP achieved a percentage of tyrosine-phosphorylated cells and a level of pY phosphorylation similar to that of untreated spermatozoa from normozoospermic samples, overcoming their original deficiency. *Statistically different from sperm incubated without PTX and dbcAMP ($P < 0.05$) ($n = 4$).

in membrane fluidity. However, even when the magnitude of the changes was similar for both groups, the overall fluidity of asthenozoospermic cells remained significantly lower ($P < 0.05$) than that attained by spermatozoa from the normozoospermic group after capacitation. These differences were observed irrespective of the method used to separate sperm from seminal plasma (data not shown).

Figure 5 Membrane fluidity of spermatozoa studied immediately after washing (T0) and after 6 h (T6) of incubation under capacitating conditions and measured by the variation of the Laurdan GP. Results are expressed as median $\pm$ S.E.M. Statistically significant: a vs b, $P < 0.01$; c vs d, $P < 0.05$; a vs c, $P < 0.01$; b vs d, $P < 0.05$.

Discussion

Asthenozoospermia is a common cause of sperm pathology and male infertility (Aitken *et al.* 1982). Except for those cases of severe alteration in which ultrastructural flagellar anomalies are detected (Chemes *et al.* 1998), the etiology of this sperm pathology likely involves an array of biochemical and functional defects. In our study, spermatozoa from asthenozoospermic patients showed a clear impairment in their motility and their capacity to develop hyperactivation, which associated with low membrane fluidity and a concomitant inability to undergo protein tyrosine phosphorylation. This was particularly evident when spermatozoa were challenged with a capacitating incubation (6 h at 37 °C, 5% CO₂ in Ham/BSA).

Semen samples from asthenozoospermic patients showed not only a low percentage of motile spermatozoa but sperm motion of poor quality, reflected by decreased VAP, VSL, VCL, and ALH. As mentioned, these spermatozoa also displayed a marked impairment in their capacity to develop hyperactivated motility. Although these defects may be the cause of the reported poor fertilizing ability of asthenozoospermic samples, the pathogenetic mechanisms leading to such alterations are, for the most part, unknown.

Several groups, including ours, have reported a close association between sperm protein tyrosine phosphorylation and motility and hyperactivation in different mammalian species (Leclerc *et al.* 1996, Nassar *et al.* 1999, Si & Okuno 1999, Luconi *et al.* 2001, Yunes *et al.* 2003, Buffone *et al.* 2004). Differences in the level of hyperactivated sperm, especially after capacitation,

could originate from the impaired capacity of these cells to phosphorylate proteins in tyrosine residues which, in turn, could be linked to reduced membrane fluidity.

Various reports have described an active participation of the sperm plasma membrane in the process of capacitation, mainly through loss of cholesterol (Davis 1981, Cross 1998, Visconti *et al.* 1999a). Cholesterol efflux produces an increase in membrane fluidity which directly or indirectly impacts protein function, such as that of surface ion channels and enzymes. Changes in ion fluxes across the membrane, mainly HCO_3^- , Na^+ , K^+ , and Ca^{2+} , have been implicated in initiating sperm capacitation as well as tyrosine phosphorylation (Yanagimachi 1994, Visconti & Kopf 1998). More specifically, an increase in intracellular HCO_3^- stimulates soluble adenylyl cyclase which rapidly increases cAMP, protein tyrosine phosphorylation, sperm motility, and acrosome reaction (Visconti *et al.* 1999b, Gadella & Harrison 2000, Luconi *et al.* 2005). The endpoint of these capacitation-associated changes, at least those related to motility and hyperactivation, appears to be an increased binding of the regulatory subunits of PKA, especially $\text{tyr}^{\text{p}}\text{ell}$, to tyrosine-phosphorylated forms of A-kinase anchoring proteins 3 and 4. Interruption of this binding leads to decreased sperm motility (Vijayaraghavan *et al.* 1997, Luconi *et al.* 2004).

Sperm tyrosine phosphorylation can be stimulated by cAMP analogs and/or phosphodiesterase inhibitors via direct PKA activation, which occurs downstream of the plasma membrane (Visconti *et al.* 1995b, Leclerc *et al.* 1996, Nassar *et al.* 1999, Bajpai & Doncel 2003, Buffone *et al.* 2004). In order to evaluate the participation of a plasma membrane defect in the inability of asthenozoospermic sperm to undergo protein tyrosine phosphorylation, we designed an experiment in which spermatozoa were treated with dbcAMP and pentoxifylline to increase their endogenous levels of cAMP. Results showed that defective tyrosine phosphorylation of asthenozoospermic spermatozoa could be overcome when those stimulators were added to the incubation medium, indicating that signal transduction mechanisms downstream of cAMP were not significantly affected in those cells.

These findings suggested that the deficiency in tyrosine phosphorylation observed in asthenozoospermic spermatozoa and, possibly, their dysfunctional motility could be associated with an alteration in the dynamics of the plasma membrane. Removal of cholesterol and increased membrane fluidity is a key step in the initiation of capacitation (Cross 1998). It decreases the cholesterol/phospholipid ratio (Davis 1981), allowing for redistribution of membrane lipids and proteins (Cross & Overstreet 1987, Gadella *et al.* 1999). Capacitation can be inhibited by adding cholesterol or cholesterol analogs to the capacitating medium (Visconti *et al.* 1999a), and can be stimulated by cholesterol acceptors such as β -cyclodextrins (Osheroff *et al.* 1999). Changes in membrane dynamics have been associated with tyrosine phosphorylation as well as sperm function and fertilizing ability (Gadella *et al.* 1999, Flesch *et al.* 2001).

In order to verify if asthenozoospermic sperm have a membrane dynamics defect that could be associated with their tyrosine phosphorylation and motility deficiencies, we have measured changes in the membrane fluidity of the sperm during a capacitating incubation, studying changes in Laurdan fluorescence (Ambrosini *et al.* 2001). Spermatozoa from the asthenozoospermic group revealed significantly less fluid membranes at the beginning of the incubation (T0). Capacitation increased the membrane fluidity of these cells, but never to the level achieved by normozoospermic spermatozoa.

Such difference in membrane fluidity could be due to the increased susceptibility of these spermatozoa to suffer peroxidative damage (Calamera *et al.* 2003), as the generation of membrane lipid hydroperoxides has been associated with membrane fluidity reduction (Aitken *et al.* 1993, 1994, Windsor *et al.* 1993). This susceptibility of asthenozoospermic sperm could be explained, in part, by their membrane composition, which is responsible for their reported higher oxidation coefficient (Calamera *et al.* 2003). Sperm membranes of asthenozoospermic samples contain high levels of polyunsaturated fatty acids, making them more prone to attack by reactive oxygen species. Since oxidizing conditions are normal during sperm capacitation and have been linked to signal transduction and tyrosine phosphorylation (Aitken *et al.* 1994, 1998), the predisposition of the asthenozoospermic samples to oxidative damage may be the origin of their membrane dysfunction, resulting in tyrosine phosphorylation deficiency and alteration of motility.

The biological and clinical significance of the above-described findings resides in the association between a common sperm pathology such as asthenozoospermia and a defective molecular mechanism, closely related with the acquisition of the sperm's fertilizing ability. 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.

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