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# Incidence of sperm-tail tyrosine phosphorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples

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**Keywords:** sperm motility, hyperactivation, asthenozoospermia, tyrosine-phosphorylation, protein-tyrosine kinase.

**ABSTRACT**: Our objective was to study the incidence of sperm-tail phosphotyrosine immunoreactivity in normozoospermic and asthenozoospermic human sperm samples, its association with sperm motion parameters, particularly hyperactivated motility, and its potential involvement in the pathogenesis of asthenozoospermia. The work was conducted as a prospective experimental study in the Sperm Biology and Andrology laboratories of the Jones Institute, a medical school-based fertility center. The study subjects were healthy fertile male donors (normozoospermic samples) and infertile patients (asthenozoospermic samples) attending the center. Recently ejaculated semen samples were washed twice to eliminate seminal plasma and a swim-up was performed to select the motile population which, in turn, was incubated up to 18 h at 37ºC in 3.5% human serum albumin-supplemented Ham's F10 to allow for capacitation. For evaluation, sperm aliquots were taken pre-swim-up ($T_o$), immediately post swim-up ($T_1$), at 6 h ($T_6$), and 18 h ($T_{18}$) of incubation. The main outcome measures were computer-analyzed sperm motion parameters and hyperactivated motility, and immunodetection of phosphotyrosine (PY)-containing proteins. During the capacitating incubation, normozoospermic samples displayed maximum motility, velocity, and hyperactivation at $T_6$, significantly decreasing their values at $T_{18}$. PY-proteins were located both at the tail and head of spermatozoa. Their expression increased progressively during the incubation, being present in about 70% of the sperm tails at $T_{18}$. Asthenozoospermic samples showed an inability to respond to capacitation with an increase in motion parameters and PY-phosphorylation. At $T_6$, both hyperactivation and PY-phosphorylation were significantly lower than in normal samples. Our results suggest that PY-phosphorylation of tail proteins is highly conspicuous in human spermatozoa, and increases its incidence in a time-dependent manner, as more sperm become capacitated. Asthenozoospermic samples displaying low percentages of motile sperm and altered motion characteristics showed a decreased incidence of PY-phosphorelated sperm. Tail protein PY-phosphorylation may be related to sperm movement, especially to hyperactivated motility and its deficiency may be associated to asthenozoospermia.

## Introduction

Abundant evidence exists about the participation of several factors in the initiation and regulation of sperm motility (Morisawa *et al*., 1990). Three of these factors, cAMP, calcium and intracellular pH have received considerable attention as regulators of flagellar motility (Majumder *et al*., 1990). Recent technical advances have accelerated the identification of specific biochemical pathways involving second-messenger modulated protein-phosphorylation. It is now clear that this phenomenon plays a pivotal role in the regulation of the mechanochemical processes underlying sperm movement (Tash, 1990; Tash and Bracho, 1994, 1998). Cyclic AMP regulates initiation and maintenance of motility, while calcium has also been postulated to be a vital regulatory component of the active sliding asymmetry that occurs during progressive sperm movement (Brokaw *et al*., 1974) and fertility-associated hyperactivation (White

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and Aitken, 1989). The major action of cAMP is stimulation of protein phosphorylation by cAMP-dependent protein kinases, which in turn phosphorylate flagellar proteins on serine or threonine residues.

Although there is considerable evidence that these type of phosphoproteins play a role in regulation of sperm motility (Tash, 1990; Chaudhry *et al*., 1995; Tash and Bracho, 1998), little is known about the participation of phosphotyrosine-containing proteins in such event. Phosphotyrosine-containing proteins have been shown to be present in spermatozoa of different species (Visconti *et al*., 1995a,b; Carrera *et al*., 1996; Luconi *et al*., 1996; Berruti and Martegani, 1989; Si and Okuno, 1999) and several reports associate them with motility (Hayashi *et al*., 1987; Yunes *et al*., 1994; Carrera *et al*., 1996; Leclerc *et al*., 1996; Vijayaraghavan *et al*., 1997; Mahony and Gwathmey, 1999; Si and Okuno, 1999; Herrero *et al*., 2001; Marín-Briggiler *et al*., 2002). Most of these reports highlight the association between PY-phosphorylation of certain proteins and general changes in sperm motility. However, little has been published about the incidence of such phenomenon, PY-phosphorylation, and its time dependent expression, in a capacitating population of human spermatozoa. Furthermore, if this association is true, we hypothesize that human asthenozoospermic samples displaying low percentages of motile spermatozoa would also show decreased incidence of tail PY-phosphorylation. Although there is a general consensus that ultrastructural anomalies underlie severe asthenozoospermia (Chemes *et al*., 1998), the etiology and pathogenesis of temporary and/or mild asthenozoospermia remains, for the most part, undefined.

We have previously reported the involvement of PY-proteins in human sperm-zona pellucida interactions (Doncel *et al*., 1993). Herein we focus on the PY-proteins of the sperm-tail and their potential association with sperm movement, particularly hyperactivated motility, under physiological and pathological conditions. The main goals of the present work are to study the incidence of sperm-tail phosphotyrosine immunoreactivity in normozoospermic and asthenozoospermic human sperm samples, its association with sperm motion parameters, particularly hyperactivated motility, and its potential involvement in the pathogenesis of asthenozoospermia.

## Material and Methods

Normal semen samples (motility ≥ 50%, motile sperm ≥ 60x10$^6$/ml) (n=16) from healthy fertile donors, and asthenozoospermic samples (motility ≤ 30%) (n= 13) obtained from patients consulting our infertility program were studied. Recently ejaculated semen samples were washed twice in Ham's F-10 medium supplemented with 3.5% of human serum albumin. After performing the swim-up separation for 1 h, the concentration was adjusted to 20x10$^6$/mL. The samples were further incubated at 37°C and 5% $CO_2$ for 6 and 18 additional hours. Small aliquots were taken after the two-washings ($T_0$), immediately post swim-up ($T_1$), at 6 h ($T_6$) and 18 h ($T_{18}$) of incubation, in order to perform indirect immunofluorescence, ATP determination, and computer-assisted sperm-motion analysis.

For the indirect immunofluorescence technique (IIF), three aliquots of 10 µl were taken from each experimental variant. Two of them were used as controls for the immuno-staining procedure. The first control group did not include the use of the first antibody (anti-phosphotyrosine monoclonal antibody py20, ICN Biomedicals, Costa Mesa, CA), and the second control group included the first antibody previously blocked with O-Phospho-DL-Tyrosine (OPT) (SIGMA, St. Louis, MO), the original antigen against which the antibody was raised (Glenney *et al*., 1988). Blocking was performed shortly before performing the IIF procedure, incubating the antibody (0.1 mg/mL) with OPT (40mM in phosphate buffered saline solution, PBS; original stock dissolved in NaOH) for 30 min at 37ºC. With the help of a radial immunodiffussion test (mouse IgG2b subclass Nanorid LL Kit, The Binding Site, Birmingham, UK) the approximate concentration of the antibody left in solution after the blocking incubation was checked. The whole IIF procedure with the first antibody, both blocked and unblocked, was performed as follows: aliquots of 10 µl were air dried on an 8-well slide and fixed with methanol for 20 min. After 10 min washing and rehydration with PBS the samples were incubated in a wet chamber for 2 h at room temperature (20 to 24°C) with the first antibody, the anti-phosphotyrosine monoclonal antibody py20 (100 µg/mL in PBS), followed for 2 PBS-BSA (PBS with 1% of bovine serum albumin) washings 10 min each. Immediately after, the samples were incubated with a fluorescein-conjugated affinity purified goat antibody to mouse IgG (50 µg/mL, Organon Teknika, Cappel Research Products, Durham, NC) for 30 min at room temperature in a wet chamber. Finally, the samples were washed three times with PBS-BSA, 10 min each, mounted and evaluated with a Nikon Microphot-FX epifluorescence microscope (600x). At least 200 cells were evaluated for each experimental variant.

The computer-assisted sperm motion analysis was

performed with a Hamilton-Thorn automated image analyzer (HTM-IVOS v. 10, Hamilton-Thorn Research, Beverly, MA). Standard motion parameters were obtained. Hyperactivated motility (HA), defined as motility with star-spin or high amplitude thrashing patterns and short distance of travel, was sorted using the following set of parameters, adapted from Burkman (Burkman, 1991): (1) curvilinear velocity (VCL, velocity calculated from the sum of trackpoint-to-trackpoint velocity), minimum 100 µm/sec, maximum 500 µm/sec.; (2) linearity (LIN, measures the departure of the cell from a straight line), minimum 0%, maximum 65%; and (3) amplitude of lateral head displacement (ALH, it corresponds to the mean width of the sperm head oscillation as the cell swims), minimum 7.5 µm, maximum 100 µm. The pertinent setting used during the motility assessment was: Frames acquired = 30; Frame rate = 60 Hz; Minimum cell size = 4 pixels; Low VAP cutoff = 5 µm/sec; Static head size = 0.2 to 2.99; Static head intensity = 0.26 to 1.31; and Static head elongation = 0 to 100.

The ATP content in the human spermatozoa was measured by a bioluminescence assay (Lemasters and Hackenbrok, 1978) after extraction by boiling in Tris-EDTA buffer for 15 min. Luminiscence was recorded after the addition of 150 µl of ATP monitoring (1243-200. Bio-Orbit. Turku, Finland). Next, 20 µl of $5 \times 10^{-6}$ M ATP (1243-1201. Bio-Orbit. Turku, Finland) was added as an internal standard. Light emission was monitored on an Berthold LB9505 C (version 4.08) luminometer at 37°C. The results were expressed as pmol/$10^6$ spermatozoa.

*Statistical Analysis*

Because some of the normal samples did not have counts taken at time zero, maximum likelihood was used to estimate the parameters for an incomplete repeated measures design. A univariate analysis of variance (ANOVA) was used to test for significant differences in tyrosine-phosphorylation, motion parameters, and HA between normal and asthenozoospermic samples along the 18 h incubation period. Post-hoc t-tests, with a Bonferroni correction for Type I error rate, were performed. An ANOVA test was also employed for ATP. Labeled spermatozoa were compared between control and treated samples by two-tailed t-test. Where necessary, percentages were transformed using arcsin before analysis. In all the cases, a value of $p < 0.05$ was considered statistically significant.

**Results**

Multiple motion parameters, provided by an HTM motion analyzer, from normozoospermic and asthenozoospermic (motility < 30%) samples were recorded after sperm were washed out of seminal plasma ($T_0$), immediately post-swim-up ($T_1$), and after 6 ($T_6$) and 18 ($T_{18}$) h of incubation in Ham's F-10 + 3.5% HSA. Some of these parameters can be seen in Table 1. Initially, $T_0$, normal samples were significantly better than asthenozoospermic ones, both in motility -by definition- and VCL. After swim-up and at 6 h of incubation, this difference was also present in ALH. However, after an over-

**TABLE 1.**

**Motion parameters of normozoospermic and asthenozoospermic samples during capacitation**

| Incubation Time (h) | Group | Motility (%) | VCL (µm/sec) | LIN (%) | ALH (µm) |
|---|---|---|---|---|---|
| 0 | Normo | 66 ± 3 | 115 ± 6 | 61 ± 2 | 4.2 ± 0.2 |
| | Astheno | 15 ± 4[a] | 87 ± 6[b] | 59 ± 3 | 4.2 ± 0.2 |
| 1 | Normo | 85 ± 2 | 142 ± 3 | 63 ± 2 | 4.6 ± 0.2 |
| | Astheno | 25 ± 9[a] | 106 ± 6[a] | 61 ± 3 | 4.3 ± 0.2[c] |
| 6 | Normo | 86 ± 3 | 160 ± 3 | 56 ± 3 | 6.2 ± 0.2 |
| | Astheno | 28 ± 9[a] | 104 ± 6[a] | 60 ± 3 | 4.6 ± 0.3[a] |
| 18 | Normo | 50 ± 5 | 92 ± 4 | 49 ± 1 | 4.1 ± 0.2 |
| | Astheno | 25 ± 8[c] | 84 ± 6 | 53 ± 3 | 3.8 ± 0.5 |

Statistical significance (normo vs. astheno):
[a] = $p < 0.0001$; [b] = $p < 0.01$; [c] = $p < 0.05$

night incubation ($T_{18}$), only the percent motility remained statistically higher for normozoospermic samples.

Concerning the incidence of hyperactivated motility (Fig. 2), again, percentages were higher for normozoospermic samples, reaching statistical significance at 6 h of incubation. Using a mathematical model, a nonlinear trend can be predicted for the data. Both groups show an "inverted U" trend with an increase up to about 8 h and then a decline to below baseline levels by 18 h. Once selected through a swim-up, the motile sperm population from the asthenozoospermic samples showed no increase in spontaneously hyperactivated sperm between $T_1$ and $T_6$. Conversely, in normozoospermic samples, this subset almost doubled during the same time-frame.

In order to localize phosphotyrosine-containing proteins in human spermatozoa, a well-characterized monoclonal antibody (py20) that recognizes phosphotyrosine (PY) but not phosphoserine or phosphothreonine (Glenney *et al*., 1988), was used in an indirect immunofluorescent technique on methanol-fixed sperm. The fluorescent label appeared on the head and/or the tail of the spermatozoa. At the head level, it was faint and mainly concentrated in the equatorial segment and/or over the acrosomal area. The incidence of these sperm-head PY-containing proteins and their involvement in human sperm-zona interaction have been already reported (Doncel *et al*., 1993). PY fluorescent label at the sperm-tail level was conspicuously bright all along the tail except for the midpiece and the end tip, where fluorescence was never seen (Fig. 1). Recognition of PY-proteins was specific since blockage of the anti-PY monoclonal antibody with 0-phospho-DL-tyrosine (40 mM) completely abolished sperm labeling. As another control, the secondary antibody, a fluorescein-conjugated anti-mouse IgG, used alone, was also negative.

Neither the microscopic intensity of the fluorescence nor the distribution of the label differed between normo- and asthenozoospermic samples. At $T_0$, the incidence of sperm displaying a detectable label at the tail level was low for both normo- and asthenozoospermic samples (Fig. 3). However, after the motile populations were

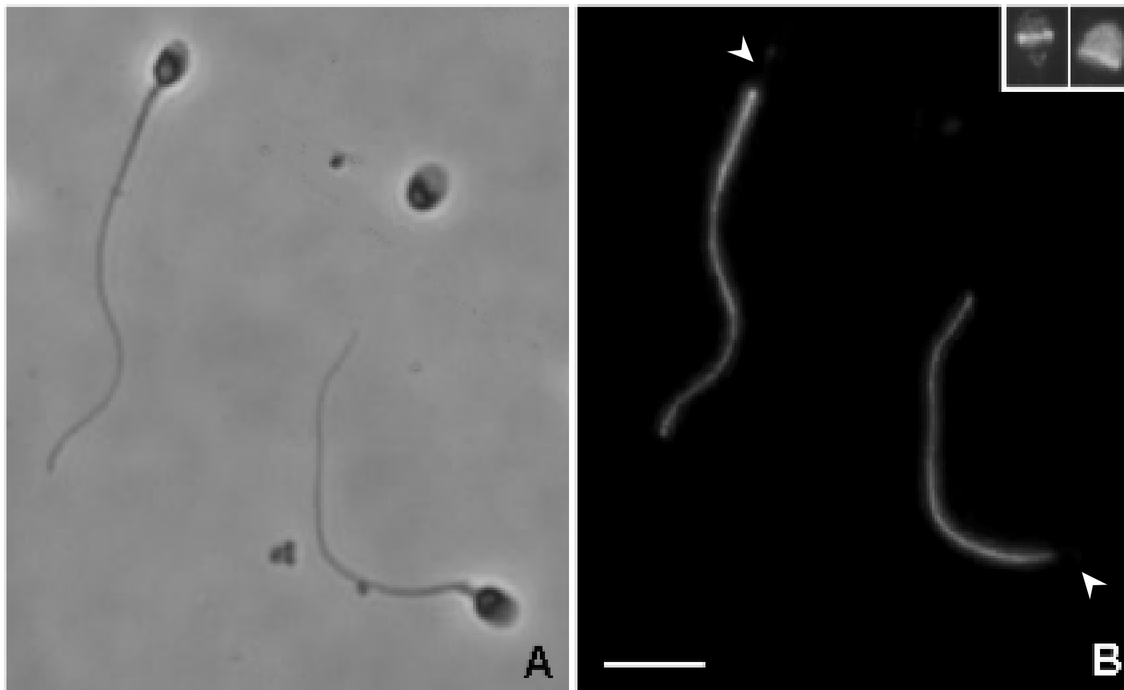


**FIGURE 1.** Photograph showing human spermatozoa from a normozoospermic sample ($T_1$) labeled with antiphosphotyrosine monoclonal antibody (400x) (panel A: phase contrast image; panel B: immunofluorescence image). Notice the bright fluorescence all along the tail excluding most of the midpiece. Some spermatozoa (<10 %) showed either a faint equatorial label at the head level (inset panel B, left) or a whole label on the acrosome region (inset panel B, right). Arrowheads show the begining of the midpiece (white bar in panel B = 10 µm). Asthenozoospermic spermatozoa do not differ qualitatively from normal spermatozoa (data not shown).

selected, the incidence of tail PY-phosphorylation increased at a significantly greater rate in the normal group. The asthenozoospermic sperm showed little increase during the incubation time. The differences in means between the two groups were not significant at $T_0$ and $T_1$, but highly significant ($p < 0.0001$) at $T_6$ and $T_{18}$.

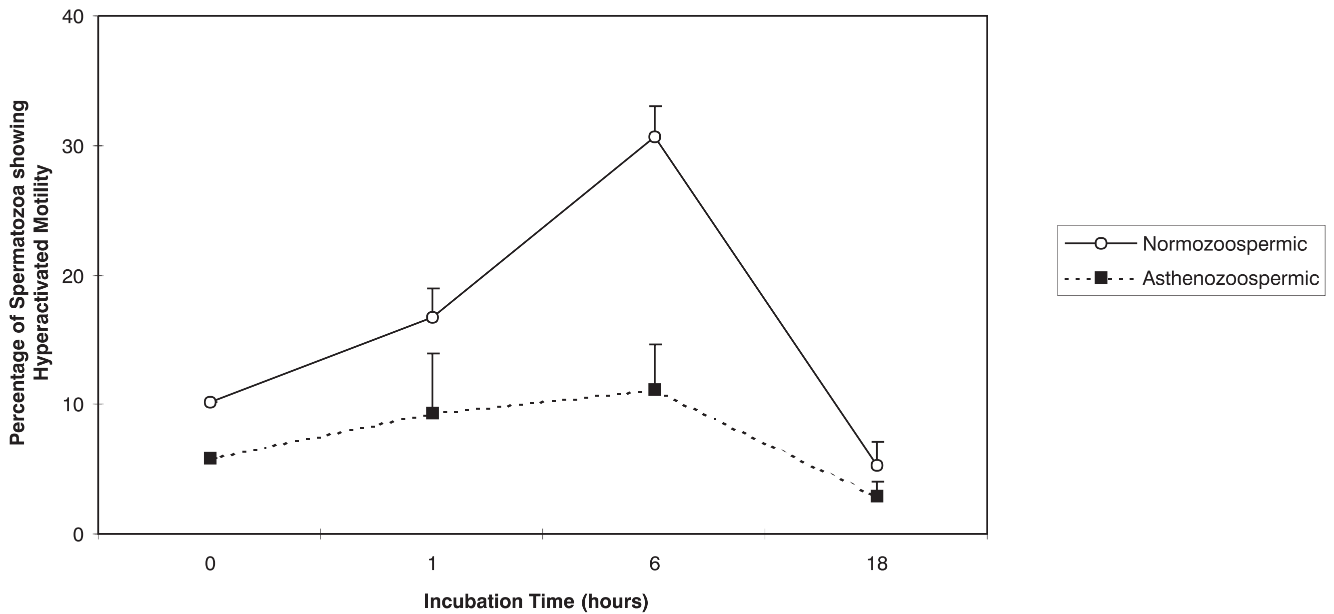


**FIGURE 2.** Incidence of sperm displaying hyperactivated motility in normozoospermic and asthenozoospermic samples during capacitation (X±SEM). The mean of the normal samples was significantly greater only at 6 hours (p < 0.001). At least 200 cells were evaluated at each time and condition.

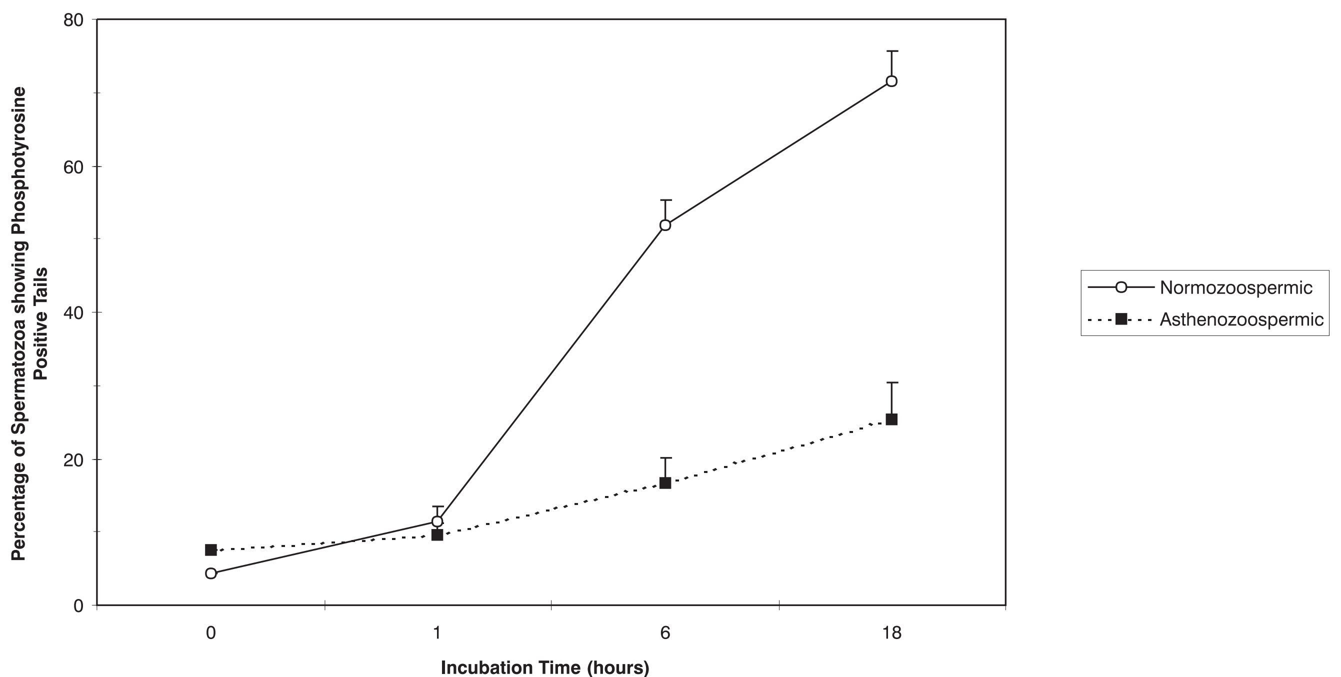


**FIGURE 3.** Incidence (expressed as percentages) of sperm-tail phosphotyrosine-containing proteins in normozoospermic and asthenozoospermic samples (X±SEM). At 6 and 18 h, the normal samples had significantly greater percentages than the asthenozoospermic ones (p < 0.0001). At least 200 cells were evaluated at each time and condition.

## Discussion

The results presented above show that: (1) tyrosine-phosphorylation is noticeable in normal human spermatozoa incubated in capacitating conditions, especially along the principal piece of the tail; (2) its incidence increases in a time-dependent manner during a capacitating incubation, particularly in normal spermatozoa; (3) asthenozoospermic samples displaying altered motion parameters also show defective PY-phosphorylation of sperm tails; and (4) overall –excluding T18–, there is an apparent association between PY-phosphorylation at the tail level and sperm motion, especially hyperactivated motility.

PY-phosphorylation has been described in sperm (reviewed in Visconti and Kopf, 1998) and several PY-proteins identified in sperm extracts (Duncan and Fraser, 1993; Visconti *et al.*,1999; Carrera *et al.*, 1996; Leclerc *et al.*, 1996; Visconti *et al.*, 1999; Pukazhenthi *et al.*, 1998; Tardif *et al.*, 2001). Most of these proteins apper to be located at the tail level. The most abundant of those proteins is AKAP4, an A kinase anchoring protein localized in the fibrous sheath (Carrera *et al.*, 1996, Johnson *et al.*, 1997).

Conclusive evidence points out that most of the sperm phosphoproteins are concerned with motility, structurally or functionally related to axonemal components (Tash, 1990; Tash and Bracho, 1998). It is also true that most of them are phosphorylated in a cAMP-dependent manner, bearing their phosphate groups on serine or threonine residues (Brokaw *et al.*, 1974; Lindemann and Kanous, 1989; Majumder *et al.*, 1990). Some tyrosine phosphorylated proteins (PY-proteins), however, have also been reported to be associated with motility (Vijayaraghavan *et al.*, 1997; Si and Okuno, 1999; Leclerc *et al.*, 1996). Interestingly, Morisawa and Hayashi (1985) identified an axonemal protein in salmonid sperm which is phosphorylated specifically when sperm motility is initiated. This protein has a molecular weight of 15,000 and is phosphorylated only at tyrosine residues. Using ion-channel blockers as well as A-kinase and tyrosine kinase specific inhibitors, these authors were able to lay out a model about sperm motility control (Morisawa and Morisawa, 1990). Ion changes through activation of adenylate cyclase cause increase in intracellular cAMP. An activated cAMP-dependent protein kinase phosphorylates/activates a tyrosine-kinase. This results in PY-phosphorylation of the 15 kDa protein previously described which, in turn, triggers a final step of sperm motility initiation. Mechanistically, the relationship between hyperactivated motility and sperm-tail PY-phosphorylation could be effected through a cAMP-dependent via, as outlined in Morisawa's model and suggested by Visconti and co-workers (1995b), or more directly by stimulation of a protein tyrosine kinase. In this regard, tyrosine kinase activity has been already reported in mammalian sperm (Berrutti and Mantegani, 1989; Leyton and Saling, 1989; Carrera *et al.*, 1996; Leclerc *et al.*, 1996; Luconi *et al.*, 1996; Vijayaraghavan *et al.*, 1997; Si and Okuno, 1999).

Here we report that PY-phosphorylation of tail proteins increases its incidence in the normal sperm population during the time and under the conditions known to favor capacitation and hyperactivation (Yanagimachi, 1994). The number of PY-phosphorylated sperm tails keeps building up from the moment they are washed out of seminal plasma, reaching about 70% after 18 h of incubation. Although the microscopic immunofluorescence technique is not sensitive enough to firmly conclude it, there appears to be no major dephosphorylation of structural PY-immunoreactive proteins in the flagellum. It might be possible that these PY-containing proteins represent an "activated state" of the sperm tail developed during capacitation, which is a basic requirement for hyperactivation to occur and/or to be regulated by finer and more sensitive mechanisms of control.

During the initial part of the incubation, there appears to be a good correlation between the number of sperm that developed hyperactivated motility and the number of spermatozoa showing PY-immunoreactive tails. At the end of the incubation, however, these two phenomena seem to dissociate. The drastic fall in hyperactivation percentages together with a general decrease in motion parameters at $T_{18}$ cannot be attributed to lack of ATP since sperm storages were slightly higher at $T_{18}$ (237 ± 21 pmoles/$10^6$ sperm) than at $T_6$ (191 ± 27 pmoles/$10^6$ sperm). Moreover, both values were significantly higher ($p < 0.05$ and 0.001 respectively) than the one obtained at $T_1$ (100 ± 12 pmoles/$10^6$ sperm). Other factors, however, may have been responsible for the drop in the quality of sperm movement, especially hyperactivated motility, since it is known than extended incubation times have deleterious effects on sperm function (Calamera *et al.*, 2001). It has been shown that the production of reactive oxygen species (ROS) is not apparently the cause of the motility drop since addition of catalase to the medium does not prevent the loss of motility despite it precludes the ROS increase (Calamera *et al.*, 2001).

Except for those rare cases of repeated severe asthenozoospermia in which ultrastructural flagellar

anomalies are detected (Chemes *et al*., 1998), the etiology of this sperm pathology remains elusive, and probably requires a complex arrange of biochemical, functional and structural deffects. Biochemical defects, antisperm antibodies, leukocytospermia, systemic drugs and smoking are among the most common culprits cited in the literature (Hargreave, 1990). Data derived from our observations in asthenozoospermic samples collected from patients consulting for infertility show that initially ($T_o$), the most conspicuous difference with normozoospermic samples resides on the number of motile cells. However, after swim-up and further incubation under capacitating conditions, the motile population from asthenozoospermic samples was still not able to reach the motion characteristics of normozoospermic samples. Particularly striking is the difference in percentages of hyperactivated sperm after 6 h of incubation. This impairment in the quality of sperm movement and in the ability to respond to capacitation-induced changes shown by asthenozoospermic sperm was associated with their incapacity to tyrosine-phosphorylate tail proteins. Conceptually, altered protein phosphorylation may well represent one of the causes of the sperm motion deficiency. In this sense, the present report appears to be the first one suggesting a molecular mechanism associated to common asthenozoospermia. At present, our research efforts are directed toward elucidating this latter possibility.

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