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Cyclic nucleotide phosphodiesterase inhibition increases tyrosine phosphorylation and hyper motility in normal and pathological human spermatozoa

Roberto Yunes, Pedro Fernández, Gustavo F. Doncel, and Aníbal A. Acosta

The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA. USA.

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ABSTRACT: Our objective was to determine the effect of phosphodiesterase (PDE) inhibition on: 1) tyrosine phosphorylation of human spermatozoa at the tail level; and 2) sperm motion parameters and hyperactivated motility. The study was conducted with normozoospermic and asthenozoospermic samples incubated under *in vitro* capacitating conditions. The main outcome measures were computer-assisted sperm motion analysis and fluorescent immunodetection of phosphotyrosine-containing proteins. Pentoxifylline (PTX) was used as PDE inhibitor because of its wide use in the clinic. PTX-treatment significantly increased sperm velocity, hyperactivated motility and tyrosine-phosphorylation, both in normo and asthenozoospermic samples. Tyrosine-phosphorylation of tail proteins was highly conspicuous in both types of samples, showing no differential pattern after PTX-treatment. Normozoospermic samples treated with pentoxifylline showed an increase in the number of spermatozoa displaying hyperactivated movement and tyrosine-phosphorylation at the tail level. Preliminary data on asthenozoospermic samples exhibiting altered motion characteristics and defective phosphorylation of sperm-tail proteins showed that both defects can be concomitantly overcome by pentoxifylline treatment. Tyrosine-phosphorylation of sperm-tail proteins is underlying the enhancement of hyperactivated motility resulting from PDE inhibition by pentoxifylline.

Introduction

Several factors have been implicated in sperm motility regulation, especially cAMP, calcium, bicarbonate and intracellular pH (Yanagimachi, 1994). The main action of cAMP would be the stimulation of protein phosphorylation by cAMP dependent protein kinases. Actually, many flagellar constituents identified in mammals are known to be or contain phosphorylated proteins (Yanagimachi, 1994). Most of them carry their phosphate groups on serine or threonine residues, showing the result of a typical protein kinase-A mediated phosphorylation.

During the past decade there have been numerous reports about the participation of phosphotyrosine-containing proteins in sperm capacitation and motility (Leyton and Saling, 1989; Berruti and Martegani 1989; Visconti *et al*., 1995a; Luconi *et al*., 1996; Visconti *et al*., 2002). Since the first report of phosphotyrosine-containing proteins in rainbow trout spermatozoa (Hayashi *et al*., 1987), the occurrence of these proteins has been reported in boar (Berruti and Martegani, 1989), mouse (Leyton and Saling, 1989), hamster (Visconti *et al*., 1999b), bovine (Galantino-Homer *et al*., 2004) and human spermatozoa (Naz *et al*., 1991). Interestingly, it is known that the initiation of motility in rainbow trout

Address correspondence to: Dr. Roberto Yunes. Area de Farmacología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, CC 33, (5500) Mendoza, ARGENTINA.
Fax: (+54-261) 449 4047. E-mail: ryunes@fcm.uncu.edu.ar
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sperm is concomitant with the tyrosine-phosphorylation of a 15kD axonemal protein (Hayashi *et al*., 1987), and that the expression of the scatter factor/hepatocyte growth factor –a natural ligand for tyrosine kinase receptors- has been proposed to be correlated with the initiation of sperm motility in the murine male genital tract (Naz *et al*., 1994). Also, it has been reported that tyrosine-phosphorylation in human sperm increases with time under *in vitro* capacitating conditions, and that this increase is related to hyperactivated motility (Yunes *et al*., 2003; Nassar *et al*., 1999a).

It is well known that pentoxifylline, a phosphodiesterase (PDE) inhibitor from the group of methylxanthines (Ward and Clissold, 1987), is a potent stimulator of human sperm hyperactivated motility (Tesarik *et al*., 1992; Pang *et al*., 1993). In fact, it has been successfully used in the *in vitro* treatment of asthenozoospermic samples for assisted reproduction (Yovich *et al*., 1990). Hyperactivated motility, usually acquired along the capacitation process, has been shown to be a crucial step in order for a spermatozoon to fertilize an egg (Yanagimachi, 1994). It is believed to be dependent on intracellular calcium concentration and cAMP levels (Yanagimachi, 1994). Since pentoxifylline is known to increase the intracellular concentration of cyclic nucleotides (Ward and Clissold, 1987), it has been suggested that the effect of pentoxifylline on cAMP levels could be the main molecular mechanism to explain the augmented hyperactivated motility of treated sperm samples (Tesarik *et al*., 1992; McKinney *et al*., 1993; Stachecki *et al*., 1994). However, the specific mechanism of such stimulation remains to be elucidated (Tesarik *et al*., 1992). Particularly intriguing is the persistence of the effect on sperm hyperactivation until at least 4 h after drug removal (Pang *et al*., 1993).

In this preliminary study our goal was to describe the effect of PDE inhibition on protein tyrosine phosphorylation and hyperactivated motility in normal and asthenozoospermic human spermatozoa, emphasizing their possible temporo-spatial association.

Material and Methods

Normal semen samples (motility $\geq$ 50%; motile sperm $\geq 60x10^6$/ml) (n=16) from healthy donors, and asthenozoospermic samples (n=5) obtained from patients consulting our infertility program were studied. Recently ejaculated semen samples were washed two times in Ham's F-10 medium supplemented with 3.5% of human serum albumin (Ham/HSA). After performing a swim-up separation for 1 h and adjusting the sperm concentration to $20x10^6$ /ml, two aliquots of 100 µl were taken, designated as control and test, and incubated for 1h in Ham/HSA. Another set of experiments was performed with washed samples only. Pentoxifylline (SIGMA, St. Louis, MO) was added to the test aliquot at a final concentration of 3mM, and both aliquots were further incubated for 1 more h at 37ºC and 5% CO_2. At the end of the incubation period, small aliquots were

TABLE 1.

Effect of pentoxifylline on motion parameters of normo- and asthenozoospermic samples

Group	PTX	Motility (%)	VCL (µm/seg)	LIN (%)	ALH (µm)
NORMO	-	85 ± 2	115 ± 4	65 ± 2	4.3 ± 0.1
	+	85 ± 2	134 ± 5[a]	53 ± 2[b]	5.2 ± 1.9
ASTHENO	-	24 ± 7	103 ± 8	60 ± 8	4.6 ± 0.4
	+	36 ± 8	136 ± 10	47 ± 5[a]	6.3 ± 1.7

PTX: pentoxifylline
Swim-up spermatozoa were incubated at 37ºC for 1 h with or without 3.0 mM PTX. Motion parameters were analyzed with a HTM motion analyzer (see text for details)
a: $p<0.05$; b: $p<0.0001$.

used to perform phosphotyrosine indirect immunofluorescence and computer-assisted sperm motion analysis (CASA).

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 pY-20, 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). The blocking procedure was accomplished shortly before performing the IIF, by incubating the antibody (0.1mg/ml) with OPT (40mM in phosphate buffered saline, PBS: original stock dissolved in NaOH) for 30 min at 37ºC. With the help of a radial immunodifussion 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 IIF procedure with the first antibody, both blocked and unblocked, was performed as follows: aliquots of 10µl were air dried on a spotted slide and fixed with methanol for 20 min. After 10 min-washing and rehydration with phosphate buffered saline solution (PBS) the samples were incubated in a wet chamber for 2 h at room temperature (20-24ºC) with the first antibody, the anti-phosphotyrosine monoclonal antibody py20 (100µg/ml in PBS), followed for 2 PBS-BSA (PBS with 1% of bovine serum albumin) washings of 10 min each. Immediately after, the samples were incubated with a fluorescein-conjugated affinity purified goat antibody to mouse IgG (50µg/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 Micro hot-FX epifluorescence microscope (600x). At least 200 cells were evaluated for each experimental variant.

The computer-assisted sperm motion analysis (CASA) was performed with a Hamilton-Thorn automated image analyzer (HTM-IVOS v.10, Hamilton-Thorn Research, Danvers, MA). Hyperactivated motility, 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 100µm/seg, maximum 500µm/seg; 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 oscillations as the cell swims), minimum 7.5 Hz, maximum 100 Hz. The pertinent setting used during the hyperactivated motility assessment were: Frames acquired = 30; Frame rate = 60Hz; Minimum cell size = 4pixels; Low VAP cutoff = 5µm/seg; Static head size = 0.2 to 2.99; Static head intensity = 0.26 to 1.31; and Static head elongation = 0 to 100.

Statistical Analysis

Mean values of movement parameters and labeled sperm were compared between control and test samples by two-tailed t-test. Where necessary, percentages were transformed using arcsin before analysis. A value of $p< 0.05$ was considered statistically significant.

Results

Pentoxifylline (PTX)-treatment did not increase the percentage of motile spermatozoa, neither in normal nor in asthenozoospermic samples (Table 1). It did, however, significantly ($p< 0.0001$) augment the incidence of hyperactivated sperm after 1h of incubation, both in normal as well as asthenozoospermic samples (8.7 ±

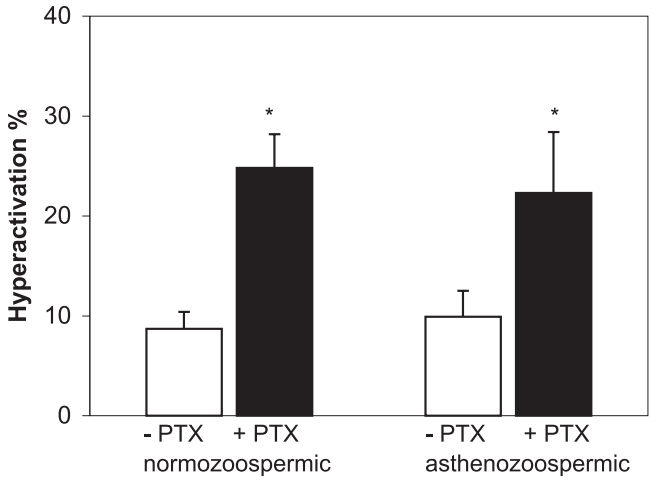

FIGURE 1. Incidence of sperm displaying hyperactivated motility in normozoospermic and asthenozoospermic samples after 1 h of incubation ($\bar{x}$±SEM), with (+) or without (–) the addition of 3 mM pentoxifylline. *Indicate conditions that are statistically significantly different.

1.3% vs. 24.8 ± 2.7%, and 9.9±3.2 vs. 22.3±5.0, respectively) (Fig. 1). After treatment with PTX, both normozoospermic and asthenozoospermic samples increased their VCL and ALH, while decreasing LIN (Table 1). These changes accounted for the net increase in the percentages of hyperactivated spermatozoa (Fig. 1).

The phosphotyrosine immunolabelling was specific since neither the blocking of the first antibody with OPT nor the group performed without using the first antibody showed any fluorescent staining. As it has been described (14), three partially overlapping fluorescent patterns were identified with the immunostaining procedure. All of them shared a homogenously distributed bright label along the tail, which excluded the midpiece, differing on the head label patterns (no label, equatorial pattern or cap). To consider a spermatozoon positive with this technique, we only evaluated presence or absence of tail label. There was, however, a significant increase ($p < 0.05$) in the number of labeled sperm in test groups in relation to control groups, both in normo and asthenozoospermic samples (44.2 ± 2.6% vs. 18.2 ± 1.7%, and 32.3 ± 6.1% vs. 16.2 ± 3.4%, respectively) (Fig. 2).

Spermatozoa from normo and asthenozoospermic samples, as well as those untreated (control) or treated with PTX (test) showed similar fluorescent patterns (Fig 3).

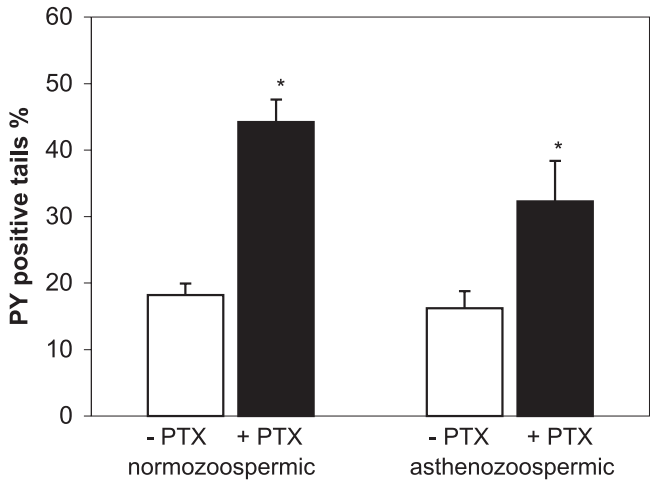

FIGURE 2. Incidence of sperm-tail phosphotyrosine-containing proteins in normozoospermic and asthenozoospermic samples after 1 h of incubation (x̄±SEM), with (+) or without (-) the addition of 3 mM pentoxifylline. * Indicate conditions that are statistically significantly different.

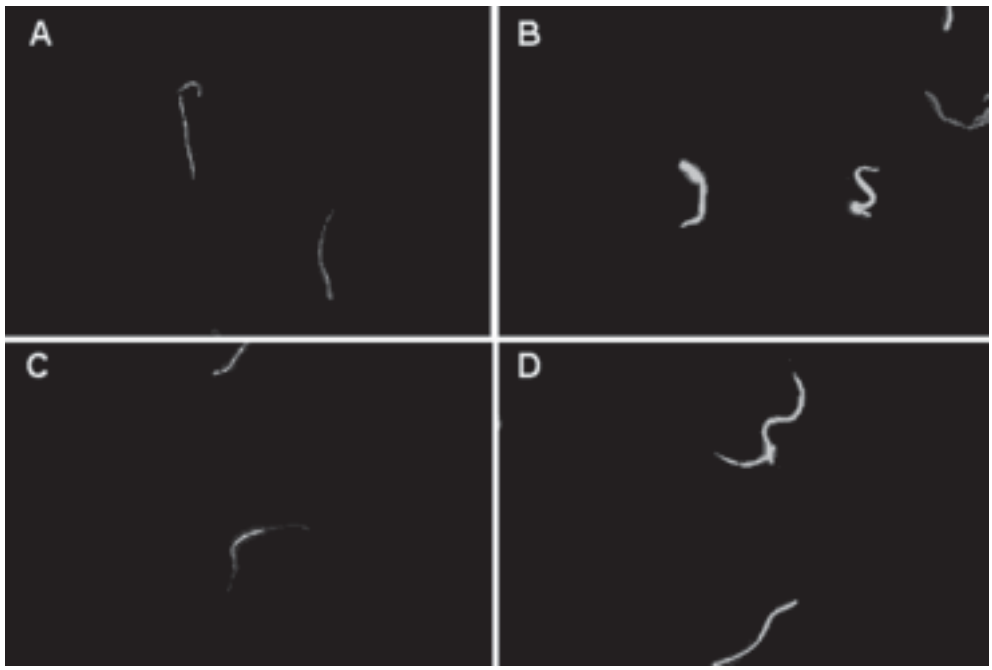

FIGURE 3. Photographs of human spermatozoa displaying tyrosine phosphorylated proteins. Normozoospermic sample untreated (A) or treated with PTX (B). Asthenozoospermic sample untreated (C) or treated with PTX (D). Magnification: X 1,000

Discussion

We reported previously that human asthenozoospermic semen samples displaying low percentages of motile sperm and altered motion characteristics showed decreased incidence of tyrosine phosphorylated sperm (Yunes *et al*., 2003). This is due to an inability of asthenozoospermic spermatozoa to respond to capacitating conditions with tyrosine phosphorylation of critical proteins having molecular masses around 80-170 kDa (Buffone *et al*., 2005). In turn, this deficiency appears to be associated to decreased membrane fluidity, which would prevent changes in the external microenvironment from triggering capacitation-associated signal transduction (Buffone *et al*, 2005).

During capacitation, sperm lose cholesterol, increasing membrane fluidity and triggering transmembrane ion fluxes (Yanagimachi, 1994; Cross, 1998). Increased intracellular levels of $NaHCO_3$ stimulate a soluble adenyl cyclase, which converts ATP into cyclic AMP (Visconti *et al*., 1999a; Luconi *et al*., 2005). Cyclic AMP stimulates serine/threonine and tyrosine phosphorylation, as well as sperm motility and acrosome reaction (Yovich *et al*., 1990; Visconti *et al*., 1995b). It is not totally clear, however, how cAMP stimulates all these events. Visconti and coworkers postulate that a cAMP-dependent kinase (PKA) is located upstream of a tyrosine kinase (PTK) and directly or indirectly stimulates sperm tyrosine-phosphorylation by activating PTK, inactivating tyrosine phosphatases, and/or activating other serine/threonine kinases (Visconti *et al*., 2002). An increase in tyrosine phosphorylation of critical proteins would lead to capacitation-associated motility changes such as hyperactivation (Visconti and Kopf, 1998). On the other hand, we have demonstrated that while PTK inhibitors such as genistein decrease capacitation-induced tyrosine phosphorylation and hypermotility in human sperm, specific PKA inhibition by H-89 had only a negligible effect (Bajpai and Doncel, 2003). H-89 did block these changes, however, when they were induced by exogenous addition of permeable analogs of cAMP. Very recently, Luconi *et al*. (2005), placing PTK activity upstream of PKA, have suggested that cAMP increases tyrosine phosphorylation of AKAP 3 which leads to increased binding/recruitment of PKA, enhanced phosphorylation efficiency, and induction of hyperactivation.

The present results confirm that increased levels of cAMP as a result of phosphodiesterase inhibition not only induce tyrosine phosphorylation and hyperactivation in normal sperm but also overcome asthenozoospermic sperm deficiency to undergo such processes. Whatever the target of cAMP is, this finding reinforces the hypothesis that the main defect in asthenozoospermic spermatozoa would be upstream of cAMP, i.e., at the soluble adenyl cyclase or phase membrane levels (Buffone *et al*., 2005).

Recently it has been demonstrated that mice deficient for adenyl cyclase (knock-out model), are infertile due to a severe sperm motility defect, which could be reversed by treating sperm with a membrane-permeable cAMP analog (Esposito *et al*., 2004). Increased plasma membrane rigidity caused by uploading of cholesterol sulfate was also shown to impede tyrosine phosphorylation and capacitation, an inhibition that could be overcome by addition of permeable cAMP analogs and a PDE inhibitor (Osheroff *et al*., 1999).

PDE inhibition in human sperm has been reported to increase acrosome reaction, motility, and fertilizing ability (Das Gupta *et al*., 1994; Nassar *et al*., 1999a,b). PDEs belong to at least 11 identified families (Soderling and Beavo, 2000). Six types of mRNA transcripts have recently been identified in human ejaculated sperm (Richter *et al*., 1999). Using selective inhibitors and specific immunolocalization, 3 isoforms, PDE 1,3, and 4, have been confirmed in mammalian sperm (Fisch *et al*, 1998; Lefievre *et al*., 2002). Both a Ca^{2+}/calmodulin-dependent PDE1 and a cAMP-dependent PDE4 are localized in the flagellum and appear to be related to motility.

PTX blocks both PDE1 and PDE4, assuring significant increase in the endogenously levels of cAMP (Ain *et al*., 1999). According to our results, PTX-reported enhancement of human sperm fertilizing ability may be due to increased tyrosine phosphorylation of critical sperm proteins, especially those related to flagellar motility. Since sperm intracellular calcium is also mobilized by PTX (Ain *et al*., 1999), we can not rule out an additional stimulatory effect of this cation on motility and hyperactivation.

Our data show that PTX enhanced sperm velocity, hyperactivated movement and protein tyrosine phosphorylation concomitantly. Furthermore, it exerted this stimulation on both normal and pathological sperm. Within this latter group, asthenozoospermic sperm were able to recover their ability to hyperactivate by phosphorylating proteins in tyrosine. PTX, however, only blocked cAMP degradation. The fact that cAMP accumulated to levels capable of inducing both hyperactivation and tyrosine phosphorylation is indicative that adenyl cyclase activity in asthenozoospermic sperm was not defective. Given the relevance of the re-

sults, and since the data from asthenozoospermic samples were obtained from five patients, further work in order to fully support this preliminary study is guaranteed.

Inhibition of sperm PDE activity and its consequent increase in cAMP and tyrosine phosphorylation may constitute a way to increase asthenozoospermic samples' motility and hyperactivation, allowing these patients to opt for less interventional and expensive assisted reproduction techniques such as intrauterine insemination and standard *in vitro* fertilization.

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