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Pathophysiology of sperm motility

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1. ABSTRACT

Mammalian spermatozoa acquire the ability to swim during their transit from the testis to the oviduct under the control of several external and intracellular factors. These factors play also a pivotal role in regulating acquisition of hyperactivated motility and during the process of chemotaxis. This review summarizes the involvement of such factors in acquisition and maintenance of sperm motility, hyperactivation and chemotaxis, focusing in particular on the molecular bases of asthenozoospermia, a pathology of seminal plasma characterized by reduced sperm motility, which is one of the main causes of male infertility. Current *in vitro* treatments of asthenozoospermia are shown, together with the most recent findings on pharmacological and physiological molecules capable of stimulating sperm motility. The structure, function and mechanism of sperm flagellum responsible for the development of active motility are also analyzed in details.

2. BIOCHEMICAL REGULATION OF SPERM MOTILITY

Upon release from the testis, human and all mammalian spermatozoa are immotile but in order to reach the oocyte and fertilize, they acquire the ability to actively swim during their transit through the epididymis and the female genital tract under the control of different external and intracellular factors (see Figure 1). All these factors are important not only for the development but also for the maintenance of sperm motility. Although these two processes seem to be regulated on the same bases, the majority of the *in vitro* studies has been focused on the maintenance of sperm motility only. In fact, ejaculated spermatozoa or epididymal spermatozoa from the cauda are currently used. In such stages of maturation spermatozoa have already started an autonomous kind of motility, whereas precocious stages, such as testicular spermatozoa, could be the best model for studying factors and events controlling the development of motility.

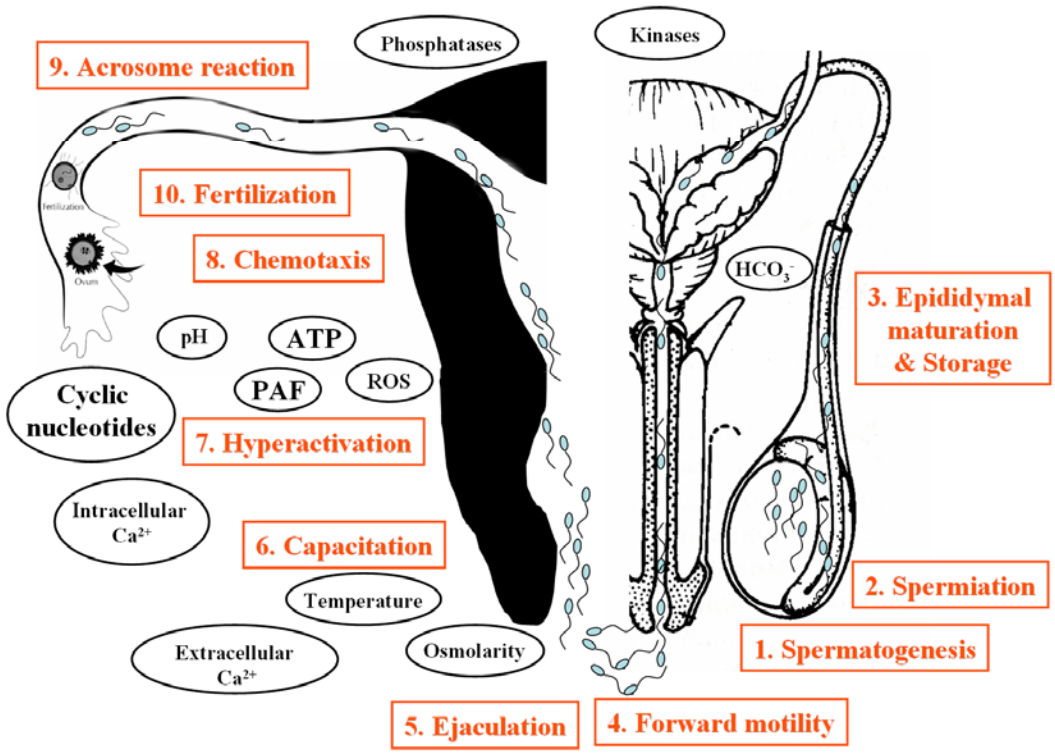

Figure 1. Factors affecting sperm motility during the "Sperm Journey" from the testis (right) to the ovary (left). External and intracellular factors affecting sperm motility are indicated (in black) together with the processes of activation (in red) that spermatozoa undergo during their "journey" from the testis to the ovary.

2.1. Calcium

In physiological conditions, calcium is one the most important ions regulating human sperm motility (1). However, the role of calcium in activating spermatozoa has been always regarded as controversial. Indeed, voltage-gated, cyclic nucleotide-gated and transient receptor potential calcium channels have been described along the plasma membrane of the entire flagellum (for rev see 2,3), thus suggesting the importance of calcium entry for motility. Transient receptor potential calcium channels have been recently demonstrated in sperm tail and are involved in stimulation of sperm motility by capacitative calcium entry (4) and knock out mice for the novel discovered CatSper calcium channel specifically expressed in the tail, are infertile due to loss of progressive motility (5). Increase in intracellular calcium levels is also indirectly implicated in activation of intracellular calcium stores via IP3 signalling (6,7). Upon its entrance, calcium may activate phospholipases and also modulate several enzyme activities. In particular, the activated calcium/calmodulin (CaM) complex has been shown to stimulate sperm motility through direct interaction with soluble adenylate cyclase (sAC) (8,9), protein kinases (10,11), phosphatases (12) and phosphodiesterases (13), finally leading to an increase in cAMP and phosphorylation of sperm proteins. CaM has been characterized in sperm axonema and proposed as the intracellular calcium sensor regulating motility (14). CaM levels are reduced in sperm from asthenozoospermic patients (15) and inhibitors of this enzyme negatively affect sperm motility (16). Among CaM target enzymes, Marin-Briggiler *et al* (10) characterized the CaM dependent protein kinase. Inhibition of the isoform IV results in a specific decrease in motility parameters and ATP levels without affecting sperm viability, protein tyrosine phosphorylation or acrosome reaction (10). Incubation of motile sperm in the absence of calcium dramatically reduces motility parameters (10), suggesting the importance of calcium in the maintenance of human sperm motility.

Although external calcium has been demonstrated to be essential for sperm motility, several evidences suggest that its intracellular concentrations must be strictly regulated to allow precise timing for sperm activation (17,18). In fact, decreasing levels of external calcium between the caput and cauda of the epididymis are associated with progressive development of sperm motility and increase in tyrosine phosphorylation of proteins (19,20). Calcium addition to demembranated human sperm suppresses motility (21), as well the increased intracellular calcium levels following cryopreservation negatively correlate with sperm motility and fertilizing ability (22). Although many papers focused on the role of calcium channels in the entry of this ion, very little is known about calcium extrusion from the cell. Recently, plasma membrane Ca^{2+}/calmodulin-dependent Ca^{2+} ATPases (PMCA) have been demonstrated to be essential for maintaining intracellular calcium homeostasis (23). Indeed, homozygous male mice with a targeted gene deletion of PMCA isoform 4, which is highly enriched in the sperm

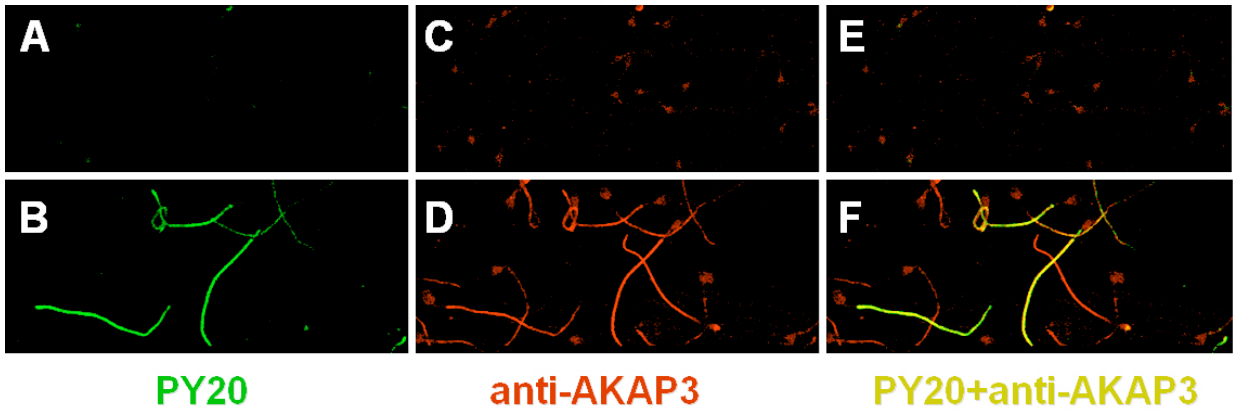

Figure 2. Immunofluorescence analysis of fixed and permeabilized human spermatozoa. Confocal microscopy of the double fluorescence for tyrosine phosphorylated proteins (PY20-HRP antibody, green) and for AKAP3 (anti-AKAP3 antibody, red) reveals positivity for both antibodies in sperm tails. Simultaneous analysis of the double fluorescence confirms that tyrosine phosphorylation mainly interests AKAP3 in the tail (double fluorescence, yellow). A,C,E: negative controls avoiding primary antibody.

tail, are infertile due to severely impaired sperm motility. Furthermore, this detrimental effect can be mimicked by inhibition of the enzyme in wild-type animals, thus supporting the hypothesis of a pivotal role of the PMCA4 on the regulation of sperm function and intracellular Ca^{2+} levels (23).

The molecular mechanisms underlying such striking stimulatory and detrimental effects of calcium on sperm motility are still obscure, although they seem to be due to activation of concurrent signaling pathways such as simultaneous activation of protein kinases and phosphatases. Indeed, calcium levels must be kept low in order to prevent activation of phosphatases such as calcineurin (18), which dephosphorylates and inactivates tail proteins involved in sperm motility (18,24). An alternative hypothesis developed by Aitken's group, suggests that keeping internal calcium homeostasis in the presence of high extracellular calcium decreases ATP availability for tyrosine phosphorylation and sperm movement (25).

2.2. Kinases and Phosphatases

Although several evidences indicate the importance of protein phosphorylation as one of the key processes in transducing the stimulatory signals to motility, little is known about the specific kinases and phosphatases involved. Generally, sperm motility has been demonstrated to be associated with increased tyrosine phosphorylation of specific protein targets in sperm tails following tyrosine and serine-threonine kinase activation, whereas it negatively associates with phosphatase activation (17,20,24,27). Tyrosine phosphorylated proteins in response to sperm capacitation are mainly localized in sperm tails (28-30). Defects in tyrosine phosphorylation in specific proteins in sperm tails in response to capacitation have been described in asthenozoospermic patients and strictly associate with reduced motility and hyperactivation in such subjects in comparison with normozoospermic and proven-fertile groups (31,32). These defects in protein activation seem to be dependent on decreased membrane fluidity found in spermatozoa from asthenozoospermic men (32). Interestingly, even in semen from normozoospermic men, different sperm subpopulations cohabit, showing different plasma membrane fluidity and ability to undergo tyrosine phosphorylation of proteins and hyperactivation in response to capacitation (33).

Sperm protein phosphorylation is regulated by a tuned balance between kinase and phosphatase activities (24,26). In particular, the adenylate cyclase/cAMP/PKA system has been demonstrated to be involved in tyrosine phosphorylation of different sperm proteins in the flagellum associated with sperm motility (18,26,28,34-40). cAMP produced by activation of adenylate cyclase binds to PKA holoenzyme inducing the release and activation of the catalytic subunit. Sperm treatments enhancing intracellular cAMP and PKA activity stimulate motility (34,41). Since protein kinase A is a serine-threonine kinase, it is conceivable that it activates some intermediate tyrosine kinases. An alternative pathway involving tyrosine kinase activation upstream to PKA has been recently reported by our and other groups (27,39,40). In fact, both inhibition of PI3K by LY294002 and physiological activation of sAC by bicarbonate (see below), stimulate an increase in intracellular cAMP levels and in tyrosine phosphorylation of the tail scaffolding protein A kinase anchoring protein 3, AKAP3. Confocal microscopy on fixed and permeabilized spermatozoa confirms that capacitation-induced tyrosine phosphorylation of sperm proteins interests mainly the tail and in particular AKAP3, since tyrosine phosphorylation co-localizes with AKAP3 positivity (Figure 2). The stimulated phosphorylation of AKAP3 results in an increased binding of PKA regulatory subunit RII beta, which is thus selectively recruited and activated in sperm tail, where it interacts locally with its targets finally resulting in an increase in sperm motility. Disruption of PKA-AKAP3 interaction results in inhibition of sperm motility (39). Sperm treatment with the PKA inhibitor H89 results in inhibition of sperm motility but not

of AKAP3 tyrosine phosphorylation (27,39), thus suggesting that PKA is involved in regulation of sperm motility downstream to tyrosine kinases. Conversely, inhibition not only of motility but also of tyrosine phosphorylation following sperm treatment with H89 has been reported by other authors, suggesting an upstream effect of PKA (37,42,43). Such discrepancy could be explained either by differences in H89 concentrations used and in the timing of H89 addition or by hypothesizing that tyrosine phosphorylation affects different targets upstream and downstream of PKA activation.

The importance of AKAP scaffolding proteins in regulating sperm motility has been recently remarked by targeted disruption of *Akap4* gene, whose product, AKAP4, is closely related to AKAP3. These mutant mice show defects in sperm flagellum and motility resulting in infertility (44). In sperm of subjects affected by dysplasia of the fibrous sheath, no alteration in AKAP3 and AKAP4 genes had been found, in particular in the PKA binding domain (45), until the appearance of a recent case report (46). Moreover, defects in the ability of such scaffolding proteins to undergo tyrosine phosphorylation, thus affecting PKA recruitment, have not been excluded.

2.3. Cell Volume and Osmolarity

During their transit and maturation through the epididymis, spermatozoa acquire the ability to regulate their cell volume, which is a very important process for fertility and correct development of motility. In fact, in the passage from the testis to the epididymis, the osmolarity of the luminal fluid increases and normal spermatozoa counteract shrinkage by increasing the uptake of organic osmolytes such as L-carnitine and aminoacids secreted by the epithelium (47). Conversely, upon ejaculation, spermatozoa are subjected to the rather hyposmotic environment of the female genital tract [osmotic pressure falls from 420 to 300 mmol/Kg passing from the epididymal cauda to the uterus (47,48)]. Thus, in order to prevent swelling, spermatozoa loose water and osmolytes acquired in the epididymis. Defects in such a tuned mechanism of volume regulation and in the epididymal osmolyte uptake cause an abnormal increase in sperm head volume and angulation of sperm tail (47), resulting in defects in sperm motility and fertility. A similar hairpin shape in sperm tail and its detrimental consequence on motility has been demonstrated also in both c-ros knock out mice and following sperm treatment with ion-channel blocker quinine (49). Interestingly, seminal plasma osmolarity (intermediate between epididymis and uterus) is significantly higher in asthenozoospermic subjects irrespective of the cause of asthenozoospermia compared to normozoospermic ones (50). Moreover, seminal osmolarity negatively correlates with sperm progressive motility and kinetic characteristics (51), thus suggesting a potential pathological role of seminal hyper-osmolarity in the reduction of sperm motility in asthenozoospermic subjects. Sperm exposure to low osmolarity media such as oviductal and uterine fluids activates an influx of Ca^{2+} through osmolarity–sensitive calcium channels (50).

The role of fluid reabsorption for sperm maturation in the apical region of epididymis has been extensively investigated (52). In particular, estrogens may control differential expression of Na^+/H^+ exchangers (53) and aquaporin channels (54) through ER alpha in the initial segment and caput of the epididymis. Aquaporin channels (AQ7) are also expressed in sperm tails and seem important for the control of the cell volume, motility and fertility (55). Thus, it is conceivable that sperm maturation in the epididymis may be modulated by active water transport at two levels: through water reasorbption by non ciliated epidydimal epithelium and at sperm plasma membrane too.

L-carnitine, which is one of the main osmolytes captured by sperm during the transit through the epididymis is essential for acyl transport in the mitochondrial beta-oxidation of long-chain fatty acids and may prevent sperm DNA and membrane damage induced by reactive oxygen species (ROS). Indeed, a positive effect of carnitine oral administration in increasing semen quality and in particular sperm forward motility in oligoasthenoteratozoospermic and asthenozoospermic patients has been reported by different trials (56,57).

2.4. Reactive Oxygen Species

Reactive oxygen species and in particular hydrogen peroxide produced either by spermatozoa and leukocytes in the seminal plasma have been described to affect different sperm functions including motility (58). Their effects seem to be dual depending on the concentration of ROS: low levels can induce the cAMP-PKA signaling cascade leading to an increase in sperm motility and tyrosine phosphorylation of proteins stimulating capacitation while high levels exert an inhibitory role (59,60). The detrimental effect of ROS on sperm motility has been associated with an increased lipid peroxidation in plasma membrane (61). High production of ROS may account for some types of asthenozoospermia as well as defects in the cell systems devoted to defence against ROS; in such cases the potential *in vitro* use of antioxidants to keep ROS at low levels in order to preserve sperm motility may be indicated (62,63). However, levels of glutathione-dependent seleno-enzymes in human spermatozoa, which are responsible for a generalized protection against reactive oxygen species, have been reported to be similar in spermatozoa purified from both normozoospermic and asthenozoospermic subjects (64).

2.5. Immunological Factors

Sperm motility is also affected by immunological factors: high levels of cytokines such as tumor necrosis factor alpha, interleukin 6 and interleukin 1 beta in seminal plasma of men with spinal cord injuries have been reported to correlate with low sperm motility in such patients (65). Inactivation of such factors by *in vitro* semen treatment with monoclonal antibodies results in a significant improvement of motility.

3. THE PROPULSION MECHANISM IN THE TAIL

Sperm swimming is characterized by a rhythmic three-dimensional and asymmetric movement of the flagellum. This peculiar movement is assured by the complex organization of the flagellum (Figure 3). With the exception of the distal part (end piece) containing the

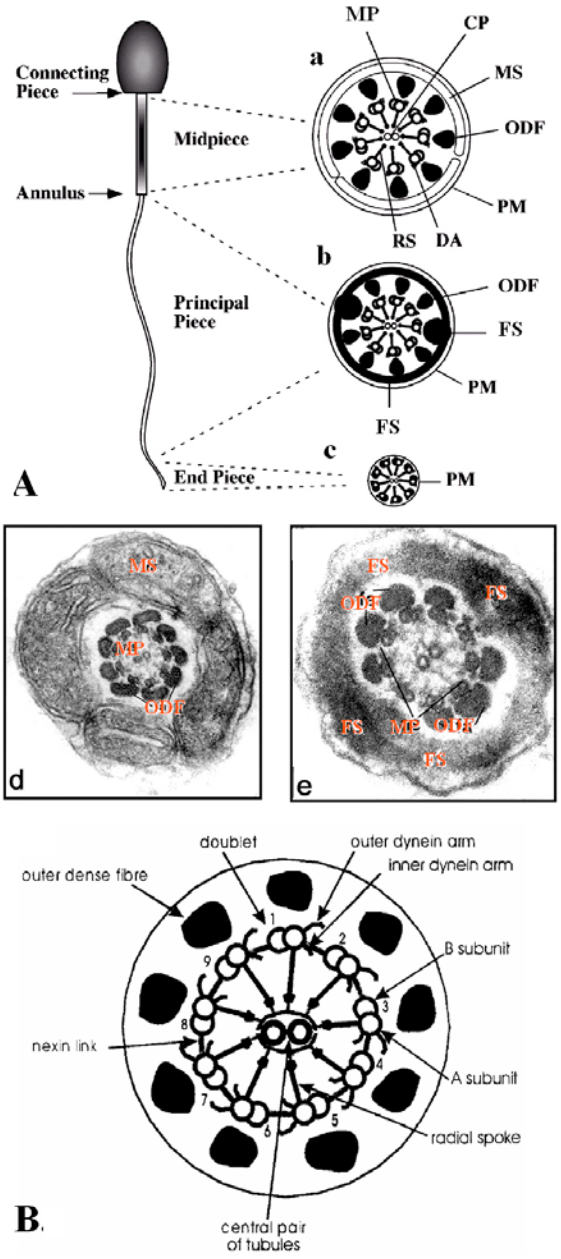

Figure 3. Schematic representation of a human sperm. A. Longitudinal section showing head, middle piece, principal piece and end piece. The insets on the right show the cytoskeletal organization of the sperm tail in transverse sections at different levels: middle (a), principal (b) and end piece (c). Electron microscopy of the transverse sections of the sperm tail at the level of the middle (d) and the principal piece (e) is reported in the lower part of the figure. B. Organization of the axonema: CP: central pair; MS: mitochondria; ODF: outer dense fibers; PM: plasma membrane; DA: dynein arms; RS: radial spoke; MP: microtubule pairs; FS: fibrous sheath. Modified with permission from Mortimer, S.T., 2000 (125).

central couple of microtubles only, the entire flagellum is organized in a peculiar cylindrical structure called axonema consisting of 9 couple of tubulin A and B microtubules connected each other by nexin arms and to the central one by radial spokes. Each microtubule doublet is externally anchored to 9 corresponding asymmetric outer dense fibers (ODF), which are surrounded by the fibrous sheath in the principal piece and by packed mitochondria in the middle piece of the flagellum (Fig.3). The base of the flagellum is thickened by the connecting piece consisting of 9 segmented columns which distally fuse with the corresponding ODFs (66) and is responsible for transmission of tail movement to the head. The reciprocal sliding of each couple of microtubules originating from the sequential anchoring of the dynein arms on the neighbor couple and ATP-dependent generation of sliding force finally result in bends of alternating direction and propagation of the oscillation along the tail (Figure 4). The asymmetry of the axonemal structure together with microtubule connection with the central couple and the anchoring to their corresponding ODFs-fibrous sheath complex confers an helical shape to the propagating flagellar beat. ODFs are essential for the development of forward motility in the mature sperm and their structure and number have been highly conserved through the evolution. In particular, their cross section area positively correlates with the length of the flagellum (67). Oscillations can originate in different regions of the flagellum, however the beat frequency seems to be controlled by the basal region, which acts as a sort of pacemaker. Although different models have been hypothesized, the mechanism underlying the initiation of a new bend at the flagellar base, involving the change in the sliding direction of the microtubule couple, is still obscure (68). A recent paper on knock out mice for the functional dynein heavy chain has demonstrated the importance of dynein inner arm heavy chain for development of sperm motility (69). In fact, KO mice for dynein inner arm heavy chain gene show asthenozoospermic conditions, with the majority of spermatozoa unable to achieve forward progressive motility. In such spermatozoa, the outer dense fibres retain attachments to the inner surface of the mitochondria. These links are essential in normal spermatozoa for midpiece development and are broken when spermatozoa acquire the ability to swim upon release from the epididymis. Conversely, disruption of dynein inner arm heavy chains in KO mice determines an insufficient force to overcome these bridges resulting in spermatozoa unable of normal tail bending.

Energy to support such a process is represented by ATP which is hydrolyzed by dynein ATPase to generate the sliding force in the microtubles. Although the main source for ATP has been long considered the oxidative phosphorylation in the midpiece mitochondria, more recently also a local production of energy through an alternative glycolitic pathway has been characterized in sperm principal piece. In fact, though reduced, motility is still present when oxidative phosphorylation is uncoupled in sperm mitochondria (70). Moreover, these two metabolic processes are strictly compartmentalized in the middle and principal piece of sperm flagellum, thus, although oxidative phosphorylation is more efficient then glycolysis to produce ATP, it is hard to think that ATP diffusion from

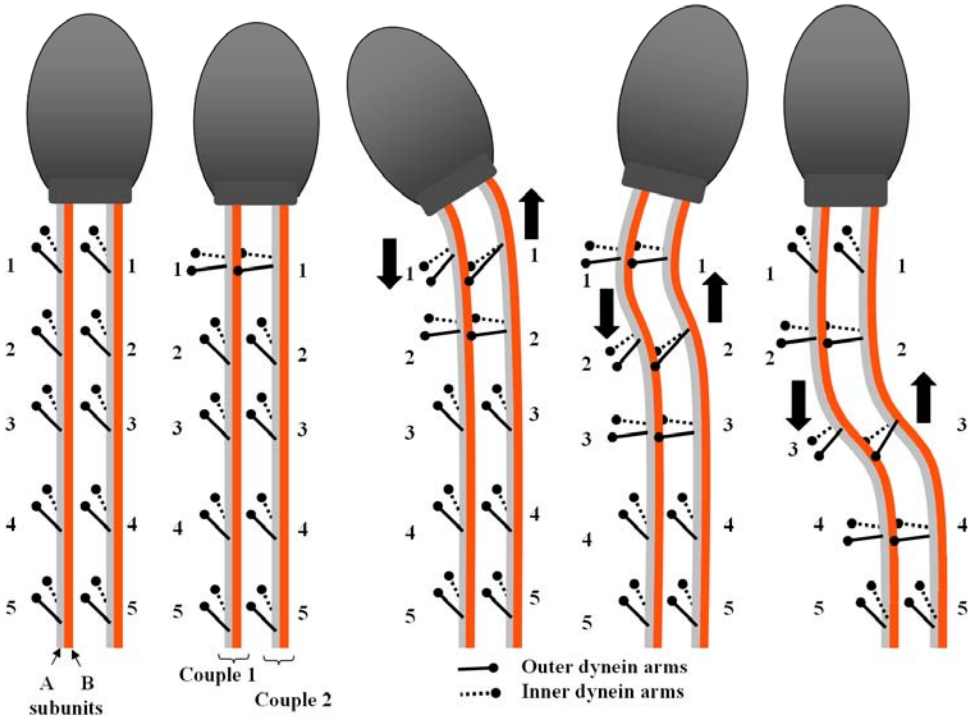

Figure 4. Mechanism of sperm tail movement based on the reciprocal sliding of two adjacent couples of microtubules. Longitudinal section of human spermatozoon. Two adjacent couples of microtubules (grey and red) are shown. The bending of the flagellum is due to the subsequent cycles of dynein arm attachment, generation of forces and detachment on the B subunit of the adjacent microtubule couple.

the former to the latter compartment could supply enough energy to support flagellar movement in the distal region of the flagellum. Miki *et al* (71) elegantly demonstrated that the sperm-specific glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS, and its human ortholog GAPD2) is necessary for sperm motility and fertility, since sperm from the knock out mice Gapds-/-, in which oxidative phosphorylation was unaffected, generate only 10.4% of ATP produced in wild type controls. Moreover, sperm motility was impaired with virtually absence of forward movement and the mice were infertile (71). Thus, glycolysis seems to be the pivotal metabolism supporting energy for sperm motility as also suggested by the presence of sperm specific isoforms of other glycolitic enzyme such as hexokinase and LDH, which are selectively expressed in sperm principal piece (72).

4. GENETIC BASES OF ASTHENOZOOSPERMIA

Any alteration in external and internal factors regulating sperm motion and in cellular structure and metabolisms involved in generating tail beat may result in defects in sperm motility and infertility. A recent study pointed out that out of 1,085 sperm samples analyzed from infertile subjects, 81% were defective in motility, being about 20% pure asthenozoospermic (73). Thus, asthenozoospermia, is one of the main male seminal pathologies underlying infertility.

In the mouse, a strict association between mutations in some genes and alteration in sperm motility has been pointed out, based on knocking out, silencing and mutagenesis technologies. Conversely, in humans such relations are more difficult to assess and define. However, in some few syndromes, involving not only defects in sperm motility, mutations in specific genes correlated to sperm motility have been identified.

Immotile cilia syndrome, also known as primary ciliary dyskinesia (PCD), is a phenotypically and genetically heterogeneous congenital disorder. The primary defect is the absence of dynein arm proteins in cilia and sperm flagella. The clinical features of PCD include recurrent sinopulmonary infections, male infertility due to absence of sperm progressive motility and laterality defects, showing situs inversus in 50% of the cases. To date, up to three genetic mutations related to dynein gene have already been identified (74,75).

Fibrous sheath dysplasia affects different cytoskeletal components of sperm tails, which appear short thickened and irregular. Abnormalities of the fibrous sheath is the common feature in all affected spermatozoa and seem to arise during sperm development rather then being due to a degeneration process. The familial clustering of the abnormal phenotype strongly suggests a genetic component of the disease (76).

Table 1. Effect of LY294002 on the number of motile spermatozoa recovered following swim up procedure in 22 severe oligoasthenozoospermic subjects.

	Control	LY294002 (10 μM)	Percentage increase over Control
Million sperm/ml	5.3±1.8	9.0±2.6[1]	+70.4%

[1] $p<0.01$ vs Control, t test for paired data. The percentage increase over Control is also indicated.

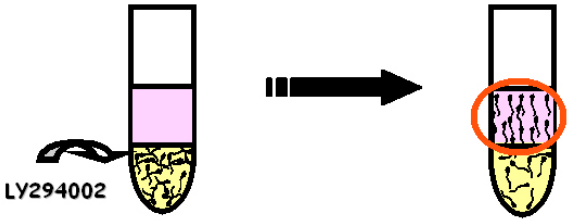

Figure 5. A schematic representation of the swim up procedure following LY294002 addition to ejaculated sperm is shown below. Sperm recovery is indicated in the red circle.

Together with genetic analysis, ultrastructural evaluation of spermatozoa by electron microscopy (76) may represent an useful diagnostic and prognostic tool in severe asthenozoospermia, since it helps to identify the ultrastructural tail defect giving the basis for hypothesizing the possible candidate gene.

5. *IN VITRO* TREATMENT OF ASTHENOZOOSPERMIA

To date a really efficient therapy is not available for asthenozoospermia, but only *in vitro* treatment of spermatozoa to improve selection for assisted reproductive technologies (ART). Thus, patients are not cured but only given a better chance to conceive in that specific session of ART.

Some molecules have been demonstrated to be capable of improving *in vitro* sperm motility, however none of them can be efficiently used in *in vitro* treatment of asthenozoospermia, as many show also detrimental effects. Among such factors, inhibitors of phosphodiesterases such as pentoxifylline (PF), analogs of cAMP and a plasma membrane phospholipid, the platelet-activating factor, which is physiologically produced and released by sperm themselves (77), have been the most studied. In particular, PF is often used in ART to improve fertilization rate and outcome in couples with male factor (78,79), since this compound not only stimulates motility in sperm obtained from asthenozoospermic subjects, but also positively affects sperm capacitation, binding to ZP and AR (80). Sperm treatment with PF before IVF has been demonstrated not to be teratogenic for the developing embryo (79), however potential toxic effects cannot be definitively ruled out (81). Moreover, the presence of non responder subjects decreases the efficacy of the treatment (82). But the most striking negative side effect exerted by the large part of these compounds including PF is their ability to stimulate also acrosome reaction (83). Unfortunately, acrosome reacted spermatozoa are unable to bind the oocyte zona pellucida, thus if acrosome reaction is induced prematurely before the contact with the oocyte, the spermatozoa are no longer able to fertilize in IVF protocols, but can be only used for ICSI.

In this scenario, during the past few years our research has been focused on two molecules which seem very quite good candidates for a potential *in vitro* therapy of asthenozoospermia. LY294002 is a pharmacological inhibitor of phosphatidyl inositol 3 kinase (PI3K), a kinase which phosphorylates in 3 OH position the inositol ring of plasma membrane phosphoinositides (84). This enzyme has been demonstrated to play a negative role in the control of sperm motility (39,85-87) and its inhibition by LY294002 stimulates a significant increase in forward and rapid motility in both ejaculated and selected human spermatozoa, independently from the technique used for selection (1,39,86). This stimulatory effect was more evident on samples from oligo-asthenozoospermic compared to normozoospermic subjects (1,85,86). In particular, direct addition of LY294002 to seminal samples of severe asthenozoospermic subjects increases the number of sperm showing forward motility recovered in swim up selection for ART (Table 1, Figure 5, 86). PI3K inhibition by LY294002 stimulates tyrosine phosphorylation of AKAP3 in the fibrous sheath of sperm tails, allowing a local recruitment and activation of PKA by increased binding of PKA regulatory subunit RIIβ to the phosphorylated form of AKAP3 (39,87). PKA activation finally results in stimulation of sperm motility and hyperactivation (39). Interestingly, at difference with the above mentioned molecules studied, LY294002 effects on sperm motility are not associated with an increase in acrosome reaction (85). Moreover, no toxic effect on embryo development has been demonstrated following either sperm, oocyte and embryo treatment with LY294002 in a mouse model (88). All these findings support the possible use of this drug as well as other PI3K inhibitors as a potential tool to improve sperm motility in ART.

Besides the use of this pharmacological tool, our group also focused attention on a physiological stimulus of sperm motility, the bicarbonate ion (HCO_3^-). Indeed, bicarbonate has been long demonstrated to improve sperm motility both *in vitro* and *in vivo* in different species (89-92). The importance of this factor in regulating sperm activation *in vivo* is also suggested by the millimolar increasing gradient of HCO_3^- that spermatozoa have to experience during their journey from the testis to the site of fertilization. The increased level of HCO_3^- in seminal plasma compared to

the epididymal fluid may allow motility to develop in the ejaculate. Okamura *et al.* (93) showed a positive correlation between lower levels of HCO_3^- in the semen of infertile men and poor sperm motility. Yet, in the male reproductive fluids, HCO_3^- levels must be kept low in order to prevent spermatozoa from undergoing premature activation and developing hyperactivated motility, processes stimulated by the 3-4 higher HCO_3^- concentrations present in the female reproductive tract (94).

The molecular mechanisms by which HCO_3^- stimulates sperm motility involve a direct activation of sperm sAC, independently of intracellular pH (95). sAC, which is insensitive to forskolin and G-protein regulation, and is selectively activated by HCO_3^- (96-98), appears to be the main AC present in mature spermatozoa, although also different isoforms of the membrane adenylate cyclase (mAC) have been described (99-101). In somatic cells, the precise compartmentalization of sAC in distinct subcellular microdomains provides the mechanism for localized cAMP rise to specifically activate PKA in different cellular compartments (102,103). In fact, unlike mAC, sAC could diffuse and generate cAMP at the site where its target enzyme, PKA, is localized (104). Although sAC activity seems to be predominantly associated with the sperm particulate fraction (105), a similar mechanism could be hypothesized to be acting also in sperm.

Mouse defective for sAC are infertile apparently due to impairment of sperm motility (106). Interestingly, motility can be restored in sAC KO mice by cAMP administration (106), whereas such a treatment does not revert either hyperactivation and tyrosine phosphorylation defects as well as sperm inability to fertilize, suggesting that sAC is necessary also for a correct spermatogenesis and/or epididymal maturation (107). Moreover, by sperm treatment with a specific inhibitor of sAC, KH7, the same authors were able to distinguish between sAC-dependent and -independent processes during capacitation in mouse sperm, showing that tyrosine phosphorylation of protein as well as sperm motility and hyperactivation are regulated by sAC, while acrosome reaction is not (107). However, the role played by mAC in controlling sperm motility cannot be ruled out. In fact, selective knock out for membrane olfactory adenylate cyclase 3 is associated with male infertility due to sperm inability to penetrate zona pellucida, since such sperm show a significant reduction in both motility and acrosome reaction (108).

We have recently demonstrated that in swim up selected human spermatozoa physiological concentrations of bicarbonate (15 and 75 mM) rapidly stimulate an increase in intracellular cAMP levels and tyrosine phosphorylation of AKAP3, the latter phenomenon resulting in an increased amount of PKA bound to this scaffolding protein, in a manner resembling LY294002 effects (39,40). The stimulatory effects of bicarbonate on both sperm motility and AKAP3 phosphorylation seem to involve the ion entry into the cell and activation of sAC respectively since they are inhibited by 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid specific blocker of bicarbonate transporter and by 2OH-estradiol, a selective inhibitor of sAC (40). Thus, our findings strongly suggest that both HCO_3^- and LY294002 increase sperm motility by converging on the same signaling pathway involving stimulation of cAMP production by sAC and tyrosine phosphorylation of AKAP3 in sperm tail. Redundancy of the signalling pathways leading to AKAP3 phosphorylation further remarks the importance of this process in regulating sperm motility. Molecules acting in promoting such phosphorylation could be potentially used for increasing the number of motile spermatozoa selected for ART, offering infertile couples better chances to enter less invasive ART programs such as IUI and IVF versus ICSI.

6. HYPERACTIVATION

Hyperactivation is a peculiar pattern of motility developed by sperm in association with the process of capacitation in the female tract. However, it can be achieved also *in vitro* by seminal plasma removal and incubation of sperm in capacitating media (109). It is characterized by a more energic and less symmetric beat of sperm flagellum, which help sperm progression in the female oviduct through the high visco-elasticity of the cervical mucus (110), as well the mechanical penetration of the cumulus oophorus and the zona pellucida surrounding the oocyte (111,112). Moreover, in species in which the oviductal isthmus represents a reservoir of spermatozoa, this peculiar swimming pattern seems to be important for sperm release from trapping in the folds and crypts of the oviductal epithelium (112). In fact, ovulation induces a modification of the carbohydrate moiety of oviductal epithelium resulting in the release of fully activated sperm which have developed hyperactivation, ensuring a precise timing for sperm fertilization potential (113).

The importance of the correct timing of hyperactivation has been demonstrated by infertility of *t* haplotype mice, whose spermatozoa undergo premature hyperactivation in the female reproductive tract (114). However, motility and hyperactivation seem to be discontinuous and reversible processes allowing sperm to switch alternatively from one pattern to the other (115). Capacitation and hyperactivation are two complementary aspects of sperm activation and develop simultaneously in physiological conditions. In particular, when considering capacitation as the complex of physiological changes enabling sperm to fertilize (109), hyperactivation should be considered as a part of such a process. However, they occur as independent processes as demonstrated by *t* haplotype mice, showing premature hyperactivation but normal timing of capacitation *in vitro*. The precise timing for development of hyperactivation is orchestrated by ovulation. Indeed, the follicular fluid has been demonstrated to have a stimulatory effect on sperm hyperactivation in a dose dependent manner (116,117), although the specific component capable of directly affecting sperm motility has not yet been isolated (83,118). Although sharing similar signalling pathways of activation, capacitation and hyperactivation are distinct processes since they show different thresholds for activating factors such as calcium. Indeed, calcium and bicarbonate concentrations required for hyperactivation are far higher than those needed for capacitation (7,111).

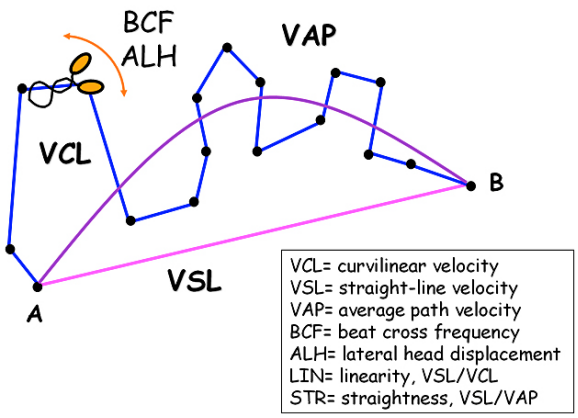

Figure 6. A schematic representation of the swim up procedure following LY294002 addition to ejaculated sperm is shown.

The molecular bases underlying hyperactivation have been investigated by different authors by the use of a demembranated sperm model, in which both plasma and mitochondrial membranes were removed by Triton X 100, leaving the axonemal structure intact and functional (111). Development of hyperactivated and activated motility shares the same signalling pathways and molecular actors, although different activation thresholds are involved. In particular, although ATP and cAMP are able to stimulate motility of demembranated spermatozoa, it is only following addition of calcium that hyperactivation begins (119), suggesting that this ion is the key regulator of the process (111).

Both external calcium and intracellular stores are important for the increase in intracellular calcium levels associated with hyperactivation. Intracellular calcium stores showing inositol 1,4,5-trisphosphate receptors (IP3R) have been demonstrated not only in the acrosome (6) but also in the neck (7). In particular, in the distal region of sperm neck, the axoneme associates with mitochondria and is surrounded by a redundant nuclear envelope, whose enlarged cisternae represent the flagellum intracellular calcium stores (7). The release of calcium from these structures through IP3-gated channels seems to directly initiate sperm hyperactivation (111,120). Moreover, hyperactivation is also modulated by calcium entry through plasma membrane specific channels such as voltage-gated, receptor-associated, store-operated, and cyclic nucleotide-gated channels (for review see 2,3). A recently discovered family of sperm specific voltage-operated calcium channels, the CatSper family, plays a pivotal role in the development and maintenance of sperm motility. The four members of the family are differentially expressed along the tail and while CatSper1 seems to regulate sperm activated motility (121), CatSper2 is important for hyperactivation, since knock out mice are infertile due to their inability to develop hyperactivation and thus penetrate zona pellucida but show normal capacitation, motility and acrosome reaction (122). Interestingly, male infertility in a mutant CatSper2 family has been recently described (123).

Similar to activated motility, also hyperactivation is regulated by a complex balance between kinase/phosphatase activity. Increased tyrosine phosphorylation of several sperm proteins in the tail has been described to be associated with physiological (36) and temperature-induced hyperactivation (124). Calmoduline kinase II is one of the few discovered calcium targets in spermatozoa. Upon its activation by the calcium/calmodulin complex it specifically stimulates hyperactivation, since sperm motility was not affected by an inhibitor of the enzyme, KN-93 (11).

7. COMPUTER ASSISTED SPERM ANALYSIS (CASA)

The hyperactivation state of a sperm population can be described by measuring through computer aided sperm analysis (CASA) some kinematic parameters associated to this peculiar pattern of motility. The sensitivity and confidence of these instruments have greatly improved in the last few years and can be now referred as potent research and clinical tools to measure both seminal conventional and hyperactivation parameters (125). Essentially, CASA allows simultaneous evaluation of kinematic values for a high number of spermatozoa in the sample covering the velocity, the width of the sperm head's trajectory and frequency of the change in direction of the sperm head (125). All parameters are measured by CASA on the sperm head instead of on the flagellum, as head movement passively reflects the flagellar beat and can be easily followed due to its lower frequency compared to the tail. The velocity values are based on curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP). VCL is referred to the real distance that the sperm head covers during the observation time, the VAP to the distance the sperm covers in the average direction of movement and the VSL to the straight-line distance between the starting and the ending points of the sperm trajectory (Figure 6). More strictly associated to sperm head characteristics, lateral head displacement (ALH) and beat cross frequency (BCF) measure respectively the width of the lateral movement and the number of times the sperm head crosses the direction of the movement. The instrument can also derived from the obtained data the sort fraction (SF), which represents the percentage of spermatozoa in the sample showing hyperactivation. The criteria for detection hyperactivation can be manually set and have been defined as VCL>150μm/s, ALH>7.0 μm, linearity LIN<50% (126) for human sperm.

Besides the undoubtful importance for research studies, the power of CASA is recently emerging also for the clinic. Indeed, understanding of the pathophysiological implications of sperm motility, allows us to make possible predictions on the chances of a man's sperm sample to penetrate the partner's cervical mucus and oocyte investments, providing essential data for choosing the best ART for the couple (125)

8. SPERM CHEMOTAXIS

Spermatozoa from invertebrates to mammals have been demonstrated to be attracted by chemoattractants secreted by the egg. This mechanism plays a pivotal role in

guiding sperm towards the oocyte, being particular important for those species characterized by external fertilization. By binding to sperm specific receptors, these molecules affect sperm motility inducing a directed movement towards the chemical gradient of the chemoattractant (chemotaxis) associated with increased flagellar bend asymmetry (hyperactivation). In sea urchin, speract secreted by the eggs induces, in a species-specific manner, a chemotactic response in the sperm by stimulating a transmembrane guanilate cyclase receptor complex associated to K+ channels prevalently localized along the flagellum, finally resulting in an increase in intracellular cAMP and calcium (127,128).

In human sperm, induction of chemotaxis by follicular fluid (FF) has been extensively demonstrated *in vitro* (129). Progesterone (130) and chemokines such as RANTES (131) have been suggested to be the active components of FF involved in sperm chemotaxis, even if the major effect of the steroid seems to be directed on sperm hyperactivation rather that on chemotaxis (132). Moreover, odorant-like molecules through their specific olfactory receptors expressed on human spermatozoa induce a membrane adenylate cyclase-dependent increase in intracellular calcium, finally resulting in redirection of sperm along the ascending gradient of the odorant (133,134). Sperm chemoattractants are secreted not only by the pre-ovulatory follicle but, as recently demonstrated, also by the mature oocyte and the surrounding cumulus (135), thus contributing to guide sperm to the site of fertilization. However, the physiological role of chemotaxis in human spermatozoa is still controversial. Rather than being important to guide sperm toward the oocyte, in humans chemotaxis seems more likely to recruit a selected activated subpopulation of spermatozoa (136,137).

9. CONCLUSIONS AND FUTURE DIRECTIONS

Much has still to be disclosed concerning the biochemical pathways that regulate and maintain sperm motility. In particular it is still unclear how spermatozoa begin to move following their release from the testis to the epididymis and which are the signals necessary for such activation. The identification of molecules involved in the process of motility appears, however, to be very hard. Genetic studies in mice show that many genes are involved in development and maintenance of sperm motility. Many of these genes are testis specific belonging to the fibrous sheet of the principal piece of the tail. Clarifying the molecular mechanisms involved in the onset of this important and vital sperm function may be of a great help for the development of possible therapeutic strategies. Indeed, although some non specific therapies (such as the use of carnitine or ROS scavengers) have been proven to be of a certain efficacy, at the moment, as mentioned above, *in vitro* treatments remain the main option for treatment of asthenozoospermia. At the same time, identification of key molecules and enzymes regulating sperm motility might be useful for male contraceptive aims.

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