[Frontiers in Bioscience 16, 1344-1363, January 1, 2011]

Markers of human sperm functions in the icsi era

Monica Muratori, Sara Marchiani, Lara Tamburrino, Gianni Forti, Michaela Luconi, Elisabetta Baldi

Department of Clinical Physiopathology, Andrology and Endocrinology Units, Center of Excellence "DeNothe", University of Florence, Italy

TABLE OF CONTENTS

1. ABSTRACT

The process of fertilization is crucial for species development and maintenance. Due to social and environmental problems, the number of infertile couples is increasing worldwide. Male and female factors contribute equally, and about 7% of men experiences problems in conceiving a child due to sperm defects. Assisted reproduction techniques (ARTs), including the most invasive intracytoplasmic sperm injection (ICSI), are the only available therapy for severe male factor infertility. Whether such techniques are associated with increased birth defects is still debated, and search for alternative options should go on. A better understanding of the molecular mechanisms involved in the process of fertilization may lead to the development of new pharmacological strategies to treat infertile men and new male contraceptive agents. In addition, in view of the low predictive power of routine semen analysis, new tests aimed to better predict the fertilization potential could be developed. The present review smmarizes current evidence of the molecular mechanisms involved in fertilization in human spermatozoa, with particular emphasis on the main post-ejaculatory maturation events, i.e. sperm capacitation and motility.

2. INTRODUCTORY REMARKS

2.1. Markers of sperm functions: is there still a role in the era of ICSI?

Couple infertility affects approximately 15% of couples and the so-called "male factor" is either the main determinant or a con-cause of infertility in approximately 50% of cases suggesting that prevalence of infertility/subfertility in men is around 7% (1), clearly exceeding that of diabetes mellitus. Male infertility is generally due to defects in spermatogenesis, mainly caused by factors acting directly at the testicular level. The etiology of testicular (reproductive) failure remains unknown in about 50% of the cases and is referred to as "idiopathic infertility". A genetic origin is likely responsible for many cases of "idiopathic" testicular failure since the number of genes involved in human spermatogenesis is expected to be over thousand. Pharmacological treatment of the male partner can only be successfully applied to non idiopathic causes (such as hypogonadropic hypogonadisms), whereas for idiopathic infertility, despite many attempts, virtually no treatment is available (1). For this reason an increasing number of couples choose Assisted Reproductive Technologies

(ARTs) to facilitate sperm/egg interaction. It is estimated that approximately 2-3% of children in industrialized countries are born following an ART procedure, with a peak of 4% in Denmark (2). Among the different ART procedures, *in vitro* fertilization of the egg and Intracytoplasmic Sperm Injection (ICSI) are those more used in case of severe male factor. ICSI consists in forced injection of a sperm into the oocyte by use of a micromanipulator. Whereas from one hand ICSI provides a new hope for couples with severe male factor, from the other hand it has dramatically changed the approach of the clinician to the infertile/subfertile male. Indeed, for many clinicians, all the efforts now are going on finding a single spermatozoon which could be available for ICSI procedure (1). Whether serious birth defects are increased in ART babies is still object of debate (3-4), despite more than 30 years passed from description of the first IVF-ET procedure and 17 from first ICSI. If birth defects after ICSI are present they are more likely due to unknown genetic/functional problems (of unknown aetiology) of the gametes used for the procedure rather than to the procedure itself. Genetic or epigenetic defects in the spermatozoa used for ICSI could impact on the health and wellbeing of the offspring in several way, not just simply by perpetuation of male infertility, and even if a phenotypic change is not present in the first generation it may appear in the future ones. It has been suggested that, to decrease the risk, invasive procedures such as ICSI should be avoided when not needed (for instance in the absence of male fertility problems). For all these reasons, the identification of molecular markers of the different sperm functions necessary for the achievement of oocyte fertilization remains a major goal of research in sperm biology even in the ICSI era. Pharmacological intervention to treat male infertility with non-ART based therapies, diagnosis of idiopathic male infertility and development of male contraception, are the three main areas of research which may benefit from the identification/characterization of molecular targets on sperm. As new and highly sophisticated tools are now available for the scientific community, a rapid progress in this field is expected.

2.2. The new reference values of the WHO guidelines: which future for routine semen analysis?

Routine semen analysis provides, among others, information about the number, motility and morphology of the ejaculated spermatozoa. It still represents the most valuable investigation in the diagnosis of male infertility and in the clinical approach to the infertile couple. The 5th edition of the World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen (5) provides new methodological advices as well as new reference intervals for values of semen parameters based on a recent study on fertile men population (6). Respect to those reported in the previous edition of the manual (7), these new reference values may represent an important improvement, as previous ones were based on reference values of different laboratories (likely obtained by using different analytical procedures) and no data about fertility of the included subjects were available at that time. In the work by Cooper *et al*, the study population consists of 4500 men from 4 continents: among them only those (n=1953, mean age 31$\pm$5) who had a time-to-pregnancy below 12 months have been chosen to define the reference values. The new reference values are far below those indicated in the previous edition. As an example, considering the 5% percentile of the distribution, total sperm motility to define a normozoospermic man falls from 50 to 32% and percentage of normal morphology from 15% to 4%. It must be considered that reference values reported in the paper by Cooper *et al* (6), are based only on male partners of couples with time-to-pregnancy below 12 months, without taking into account the female fertility status. In addition, the data derive from 4 studies obtained in different laboratories and not all the semen parameters are available form all the laboratories with a decrease of number of subjects on which some of the cutoff value are based on. Finally, no data are available about how much time has passed from the last conceivement of the so called “fertile” individuals included in the study.

In any case, these new reference values have the potential to greatly affect the way the physician will approach the infertile couple. Indeed, whereas from one hand these new values may reflect the trend to a decline of semen quality evidenced in some studies (8), as well as the general improvement of the techniques to analyse semen quality, from the other they seem to decrease the overall predictive value (by the way very weak) of semen analysis for male fertility potential, becoming, for the physician, only an overall indication of the fertility status. Moreover, as evidenced by Skakkebaek (9) in a recent editorial, lower cutoff values may lead to inappropriate andrological help for subfertile men. It appears evident the need for additional tests complementary to semen analysis (10) for those couples who are searching for answers to their infertility problems and not a simple solution such as finding a single spermatozoon (whatever is the provenience) for ICSI. Such tests should be more predictive of ART outcome, being based on molecular markers of post-ejaculatory sperm functions, which are necessary for completion of the process of fertilization, or on assessment of nuclear/chromatin status (such as determination of sperm DNA fragmentation, or occurrence of aneuploidies).

3. MOLECULAR MARKERS OF POST-EJACULATORY MATURATION EVENTS

The process of fertilization allows two terminally-differentiated cells (a sperm and an egg) to form a zygote, a totipotent cell which may give rise to a new individual. Before and during fertilization, many different steps (Figure 1) need to be accomplished by the two individual gametes, and if even one of these steps is not achieved, a failure in the process occurs.

Concerning spermatozoa, these steps are activated by process including post-translational modifications of proteins such as phosphorylation and glycosilation as the cell cannot respond to stimuli by modulation of protein synthesis. Indeed, in order to preserve the integrity of its genoma, which at fertilization has to be delivered to the

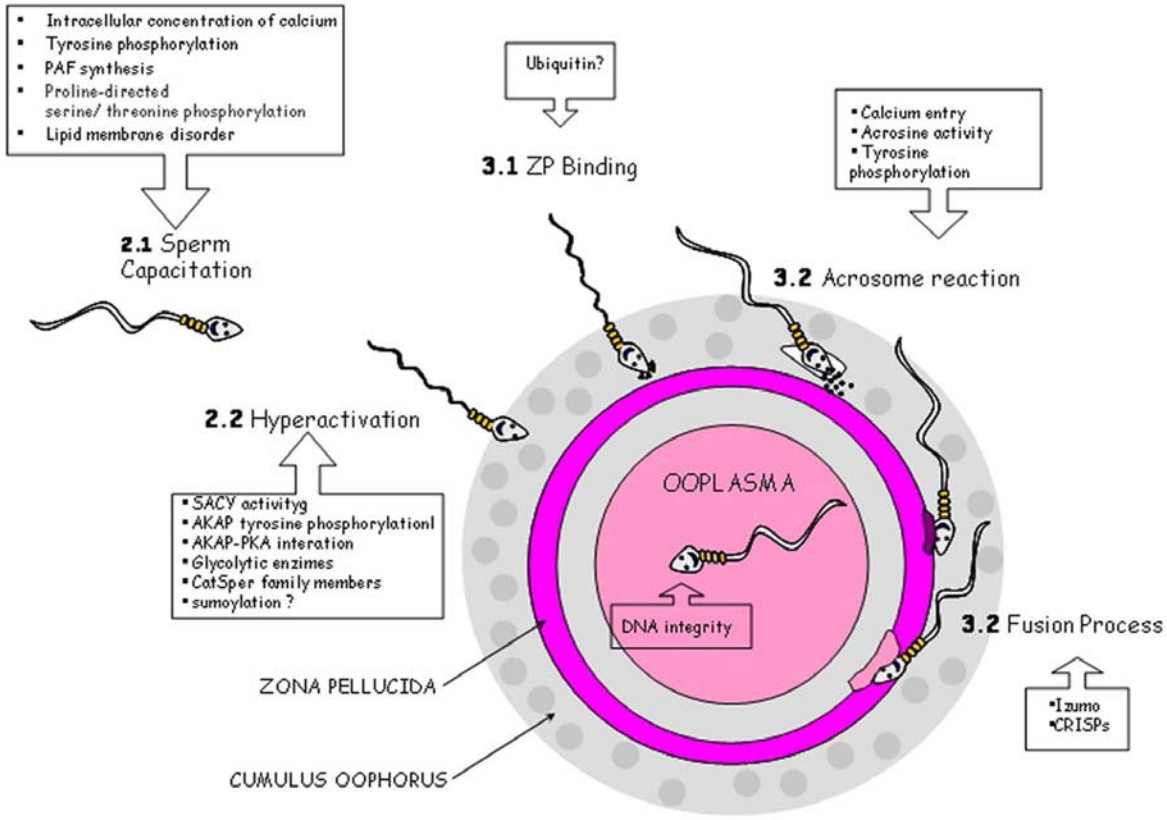

Figure 1. Schematic representation of the main steps of the fertilization process. Sperm capacitation and development of hyperactivated motility occur both at various levels of the female genital tract and are needed both to cross the oocyte cumulus and zona pellucida. Physiological acrosome reaction occurs following the contact with the zona. In the boxes, the possible molecular markers for each step are indicated.

oocyte, the spermatozoon has a high degree of DNA condensation resulting in no occurrence of transcription and translation processes in the mature gamete.

To accomplish the fertilization process, spermatozoa need progressive motility to "travel" in the female genital tract to reach the oocyte. In addition, spermatozoa are activated during their sojourn in the female reproductive tract. Such activation is known as capacitation and gives the sperm the ability to bind to the zona and to respond to stimuli inducing acrosome reaction (AR), the exocytotic process occurring after sperm-zona binding. During capacitation, a particular type of motility (known as hyperactive motility) develops, allowing the sperm to penetrate oocyte vestments and reach the oolemma. Sperm binding to the oolemma as well as fusion of the two gametes are mediated by functional proteins present on both gametes. It appears quite clear that semen analysis approach, *per se*, cannot give any information on the sperm ability to encompass the above-described steps, which require activation of several intracellular pathways as well as integrity and function of several sperm proteins. Functional tests investigating the ability of spermatozoa to accomplish each of the single steps leading to fertilization have been developed in the past in order to overcome such limitations (11), but some of these require expensive equipments and notable technical ability with difficult application for routine purposes.

Studies aimed at identifying molecular markers of sperm function related to fertilization have mostly employed animal models of male infertility and *in vitro* experimental conditions mimicking the process of fertilization. Both approaches have limitations. Animal models should be validated in human, as differences in the pathways involved in each sperm function have been observed and are expected among species. For these reasons, gene KO inducing male infertility and subfertility in animals needs clinical confirmation. An example of gene KO causing male infertility in mouse and whose effect was confirmed also in human is CATSPER1 gene, coding for a sperm calcium channel (12, see below). In addition, studies in the human are mostly performed in ejaculated sperm whereas those in animals in epididymal ones, which have a different level of maturation and have not been mixed with seminal plasma. On the other hand, studies conducted *in vitro* may suffer from the inappropriate environment. In addition, experimental conditions may vary from one study to another (use of different media, use of demembranated sperm, different incubation times, sperm selection etc) making difficult the comparisons of the results. In the

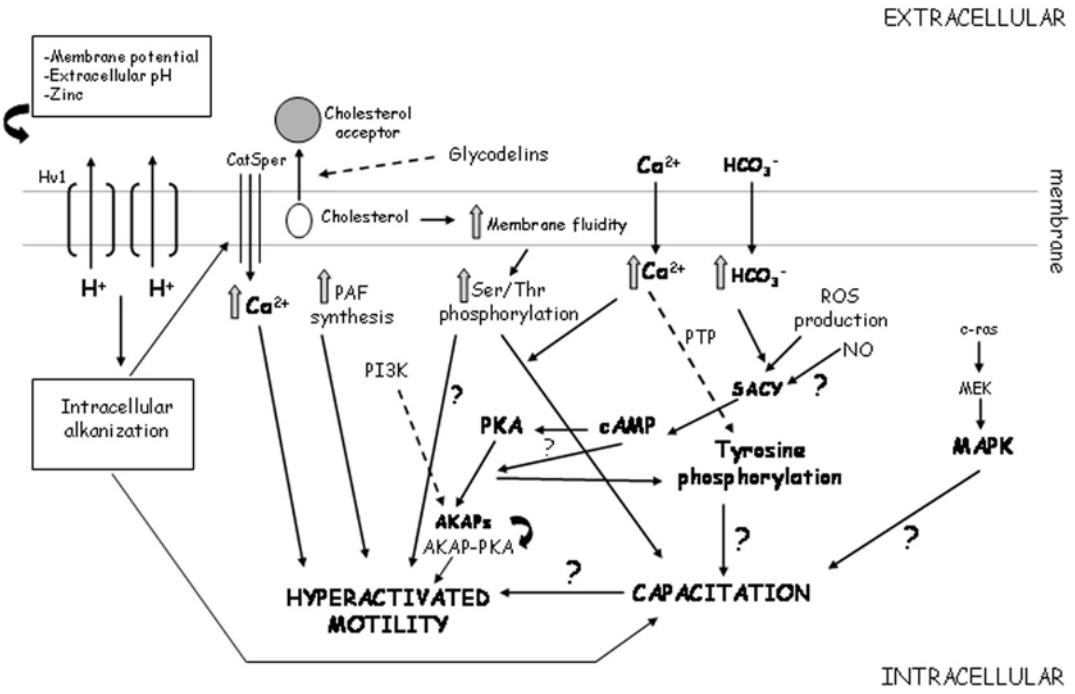

Figure 2. Schematic representation of the main intracellular signalling pathways that are activated during the process of capacitation and development of hyperactivated motility. Early events during the process of capacitation include cholesterol removal from the membrane, a process which is stimulated by an extracellular cholesterol acceptor (physiologically albumin) and inhibited by glycodelins, entry of calcium (Ca^{2+}) and bicarbonate (HCO^{3-}). Another early event is represented by intracellular alkalinization (regulated by membrane potential extracellular pH and zinc), which is involved both in calcium and tyrosine phosphorylation increase occurring later. HCO^{3-} leads to activation of Sacy and the cAMP/PKA pathway. Autocrine production of reactive oxygen species (ROS), nitric oxide (NO) and PAF has been demonstrated during capacitation. All these pathways converge in an increase of tyrosine phosphorylation of proteins. The increase of intracellular Ca^{2+} limits the increase of tyrosine phosphorylation by a poorly defined mechanism that may involve PTP. ras-MEK/MAPK and PKC activation and serine/threonine phosphorylation of proteins have been shown to occur during capacitation but their role in the process remains to be clearly defined.Pathways involved in development of sperm hyperactivated motility, include entry of Ca2+ through CatSper calcium channels whose role in motility has been recently disclosed in a KO mouse model, and autocrine synthesis of PAF which exert a positive effect. Hyperactivated motility is stimulated by inhibitors of PI3K through increase AKAP tyrosine phosphorylation and AKAP-PKA interaction. CatSper can be also activated by intracellular alkalinization due to the outward transport of protons through Hv1 Voltage-Gated channel.Dotted arrows indicate inhibiting pathways. Interrogation marks indicate lack of definitive proofs of the involvement of a given pathway in the process. Abbreviations: SACY, soluble adenylate cyclase; AKAPs, A-kinase-anchoring proteins; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NO, nitric oxide; PAF, platelet-activating factor; PDE, Phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PI3K, phosphatydil inositol 3 kinase; PTK, protein tyrosine kinase; PTP protein tyrosine phosphatase; Src, cellular homologue of transforming gene of Rous sarcoma virus; reactive oxygen species (ROS).

following paragraph, we will discuss these studies and identify possible molecular markers of each sperm function needed for the fertilization process by mostly relying on studies on human sperm.

3.1. Sperm capacitation

Defined as the process that allows spermatozoa to achieve the final competence to fertilize the oocyte, sperm capacitation is a highly complex event involving membrane and intracellular modifications. Capacitation is time-related and is initially promoted by loss of decapacitating factors (sperm membrane binding proteins secreted from the epididymis and accessory glands) from the sperm surface (13). The timing of the process may vary *in vitro* in sperm from subfertile subjects (14). For this reason, it may be useful for the clinician to monitor the event with specific markers of the process before application of *in vitro* fertilization or other ART techniques.

3.1.1. Intracellular pathways activated during capacitation

Results of *in vitro* studies have documented the activation of many signalling pathways during the process of capacitation in human sperm (Figure 2), but which pathway is absolutely required for the completion of the process remains to be established. Moreover, it is possible that some of the pathways are redundantly activated. Help in this matter might come from animal models of altered gene function and from proteomic analysis of proteins

associated with the specific event. A list of mouse gene KO leading to male and female fertility problems has been recently published (15). However, in most of these studies whether capacitation was fully attained in KO sperm has not been determined. Indeed, animal models of altered gene function that result in clear capacitation defects are few and not really conclusive. Such studies indicate a role for the A1 adenosine receptor (16), for platelet activating factor receptor (17), for fibroblast growth factor receptor (18) and for the calcium dependent serine proteinase Pcsk4 (19) in the completion of the process. However, sperm from these mice show mild capacitation-related problems, indicating that alternative pathways, equally efficient to reach the capacitation end points, are activated. Moreover, sperm from these mice are characterized by multiple defects due to alterations of spermatogenesis, which may be also responsible for the reduced fertilizing ability. KO models where sperm capacitation occurs precociously with respect to wt mice have been also described. In one of these models, the lack of the long isoform of beta1,4-galactosyltransferase I, a sperm surface proteins indicated as a possible ZP3 receptor, prevents binding of de-capacitating factors during epididymal transit of sperm, resulting in accelerated capacitation due to constitutive activation of intracellular pathways such as those mediated by calcium and cAMP (20). Conversely, the PCSK4 KO model shows enhanced tyrosine phosphorylation of proteins and altered proteins proteolysis (21) which may be related to the reduced fertilizing potential of these mice.

Proteomic analysis is potentially useful for the identification of the proteins that undergo post-translation changes and/or modifications during capacitation as well as for the identification of the enzymes involved in the process. However, although the advent of proteomics has allowed efficient global profiling of proteins, isolation and separation of plasma membrane proteins or proteins with a large molecular mass still represent the Achilles' heel of these techniques and complementary approaches are often needed to provide a detailed picture. In view of the role played by membrane proteins in sperm functions, a technology able to provide a detailed profile of proteins exposed on the surface of the plasma membrane appears essential to disclose eventual capacitation-related modifications of sperm surface proteins. In a recent comprehensive review, Brewis and Gadella (22) outlined the most important challenges related to sperm proteomic, with particular attention to surface proteins. Modifications of human sperm proteins during capacitation by a proteomic approach have been recently determined by Secciani *et al* (23). Of interest, several proteins undergo modifications of their global amount during the process. Among those proteins whose amount appears to increase, some are related to cell-stress events, whereas among those decreasing, many are involved in protein metabolism and energy production. Interestingly, flagellar proteins involved in motility, such as AKAP4, tubulin and actin, also show quantitative decrease following capacitation. Although these results await confirmation, they may indicate the occurrence of fragmentation of flagellar proteins as a consequence of the capacitation process (23).

An important point concerns the detailed time course of events depicted in Fig.2. Loss of membrane cholesterol, driven by albumin present in the extracellular environment, is believed to enhance membrane fluidity and/or to directly affect membrane proteins involved in signalling, enabling capacitation to occur (24). Cholesterol is concentrated in raft microdomains, which undergo a heavy re-organization during capacitation, leading to a decrease in membrane raft content in human sperm (25). Determining the exact role (mediating cholesterol efflux? Facilitating interaction of signalling molecules? Terminating signalling?), if any, of lipid rafts during capacitation appears to be a complex issue both because of methodological problems in raft isolation as well as because of their variable composition within sperm (25). Ca^{2+} and bicarbonate increase in the cytoplasm as well as ROS generation and activation of a soluble adenylate cyclase (26) appear to be upstream of tyrosine phosphorylation of proteins. However, whereas bicarbonate and ROS, trhough activation of a soluble adenylate cyclase (26), lead to increased tyrosine phosphorylation of proteins, Ca^{2+} ultimately leads to a limitation of the phenomenon at least in human sperm (27-28). The central role of soluble adenylate cyclase (SACY) in the process is demonstrated by studies in $SACY^{-/-}$ mice which are infertile due to inability to develop hyperactivated motility as well as lack of tyrosine phosphorylation increase in response to bicarbonate (29). Recently, a major breaktrough in the field is represented by the demonstration that alkaliniziation of sperm cytoplasm (occurring during capacitation) is mediated by a proton channel (named Hv1) (30). Hv1 is located in the principal piece of the flagellum and is activated by membrane depolarization. Although further studies are needed to define the exact role of this protein channel in sperm capacitation, the evidence so far suggests its involvement in the completion of the process at least in human sperm (30) .

The rather long list of signalling events demonstrated during sperm capacitation (Figure 2) is indicative of the complexity of the process. As mentioned above, some level of redundancy may exist. In addition, some of these pathways may be secondarily activated to produce development of hyperactivated motility, a process which is coincident to that of capacitation (see below) but whose dependence on capacitation is discussed.

In the following paragraphs, the two best studied capacitation-related events, namely intracellular calcium and protein phosphorylation, are reviewed.

3.1.2. Intracellular calcium

An increase in intracellular free Ca^{2+} concentration is characteristic of early phases of capacitation (31). Because of its fusogenic properties, Ca^{2+} is believed to play an essential role in acrosome reaction (AR) of spermatozoa and in the subsequent process of fusion with the oolemma, but the exact role of the ion in the process of capacitation is less clear. It is possible that the

time-related increase of intracellular calcium during capacitation prepares the membrane to the fusogenic process. In addition, in some species, calcium may be involved in stimulation of a bicarbonate- and calcium-sensitive SACY (32) which finally results in the increase of tyrosine phosphorylation (33). In other species including the human, such a role of Ca^{2+} is less clear, as eliminating the ion from the capacitating medium enhances tyrosine phosphorylation (27-28; 34). Ca^{2+} regulation of ATP levels (34) and Ca^{2+}-regulated calmodulin activation (28) have been suggested as possible mediators of this effect.

The increase of intracellular calcium occurring during capacitation seems to control superoxide and nitric oxide formation during the process in a complex way (35). Since small amounts of reactive oxygen species are required in order to complete the process of capacitation (35), it is possible that the initial rise of calcium may prime oxidase and NOS activity in sperm to keep the process going on.

The mechanisms that trigger the spontaneous rise of intracellular Ca^{2+} occurring during capacitation are not completely understood. Hypotheses include inhibition of Ca^{2+}-ATPase (36), increased permeability to Ca^{2+} due to cholesterol loss from the membrane (37), or activation of unidentified channels. In addition, in view of the recent identification of intracellular calcium stores (38, 39) in spermatozoa, it can be hypothesized a role of polyphosphoinositides–mediated calcium release from these stores in the process. A large variety of Ca^{2+} channels has been demonstrated in mammalian spermatozoa (for rev see 38) which are involved in different cellular responses. Evidence exists indicating that voltage-dependent T-type Ca^{2+} channels may be involved in capacitation (40). Recently, in a KO mouse model of CATSPER1 Ca^{2+} channel, Xia and Ren (41) demonstrated that the increase of intracellular calcium stimulated by addition of BSA (considered to be involved in the initial phases of capacitation) is lost, suggesting the involvement of this sperm specific weakly voltage-dependent channel at least in initial phases of capacitation. Interestingly, CATSPER1 mutations have been recently detected in members of two consanguineous families segregating autosomal recessive male infertility characterized by mild-to-severe oligoasthenozoospermia (12) suggesting that CATSPER1 is required for normal sperm functions in humans. Another possible mechanism involves a sperm Ca^{2+}-ATPase which is activated by decapacitating factors in uncapacitated sperm and that become inactivated following their removal from the surface (42). The demonstration of IP3-gated internal Ca^{2+} stores both in the head (39) and at the base of the flagellum (43) and their involvement in development of hyperactivated motility (44), suggest that Ca^{2+} entry during capacitation may be also regulated by these Ca^{2+} storage sites, but a clear-cut demonstration of their involvement is lacking so far.

3.1.3. Protein phosphorylation

The increase in tyrosine phosphorylation of proteins during capacitation is a well documented process that involves several proteins. In human and boar spermatozoa most of these proteins are located in the tail (45-47), whereas in other species, such as ram (48), the increase in tyrosine phosphorylation occurs also in the acrosome. Identification of proteins undergoing tyrosine phosphorylation during capacitation is, at the moment, incomplete, whereas it is expected that only the complete identification of these proteins will disclose the biological meaning of the increase of tyrosine phosphorylation during capacitation will be revealed. So far identified tyrosine phosphorylated proteins in human sperm, include ion channels, metabolic enzymes and structural proteins, mainly located in the flagellum (49), CABYR, a calcium-binding protein localized in the principal piece of the tail in association with the fibrous sheath (50), and members of the ERK (extracellular-signal regulated kinase) family, (51-52). The family of A-kinase-anchoring proteins (AKAPs) are the main tyrosine phosphorylated structural proteins of the fibrous sheath (49) and their involvement in motility has been well defined (see below).

Tyrosine phosphorylation of proteins in human sperm does not require Ca^{2+} in the extracellular medium (27-28), whereas albumin and bicarbonate are required. A decrease of membrane cholesterol content appears to promote the phenomenon, (53) and high cholesterol content in the membrane may underlie a defect of phosphorylation in subfertile/infertile men (54). Despite the demonstration that the process is regulated by a cAMP-dependent pathway (33), the involvement of the serine/threonine kinase protein kinase A (PKA) upstream of the process is not clear (discussed in paragraph 2.2), and the downstream tyrosine kinase(s) (TKs) which link(s) the cAMP pathway to tyrosine phosphorylation increase remain(s) elusive. Evidence in murine (55) and bovine (56) sperm pointed to c-src as one of the kinases which might cover this role. In human sperm, c-src inhibition has been shown to decrease tyrosine phosphorylation during capacitation (57). However, it has been recently demonstrated that the src inhibitor used in these studies, shows a low degree of specificity and that c-src-KO mice undergo normal capacitation process including tyrosine phosphorylation increase (58), ruling out an involvement of the kinase in the process.

Whatever the role of tyrosine phosphorylation in capacitation, its level in human sperm correlates strongly with sperm-zona binding capacity (46) and alterations in its increase have been found in subfertile subjects (54) pointing out its physiological role in fertilization.

In spite of the numerous studies on tyrosine phosphorylation, only few studies have been published on phosphorylation of ser/thr residues, reflecting difficulties in performing such studies. An increase in proline-directed serine/threonine phosphorylation during capacitation has been demonstrated in human sperm (59), but further studies are waited in order to understand the role of this pathway in capacitation.

In conclusion, occurrence of capacitation in sperm in a given subject could be monitored by measuring intracellular Ca^{2+} ($[Ca^{2+}]_i$) using fluorescent Ca^{2+} indicators

and evaluating both basal and agonist-stimulated increase as well as by evaluating the increase of tyrosine phosphorylation by western blot analysis comparing basal and capacitated levels (60).

3.2. Sperm motility

The male gamete is uniquely characterized by active motility. The immotile testis spermatozoa are passively transported through the epididymis where they undergo a final process of maturation, acquiring an independent motility under the control of several environmental and intracellular factors in the caudal part of this structure (61). Besides forward motility displayed by human spermatozoa in the ejaculate, capacitation occurring following sperm permanence in the female genital tract enables sperm to develop a different kind of motility, called hyperactivation, characterized by high velocity, large amplitude and asymmetry of the flagellar beat, which helps the male gametes to detach from the oviductal epithelium, progress in the more viscous oviductal fluid and eventually penetrate the zona pellucida and the other oocyte vestments. Hyperactivation is easily achieved *in vitro* by incubating spermatozoa in defined media supplemented with the same factors that regulate the process of capacitation.

Alterations and defects in sperm motility (different grades of asthenozoospermia) is one of the main causes of male infertility based on sperm factors. Our present knowledge of the mechanisms and factors concurring to sperm motility reveals that this process is extremely complex (Figure 2) and involves a plethora of signaling molecules and structural proteins, organized in redundant pathways justified by the importance of motility in the fertilization process.

Enhancement of the mechanisms underlying sperm motility is one of the goal of the research in the field of assisted reproductive technologies to improve *in vitro* sperm quality and recovery prior to applying ART (62).

3.2.1. Molecular bases of sperm motility

Both structural proteins and signaling molecules concur to the correct development and maintenance of sperm motility. Moreover, the peculiar sperm glycolytic enzymes present in the principal piece of sperm tail (63) and the mitochondrial proteins of the oxidative phosphorylating cascade in the midpiece generate ATP molecules (64) necessary for microtubules reciprocal sliding in the principal piece of sperm tail sustaining the flagellar beat.

3.2.2. Structural and scaffolding proteins

Among structural proteins, the constituents of the fibrous sheath play a pivotal role in orchestrating sperm motility. In fact, the peculiar organization of the axonemal structure in the sperm flagella is characterized in the principal piece by a 9+2 axoneme structure surrounded by the fibrous sheath, consisting of columnar outer dense fibers surrounded by a transverse ribs anchored to 2 longitudinal columns (63). The anchoring of axonemal structure to the rigid fibrous sheath results in deforming the planar bend of the tail supported by the reciprocal sliding of adjacent couples of microtubules. Thus, the peculiar asymmetric and tridimensional movement of sperm tail relays on fibrous sheath interactions with the central axoneme.

Defects in these components, such as outer dense fibers and AKAPs, result in several morphological and functional alterations relevant to sperm asthenozoospermia and reduced fertility (65). AKAP 3 and 4 are not only the main structural components of sperm flagellum, but also act as scaffolding proteins coordinating activity of docked enzymes and second messengers. In fact, AKAPs can specifically recruit their protein targets, such as kinases, phosphatases and other enzymes, selectively compartmentalizing such proteins and enabling them to interact in super-molecular complexes with their own substrates anchored themselves on AKAPs. Thus AKAPs can spatio-temporally activate specific and focused

signaling cascades initiated by protein-protein interactions occurring on AKAP scaffold (65).

Therefore, defects in structure and function of such proteins are likely to be associated to defects in sperm motility. Fibrous sheath dysplasia characterized by thickened and dysmorphic sperm flagella, involves a plethora of defects in different components of the fibrous sheath arising during spermiogenesis (66). Although AKAP4 KO mice show defects in sperm motility and are infertile, in humans, dysplasia of the fibrous sheath (FSD) does not seem to associate with alterations in AKAP3 and 4 genes, in particular in the PKA binding domain (67). However, a recent paper from Baccetti's group reported a genetic defect (complete deletion) in AKAP4 gene in a patient with FSD (68).

Tyrosine phosphorylation in the AKAP3 following treatment with the phosphatidylinositol 3-kinase inhibitor LY294002, which has been shown to enhance motility, or physiological stimulation *in vitro* with bicarbonate, has been recently demonstrated to specifically recruit activated PKA in sperm tails resulting in a significant increase in sperm motility and hyperactivated parameters (69-70), providing an interesting link between tyrosine phosphorylation and function of the protein. Hence, absent or poor tyrosine phosphorylation of AKAP may mark patients with different grades of asthenozoospermia, underlying a possible defect in the ability of the protein to undergo phosphorylation or in the signaling machinery responsible for its phosphorylation (see below).

3.2.3. Kinase and phosphatase system

The kinase/phosphatase system exerts a significant role in regulation of sperm motility (Figure 2) by a rapid and reversible modulation of several signaling cascades. The SACY/cAMP/PKA and the connected tyrosine phosphorylation systems have been extensively studied in the last decades.

In spermatozoa, cAMP is predominantly produced by SACY, although membrane isoforms have also been described (71). SACY and PKA interaction with

compartmentalized AKAP initiate a focused and spatio-temporal activation of signalings. This specific compartmentalization of SACY in distinct subcellular domains may lead to activation of PKA by locally produced cAMP (61; 70), in particular in sperm tail, where SACY is predominantly associated with fibrous sheath. KO for SACY results in infertility due to impairment in sperm motility (72). Interestingly, in spermatozoa from this KO model, cAMP administration restores forward (but not hyperactivated) motility, tyrosine phosphorylation as well as fertility (72), further confirming the importance of the tyrosine kinase pathway downstream of SACY (70). PKA role in regulation of sperm motility has been investigated in details by the use KO mouse models for different PKA catalytic and regulatory subunits (for rev see 61;73). However, results of selective KO for different PKA subunits led to controversial results representing one of the best example of the problems linked to the use of KO animals (74). In particular, selective KO for catalytic PKA subunits results in impaired sperm motility (74), which is not affected, conversely, by KO of regulatory subunits, despite a delocalization of PKA catalytic activity from the fibrous sheath can be observed in this mouse model (75). Furthermore, null mice for the sperm specific PKA catalytic subunit Cα2 display normal ability to initiate motility although bicarbonate failed to induce hyperactivation and tyrosine phosphorylation of proteins (76), pointing out the possible specific role of this sperm specific PKA in hyperactivation. Compensatory increases of the alternative isoforms of PKA subunits could account for the absence of a specific motile phenotype in such KO models. In order to circumvent such compensatory bias which may occur in KO models and to overcome problems associated with lack of specificity in the use of PKA inhibitors to study the role of this enzyme in sperm motility and capacitation, the group of McKnight, recently developed a conditional tissue-specific PKA inhibition using a chemical genetic approach (77). The authors engineered the mouse Pkraca gene to introduce a mutation in the ATP binding pocket of PKA catalytic subunit (Cα) conferring sensitivity to the pyrazolo[3,4-d]pyrimidine inhibitor, 1NM-PP1. In these mice, the CαM120A mutant protein is expressed and functional, while the wild-type Cα is turned off. When animals are treated with 1NM-PP1, this compound specifically inhibits PKA activity only, without affecting activity of other kinases. By the use of this conditional KO mouse, they could dissect PKA role in early and late phases of capacitation (77), demonstrating that acute PKA inhibition prevents bicarbonate-induced increase in flagellar beat frequency during early stage of capacitation and prolonged PKA inhibition also affects late stages of capacitation, supporting the asymmetrical flagellar beat, characterizing hyperactivation. Conversely, acute PKA inactivation in late stages of fully capacitated sperm does not affect protein tyrosine phosphorylation or hyperactivated flagellar asymmetry. These findings clearly support a biphasic role of PKA in controlling motility associated with capacitation: a ‘‘rapid’’ action required for the activation of flagellar beat; and a ‘‘slow’’ action such as the change in the flagellum waveform symmetry, that needs PKA to be long lasting active (78).

Tyrosine phosphorylation of sperm proteins represents the main post-translational modification underlying regulation of sperm activation. Tyrosine phosphorylation of proteins, in particular in sperm tails, occurs during capacitation (33; 78). In particular, the fine equilibrium between tyrosine kinase and phosphatase activity is the central key controlling activation and maintenance of activated and hyperactivated sperm motility (61;79). Reduction of tyrosine phosphorylation of sperm proteins, in particular in the same molecular weight range of AKAP3 and 4, seems to be strictly associated with sperm motility defects in semen of asthenozoospermic subjects compared to normozoospermic and fertile subjects (80). PI3K inhibition as well as bicarbonate-stimulated SACY activation have been demonstrated to induce a significant increase in cAMP and tyrosine phosphorylayion of AKAP3 in sperm tail, which results in a significant increase in sperm motility and hyperactivation (69-70). The evaluation of tyrosine phosphorylation of AKAPs in subjects with different motility defects is mandatory to establish whether it may represent a valuable marker of sperm motility, although absence or poor phosphorylation may reflect defects at different levels (membrane bicarbonate transport, cAMP production, kinase activation, AKAP, etc). However, no quantitative and qualitative alteration in AKAP3 and in AKAP4 and its precursor pro-AKAP4 has been found in dysplasia of the fibrous sheath (81) and stump tail (82) pathologies.

Among kinases which are either regulated by tyrosine phosphorylation or can phosphorylate in tyrosine residues their substrates, PI3 kinase, Src and MAPK have been extensively investigated for their possible involvement in regulation of sperm motility.

Following initial studies demonstrating that the use of the PI3K inhibitor LY294003 induces an increase of motility (69;83), recent papers, employing wortmanin as PI3K inhibitor, demonstrate that the lipid kinase is involved in induction of AR (84) and capacitation (85-86) of mammalian sperm. In addition, Nauc *et al* (87) have shown that incubation of human sperm with LY294002 induces capacitation and tyrosine phosphorylation of proteins, whereas wortmannin had opposite effects. Although our findings suggest that incubation of sperm with LY294002 produces a decrease of PI3K activity (69), it is possible that unrecognized non specific effects of the inhibitor are also involved. Whatever is the mechanism, the effects on motility (69; 83; 88), capacitation (87), tyrosine phosphorylation of proteins (69; 87) and AKAP-PKA interaction (69) observed with LY294002 deserve additional efforts to clarify the mechanisms involved as the compound may be the considered the prototype for future pharmacological tools to increase sperm functions *in vitro*.

An interesting crosstalk between PI3K, PKA and PKC signaling pathways which crucially converges downstream on Glycogen Synthase Kinase-3A (GSK3A) inactivation has been described to concur to sperm motility (88). According to such model, PKA activation through bicarbonate-induction of SACY or by PI3K inhibition, results in a direct or PKC-mediated

phosphorylation/inhibition of downstream GSK3A which finally results in a significant increase in boar sperm motility.

An apparently opposite involvement of PI3K in positive control of sperm capacitation has been described by Breitbart's group in boar sperm (89). In fact, PKA seems to activate PI3K in the late phase of capacitation by direct phosphorylation and by stimulating degradation of PKCα and PP1γ2 which restrain PI3K activity. However, such discrepancies in PI3K invovement could be explained by the timing of enzyme activation/inhibition during capacitation. In fact, at the beginning of capacitation both PKCα and PI3K are active, and the high activity of PKCα/PP1γ2 prevents PKA from inhibiting PI3K. But towards the end of capacitation, phospho-PKCα undergoes dephosphorylation and both PKCα and PP1γ2 are degraded, allowing the PKA-mediated-PI3K-phosphorylation/activation to occur. However, while the early events of PI3K low activity and rapid PKA activation occurring at the beginning of capacitation could be mainly involved in initiating and maintaining motility, the late events involving a PKA feed back inhibition of PI3K may be limited to the control of acrosome reaction (86;89). These findings further confirm the 2 step process of capacitation above described (78), consisting of early and late events.

In the seek for the intermediate tyrosine kinase(s) which may transduce PKA stimulatory regulation on sperm motility, the tyrosine kinase Src has been suggested to be involved in PKA stimulation of hyperactivation in the mouse spermatozoa (55). However, this role was not confirmed in human sperm (57; 90) where, moreover, a different Src isoform showing a higher molecular weight than the somatic one has been described (57). In human sperm, Src seems to be mainly involved in supporting tyrosine phosphorylation and progesterone-stimulated acrosome reaction, rather than being involved in sperm motility (57). Moreover, a direct interaction with PKA has been excluded (57), whereas the contribute of Src to the increased tyrosine phosphorylation of sperm proteins occurring during capacitation is rather due to a PKA parallel inhibition of Ser-Thr phosphatases, thus resulting in potentiating PKA effects (58).

Finally, MAPK contribute to sperm motility is rather controversial. In fact, whereas some authors localize ERK1/2 in the tail of mature human spermatozoa downstream of PKC activation (91), others did not find ERK expression in sperm flagellum, but rather in the post-acrosomal region of sperm head, being involved in other tyrosine phosphorylation events occurring during capacitation (51). Interestingly, spematozoa express both ERK and p38 MAPK isoforms, which regulate spermatozoon forward and hyperactivated motility in an opposing fashion. Following phosphorylation and activation, ERK was found to stimulate, whereas p38 MAPK inhibits forward and hyperactivated motility (91). Moreover, the expression level of ERK1 rather than ERK2 isoforms as well as the phosphorylation status of p38 inversely correlate with forward and hyperactivated motility (91), suggesting a prognostic value of p38 and ERK levels for sperm motility defects.

3.2.4. Calcium

Several signaling pathways and intracellular second messengers are common to the development of activated and hyperactivated motility, however, some are uniquely essential for hyperactivation (92). In particular, Ca2+ is essential for the development of sperm motility, and its intracellular levels are tightly regulated by several Ca2+ channels (93). Although Ca2+ influx is associated with sperm capacitation and tyrosine phosphorylation of proteins (see above), intracellular free calcium must be kept at low levels in order to allow precise timing of sperm activation (27) and ATP to be used for hyperactivation and tyrosine phosphorylation processes (34). Sperm has the highest expression and variety of calcium channels, mainly expressed in the tail, which strongly suggests their involvement in regulation of sperm motility. Catsper, a novel class of tetrameric voltage-gated Ca2+ channels expressed in sperm tail, is essential for the development of hyperactivation. Indeed, KO mice for genes encoding these channels display absence of such motility, despite normal activated motility, finally resulting in infertility (92). Mutations of Catsper1 paralogue gene were found in two Iranian families resulting in male infertile phenotype characterized by reduced sperm motility and abnormal sperm morphology (12), confirming the pivotal role of Catsper1 in motility. Unfortunately, no further investigation of hyperactivation parameters in semen samples was performed in that study. In addition, infertile men who lack sperm motility exhibit decreased catsper gene expression compared with men infertile for other reasons (94). Besides the first characterized Catsper 1 and 2 (95), two other related genes, Catsper 3 and 4, showing a similar expression on sperm tail plasma membrane, have more recently been described (92). However, the involvement of this class of Ca2+ channel in development of hyperactivated motility in human sperm has not been conclusively addressed despite demonstration of expression of mRNA for catsper family members by RT-PCR (94). In conclusion, the role of calcium in regulating sperm motility and hyperactivation is quite controversial, since from one hand, high Ca^{2+} levels deplete intracellular ATP (important for protein phosphorylation and thus for capacitation and development of motility) (34), and from the other hand, Ca^{2+} influx appears essential for inducing hyperactivation (92). Progesterone evokes a defective calcium entry in sperm from asthenozoospermic compared to normomotile subjects (96). Intracellular sperm alcalinization is one of the other ion mechanism occurring during passage in the female genital tract, which controls sperm activation, including sperm development of motility (97). However, the mechanism responsible for transmembrane proton extrusion has remained elusive, due to inability to measure membrane conductance in human sperm. Successful patch clamping of human spermatozoa has recently enabled the discovery of proton channel Hv1 confined to the principal piece of sperm tail, to be responsible for the dominant proton conductance (30). This voltage-gated proton channels are expressed at unusual high density in the sperm tails and are activated by membrane depolarization,

alkaline environment, removal of extracellular zinc, the potent Hv1 blocker. Since the flagellar CatSper channel, which is the major Ca2+ entry pathway into spermatozoa, is activated by intracellular alkalinization (98), Hv1, which localizes in the same principal piece of sperm tail, can be hypothesized to control CatSper activity and regulate intracellular Ca2+ concentration.

3.2.5. Sperm motility and ART

A better knowledge of the molecular mechanisms underlying developing and maintenance of sperm motility will enables us to design sperm treatments which can improve this function. Retrieval of a higher number of spermatozoa with a better quality from ART sperm separation techniques could affect the choice of the techniques the unfertile couple will undergo in ART protocols. Different *in vitro* approaches to enhance sperm motility in semen samples used for ART have been developed (62). Inhibition of phosphodiesterase with pentoxifylline (99) and tadalafil (100), resulting in sustained levels of cAMP has been demonstrated to improve sperm motility in asthenozoospermic subjects. Moreover, a signiificant improvement in sperm motility, more evident in asthenozoospermic samples, can be achieved by PI3K inhibition and SACY stimulation by bicarbonate through increased levels of cAMP and tyrosine phosphorylation of AKAP3 in sperm tails (69-70). In particular, a significant increase in the number of spermatozoa recovered by swim up procedures has been observed in the presence of the PI3K inhibitor LY294002 (61). However all these sperm treatments have to be proven to have no toxic effect (101-102) on the developing embryo in order to be applied in ART.

4. MOLECULAR MARKERS OF SPERM-OOCYTE INTERACTION

4.1. Sperm-zona binding and sperm-oocyte fusion

Interaction between sperm and oocyte initiates at the cumulus level and terminates after the sperm has completely fused with the oocyte. The identity of the sperm receptor(s) responsible for the attachment to the zona and initiation of the signal transduction cascade leading to acrosomal exocytosis, remains obscure. Many different molecules and many models have been developed in the last decades (for rev see 15), involving different carbohydrates and proteins. KO mouse models for many of these proteins (reviewed in 15) pointed toward ADAM3 as the most important factor in sperm-zona binding. However, further studies are needed to demonstrate that this protein is operative in the process of sperm-zona binding in human beings. The possibility that a direct sperm-ZP interaction is not required for induction of acrosome reaction has been also recently discussed in the comprehensive review (15).

A similar situation applies for the molecular mechanism of sperm-egg membrane fusion, where many different molecules have been suggested to play a role (15). The identification, by studies on mice with altered gene function, of Izumo on sperm and of CD9 on oocyte as the two major players in the game (15) represents now a milestone in the field. Evaluation of Izumo protein expression in the sperm membrane and a search for mutations of the gene might reveal alterations in the ability of sperm to fuse. So far, however, no patient negative for Izumo expression (detected by immunocytochemistry) has been identified among 25 affected by severe oligozoospermia or selected for a previous history of fertilization failure (103). More recently, the occurrence of four polymorphisms of *Izumo* in patients with fertilization failure has been reported, without however, any apparent effect in the fertilization process (104). However, the weakness of these studies is reflected in the low number of subjects examined. The identification of an inactivating mutation of *Izumo* in men with defects of sperm-oocyte fusion is awaited before any definitive conclusion about the involvement of this protein in sperm-oocyte fusion in humans may be drawn.

4.2. Sperm acrosome reaction

Whether it occurs following binding to the zona or during transit in the cumulus matrix (15) AR appears to be a necessary event for the sperm in order to fertilize the egg. AR is mediated by large influxes of Ca^{2+} and activation of kinases (38). Many different molecules present in the female genital tact, including steroids, phospholipids, small peptides and growth factors, have been shown to induce AR. Recently, the classical view that sperm binding to zona triggers the AR has been disputed by demonstrating, in a murine model, that AR occurs much later following sperm adhesion to zona and hypothesising that mechanosensory signals which develop during zona penetration trigger the process (105). Such a model, although fascinating, needs to be confirmed in sperm from other species and human in particular.

Until the receptor(s) responsible for sperm-zona binding and induction of AR has been unequivocally characterized (15), markers of AR are represented by signalling events stimulated by the two physiological AR inducers, i.e. zona proteins and progesterone (P). Actually, in view of the limited efficacy of recombinant ZP proteins in stimulating AR, most of the knowledge on the molecular signalling occurring during AR in human sperm derives from studies employing P. P induces Ca^{2+} influx, modifies the fluxes of other ions (106) and induce tyrosine phosphorylation of proteins (107). P is present at micromolar concentration in the follicular fluid (108) and thus sperm are engaged with high levels of the steroid at the site of fertilization. The fact that pre-treatment with P "primes" sperm to subsequent response to zona proteins (109) suggests that the steroid may be involved in sensitizing sperm to the AR process while they cross the cumulus matrix. Responsiveness to P is decreased in subfertile men (96) and correlates with *in vitro* fertilizing ability of sperm (110), indicating a physiological role of the steroid in the process of fertilization. The involvement of a receptor-mediated mechanism in the action of P has been proposed based on several evidence (111). Several candidates have been proposed to cover this role (reviewed in 112), including a truncated form of the classic P receptor, and novel P membrane receptors. Recently, by a detailed proteomic analysis of human spermatozoa, an extragenomic P receptor was identified (113), although

proofs of its involvement in the P-inducing effects on sperm are lacking. Despite all these evidence, the possibility that a non-receptor mechanism is involved in P action in sperm cannot be excluded. Evidence against a receptor mechanism include the concentrations (from 0.1- to 10 μM) of the steroid needed to induce the effects, which are highly above those needed for receptor-mediated responses.

5. OTHER MARKERS OF SPERM FUNCTIONS

5.1. Sperm DNA integrity

DNA integrity is a pivotal feature of sperm as the delivery of paternal genome to the oocyte represents the main function of the male gamete. In both natural and assisted reproduction DNA integrity is considered a crucial determinant of normal fertilization, of embryo and fetal development and of child well being. Sperm chromatin has a unique organization, being six times more condensed respect to mitotic chromosomes. Such tight chromatin package is achieved by the replacement of somatic-type histones by protamines (basic protein specific of sperm nuclei) and the consequent DNA condensation into compact doughnuts (114). Despite of the unique chromatin architecture, there are some reasons that make sperm specifically susceptible to DNA damage. First, they progressively lose the DNA repair systems in late spermatogenesis (115). In addition, cells with potential DNA damage may be found in the ejaculate because of a failure in the completion of the process of apoptosis [as suggested by the abortive apoptosis theory developed by Sakkas *et al*, (116)] or of a physiological loss of the apoptotic machinery during sperm maturation, as suggested by studies on animal models (117). Further, in human sperm, chromatin structure is less compact than in other mammals as a consequence of both the retention of 15% histones and a lower amount of disulphide cross linking between protamins, due to the presence of the cystine residues deficient protamine P2 (118). Finally, the high content of membrane unsaturated fatty acids and the loss of cytoplasmatic antioxidant enzymes during maturation, make sperm DNA a susceptible target for ROS attack.

Several chromatin abnormalities have been described in human sperm, including chromosomal aneuploidy (119), poor protamination (120), increased DNA susceptibility to denaturation induced by acid or heat (121), occurrence of modified DNA bases (122) and of double and/or single strand breaks in DNA (from herein indicated as DNA fragmentation, 123). Among such anomalies, DNA fragmentation and its impact on reproduction (assisted or natural) are the most extensively investigated. Several authors propose that the assessment of sperm DNA fragmentation could be a good candidate parameter to diagnose male fertility potential and to predict reproduction outcomes. However, the scientific community has not yet reached the consensus on the clinical value of determining sperm DNA integrity. Indeed, studies on the impact of such parameter on outcome of natural and assisted reproduction are conflicting. Even if several investigations have shown that percentage of sperm with DNA damage is greater in infertile rather than in fertile men (118; 124), controversial data have been published on the relationship between sperm DNA integrity and various indices of fertility, including fertilization, embryo cleavage, implantation, pregnancy and live birth rates of the offspring (for review see 125), both after IVF and ICSI. Conflicting results may be also related to the heterogeneous characteristics of the studies, since many factors exist that potentially affect the predictive power of tests determining sperm DNA damage (126). Among these, a very important one is the ability of the oocyte to repair sperm DNA damage. Such ability is, however, difficult to determine by current tools and in turn is assumed to depend on other factors including maternal age, the extension and the type of sperm DNA damage (127). Another important factor affecting the predictive power of the levels of sperm DNA damage is represented by the methods used to detect such damage (125), that are many and not equivalent. For instance, the two most popular techniques, TUNEL assay and SCSA, albeit well correlating (121), reveal different types of damage. Whereas TUNEL assay directly detects the DNA breakage, SCSA detects the ratio of denaturated DNA/ total DNA at cell level, induced by an acid treatment. Another important aspect is the lack of a standardized procedure for most of the technique, including TUNEL assay, that is expected to heavily flaw the reproducibility of the measures (128; 129). In addition, our group has shown that the presence in semen of variable amounts of M540 (apoptotic) bodies (45; 130) may affect the values of DNA fragmentation in subfertile patients, when assessed by flow cytometry. The issue of the technique points out the need for the an agreement on the standardized method to be used to decide whether or not sperm DNA damage could be have a clinical utility in the management of couple infertility.

Thanks to new tools to reveal 8-OHG, studies on sperm DNA damage due to oxidative stress are emerging (122; 131). Oxidative stress is one of the possible cause of sperm DNA fragmentation (132-133), inducing first 8-OHG and then DNA breakage (126).

Gaining insight on which, among the proposed mechanisms, is the main determinant of sperm DNA fragmentation could be important for setting new strategies to decrease the levels of DNA fragmentation in semen. Presently, only antixodants are available for clinical purpose. However, whether the administration of antioxidant is of clinical utility performed in the management of male infertility and to decrease sperm DNA fragmentation is an open question, as results of clinical studies so far showed limited efficacy.

5.2. Post-translational modifications of proteins by ubiquitination and sumoylation

Ubiquitination and sumoylation are post-translational modifications of proteins consisting of the reversible covalent attachment of the small peptides ubiquitin and SUMO (small ubiquitin related modifier) to the target proteins. These two modifications are implicated in the regulation of many cellular processes and in some cases they interact, resulting in antagonistic or synergistic effects (134).

Ubiquitin and SUMO create an isopeptide bond between their C-terminal glycine residue and the ε-amino group of a lysine residue of the target proteins. In both conjugation processes three classes of enzymes are involved: activating (E1) and conjugating (E2) enzymes and ligases (E3).

5.2.1. Ubiquitination

In somatic cells ubiquitin marks defective proteins committing them to 26S proteasome or lysosomes degradation (135); ubiquitin-proteasome pathway is implicated in a variety of cellular functions, such as membrane receptor-endocytosis (136), cell cycle progression (137), antigen presentation and retroviral infections (138). Moreover ubiquitination acts as a signal for the internalization and sorting of plasma membrane proteins (139).

Many studies focused on ubiquitin system in spermatogenesis and fertilization, proposing different functions at different levels (140). Ubiquitin is highly expressed in spermatogonia, spermatocytes, spermatids and Sertoli cells (141) and histone ubiquitination seems to have an important role during chromatin remodelling and gene expression at various levels of spermatogenesis (141-142).

Sutovsky's group proposed ubiquitination as a sperm quality control operating in the phase of sperm maturation during transit in epididymis. According to this hypothesis, surface defective spermatozoa are ubiquitinated for subsequent degradation and phagocytosis by epididymal cells (143). Ubiquitinated sperm found in the ejaculate may thus represent a portion of spermatozoa escaped from epididymal phagocytosis (143). The role of epididymis in sperm quality control is contested by Cooper *et al* (144), who argue that there are no evidences of a sperm loss during epididymal transit; moreover, histological and ultrastrucutral feautures of epididymal epithelial cells that resembling those of phagocytosis have never been reported.

In agreement with the occurrence of quality control of epididymal sperm and its failure in delete defective sperm, it has been showed that the global amount of ubiquitination in human semen negatively correlates with semen quality (145) and outcome of ART (146). However, in most of these studies, ubiquitination was evaluated in the total semen by flow cytometry, without selecting the population of interest (sperm) and without eliminating all the interferents (round cells, debris or M540 bodies) which may be present. In a study performed by our group on sperm ubiquitination in 45 subfertile subjects, the parameter was calculated in the population formed by only sperm (147). Our study demonstrated that M540 bodies, [see above (45,130)] are ubiquitinated and the ubiquitination of such elements correlates to poor semen quality (147). On the other hand, when only ubiquitinated spermatozoa are considered, positive correlations with semen quality are found (147). However, both normal and morphologically abnormal sperm are ubiquitinated (68; 145; 147), suggesting that ubiquitination may have additional and even opposite roles in sperm functions. To make the issue even more complex, in a recent study evaluating sperm head ubiquitination (excluding sperm labelled in other structures), within the population formed by only sperm, an association with unsuccessful outcome of ART was found (146), supporting the hypothesis that ubiquitination may have different roles depending on the sperm structures that are marked.

Evidences that ubiquitin-proteasome pathway is also implicated in zona pellucida penetration have been provided (148-149). Indeed zona pellucida proteins are ubiquitinated and are degradated upon sperm binding by sperm proteasomes (150). Moreover it has been recently suggested that acrosomal proteins of capacitated sperm could be ubiquitinated and then dagradated by acrosomal proteasomes to promote acrosomal exocytosis upon sperm contact with zona pellucida (148). A further possible function for ubiquitination has been proposed in the process of sperm mitochondria degradation occurring following fertilization to allow almost exclusive mitochondrial maternal inheritance (151). Ubiquitinated mitochondria may thus represent a target for the oocyte proteasoma.

In conclusion, further studies are necessary to determine and substantiate the hypothesis on the roles of ubiquitination in spermatogenesis, sperm functions and fertilization processes. In addition the identification of ubiquitinated proteins could be helpful to explain these ubiquitin functions.

5.2.2. Sumoylation

About a decade ago, a novel protein modification was discovered by Melchior' Laboratory (152). The new peptide shares 18% of overall identity with ubiquitin and for this reason it was named SUMO: Small Ubiquitin related Modifier. SUMO is a protein of ~11 kDa, highly conserved in eukaryotic cells (153). Four SUMO isoforms are present in mammals: SUMO1, 2, 3 and 4. While SUMO1 has a sequence identity of 50% with SUMO2 and SUMO3, SUMO2 and 3 have a 95% similarity (154) and for this reason are referred as SUMO2/3. SUMO removal from proteins is catalyzed by a family of sentrin/SUMO-specific proteases (SENPs) (155).

SUMO modifications are involved in different nuclear functions: transcriptional regulation (156), DNA repair (157) and protein trafficking (158). Besides nuclear functions, new roles for SUMO are suggested in other cellular compartments, although less studied for the moment. As an example, sumoylation of the mitochondrial protein DRP1 (dynamin-like GTPase) seems to be involved in the fragmentation of these organelles (159), maintaining the balance between mitochondrial fission and fusion.

A role in spermatogenesis has been also suggested for SUMO1 both in human (160) and in other species (161-162). SUMO1 was found localized to specific chromatin and other cellular domains in germ (spermatocytes and spermatids), mature (163) and testicular somatic (Leydig, Sertoli and peritubular myoepithelial cells) cells, supporting multiple and non-identical functions of SUMO during spermatogenesis

(160;162;164-165). Indeed, an involvement of this protein both in heterochromatin organization (160), meiotic sex chromosome inactivation (165) and regulation of gene expression (160) has been postulated. Moreover, it has been found an increase of sumo-conjugated proteins and the presence of SUMO close to the sites of DNA breaks in response to oxidative stress in mouse germ cells (166), suggesting a role for sumoylation both in DNA damage repair and in association with the cellular response to DNA damage, consistent with those reported in non-testicular cells (157).

Besides SUMO1, another isoform of the protein, SUMO2/3, has been detected in human germ cells, where hypo- or hyper-sumoylation is related to abnormal spermatogenesis (164). Recently, our group found the presence of both SUMO1 and SUMO2/3 in human ejaculate (163 and unpublished data). In particular our study demonstrated the occurrence of SUMO1 staining in live spermatozoa and its negative correlation with motility in asthenozoospermic subjects. SUMO1 is mainly localized in nucleus and it is occasionally found in the midpiece of ejaculated sperm. Overall, our results suggest a possible role for SUMO in the regulation of sperm motility (163).

These studies may give input to further research on the role of sumo-related modifications of proteins in spermatogenesis revealing new possible causes of male infertility.

6. CONCLUSIONS

In conclusion, several molecular markers can be proposed for each of the sperm functions necessary to fertilize the oocyte in human (see fig.1). For many of these markers, however, further studies are needed to identify the genetic disorder(s) underlying the lack of function in infertile men. Studies carried out in animal models need to be verified in human as difference in achieving the competence to fertilize have been demonstrated among mammalian species. Some of the genes which appear to be essential in animal KO models may be unessential or redundant in human. At the same time, knowledge deriving from *in vitro* studies, even if conducted with human spermatozoa, suffers from the important bias that the natural process occurs *in vivo* and thus in a likely different environment. In any case, even if ICSI appears at present a sort of panacea in the treatment of mild/severe male infertility, genetic and molecular studies on sperm functions are encouraged in order to develop new and less invasive strategies for the treatment of the infertile man.

7. ACKNOWLEDGMENT

Supported by a grant from Ministry of University and Scientific Research (PRIN project to E. Baldi), Ente Cassa di Risparmio di Firenze (project to Gianni Forti) and Tuscany Region (project to Gianni Forti).

8. REFERENCES

1. S Bhasin: Approach to the infertile man. *J Clin Endocrinol Metab* 92, 1995-2004 (2007)

2. F Shenfield, J de Mouzon, G Pennings, A P Ferraretti, A N Andersen, G de Wert, V Goossens; ESHRE Taskforce on Cross Border Reproductive Care: Cross border reproductive care in six European countries. *Hum Reprod* 25, 1361-1368 (2010)

3. W Verpoest, H Tournaye: ICSI: hype or hazard? *Hum Fertil* 9, 81-92 (2006)

4. M M Matzuk, D J Lamb: The biology of infertility: research advances and clinical challenges. *Nat Med* 14, 1197-1213 (2008)

5. World Health Organization: WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th edition, WHO Press, Geneva, Switzerland (2010)

6. T G Cooper, E Noonan, S von Eckardstein, J Auger, H W Baker, H M Behre, T B Haugen, T Kruger, C Wang, M. T Mbizvo, K M Vogelsong: World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 16, 231-245 (2010)

7. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions. 4th edition, Cambridge University Press, Cambridge, UK (1999)

8. R Menkveld: Clinical significance of the low normal sperm morphology value as proposed in the fifth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen. *Asian J Androl* 12, 47-58 (2010)

9. N E Skakkebaek: Normal reference ranges for semen quality and their relations to fecundity. *Asian J Androl* 12, 95-98 (2010)

10. D J Lamb: Semen analysis in 21st century medicine: the need for sperm function testing. *Asian J Androl* 12, 64-70 (2010)

11. R J Aitken: Sperm function tests and fertility. *Int J Androl* 29, 69-75 (2006)

12. M R Avenarius, M S Hildebrand, Y Zhang, N C Meyer, L L Smith, K Kahrizi, H Najmabadi, R J Smith: Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 84, 505-510 (2009)

13. B Nixon, D A MacIntyre, L A Mitchell, M K O'Bryan and R J Aitken: The identification of mouse sperm surface-associated proteins and characterization of their ability to act as decapacitation factors. *Biol Reprod* 74, 275-287 (2006)

14. S D Kholkute, P Meherji, C P Puri: Capacitation and the acrosome reaction in sperm from men with various semen profiles monitored by a chlortetracycline fluorescence assay. *Int J Androl* 15, 43-53 (1992)

15. M Ikawa, N Inoue, A M Benham, M Okabe: Fertilization: a sperm's journey to and interaction with the oocyte. *J Clin Invest* 120, 984-994 (2010)

16. A Minelli, L Liguori, I Bellazza, R Mannucci, B Johansson, B B Fredholm: Involvement of A1 adenosine receptors in the acquisition of fertilizing capacity. *J Androl* 25, 286-292 (2004)

17. C Wu, T Stojanov, O Chami, S Ishii, T Shimizu, A Li, C O'Neill: Evidence for the autocrine induction of capacitation of mammalian spermatozoa. *J Biol Chem* 276, 26962-26968 (2001)

18. L Cotton, G M Gibbs, L G Sanchez-Partida, J R Morrison, D M de Kretser, M K O'Bryan: FGFR-1 [corrected] signaling is involved in spermiogenesis and sperm capacitation. *J Cell Sci* 119, 75-84 (2006)

19. C Gyamera-Acheampong, M Mbikay: Proprotein convertase subtilisin/kexin type 4 in mammalian fertility: a review. *Hum Reprod Update* 15, 237-247 (2009)

20. C Rodeheffer, B D Shur: Sperm from beta1,4-galactosyltransferase I-null mice exhibit precocious capacitation. *Development* 131, 491-501 (2004)

21. C Gyamera-Acheampong, J Vasilescu, D Figeys, M Mbikay: PCSK4-null sperm display enhanced protein tyrosine phosphorylation and ADAM2 proteolytic processing during *in vitro* capacitation. *Fertil Steril* 93, 1112-1123 (2010)

22. I A Brewis, B M Gadella: Sperm surface proteomics: from protein lists to biological function. *Mol Hum Reprod* 16, 68-79 (2010)

23. F Secciani, L Bianchi, L Ermini, R Cianti, A Armini, G B La Sala, R Focarelli, L Bini, F Rosati: Protein profile of capacitated versus ejaculated human sperm. *J Proteome Res* 8, 3377-3389 (2009)

24. A J Travis, G S Kopf: The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *J Clin Invest* 110, 731-736 (2002)

25. N L Cross: Reorganization of lipid rafts during capacitation of human sperm. *Biol Reprod* 71, 1367-1373 (2004)

26. B S Jaiswal, M Conti: Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. *J Biol Chem* 276, 31698-31708 (2001)

27. M Luconi, C Krausz, G Forti, E Baldi: Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod* 55, 207-216 (1996)

28. A Carrera, J Moos, X P Ning, G L Gerton, J Tesarik, G S Kopf, S B Moss: Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. *Dev Biol* 180, 284-296 (1996)

29. F Xie, M A Garcia, A E Carlson, S M Schuh, D F Babcock, B S Jaiswal, J A Gossen, G Esposito, M van Duin, M Conti: Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. *Dev Biol* 296, 353-362 (2006)

30. P V Lishko, I L Botchkina, A Fedorenko, Y Kirichok: Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell* 140, 327-337 (2010)

31. E Baldi, R Casano, C Falsetti, C Krausz, M Maggi, G Forti: Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl* 12, 323-330 (1991)

32. K C Hess, B H Jones, B Marquez, Y Chen, T S Ord, M Kamenetsky, C Miyamoto, J H. Zippin, G S Kopf, S S Suarez, L R Levin, C J Williams, J Buck, S B Moss: The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev Cell* 9, 249-259 (2005)

33. P E Visconti, J L Bailey, G D Moore, D Pan, P Olds-Clarke, G S Kopf: Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 121, 1129-1137 (1995)

34. M A Baker, L Hetherington, H Ecroyd, S D Roman, R J Aitken: Analysis of the mechanism by which calcium negatively regulates the tyrosine phosphorylation cascade associated with sperm capacitation. *J Cell Sci* 117, 211-222 (2004)

35. C O'Flaherty, E de Lamirande, C Gagnon: Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radic Biol Med* 41, 528-540 (2006)

36. H Breitbart, S Rubinstein, L Nass-Arden: The role of calcium and Ca2+-ATPase in maintaining motility in ram spermatozoa. *J Biol Chem* 260, 11548-11553 (1985)

37. S A Belmonte, C I Lopez, C M Roggero, G A De Blas, C N Tomes, L S Mayorga: Cholesterol content regulates acrosomal exocytosis by enhancing Rab3A plasma membrane association. *Dev Biol* 285, 393-408 (2005)

38. S J Publicover, L C Giojalas, M E Teves, G S de Oliveira, A A Garcia, C L Barratt, C V Harper: Ca2+ signalling in the control of motility and guidance in mammalian sperm. *Front Biosci* 13, 5623-37 (2008)

39. L D Walensky, S H Snyder: Inositol 1,4,5-trisphosphate receptors selectively localized to the

acrosomes of mammalian sperm. *J Cell Biol* 130, 857-869 (1995)

40. R Felix: Molecular physiology and pathology of Ca2+-conducting channels in the plasma membrane of mammalian sperm. *Reproduction* 129, 251-262 (2005)

41. J Xia, D Ren: The BSA-induced Ca2+ influx during sperm capacitation is CATSPER channel-dependent. *Reprod Biol Endocrinol* 7, 119 (2009)

42. S A Adeoya-Osiguwa, L R Fraser: Evidence for Ca(2+)-dependent ATPase activity, stimulated by decapacitation factor and calmodulin, in mouse sperm. *Mol Reprod Dev* 44, 111-120 (1996)

43. H C Ho, S S Suarez: Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction* 122, 519-526 (2001)

44. B Marquez, G Ignotz, S S Suarez: Contributions of extracellular and intracellular Ca2+ to regulation of sperm motility: Release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. *Dev Biol* 303, 214-221 (2007)

45 M Muratori, I Porazzi, M Luconi, S Marchiani, G Forti, E Baldi: AnnexinV binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 25, 797-810 (2004)

46. D Y Liu, G N Clarke, H W Baker: Tyrosine phosphorylation on capacitated human sperm tail detected by immunofluorescence correlates strongly with sperm-zona pellucida (ZP) binding but not with the ZP-induced acrosome reaction. *Hum Reprod* 21, 1002-1008 (2006)

47. G Berruti, E Martegani: Identification of proteins cross-reactive to phosphotyrosine antibodies and of a tyrosine kinase activity in boar spermatozoa. *J Cell Sci* 93, 667-674 (1989)

48. P Grasa, C Colas, M Gallego, L Monteagudo, T Muiño-Blanco, J A Cebrián-Pérez: Changes in content and localization of proteins phosphorylated at tyrosine, serine and threonine residues during ram sperm capacitation and acrosome reaction. *Reproduction* 137, 655-667 (2009)

49. S Ficarro, O Chertihin, V A Westbrook, F White, F Jayes, P Kalab, J A Marto, J Shabanowitz, J C Herr, D F Hunt, P E Visconti: Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *J Biol Chem* 278, 11579-11589 (2003)

50. S Naaby-Hansen, A Mandal, M J Wolkowicz, B Sen, V A Westbrook, J Shetty, S A Coonrod, K L Klotz, Y H Kim, L A Bush, C J Flickinger, J C Herr: CABYR, a novel calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein involved in capacitation. *Dev Biol* 242, 236-254 (2002)

51. M Luconi, T Barni, G B Vannelli, C Krausz, F Marra, P A Benedetti, V Evangelista, S Francavilla, G Properzi, G Forti, E Baldi: Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biol Reprod* 58, 1476-1489 (1998a)

52. M Luconi, C Krausz, T Barni, G B Vannelli, G Forti, E Baldi: Progesterone stimulates p42 extracellular signal-regulated kinase (p42erk) in human spermatozoa. *Mol Hum Reprod* 4, 251-258 (1998b)

53. S Shadan, P S James, E A Howes, R Jones: Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biol Reprod* 71, 253-265 (2004)

54. M G Buffone, S V Verstraeten, J C Calamera, G F Doncel: High cholesterol content and decreased membrane fluidity in human spermatozoa are associated with protein tyrosine phosphorylation and functional deficiencies. *J Androl* 30, 552-558 (2009)

55. M A Baker, L Hetherington, R J Aitken: Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. *J Cell Sci* 119, 3182-3192 (2006)

56. C Lalancette, R L Faure, P Leclerc: Identification of the proteins present in the bull sperm cytosolic fraction enriched in tyrosine kinase activity: a proteomic approach. *Proteomics* 6, 4523-4540 (2006)

57. G Varano, A Lombardi, G Cantini, G Forti, E Baldi, M Luconi: Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa. *Hum Reprod* 23, 2652-2662 (2008)

58. D Krapf, E Arcelay, E V Wertheimer, A Sanjay, S H Pilder, A M Salicioni, P E Visconti: Inhibition of Ser/Thr phosphatases induces capacitation-associated signaling in the presence of Src kinase inhibitors. *J Biol Chem* 285, 7977-7985 (2010)

59. K N Jha, A M Salicioni, E Arcelay, O Chertihin, S Kumari, J C Herr, P E Visconti: Evidence for the involvement of proline-directed serine/threonine phosphorylation in sperm capacitation. *Mol Hum Reprod* 12, 781-789 (2006)

60. M Muratori, M Luconi, S Marchiani, G Forti, E Baldi: Molecular markers of human sperm functions. *Int J Androl* 32, 25-45 Review (2009)

61. M Luconi, G Forti, E Baldi: Pathophysiology of sperm motility. *Front Biosci* 11, 1433-1447 (2006)

62. M Wald: Enhancement of human sperm motility. *J Urol* 180, 442-443 (2008)

63. E M Eddy, K Toshimori, D A O'Brien: Fibrous sheath of mammalian spermatozoa. *Microsc Res Tech* 61, 103-115 (2003)

64. J M Nascimento, L Z Shi, J Tam, C Chandsawangbhuwana, B Durrant, E L Botvinick, M W Berns: Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. *J Cell Physiol* 217, 745-751 (2008)

65. M Luconi, G Cantini, E Baldi, G Forti: Role of A-kinase anchoring proteins (AKAPs) in reproduction. *Front Biosc* in press (2010)

66. H E Chemes, Y V Rawe: Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperm phenotypes in infertile men. *Hum Reprod Update* 9, 405-428 (2003)

67. R M Turner, M P Musse, A Mandal, K Klotz, F C Jayes, J C Herr, G L Gerton, S B Moss, H E Chemes: Molecular genetic analysis of two human sperm fibrous sheath proteins, AKAP4 and AKAP3, in men with dysplasia of the fibrous sheath. *J Androl* 22, 302-315 (2001)

68. B Baccetti, G Collodel, L Gambera, E Moretti, F Serafini, P Piomboni: Fluorescence *in situ* hybridization and molecular studies in infertile men with dysplasia of the fibrous sheath. *Fertil Steril* 84, 123-129 (2005)

69. M Luconi, V Carloni, F Marra, P Ferruzzi, G Forti, E Baldi: Increased phosphorylation of AKAP by inhibition of phosphatidylinositol 3-kinase enhances human sperm motility through tail recruitment of protein kinase A. *J Cell Sci* 117, 1235-1246 (2004)

70. M Luconi, I Porazzi, P Ferruzzi, S Marchiani, G Forti, E Baldi: Tyrosine phosphorylation of the a kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. *Biol Reprod* 72, 22-32 (2005a)

71. R W Baxendale, L R Fraser: Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. *Mol Reprod Dev* 66, 181-189 (2003)

72. G Esposito, B S Jaiswal, F Xie, M A Krajnc-Franken, T J Robben, A M Strik, C Kuil, R L Philipsen, M van Duin, M. Conti, J A Gossen: Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proc Natl Acad Sci USA* 101, 2993-2998 (2004)

73. K A Burton, G S McKnight: PKA, germ cells, and fertility. *Physiology* 22, 40-46 (2007)

74. B S Skålhegg, Y Huang, T Su, R L Idzerda, G S McKnight, K A Burton: Mutation of the Calpha subunit of PKA leads to growth retardation and sperm dysfunction. *Mol Endocrinol* 16, 630-639 (2002)

75. K A Burton, B Treash-Osio, C H Muller, E L Dunphy, G S McKnight: Deletion of type IIalpha regulatory subunit delocalizes protein kinase A in mouse sperm without affecting motility or fertilization. *J Biol Chem* 274, 24131-24136 (1999)

76. M A Nolan, D F Babcock, G Wennemuth, W Brown, K A Burton, G S McKnight: Sperm-specific protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. *Proc Natl Acad Sci USA* 101, 13483-13488 (2004)

77. D J Morgan, M Weisenhaus, S Shum, T Su, R Zheng, C Zhang, K M Shokat, B Hille, D F Babcock, G S McKnight: Tissue-specific PKA inhibition using a chemical genetic approach and its application to studies on sperm capacitation. *Proc Natl Acad Sci U S A* 105, 20740-20745 (2008)

78. P E Visconti: Understanding the molecular basis of sperm capacitation through kinase design. *Proc Natl Acad Sci U S A* 106, 667-668 (2009)

79. R M Turner: Moving to the beat: a review of mammalian sperm motility regulation. Reprod Fertil Dev 18, 25-38 (2006)

80. R Yunes, G F Doncel, A A Acosta: Incidence of sperm-tail tyrosine phosphorylation and hyperactivated motility in normozoospermic and asthenozoospermic human sperm samples. Biocell 27, 29-36 (2003)

81. R M Turner, M P Musse, A Mandal, K Klotz, F C Jayes, J C Herr, G L Gerton, S B Moss, H E Chemes: Molecular genetic analysis of two human sperm fibrous sheath proteins, AKAP4 and AKAP3, in men with dysplasia of the fibrous sheath. J Androl 22, 302-315 (2001a)

82. R M Turner, J A Foster, G L Gerton, S B Moss, P Patrizio: Molecular evaluation of two major human sperm fibrous sheath proteins, pro-hAKAP82 and hAKAP82, in stump tail sperm. *Fertil Steril* 76, 267-274 (2001b)

83. I M Aparicio, M C Gil, M Garcia-Herreros, F J Pena, L J Garcia-Marin: Inhibition of phosphatidylinositol 3-kinase modifies boar sperm motion parameters. *Reproduction* 129, 283-289 (2005)

84. L Liguori, E de Lamirande, A Minelli, C Gagnon: Various protein kinases regulate human sperm acrosome reaction and the associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif. *Mol Hum Reprod* 11, 211-221 (2005)

85. N Etkovitz, S Rubinstein, L Daniel, H Breitbart: Role of PI3-kinase and PI4-kinase in actin polymerization during bovine sperm capacitation. *Biol Reprod* 77, 263-273 (2007)

86. M K Jungnickel, K A Sutton, Y Wang, H M Florman: Phosphoinositide-dependent pathways in mouse sperm are regulated by egg ZP3 and drive the acrosome reaction. *Dev Biol* 304, 116-126 (2007)

87. V Nauc, E de Lamirande, P Leclerc, C Gagnon: Inhibitors of phosphoinositide 3-kinase, LY294002 and wortmannin, affect sperm capacitation and associated

phosphorylation of proteins differently: Ca2+-dependent divergences. *J Androl* 25, 573-585 (2004)

88. M J Bragado, I M Aparicio, M C Gil, L J Garcia-Marin: Protein kinases A and C and phosphatidylinositol 3 kinase regulate glycogen synthase kinase-3A serine 21 phosphorylation in boar spermatozoa. *J Cell Biochem* 109, 65-73 (2010)

89. H Breitbart, T Rotman, S Rubinstein, N Etkovitz: Role and regulation of PI3K in sperm capacitation and the acrosome reaction. *Mol Cell Endocrinol* 314, 234-238 (2010)

90. L A Mitchell, B Nixon, M A Baker, R J Aitken: Investigation of the role of SRC in capacitation-associated tyrosine phosphorylation of human spermatozoa. *Mol Hum Reprod* 14, 235-243 (2008)

91. T Almog, S Lazar, N Reiss, N Etkovitz, E Milch, N Rahamim, M Dobkin-Bekman, R Rotem, M Kalina, J Ramon, A Raziel, H Breitbart, R Seger, Z Naor: Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality. *J Biol Chem* 283, 14479-14489 (2008)

92. H Qi, M M Moran, B Navarro, J A Chong, G Krapivinsky, L Krapivinsky, Y Kirichok, I S Ramsey, T A Quill, D E Clapham: All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci USA* 104, 1219-1223 (2007)

93. S Publicover, C V Harper, C Barratt: [Ca2+]i signalling in sperm--making the most of what you've got. *Nat Cell Biol* 9, 235-242 (2007)

94. P Nikpoor, S J Mowla, M Movahedin, S A Ziaee, T Tiraihi: CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. *Hum Reprod* 19, 124-128 (2004)

95. T A Quill, S A Sugden, K L Rossi, L K Doolittle, R E Hammer, D L Garbers: Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci USA* 100, 14869–14874 (2003)

96. J Espino, M Mediero, G M Lozano, I Bejarano, A Ortiz, J F García, J A Pariente, A B Rodríguez: Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic patients. *Reprod Biol Endocrinol* 7, 11 (2009)

97. A Darszon, J J Acevedo, B E Galindo, E O Hernández-González, T Nishigaki, C L Treviño, C Wood, C Beltrán: Sperm channel diversity and functional multiplicity. *Reproduction* 131, 977-988 (2006)

98. Y Kirichok, B Navarro, D E Clapham: Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature* 439, 737-740 (2006)

99. J L Yovich: Pentoxifylline: actions and applications in assisted reproduction. *Hum Reprod* 8, 1786-1791 (1993)

100. T Mostafa: Tadalafil as an *in vitro* sperm motility stimulant. *Andrologia* 39, 12-15 (2007)

101. G M Centola, R J Cartie, C Cox: Differential responses of human sperm to varying concentrations of pentoxyfylline with demonstration of toxicity. *J Androl* 16, 136-142 (1995)

102. M Luconi, S Torcia, D Grillo, M T Fiorenza, G Forti, F Mangia, E Baldi: Enhancement of mouse sperm motility by the PI3-kinase inhibitor LY294002 does not result in toxic effects on preimplantation embryo development. *Hum Reprod* 20, 3500-3504 (2005b)

103. S Hayasaka, Y Terada, N Inoue, M Okabe, N Yaegashi, K Okamura: Positive expression of the immunoglobulin superfamily protein IZUMO on human sperm of severe male infertile patients. *Fertil Steril* 88, 214-216 (2007)

104. V Granados-Gonzalez, I Aknin-Seifer, R L Touraine, J Chouteau, J P Wolf, R Levy: Preliminary study on the role of the human IZUMO gene in oocyte-spermatozoa fusion failure. *Fertil Steril* 90, 1246-1248 (2008)

105. B Baibakov, L Gauthier, P Talbot, T L Rankin, J Dean: Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. *Development* 134, 933-943 (2007)

106. K Bedu-Addo, C L Barratt, J C Kirkman-Brown, S J Publicover: Patterns of [Ca2+](i) mobilization and cell response in human spermatozoa exposed to progesterone. *Dev Biol* 302, 324-332 (2007)

107. M Luconi, L Bonaccorsi, C Krausz, G Gervasi, G Forti, E Baldi: Stimulation of protein tyrosine phosphorylation by platelet-activating factor and progesterone in human spermatozoa. *Mol Cell Endocrinol* 108, 35-42 (1995)

108. J L Frederick, M M Francis, T M Macaso, R A Lobo, M V Sauer, R J Paulson: Preovulatory follicular fluid steroid levels in stimulated and unstimulated cycles triggered with human chorionic gonadotropin. *Fertil Steril* 55, 44-47 (1991)

109. E R Roldan, T Murase, Q X Shi: Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science* 266, 1578-1581 (1994)

110. C Krausz, L Bonaccorsi, P Maggio, M Luconi, L Criscuoli, B Fuzzi, S Pellegrini, G Forti, E Baldi: Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Hum Reprod* 11, 1661-1667 (1996)

111. E Baldi, M Luconi, M Muratori, S Marchiani, L Tamburrino, G Forti: Nongenomic activation of

spermatozoa by steroid hormones: facts and fictions. *Mol Cell Endocrinol* 308, 39-46 (2009)

112. J N Correia, S J Conner, J C Kirkman-Brown: Non-genomic steroid actions in human spermatozoa. "Persistent tickling from a laden environment". *Semin Reprod Med* 25, 208-219 (2007)

113. M A Baker, G Reeves, L Hetherington, J Müller, I Baur, R J Aitken: Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin appl* 1, 524-532 (2007)

114. J A Shaman, W S Ward: Sperm chromatin stability and susceptibility to damage in relation to its structure. In: The sperm cell: production, maturation, fertilization, regeneration. Eds: C De Jonge, C Barrat, Cambridge University press, pp. 31-48 (2006)

115. S E Lewis, R J Aitken: DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* 322, 33-41 (2005)

116. D Sakkas, E Mariethoz, G Manicardi, D Bizzaro, P G Bianchi, U Bianchi: Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 4, 31-37 (1999)

117. J Blanco-Rodríguez, C Martínez-García: Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids. *Biol Reprod* 61, 1541-1547 (1999)

118. J Erenpreiss, M Spano, J Erenpreisa, M Bungum, A Giwercman: Sperm chromatin structure and male fertility: biological and clinical aspects. *Asian J Androl* 8, 11-29 (2006)

119. Q Shi, R H Martin: Aneuploidy in human sperm: a review of the frequency and distribution of aneuploidy, effects of donor age and lifestyle factors. *Cytogenet Cell Genet* 90, 219-226 (2000)

120. G C Manicardi, A Tombacco, D Bizzaro, U Bianchi, P G Bianchi, D Sakkas: DNA strand breaks in ejaculated human spermatozoa: comparison of susceptibility to the nick translation and terminal transferase assays. *Histochem J* 30, 33-39 (1998)

121. G R Aravindan, J Bjordahl, L K Jost, D P Evenson: Susceptibility of human sperm to *in situ* DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res* 236, 231-237 (1997)

122. G N De Iuliis, L K Thomson, L A Mitchell, J M Finnie, A J Koppers, A Hedges, B Nixon, R J Aitken: DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 81, 517-524 (2009)

123. M Muratori, P Piomboni, E Baldi, E Filimberti, P Pecchioli, E Moretti, L Gambera, B Baccetti, R Biagiotti, G Forti, M Maggi: Functional and ultrastructural features of DNA fragmented human sperm. *J Androl* 21, 903–912 (2000)

124. M Sergerie, G Laforest, L Bujan, F Bissonnette, G Bleau: Sperm DNA fragmentation: threshold value in male fertility. *Hum Reprod* 20, 3446-3451 (2005)

125. A Zini, J M Boman, E Belzile, A Ciampi: Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 23, 2663-2668 (2008)

126. D Sakkas, J G Alvarez: Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 93, 1027-1036 (2010)

127. Y Menezo, B Dale, M Cohen: DNA Damage and Repair in Human Gametes and Embryos: A Review. *Zygote* in press (2010)

128. M Muratori, L Tamburrino, V Tocci, A Costantino, S Marchiani, C Giachini, I Laface, C Krausz, M C Meriggiola, G Forti, E Baldi: Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl* in press (2009)

129. M Muratori, L Tamburrino, S Marchiani, C Guido, G Forti, E Baldi: Critical Aspects of detection of sperm DNA fragmentation by TUNEL/Flow cytometry. *Syst Biol Reprod Med* 56, 277-285 (2010)

130. S Marchiani, L Tamburrino, A Maoggi, G B Vannelli, G Forti, E Baldi, M Muratori: Characterization of M540 bodies in human semen: evidence that they are apoptotic bodies. *Mol Hum Reprod* 13, 621-631 (2007)

131. R Santiso, M Tamayo, J Gosálvez, M Meseguer, N Garrido, J L Fernández: Simultaneous determination *in situ* of DNA fragmentation and 8-oxoguanine in human sperm. *Fertil Steril* 93, 314-318 (2010)

132. M Muratori, S Marchiani, M Maggi, G Forti, E Baldi: Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 11, 1491-1499 (2006)

133. R J Aitken, G N De Iuliis: On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 16, 3-13 (2010)

134. A Denuc, G Marfany: SUMO and ubiquitin paths converge. *Biochem Soc Trans* 38, 34-39 (2010)

135. M Hochstrasser: Ubiquitin-dependent protein degradation. *Annu Rev Genet* 30, 405–439 (1996)

136. G J Strous, R Govers: The ubiquitin –proteasome system and endocytosis. *J Cell Sci* 112, 1417-1423 (1999)

137. M Glotzer, A W Murray, M W Kirschner: Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132-138 (1991)

138. D E Ott, L V Coren, T D Copeland, B P Kane, D G Johnson, R C Sowder 2nd, Y Yoshinaka, S Oroszlan, L O Arthur, L E Henderson: Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. 1. *J Virol* 72, 2962-2968 (1998)

139. F Acconcia, S Sigismund, S Polo: Ubiquitin in trafficking: the network at work. *Exp Cell Res* 315, 1610-1618 (2009)

140. P Sutovsky: Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization and sperm quality control: killing three birds with one stone. *Microsc res tech* 61, 88-102 (2003)

141. W M. Baarends, J W Hoogerbrugge, H P Roest, M Ooms, J Vreeburg, J H Hoeijmakers, J A Grootegoed: Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. *Dev Biol* 207, 322-333 (1999)

142. J Y An, E A Kim, Y Jiang, A Zakrzewska, D E Kim, M J Lee, I Mook-Jung, Y Zhang, Y T Kwon: UBR2 mediates transcriptional silencing during spermatogenesis via histone ubiquitination. *Proc Natl Acad Sci U S A* 107, 1912-1917 (2010)

143. P Sutovsky, R Moreno, J Ramalho-Santos, T Dominko, W E Thompson, G Schatten: A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci* 114, 1665-1675 (2001)

144. T G Cooper, C H Yeung, R Jones, M C Orgebin-Crist, B Robaire: Rebuttal of a role for the epididymis in sperm quality control by phagocytosis of defective sperm. *J Cell Sci* 115, 5-7 (2001) Comment on*: J Cell Sci* 114, 1665-1675 (2002)

145. S Varum, C Bento, A P Sousa, C S Gomes-Santos, P Henriques, T Almeida-Santos, C Teodósio, A Paiva, J Ramalho-Santos: Characterization of human sperm populations using conventional parameters, surface ubiquitination, and apoptotic markers. *Fertil Steril* 87, 572-583 (2007)

146. C Ozanon, J Chouteau, P Sutovsky: Clinical adaptation of the sperm ubuquitin tag immunoassay (SUTI): relationship of sperm ubiquitylation with sperm quality in gradient-purified semen samples from 93 men from a general infertility clinic population. *Hum Reprod* 20, 2271-2278 (2005)

147. M Muratori, S Marchiani, G Forti, E Baldi: Sperm ubiquitination positively correlates to normal morphology in human semen. *Hum Reprod* 20, 1035-1043 (2005)

148. S Zimmerman, P Sutovsky: The sperm proteasome during sperm capacitation and fertilization. *J Reprod Immunol* 83, 19-25 (2009)

149. R K Naz, L Dhandapani: Identification of human sperm proteins that interact with human zona pellucida3 (ZP3) using yeast two-hybrid system. *J Reprod Immunol* 84, 24-31 (2010)

150. H Sawada, N Sakai, Y Abe, E Tanaka, Y Takahashi, J Fujino, E Kodama, S Takizawa, H Yokosawa: Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. *Proc Natl Acad Sci U S A* 99, 1223-1228 (2002)

151. P Sutovsky, R D Moreno, J Ramalho-Santos, T Dominko, C Simerly, G Schatten: Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biol Reprod* 63, 582-590 (2000)

152. R Mahajan, C Delphin, T Guan, L Gerace, F Melchior: A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88, 97-107 (1997)

153. A M Andreou, N Tavernarakis: Sumoylation and cell signalling. *Biotechnol J* 4, 1740-1752 (2009)

154. H Saitoh, J Hinchey: Functional heterogeneity of small ubiquitin related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol* Chem 275, 6252-6258 (2000)

155. E T Yeh: SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem* 284, 8223-8227 (2009)

156. D W Girdwood, M H Tatham, R T Hay: SUMO and transcriptional regulation. *Semin Cell Dev Biol* 15, 201-210 (2004)

157. S Bergink, S Jentsch: Principles of ubiquitin and SUMO modifications in DNA repair. *Nature* 458, 461-467 (2009)

158. M J Matunis, E Coutavas, G Blobel: A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *Cell Biol* 135, 1457-1470 (1996)

159. R Zunino, A Schauss, P Rippstein, M Andrade-Navarro, H M McBride: The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. *J Cell Sci* 120, 1178-1188 (2007)

160. M Vigodner, T Ishikawa, P N Schlegel, P L Morris: SUMO-1, human male germ cell development, and the androgen receptor in the testis of men with normal and abnormal spermatogenesis. *Am J Physiol Endocrinol Metab* 290, E1022-1033 (2006)

161. R S Rogers, A Inselman, M A Handel, M J Matunis: SUMO modified proteins localize to the XY body of pachytene spermatocytes. *Chromosoma* 113, 233-243 (2004)

162. M Vigodner, P L Morris: Testicular expression of small ubiquitin-related modifier-1 (SUMO-1) supports multiple roles in spermatogenesis: silencing of sex chromosomes in spermatocytes, spermatid microtubule nucleation, and nuclear reshaping. *Dev Biol* 282, 480-492 (2005)

163. S Marchiani, L Tamburrino, L Giuliano, D Nosi, V Sarli, L Gandini, P Piomboni, G Belmonte, G Forti, E Baldi, M Muratori: Sumo1-ylation of human spermatozoa and its relation with semen quality. *Int J Androl* in press (2010)

164. P W Brown, K Hwang, P N Schlegel, P L Morris: Small ubiquitin-related modifier (SUMO)-1, SUMO-2/3 and SUMOylation are involved with centromeric heterochromatin of chromosomes 9 and 1 and proteins of the synaptonemal complex during meiosis in men. *Hum Reprod* 23, 2850-2857 (2008)

165. M Vigodner: Sumoylation precedes accumulation of phosphorylated H2AX on sex chromosomes during their meiotic inactivation. *Chromosome Res* 17, 37-45 (2009)

166. V Shrivastava, M Pekar, E Grosser, J Im, M Vigodner: SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells. *Reproduction* in press (2010)

Key Words: Acrosome Reaction, Capacitation, Sperm-Zona Interaction, Sperm-Oocyte Fusion

Send correspondence to: Elisabetta Baldi, Dept. of Clinical Physiopathology, Andrology Unit,University of Florence, Viale Pieraccini, 6, I50139 Florence, Italy, Tel: 390554271486, Fax: 390554271371, E-mail: e.baldi@dfc.unifi.it

http://www.bioscience.org/current/vol16.htm