7

A Systems Biology Approach to Understanding Male Infertility

Nicola Bernabò, Mauro Mattioli and Barbara Barboni
University of Teramo
Italy

1. Introduction

Once ejaculated, mammalian spermatozoa have undergone spermatogenesis and spermiogenesis but are still unable to fertilize the oocyte. In many species, including human, male gametes become able to fertilize only upon they reside in the female genital tract for a finite period, ranging from hours to days. During this window of time important physical-chemical modifications, collectively known as "capacitation", occur, involving all the sperm biochemical machinery and conferring to male gametes the ability to fertilize. During capacitation, in one hand, spermatozoa gradually lose the decapacitating factors of epididimal origin and, on the other hand, progressively interact with regulating (either inhibiting and activating) factors present in oviductal fluid and with the tubal epithelium. Capacitation is completed when the sperm cells can successfully recognise the oocyte and extrude the acrosomal vescicle content (acrosome reaction, AR), thus penetrating the zona pellucida (ZP) and reaching the oocyte membrane.

During capacitation spermatozoa undergo important biochemical and structural modifications involving sperm head as well as sperm tail. In particular, at the head level, the membranes markedly change their architecture, tanks to their highly dynamical structure. In fact, differently from what has been believed until about ten years ago, the sperm membrane organization differs from the classical fluid mosaic model that predict a random lateral diffusion of lipids and proteins (Abou-haila & Tulsiani, 2009; Nixon & Aitken, 2009). The data from several Laboratories converge in depicting the sperm membranes as highly asymmetrical structures, either in longitudinal (membrane domains and sub-domains) (Bruckbauer et al., 2010; Gadella et al.,2008; Kotwicka et al. 2011; Venditti & Bean, 2009) and transversal (inner and outer leaflet) direction (Gadella & Harrison 2000, 2002; Harrison & Gadella 2005). Particularly in the head plasma membrane (PM) it is possible to recognize different domains, with different chemical-physical and functional proprieties: the apical ridge area, the pre-equatorial area, the equatorial area and the post-equatorial area. The apical ridge is involved in sperm-ZP binding and contains specific zona binding proteins (O'Rand & Fischer, 1987). The pre-equatorial surface is the site where the fusion between PM and outer acrosome membrane (OAM) takes place during AR, while the equatorial surface area of sperm head does not participate to the AR, but it is characterized by the presence of a hairpin like structure that is involved in the fusion of sperm and oocyte membranes at the moment of fertilization (for review see Yanagimachi, 1994 and Gadella et

al., 2008). Each domain, in turn, contains specialized areas, known as microdomains. These microdomains could be isolated from the membrane, by using 0.1% Triton X-100 or similar detergent at 4°C in a discontinuous density gradient, as detergent resistant membrane (DRM). DRMs are small lipid ordered portions of membrane that contain larger amounts of cholesterol, sphingomyelin, gangliosides, phospholipids with saturated long-chain acyl chains, and proteins such as GPI anchored proteins, caveolin and flotillin. During capacitation their organization changes, leading the association and activation of proteins involved in signal transduction, such as CB1R, TRPV1, (Botto et al., 2010) and in membrane fusion (Asano et al., 2010) (see Brewis & Gadella 2010 for review).

In addition, as in others mammalian cell types, the different composition of inner and outer leaflets is different (Gadella et al., 2008; Hickey & Buhr 2011; Müller et al., 1996). Particularly the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are concentrated in the inner leaflet and the choline phospholipids sphingomyelin (SM) and phosphatidylcholine (PC) in the outer leaflet. This asymmetry is established and maintained by the action of several translocating enzymes with differing phospholipid specificities (for review, see Bevers et al., 1998). For instance aminophospholipid translocase (also known as flippase), is responsible for transfer of PS and PE from the outer to the inner lipid leaflet, 'floppase' transfers phospholipids from inner to outer leaflet and scramblase acts as a bi-directional carrier with little specificity, simply moving all four phospholipid species in both directions (inward and outward) across the membrane lipid bilayer, thus reducing phospholipid asymmetry ('lipid scrambling'). This last event plays a key role in the acquisition of PM and OAM tendency to fuse each other (fusogenicity), increasing their fluidity.

Lipid remodeling of sperm membranes is controlled by an integrate dialogue between activating and inhibiting stimuli. In particular after ejaculation, before the seminal plasma is completely removed, the endocannabinoids concentration is high, the phospholipid scrambling is blocked and cholesterol has a wide-spread lateral localization in the sperm head PM. After the complete removal of seminal plasma and with the contact with female genital tract secretion, the concentration of endocannabionoids gradually decreases while the bicarbonate increases. As a consequence soluble adenylate cyclase (sAC) is activated in the sperm head and cAMP/PKA pathway becames effective stimulating phospholipid scrambling in the apical plasma membrane of the sperm head (Gadella & Harrison 2000). This process enable the extracellular protein to extract cholesterol, thus the membranes can complete its reorganization with the increase of the PM and OAM fusogenicity, which is the necessary prerequisite for AR.

This dynamical evolution of membranes is paralleled by a massive reorganization of other cellular components, in particular of head cytoskeleton. In fact in spermatozoa the cytosol is virtually absent, thus the PM is in direct contact with the diverse underlying cytoskeleton structures, which are highly organized and whose architecture changes during capacitation. It is believed that actin cytoskeleton may have a structural role during capacitation and AR: when fusogenicity of PM and OAM increases, a network of actin develops acting as a diaphragm between the two membranes avoiding their premature fusion. Once the capacitation was completely achieved and the physiological stimulus (ZP proteins) was detected, the ZP-induced calcium peak causes the fast depolymerisation of actin structure, thus allowing fusion of PM and OAM and, ultimately, AR (Breitbart et al., 2005; Breitbart & , Etkovitz, 2011).

In sperm tail the most evident changes are related to the motility pattern. When stored in epididymis spermatozoa are completely immotile or weakly motile, while immediately after ejaculation, they begin to swim with a species-specific pattern. Once exposed to capacitating condition, either in physiological context or in an artificial environment, male gametes gradually express a new pattern of motion, the hyperactivated motility. This process was first decribed in 1969 by Yanagimachi (Yanagimachi, 1969), who reported that the spermatozoa able to fertilize the oocyte showed a different and more vigorous swimming pattern than those functionally immature. In human, the flagella of hyperactivated spermatozoa beat less symmetrical than flagella of ejaculated spermatozoa and, as a result, they tend to swim vigorously in circles. It was proposed that the acquisition of this motility pattern is functional to allow spermatozoa to penetrate the oviductal mucus, the cumuls-oocyte complex extracellular matrix and, finally, the ZP (Chang & Suarez, 2010; Suarez 2008).

The onset of hyperactivated motility is due to the activation of a complex biochemical pathway, still under investigation (see Suarez 2008 for review). At present it is known that the interaction with female environment stimulates a signaling cascade that leads to the increase in intracellaular concentration of second messengers, in particular of Ca^{2+} and cAMP. Specifically the phospholipase C (PLC) is activated through a heterotrimeric G protein (Gq/11)-coupled receptor (R1) and produces IP3. Binding of IP3 to its receptors (IP3R) causes an increase in cytoplasmic Ca^{2+}. The activation of membrane-associated adenylyl cyclase (AC) through high cytoplasmic Ca^{2+}, G proteins and membrane potential increases intracellular cAMP. In parallel the bicarbonate may also cause an increase in cAMP by activating the soluble form of adenylyl cyclase (sAC) directly. The increase in cAMP concentration activates cyclic nucleotide-gated channels (CNG) thus promoting the Ca^{2+} influx. In addition increased cAMP activates protein kinase A (PKA) to phosphorylate axonemal or fibrous sheath proteins and results in flagellar beating. It is thought that high cytoplasmic Ca^{2+} and Ca^{2+}–calmodulin complex are responsible for asymmetrical bending of flagella that is characteristic of hyperactivation.

Anyway at the end of capacitation, of the millions of sperm normally ejaculated, only thousands reach the isthmus of the oviduct and only a few reach the ampulla, where the fertilization takes place. During this process two important functional events are accomplished: only the more motile spermatozoa are selected, thus increasing the success rate of fertilization and decreasing the risk of polyspermy, and their fertilizing ability is maintained for a relatively long period, until the oocyte is ovulated.

Because of its important implications both for basic (developmental biology, endocrinology, biochemistry) and applied science (andrology, male infertility, contraception) these events are under the attention of Researcher starting from the pionieristic works of Austin (Austin, 1951) and Chang (Chang, 1951), that, contemporaneously and independently, proposed the concept of capacitation in the far 1951. During last 60 years a myriad of molecular data was obtained and the knowledge of biochemical aspects of capacitation was markedly increased but, despite the large amount of literature and the amazing diffusion of reproduction biotechnologies, many aspects of these events are still unsatisfactory known. In particular, this deficiency is made evident by the impossibility to *a priori* identify a capacitated spermatozoon. At present the only available tests in andrology are able to *ex post* evaluate the ability of spermatozoa to acquire a particular staining pattern (CTC) or to respond to

different physiological stimuli (induction of AR, IVF). The laboratory tests usually carried out in andrology laboratories are:

- assessment of chlortetracycline staining pattern. The chlortetracycline (CTC), a UV fluorescent antibiotic, yields different patterns of distribution on the sperm surface depending on the time and conditions of the sperm incubation. These different patterns were first described in the mouse by Saling and Storey (1979), and the correlation of these patterns with the capacitation status of the sperm was subsequently empirically defined by different Authors in several species including Human (Perry et al., 1995), mice (Fraser & Herod, 1990), bull (Fraser et al., 1995), boar (Mattioli et al., 1996), etc … The advantage of this method is that it seems to relate with the capacitation status of the sperm independently of the acrosome reaction. The disadvantage is that the mechanism by which CTC yields the different patterns is not clearly understood, and the biochemical events involved in capacitation that give rise to these patterns are completely unknown. It has been suggested that changes in the distribution of Ca^{2+}-CTC complexes bound to phospholipids in the plasma membrane are responsible for the different patterns observed, thus any compound that interferes whit this event without affecting the acquisition of spermatozoa fertilizing ability, could potentially be interpreted as changing the sperm capacitation state. In addition it was found that spermatozoa treated with cytochalasin D, a specific actin polymerization inhibitor, that display a capacitation-related pattern of fluorescence, were unable to undergo AR, when tested by induction of acrosome reaction by homologous ZP (Bernabò et al., 2011). This finding suggests the idea that CTC could changes as a consequence of the membrane lipid remodeling occurring during capacitation rather than depending on the real capacitation status of male gametes.
- induction of the Acrosome Reaction. The capacitation could be defined as the ability of a spermatozoon to undergo AR when stimulated by more or less physiologically relevant agent, such as the calcium ionophore A23187, homologous ZP or progesteron. A problem with these assays is that compounds that are able to stimulate or inhibit the acrosome reaction cannot be assumed to do so by stimulating or inhibiting capacitation. In other words, acrosome reactions might be able to occur in uncapacitated sperm when the cells are treated with compounds that bypass capacitation.
- zona-free hamster oocyte penetration test: this test is aimed to provides information on the fusinogenic nature of capacitated sperm head membranes. The fusion of human spermatozoa with the hamster oocyte is functionally the same as that with the human vitelline membrane, since it is initiated by the plasma membrane overlying the equatorial segment of acrosome-reacted human spermatozoa (World Health Organization [WHO], 2010). Unfortunately, this test may result in two types of criticism: 1) it differs from the physiological situation in that the ZP is present; 2) the results of conventional hamster oocyte test depends on the occurrence of spontaneous acrosome reactions in populations of spermatozoa incubated for prolonged periods in vitro. Since this procedure is less efficient than the biological process and may involve different mechanisms, false-negative results (men whose spermatozoa fail in the hamster oocyte test but successfully fertilize human oocytes in vitro or in vivo) have frequently been recorded (WHO,1986).
- in vitro fertilization (IVF). The most reliable way to assess capacitation is to perform *in vitro* fertilization assays. In this case also, it cannot be assumed that if a specific

compound/incubation condition inhibits *in vitro* fertilization it really inhibits capacitation, since fertilization is a multistep process that involves various aspects of sperm physiology (e.g., motility, acrosome reaction) as well as the interaction with the oocyte (e.g., ZP binding, plasma membrane binding, and/or fusion). Moreover, the concentration of sperm used in the *in vitro* fertilization assays may dramatically impact interpretation of results since these assays normally use a much higher sperm/egg ratio than is normally encountered *in vivo*. It is also time-consuming and expensive to perform and, finally, has evident ethical limitation in Humans.

From these consideration it is evident that, at the present, a reliable *a priori* marker of capacitation is unavailable. Thus, in several cases, it is impossible for clinicians and andrologists to perform an adequate diagnosis (and as a consequence a therapy and a prognosis) after seminal and clinical investigations, as it happens in the case of unexplained infertility of male origin. Recent data reported that infertility affects about 7% of all men. The etiology of this pathological condition can involve factors acting at pre-testicular, post-testicular or directly at the testicular level. Primary testicular failure accounts for about 75% of all male factor infertility. Despite the recent progress in medicine of reproduction field its etiology is still unknown in about 50% of cases, in which the only possible diagnosis is "idiopathic infertility" (Krausz, 2011).

In our opinion the inability to perform a diagnosis is not due to the insufficient amount of knowledge about sperm physiopathology. During last 60 years the data concerning lipids, proteins, glycids and ions involved capacitation are amazingly grown. Each year new molecules and new function of already known molecules are discovered thanks to the adoption of continuously evolving analytical technology (2D electrophoresis, proteomic techniques, knock-out animals, development of in vitro systems,…). The reason of this failure must be sought in the approach until now adopted, that could be considered reductionist. From a philosophical point of view, the Reductionism is a way to understand the nature of complex things, such as living beings, by reducing them to the interactions of their parts, or to simpler or more fundamental things (http://en.wikipedia.org/wiki/Reductionism). Thus a "reductionist believes that a complex system is nothing but the sum of its parts. An account of it can be reduced to accounts of individual constituents". (Interdisciplinary Encyclopaedia of Religion and Science, http://www.disf.org/en/Voci/104.asp). Until now scientists simply have studied all the molecules involved in spermatozoa post-ejaculatory maturation separately: their attention has been paid to the study of single molecular determinants. Unfortunately the knowledge arising from the so obtained data fails to give information about the whole phenomenon. This could be due to the fact that spermatozoa, as well as all other cells and all living beings, behave as complex systems, i.e. as systems constituted by a network of heterogeneous components that interact nonlinearly, to give rise to emergent behaviour. In particular, as physics theory of complexity says, in a complex system the whole system is more than the sum of its single components. This concept is well highlighted by the etymology of the word "complexity": it derives from Latin *cum* = together and *plecto* (that in turn derives from the ancient Greek πλέκω) = plait, weave, braid, twist, turn; thus giving the idea of interconnected and not separable things. For instance it is noteworthy the difference of meaning between "complex" and "complicated". "Complicated" derives from the Latin *cum* and *plicare* (to fold); the solution of a complicated problem is the explication (from Latin *explicare* = to unfold), on the contrary it is impossible to "unfold" the complexity. Complex systems have some defined and peculiar features:

- the components of a complex system may themselves be complex systems. For example, an organism is made up of tissues, which are made up of cells, which are in turn made up of organelles and macromolecular complexes - all of which are complex systems;
- relationships are non-linear. This implies that a small perturbation may cause dramatic effects (see butterfly effect), proportional effects, or even no effect at all;
- relationships may contain feedback loops: both negative (damping) and positive (amplifying) feedback are found in complex systems. The effects of each element is fed back to others, in such a way that the element itself can be altered;
- complex systems may exhibit behaviors that emerge depending on the order of magnitude of descriptor point of view. It means that the study of system basic constituents can fail to give information about the behavior of whole system, that can only be studied at a higher level: for instance the description of human physiology, biochemistry and biological development (that are at one level of analysis) fail to explain human society dynamics. This is because it is a property that emerges from the collection of Humans and needs to be analyzed at a different level.

From that it is evident that the intrinsic nature of complex systems implies many difficulties with their formal modelling and simulation. In recent years a new branch of mathematics, the science of networks, was adopted to this aim representing and studying them as graphs.

2. From the Köenigsberg bridges to the genome analysis passing trough WWW

The idea to use a model, and in particular a set of nodes connected by links (a graph), is actually no new, dating back to the 1736, when Leonhard Euler used this approach to solve the Seven Bridges of Königsberg problem. The city of Königsberg in Prussia (now Kaliningrad, Russia) was set on both sides of the Pregel River, and included two islands connected to each other and to the mainland by seven bridges. The problem was to find a walk through the city that would cross each bridge once and only once. The islands could not be reached by any route other than the bridges, and every bridge must have been crossed completely every time. Euler for the first time represented the mainland and the islands as nodes and the bridges as edges connecting them. Using this modeling strategy provided the evidence that the problem had no solution.

More than two hundred and fifty years later, the science of networks was used to explore several different phenomena, from the WWW architecture (Barabási et al., 2000; Barabási 2001) to the physical connection of computers through the world (Yook et al., 2002), from the actors collaboration chains to the company market (Barabási, 2003), pointing out as some similar features seem to be shared by the most of them. Obviously one of the research fields that have benefit more from this approach was the biology. In fact the modern technologies offer to Biologists an amazing amount of data, unimaginable until a few years ago, that are increasingly difficult to interpret because of their complexity.

In particular systems biology is the discipline that aims to collect and interpret the data from high throughput techniques, viewing at the organism as an integrated network of interacting molecules. These interactions are liked to be ultimately responsible for the organism form and functions. As R. Albert recently stated "transcription factors can activate or inhibit the transcription of genes to give mRNAs. Since transcription factors are themselves products of

genes, the ultimate effect is that genes regulate each other's expression as part of gene regulatory networks. Similarly, proteins can participate in diverse post-translational interactions that lead to modified protein functions or to formation of protein complexes that have new roles; the totality of these processes is called a protein-protein interaction network. The biochemical reactions in cellular metabolism can likewise be integrated into a metabolic network whose fluxes are regulated by enzymes catalyzing the reactions. In many cases these different levels of interaction are integrated – for example, when the presence of an external signal triggers a cascade of interactions that involves both biochemical reactions and transcriptional regulation"(Albert, 2005). Aimed to provide a strategy able to study the complexity of these phenomena, systems biology brings together biology, physics, statistic and computer science, opening new perspectives in virtually all the branch of the study of life. For instance it was adopted to investigate the organization of metabolic networks, comparing the data from several organisms belonging to the three domains of life, showing as they share a common topography and similar proprieties (Jeong et al., 2000). At the same time the description of yeast proteome, carried out with the same approach, pointed out as the correlation between the connectivity and indispensability of proteins confirms that, despite the importance of individual biochemical function and genetic redundancy, the robustness against mutations is derived from the organization of interactions and the topological architecture of the network (Wagner, 2000). More recently the network representing disorders and disease genes linked by known disorder–gene associations was realized, thus offering a platform to explore all known phenotype and disease gene associations, indicating the common genetic origin of many diseases (Goh et al., 2007).

3. Systems biology of spermatozoa: The realization of a computational model

In 2010 our group, for the first time, started to apply a systems biology-based approach to the study of capacitation (Bernabò et al., 2010a). As first the human spermatozoa post-ejaculatory maturation has been modelised as a biological network (see Figure 1). To this aim, since at present a database containing the information about the molecular events occurring during this process does not exist, a new database has been realized using Microsoft Office Excel 2003. The data were obtained from peer-reviewed papers published in latest 10 years on PubMed (www.ncbi.nlm.nih.gov/pubmed/) concerning human spermatozoa. When the data are lacking or to fill incomplete pathways the data from other mammals, such as mouse, horse, pig, bull, etc.. were used, only if confirmed by a large consensus. The freely available and diffusible molecules such as H_2O, CO_2, P_i, H^+,O_2 were omitted, when not necessary and, in some cases, the record did not represent a single molecule but complex events, such as "membrane fusion" or "protein tyrosine phosphorylation" because all the single molecular determinants of the phenomenon are still unknown. The fields of the database were:

Source molecule: i.e. the molecule source of interaction.
Interaction: i.e. the nature of interaction (activation, inhibition, ...).
Target molecule: i.e. the molecule target of the interaction.
Biological function: i.e. the functional meaning or the contest of interaction (glycolysis, lipid remodelling, oxidative phosphorylation, …).
Reference: i.e. the bibliographic source of information.
Notes: i.e. all the notation such as the presence of synonyms or the explanation of complex cellular events.

These data were used to build the capacitation network by the Cytoscape 2.6.3 software (http://www.cytoscape.org). The network was spatially represented using the Cytoscape Orthogonal Layout. The node size was proportional to the connection number and the node color gradient was dependent from the closeness centrality. This parameter is computed as: $C_c(n) = 1/\text{avg}(L(n, m))$, were L (n, m) is the length of the shortest path between two nodes *n* and *m*. The closeness centrality of each node ranges from 0 to 1 and it is a measure of how fast information spreads from a given node to the others nodes. The statistical and topological analyses of networks were carried out considering the networks as directed by the Cytoscape plugin Network Analyzer (http://med.bioinf.mpi-inf.mpg.de/netanalyzer/help/2.6.1/index.html).

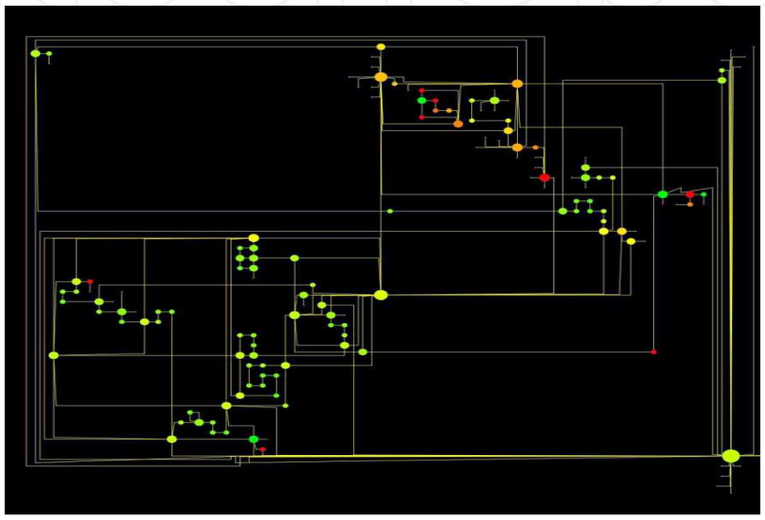


Fig. 1. Diagram showing the structure of the human spermatozoa capacitation network (see text for explanation).

Once the network was created a statistical analysis was carried out to measure the network most relevant topological proprieties (see Table 1).

The distribution of node linkages followed a power law, represented by the generic equation:

$$y = a\, x^{-b}$$

The r, R^2 and b coefficients of IN and OUT network were tabulated in Table 2.

The clustering coefficient distribution does not follow a power law, as demonstrated by the results of power law fitting of clustering coefficient distribution (r = 0.183, R^2 = 0.217).

	capacitation
N° nodes	151
N° edges	202
Clustering coefficient	0.028
Diameter	20
Averaged n° neighbours	2.662
Char. path length	6.546

Table 1. Main topological parameters of capacitation network.

The number of nodes represent the total number of molecules involved, the number of edges represents the total number of interaction found, the clustering coefficient is calculated as CI = 2nI/k(k–1),where nI is the number of links connecting the kI neighbours of node I to each other, the network diameter is the largest distance between two nodes, the Averaged n° neighbours represent the mean number of connection of each node, the Char. path length gives the expected distance between two connected nodes.

	capacitation	
	in	out
r	0.988	0.997
R^2	0.890	0.828
b	-1.542	-1.993

Table 2. Result of power law fitting of IN and OUT capacitation network.

As it is known, the analysis of networks topology made possible to recognize different architecture typologies (Barabási & Oltvai, 2004). The simpler is that of random networks, described by the Erdös–Rényi (ER) model. In this case the network has N nodes connected with probability p, which creates a graph with approximately pN(N–1)/2 randomly placed links. The node degrees follow a Poisson distribution, which indicates that most nodes have approximately the same number of links, close to the average degree, that define the scale of the network. The tail (high k region) of the degree distribution $P(k)$ decreases exponentially, thus indicating that the nodes that significantly deviate from the average are extremely rare. The clustering coefficient is independent of a node degree, and the mean path length is proportional to the logarithm of the network size, $l \sim \log N$.

Scale-free networks, following the Barabási–Albert (BA) model, are characterized by a power-law degree distribution, thus the probability that a node has k links follows $P(k) \sim k^{-\gamma}$, where γ is the degree exponent. In other word, the probability that a node is highly connected is statistically more significant than in a random graph. This imply that the network properties are strongly determined by a relatively small number of highly connected nodes (the hubs) and that a "typical" node does not exist (scale free topology). These network do not have an inherent modularity, i.e. $C(k)$ is independent of k. In addition scale-free networks have the average path length following $l \sim \log \log N$, which is significantly shorter than log N that characterizes random networks.

Modularity, local clustering and scale-free topology coexist in hierarchical networks, that integrate a scale-free topology with an inherent modular structure. The most important feature of hierarchical modularity is the scaling of the clustering coefficient, and the hierarchical architecture implies that sparsely connected nodes are part of highly clustered areas, with communication between the different highly clustered neighborhoods maintained by a few hubs.

From our results it was evident that capacitation network follows the BA model (scale free network) as pointed out by the power law that links the number of edges to the node frequency and the dispersion of clustering coefficient.

This particular kind of topology confers to the network some important biological characteristics. For instance, from a theoretical point of view, the marked heterogeneity in

the number of links per node could justify the different consequences of the removal of differently linked nodes: if highly linked nodes will be removed, network topology will be strongly affected; on the contrary if the removal will involve the less linked ones, the network structure will undergo not significative alterations (Albert et al., 2000). To test if our model behaves as predicted, the most linked nodes ($[Ca^{2+}]_i$ and the ATP-ADP system) or two or four randomly selected nodes were eliminated from the network. In the first case, the network structure collapsed (Figure 2), in the second one the variations of network topology were minimal (data not shown).

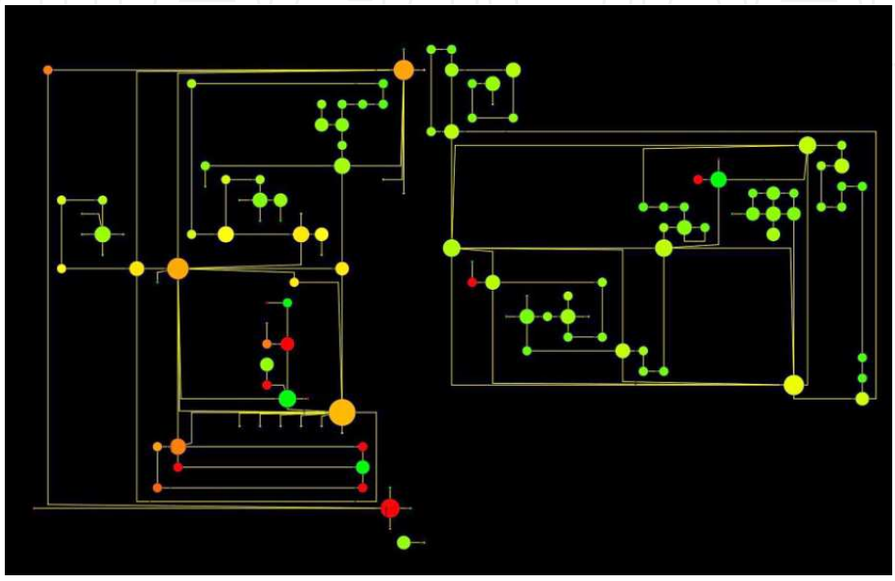


Fig. 2. Diagram showing the structure of the human spermatozoa capacitation network after [Ca2+]i and ATP/ADP nodes removal (see text for explanation).

Reasonably this particular behaviour could offer an important evolutionary advantage: the robustness against random failure. In fact a random perturbation will involve the most frequent typology of nodes, i.e. the less connected ones, with negligible consequences on network architecture, that is on whole cellular function. In our model we identified 6 hubs, thus it exist only a probability <5% (6/151) that one of them will be involved in random damages.

In detail these hubs, as shown in Table 3, are $[Ca^{2+}]_i$, ATP-ADP system, protein kynase A (PKA), Tyr phosphorylation and phospholipase D1 (PLD1).

Node	Number of links
$[Ca^{2+}]_i$	25
ATP	14
Tyr phosphorylation	13
PKA	9
ADP	8
PLD1	8

Table 3. Most connected nodes (the hubs) of capacitation and AR networks.

It is not surprising to note that in absolute the most linked node is $[Ca^{2+}]_i$. As it is well known, capacitation is a Ca^{2+}-dependent process: during this process $[Ca^{2+}]_i$ increases and the capacitation does not take place in the Ca^{2+} absence (Bretibert, 2002 ; Florman et al., 2008). Particularly the calcium homeostasis is ensured by four major Ca^{2+} clearance mechanisms, two acting on the PM and two on intracellular organelles. The PM Ca^{2+}-ATPase exports a cytoplasmic Ca^{2+} ion and imports one or two extracellular protons at the expense of ATP. When $[Ca^{2+}]_i$ is too elevated, the plasma membrane Na^+-Ca^{2+} exchanger operates in forward mode exporting an intracellular Ca^{2+} ion and importing approximately three Na^+ ions at the expense of the Na^+ gradient (Blaustein & Lederer, 1999; Fraser et al., 1993). The best characterised organellar clearance mechanisms are the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, located at the acrosomal level, and the mitochondrial Ca^{2+} uniporter (MCU), whose contribute is mainly directed to the maintenance of $[Ca^{2+}]_i$ in the midpiece of spermatozoa (Wennemuth et al., 2003). During capacitation the Ca^{2+} behaves as a second messenger converting extracelluar stimuli to chemical response in a myriad of molecular system, such as, protein kynase C (PKC), protein kynase C (PKA), actin, lipases and many others.

The ATP-ADP system is the main energetic source of spermatozoa. In fact in male gametes, the metabolic energy production is guaranteed by the glycolysis exclusively, by mitochondrial oxidative phosphorylation exclusively, or by a combination of both the pathways, depending on the species (Storey, 2008).

PKA is involved in several biochemical events, cross linking different pathways. In datail the HCO_3^- and Ca^{2+} are transported by a Na^+/HCO_3^- cotransporter (NBC) and a sperm-specific Ca^{2+} channel (CatSper) and stimulate, via soluble adenylyl cyclase (sAC), PKA activity. In addition this enzyme is involved in late events leading spermatozoa to acquire the hyperactivated motility. PKA activation is correlated with an increase in tyrosine phosphorylation dependent on the presence of cholesterol acceptors in the capacitation medium (Breitbart et al., 2006; Visconti, 2009).

Protein tyrosine phosphorylation pattern of spermatozoa changes during the capacitation in several species such as human, mice, cattle, pigs, hamsters and cats (Barbonetti et al., 2008; Urner & Sakkas, 2003; Visconti et al., 1995). This process appears to be a necessary prerequisite for a spermatozoon to fertilize an egg and has been demonstrated to increase either in flagellum and in sperm head. At present the molecular targets of protein tyrosine phosphorylation are still largely unknown (Naz & Rajesh, 2004).

PLD1 plays a pivotal role in actin polymerization, that is one of the most important events related with the acquisition of capacitation. In particular MAP-kinase, tyrosine kinase, and ADP-ribosylation factor are involved in PLD activation, leading to phosphatidyl-choline hydrolysis to produce phosphatidic acid, which mediates polymerization of G-actin to F-actin (Breitbart et al., 2005; Cohen et al., 2004; Gomez-Cambronero & Keire, 1998).

Other highly linked nodes are those related to terminal events (such as protein phosphorylation or membrane fusion). Reasonably this is due to the redundancy of biochemical signalling, as a safety strategy to overlap partial failure of the system.

It is of interest, also, to note that almost half of the nodes (40-45%) have two links: one in input and the other in output. This finding, together with the low value of clustering coefficient, is in agreement with the concept that the network had a signalling transduction-

dedicated structure: the molecular message is carried, directionally, from the beginning to the end of the chain, avoiding the presence of loop or clusters that could to slow and to interfere with the propagation of messages.

In this context, the characteristic path length (~6.5) may have two important consequences on spermatozoa physiology. Firstly, if any molecule interacts with any other in a small number of passages, the loss of information due to the signal decrease is minimized and, consequently, the signal efficiency is maximized. Secondly, any local perturbation in signalling system could reach the whole network in a short time, thus increasing the system responsiveness to intracellular and extracellular stimuli.

It was also possible to isolate the nodes with only one link. The classical scale free model indicates that these nodes are the most peripheral and, as a consequence, less important ones. For instance in the WWW the sites with only one link are the most marginal in the network and are likely destined to disappear (Barabási, 2003). On the contrary, in sperm capacitation, the nodes with one link are often the network input terminal, These nodes are able to receive links and to interact intracellular signalling machinery with external environment (female environment, seminal fluid, artificial media in laboratory technologies, ...).

A further peculiar characteristic of this network is that the activating signals are markedly most expressed than the inhibiting ones (~ 95% vs. ~ 5%). This evidence could have two different explanations:

- it is possible that the interest to recreate in vitro sperm capacitation in the contest of Assisted Reproductive Technologies leads the Researchers to study and to describe the mainly capacitation-promoting events. Thus the activating signals are not the most expressed but the most studied and, as a consequence, the most represented in scientific literature;
- the spermatozoa are functionally disposable cells. From a teleological point of view their fate is the completion of capacitation and, after all, the AR and the fertilization. Thus, it is possible that most of the biochemical pathways are objective-oriented leading sperm cells to recognise and to bind ZP and to undergo AR.

Taken altogether these finding seem to suggest that the system biology-based approach adopted could be useful to explore the spermatozoa signalling machinery, giving us new information on its physiology. It could be possible complete the dialectic of *in vivo – in vitro* models adding *in silico* model to increase the resources available to study a complex phenomenon such as the function of male gamete. In addition it could be possible to explain many biological aspects of spermatozoa biology that are out of focus looking at the single molecular determinant, thus overcoming the reductionist approach until now adopted, which did not consider the proprieties that emerge from the interaction among the different molecules involved in capacitation.

4. From the chip to the bench: Experimental validation of a model-based hypothesis

More recently an experimental set-up was carried out to empirically validate this modeling approach. An in silico and in vitro experiment was carried out on an animal model (Bernabò et al., 2011). To this aim the biological network representing boar sperm capacitation was

realized, as previously described, and its nodes were separated depending on their subcellular compartment (see Table 4 and Figure 3) using the Cytoscape plugin Cerebral v.2 (http://www.pathogenomics.ca/cerebral/). The statistical and topological analyses of the network were carried out, considering the network as undirected, by the Cytoscape plugin Network Analyzer (http://med.bioinf.mpiinf.mpg.de/netanalyzer/help/2.6.1/index.html).

Parameter		Value
N° nodes		153
N° edges		204
Clustering coefficient		0.056
Diameter		12
Averaged n° neighbours		2.654
Char. path length		4.995
Most connected nodes (n° of links)	$[Ca^{2+}]_i$	(28)
	ATP	(15)
	Tyr phosphorylation	(13)
	PKA	(9)
	ADP	(8)
	PLD1	(8)
	NADH	(8)
	Actin polymerization	(8)

Table 4. Main topological parameters of boar spermatozoa capacitation network. The number of nodes represent the total number of molecules involved, the number of edges represents the total number of interaction found, the clustering coefficient is calculated as $CI = 2nI/k(k-1)$,where nI is the number of links connecting the kI neighbours of node I to each other, the network diameter is the largest distance between two nodes, the Averaged n° neighbours represent the mean number of connection of each node, the Char. path length gives the expected distance between two connected nodes
(adapted, from Bernabò et al., 2011).

From the network analysis it was immediately evident that the "actin polymerization" node has two important and unique features:

- it is one of the most connected nodes (a hub);
- it links in a specific manner all the intracellular compartments.

To best characterize its role in capacitation-related signaling, a computational experiment was performed: the "actin polymerization" was removed from the network and the consequences on its architecture were assessed. It was found that its removal did not affect the global network topology (data not shown) but caused the loss of five important nodes (and among them the "PM and OAM fusion").

The analysis of theoretical data suggested that actin polymerization could be involved in the coordination of signaling among different subcellular districts, and that its functional ablation could compromise spermatozoa ability to complete the capacitation avoiding PM and OAM fusion, without affecting the other main signaling pathway.

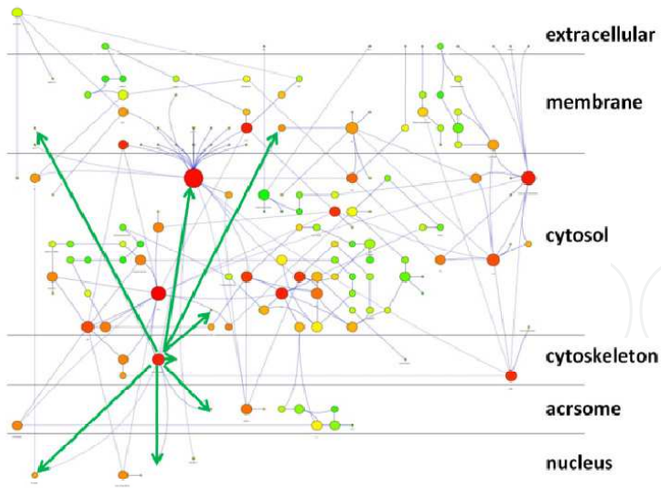


Fig. 3. Diagram showing the structure of the boar spermatozoa capacitation network and the subcellular localization of nodes (see text for explanation). The links of actin polymerization node are indicated by green arrows.

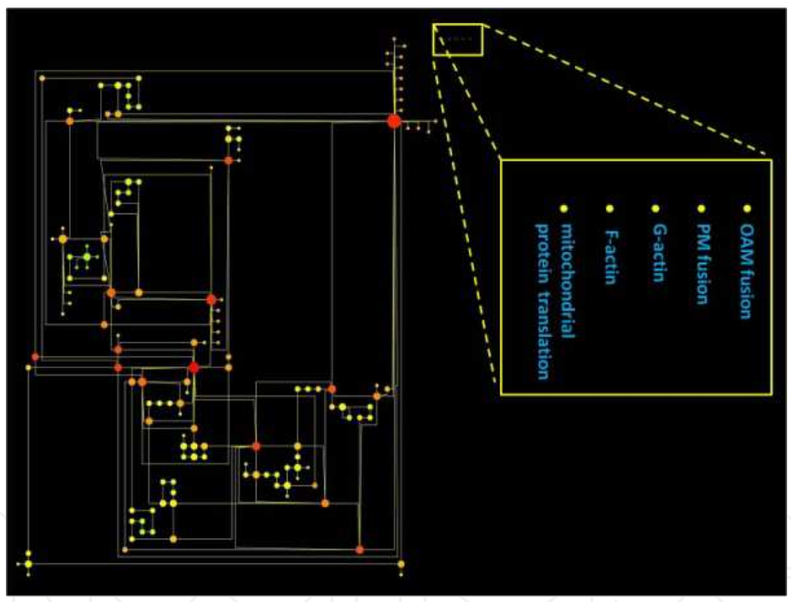


Fig. 4. Diagram showing the structure of the boar spermatozoa capacitation network after "actin polymerization" node removal (see the text for explanation).

To empirically validate this hypothesis an in vitro experiment was performed: the actin polymerization was inhibited, by administrating the cytochalasin D, a specific actin polymerization inhibitor, to boar spermatozoa incubated under in vitro capacitating conditions. Then the effects of this treatment on spermatozoa capacitation status (ZP-induced AR), and on the major cellular events involved in the acquisition of full fertilizing ability, such as membrane acquisition of chlortetracycline pattern C, protein tyrosine phosphorylation, phospholipase C-γ1 relocalization, intracellular calcium response to ZP, were assessed.

To this aim semen samples were collected and processed by an already validated protocol (Bernabò et al, 2010b) and were incubated under control conditions (CTR) or were constantly maintained in the presence of 20 µM of citochalasin D (CD).

The competence of in vitro incubated spermatozoa to undergo AR in response to solubilised zonae pellucidae (ZP) co-incubation was further evaluated as a functional endpoint of the capacitative state. As depicted in Figure 5 it was evident that:

- the percentage of spermatozoa undergoing spontaneous AR was unaffected by the treatment;
- the percentage of spermatozoa able to respond with AR to the ZP coincubation was markedly reduced by the CD administration.

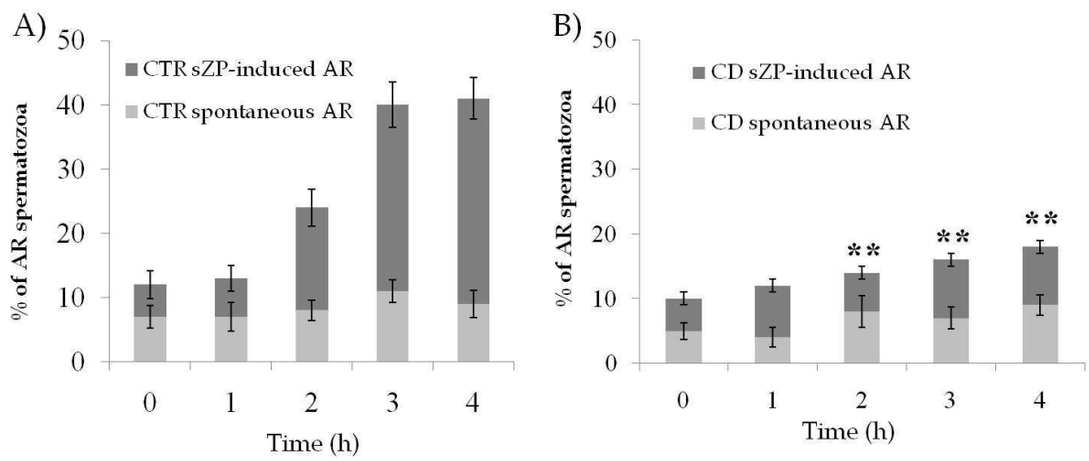


Fig. 5. Histogram representing the percentage of spermatozoa undergoing spontaneous and sZP-induced AR in CTR (A) and CD (B) treated spermatozoa. All the values are represented as mean ± SD. ** = $p<0.01$ vs. CTR, ANOVA test (from Bernabò et al., 2011).

The chlortetracycline stain (CTC) was used to evaluate the completion of calcium-dependent membrane remodelling, in keeping with Mattioli et al. (Mattioli, 1996). For each sample were assessed at least 200 spermatozoa and the percentage of spermatozoa displaying fluorescence pattern C indicative of capacitation (CTC fluorescence over the post acrosomal area) was calculated. As shown in Figure 6 the CD treatment did not affected the kinetic of acquisition of CTC pattern C.

The biochemical and ultrastructural localization of PLC-γ1 was studied by western blotting and by transmission electron microscopy on control and cytocalasin D treated spermatozoa. As a result it was found that this enzyme migrates from cytosol to the active site (the membrane) independently from CD treatment (see Figures 7 and 8)

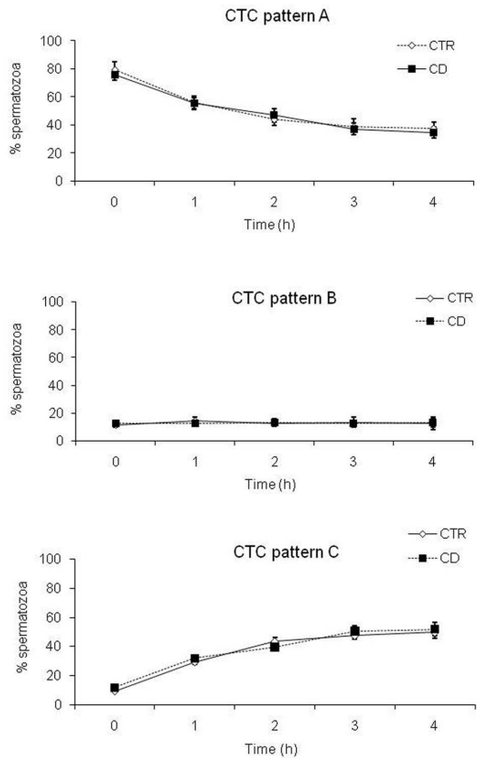


Fig. 6. Kinetic of acquisition of CTC staining pattern A, B and C in CTR and CD treated spermatozoa.
Graphic showing the percentage of spermatozoa displaying the CTC pattern A, B and C, during the 4h of incubation under control conditions (dark continue line) or in the presence of CD (dark dot line). All the values are represented as mean ± SD (from Bernabò et al., 2011)

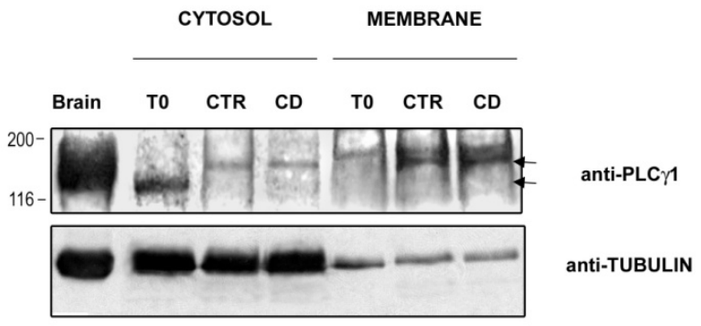


Fig. 7. Capacitation-dependent PLCγ1 relocalization.
Western Blot analysis of PLCγ1 localization in cytosolic and membrane fractions of freshly ejaculated male gametes (T0) or in spermatozoa incubated under control condition (CTR) or in the presence of CD (CD). The data showing the capacitation-dependent translocation of PLC-γ1 (arrows) from cytosol to membrane. Brain proteins were used as positive control. The filter was normalized on Tubulin expression. The data shown were representative of four independent experiments (from Bernabò et al., 2011).

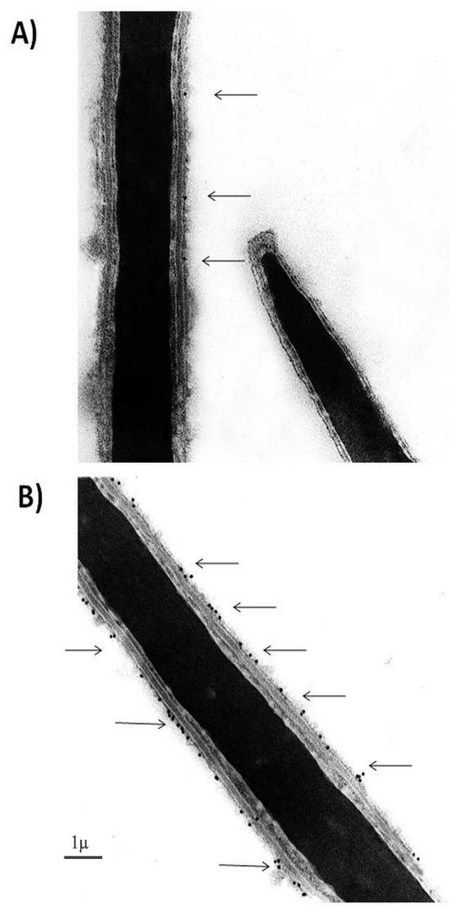


Fig. 8. Transmission electron microscopy pictures demonstrating the topography of capacitation-dependent PLCγ1 relocalization. Panel A): ejaculated spermatozoon displaying few gold particles (arrows) indicative of a low localization of PLCγ1 protein over cell membranes. Panel B): in vitro capacitated spermatozoon incubated in the presence of CD that localized on its membrane several gold particles (arrows) (from Bernabò et al., 2011).

The pattern of tyrosine phosphorylation was evaluated by western blotting on control and cytocalasin D treated spermatozoa. In this case also the CD treatment did not exerted any effect (Figure 9).

The calcium probe fluo-3-AM was used to assess the variations in the intracellular calcium concentration in response to the sZP coincubation and it was found that the physiological agonist of AR maintained its ability to stimulate a rapid rise in intracellular calcium concentration also in the CD treated spermatozoa (Figure 10).

As hypothesized from the *in silico* experiment, the treatment had a duplex effect: in one hand it completely inhibited the acquisition of fertilizing ability, in the other one all the

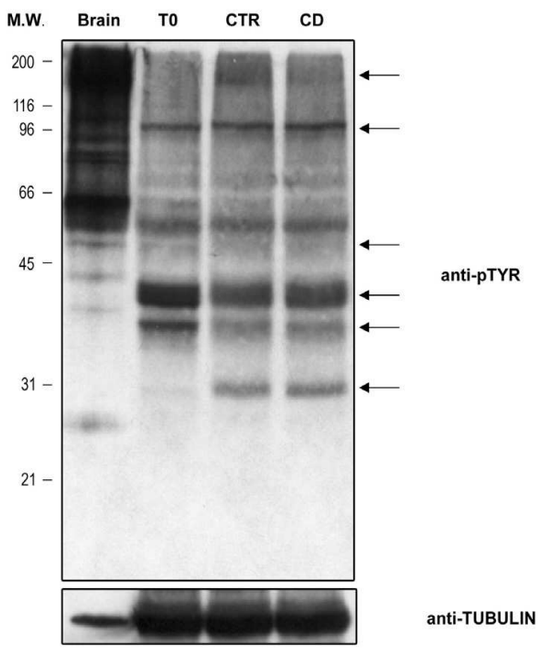


Fig. 9. Western Blot analysis of tyrosine phosphorylation pattern in total lysate of freshly ejaculated male gametes (T0) or in spermatozoa incubated under control condition (CTR) or in the presence of CD (CD). The arrows indicate the capacitation-related changes in P-Tyr. Brain proteins were used as positive control. The filter was normalized on the Tubulin expression (from Bernabò et al., 2011).

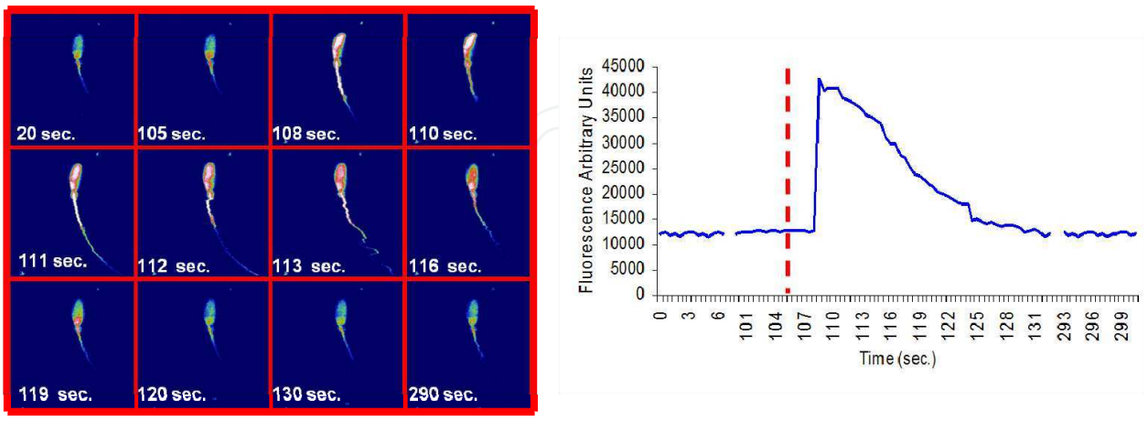


Fig. 10. An example of confocal image gallery of CD treated spermatozoa loaded with Fluo-3AM exposed to sZP (vertical red line). Notice that the rise in the $[Ca^{2+}]_i$ in spermatozoon is evident after <10 sec. from sZP addition, whereas return on the baseline occurs after about 30 sec. (adapted, from Bernabò et al., 2011).

examined pathways seemed to be unaffected by the inhibition of actin polymerization. This finding allowed us to propose some considerations:

- as already discussed the data until now available attribute to the actin dynamics a structural role during capacitation: a network of actin acts as a diaphragm between the PM and OAM avoiding their fusion. At the moment of AR this physical barrier was rapidly removed and the fusion between the two membranes can happen. If this model was correct it was reasonably to expect that the block of actin polymerization (i.e. the treatment of spermatozoa with CD) in early phases of capacitation should lead to the increase in the percentage of spermatozoa undergoing AR because of the inefficiency of PM and OAM separation. In our experiments, on the contrary, it was found that, even in the absence of the actin network, the PM and OAM did not fuse. Thus, it possible to hypothesize that in spermatozoa, as demonstrated in other cells, the role of actin cytoskeleton overcomes this merely structural function, having a more complex role. Particularly in the light of the finding that "actin polymerization" node links three hubs of the system it is possible to propose that this event could play an important role in coordination of information flow. This supposition is in keeping with the newly emerging evidence that in different cellular systems the cytoskeleton is not only a mechanical support for the cell, but it exerts a key role in signaling. As proposed by Janmey, "independent of its mechanical strength, the filaments of the cytoskeleton form a continuous, dynamic connection between nearly all cellular structures, and they present an enormous surface area on which proteins and other cytoplasmic components can dock" (Janmey, 1998). This concept is in keeping with the observation that plasma membrane surface area of a 20-μm-diameter generic cell is on the order of 700 μm^2, in contrast, the total surface area of a typical concentration of 10 mg/ml F-actin is 47,000 μm^2 (Janmey, 1998) and that the diffusion along cytoskeleton tracks could be a reliable alternative to other established ways of intracellular trafficking and signaling, and could therefore provide an additional level of cell function regulation (Shafrir et al., 2000). One implication of this role is that the actin cytoskeleton might provide a signal transduction route and macromolecular scaffold, which, during capacitation, contributes to the spatial organization of signaling pathways (Forgacs et al., 2004).
- the results obtained by the *in silico* experiments are perfectly in agreement with those from the *in vitro* approach. This datum evidences that the model seems to mimic the behavior of real system, in other words it could be possible to assume that the information inferred by the model is correct.

5. Conclusions

In our opinion, the ability to realize a reliable computational model representing main molecular events occurring during spermatozoa capacitation could be an important tool in understanding male gametes physiology and, as a consequence, their pathology. These cells are an ideal candidate to this kind of approach for several reasons:

- one of the most important problems in cell modelization is the continuous modification in cellular protein content and in molecular interactions due to the dynamical regulation of genes expression and protein transcription. The molecular composition of male gametes is stable since they are transcriptionally silent with the exception of the 55S mitochondrial ribosome-dependent protein translation of nuclear-encoded proteins.

- differently from the most of other cellular types, it is possible to empirically evaluate the functional status of the system. In fact it is possible, using for instance an animal model, to verify if the spermatozoa completed their maturation process, testing the ability of spermatozoa to complete the capacitation and, subsequently, to undergo AR by in *in vitro* fertilization assay or by *in vivo* fertilization trials: only the spermatozoa that successfully fertilize an oocyte can be considered fully competent.
- finally the spermatozoa are the only cellular type, produced in an organism, that exert their function in another one. As a consequence they are capable of independent life (unlike the other cells) and it is possible to manage them outside the organism without loss of the cell function.

We think that the adoption of this modelization strategy could be of great importance in the study of capacitation. Sperm cells are complex systems, thus to really understand their behavior it is necessary to consider them as a network of molecules interacting with each other. It is impossible to get complete information on their functional status looking only at the molecular level: the capacitation, as well the fertility, are emergent proprieties of the system, thus it is necessary to study them at a supra-molecular level. As a consequence we propose to adopt, together with the traditional methodologies, a computational-based approach to explore spermatozoa biology and, ultimately, male fertility and infertility.

Ultimately, we fully agree with the WHO statement that "advances in our understanding of the signal transduction pathways regulating sperm function will have implications for the development of diagnostic tests capable of generating detailed information on the precise nature of the processes that are defective in the spermatozoa of infertile men"(WHO, 2010).

6. References

Abou-haila, A. & Tulsiani D.R. (2009) Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Archives of Biochemistry and Biophysics,* Vol. 485, No. 1 (May 2009), pp. 485, 72-81, ISSN 0003-9861

Albert, R., Jeong, H. & Barabasi, A.L. (2000) Error and attack tolerance of complex networks. *Nature, Vol.* 406, No. 6794 (July 2000), pp. 378-382, ISSN : 0028-0836

Albert, R. (2005) Scale-free networks in cell biology. *Journal of Cell Science, Vol.* 118, No. 21 (November 2005),pp. 4947-4957, ISSN 0021-9533

Asano, A., Nelson, J.L., Zhang, S. & Travis, A.J. (2010) Characterization of the proteomes associating with three distinct membrane raft sub-types in murine sperm. *Proteomics, Vol.* 10, No. 19 (October 2010), pp. 3494-3505, ISSN: 1862-8354

Austin, C.R.(1951) Activation and the correlation between male and female elements in fertilization. *Nature*, Vol. 168, No. 4274 (September 1951), pp. 558-559, ISSN : 0028-0836

Barabási, A. L. (2003) "Linked: How Everything is Connected to Everything Else and What It Means for Business, Science, and Everyday Life." New York: Plume, ISBN 10: 0452284392

Barabási, A.L. & Oltvai, Z.N. (2004) Network biology: understanding the cell's functional organization. *Nature Reviews Genetics, Vol.* 5, No. 2 (Febuary, 2004), pp. 101-113, ISSN : 1471-0056

Barabási, A.L. (2001) The physics of the Web. *Physics World*, Vol. 14 (July 2001), pp. 33-38, ISSN: 0953-8585

Barabási, A.L., Albert, R. & Jeong, H. (2000) Scale-free characteristics of random networks: the topology of the world wide web. *Physica A, Vol.* 281, pp. 69-77, ISSN: 0378-4371

Barbonetti, A., Vassallo, M.R., Cinque, B., Antonangelo, C., Sciarretta, F., Santucci, R., D'Angeli, A., Francavilla, S. & Francavilla, F. (2008) Dynamics of the global tyrosine phosphorylation during capacitation and acquisition of the ability to fuse with oocytes in human spermatozoa. *Biology of Reproduction*, Vol. 79, No. 4 (October 2008), pp. 649-56, ISSN: 0006-3363

Bernabò, N., Berardinelli, P., Mauro, A., Russo, V., Lucidi, P., Mattioli, M. & Barboni, B. (2011) The role of actin in capacitation-related signaling: an in silico and in vitro study. *BMC Systems Biology, Vol.* 30, No. 5 (March 2011), p. 47, ISSN 1752-0509

Bernabò, N., Mattioli, M. & Barboni, B. (2010a) The spermatozoa caught in the net: the biological networks to study the male gametes post-ejaculatory life. *BMC Systems Biology*, Vol. 4 (June 2010), p. 87, ISSN 1752-0509

Bernabò, N., Pistilli, M.G., Mattioli, M. & Barboni, B. (2010b) Role of TRPV1 channels in boar spermatozoa acquisition of fertilizing ability. *Molecular and Cellular Endocrinology* Vol. 323, No. 2 (July 2010), pp. 224-231, ISSN: 0303-7207

Bevers, E. M., Comfurius, P., Dekkers D. W. C., Harmsma, M. and Zwaal R. F. A. (1998) Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. *Biological Chemistry, Vol.* 379, No. 8-9 (August-September 1998), pp. 973-986, ISSN 0021-9258

Blaustein, M.P. & Lederer, W.J. (1999) Sodium/calcium exchange: its physiological implications. *Physiological Review, Vol.* 79, No. 3, pp. 763–854, ISSN: 0031-9333

Botto, L., Bernabò, N., Palestini, P & Barboni, B. (2010) Bicarbonate induces membrane reorganization and CBR1 and TRPV1 endocannabinoid receptor migration in lipid microdomains in capacitating boar spermatozoa. *Journal of Membrane Biology, Vol.* 238, No. 1-3, pp. 33-41, ISSN: 1432-1424

Breitbart, H. (2002) Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Molecular and Cellular Endocrinology, Vol.* 187, No. 1-2 (Febuary 2002), pp. 139-144, ISSN: 0303-7207

Breitbart, H., Cohen, G. & Rubinstein, S.(2005) Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. *Reproduction*, Vol. 129, No. 3 (March 2005), pp. 263-268, ISSN: 1470-1626

Breitbart, H., Rubinstein, S. & Etkovitz, N. (2006) Sperm capacitation is regulated by the crosstalk between protein kinase A and C. *Molecular and Cellular Endocrinology,* Vol. 252, No. 1-2 (June 2006), pp. 247-249, ISSN: 0303-7207

Breitbart,H. & Etkovitz, N. (2011) Role and regulation of EGFR in actin remodeling in sperm capacitation and the acrosome reaction. *Asian Journal of Andrology,* Vol. 13, No. 1 (January 2011), pp. 106-110, ISSN: 1745-7262

Brewis, I.A., Gadella, B.M.(2010) Sperm surface proteomics: from protein lists to biological function. *Molecular Human Reproduction,* Vol. 16, No. 2 (Febuary 2010), pp. 68-79, ISSN: 1360-9947

Bruckbauer, A., Dunne, P.D., James, P., Howes, E., Zhou, D., Jones, R. & Klenerman, D. (2010) Selective diffusion barriers separate membrane compartments. *Biophysical Journal,* Vol. 99, No. 1 (July 2010), pp. 1-3, ISSN: 0006-3495

Chang, H. & Suarez, S.S. (2010) Rethinking the relationship between hyperactivation and chemotaxis in mammalian sperm. *Biology of Reproduction,* Vol. 83, No. 4 (October 2010), pp. 507-513, ISSN: 0006-3363

Chang, M.C.(1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature, Vol.* 168, No. 4277 (October 1951), pp. 697-698, ISSN : 0028-0836

Cohen, G., Rubinstein, S., Gur, Y. & Breitbart, H. (2004) Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. *Developmental Biology*, Vol. 267, No. 1 (March 2004), pp. 230–241, ISSN: 0012-1606

Florman, H.M., Jungnickel, M.K. & Sutton, K.A. (2008) Regulating the acrosome reaction. *International Journal of Developmental Biology*, Vol. 52, No. 5-6, pp. 503-510, ISSN: 0214-6282

Forgacs, G., Yook, S.H., Janmey, P.A., Jeong, H. & Burd, C.G. (2004) Role of the cytoskeleton in signaling networks. *Journal of Cell Science*, Vol. 117, No. 13 (May 2004), pp. 2769-2775, ISSN 0021-9533

Fraser, L.R. & Herod, J.E. (1990) Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. *Journal of Reproduction and Fertility,* Vol. 88, No. 2 (March 1990), pp. 611-621, ISSN: 0022-4251

Fraser, L.R., Abeydeera, L.R. & Niwa, K. (1995) Ca(2+)-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Molecular Reproduction and Development,* Vol. 40, No. 2 (Febuary 1995), pp. 233-241, ISSN: 1098-2795

Fraser, L.R., Umar, G. & Sayed, S. (1993) Na(+)-requiring mechanisms modulate capacitation and acrosomal exocytosis in mouse spermatozoa. *Journal of Reproduction and Fertility*, Vol. 97, No. 2 (March 1993), pp. 539-549, ISSN: 0022-4251

Gadella, B.M. & Harrison, R.A. (2000) The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development*, Vol. 127, No. 11 (June 2000), pp. 2407-2420, ISSN: 1011-6370

Gadella, B.M. & Harrison, R.A. (2002) Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biology of Reproduction,* Vol. 67, No. 1 (July 2002), pp. 340-350, ISSN: 0006-3363

Gadella, B.M., Tsai, P.S., Boerke, A. & Brewis, I.A. (2008) Sperm head membrane reorganization during capacitation. *International Journal of Developmental Biology,* Vol. 52, No. 5, pp. 473-480, ISSN: 0214-6282

Goh, K.I., Cusick, M.E., Valle, D., Childs, B., Vidal, M. & Barabási A.L. (2007) The human disease network. *Proceedings of National Academy of Science U S A, Vol.* 104, No. 21 (may 2007), pp. 8685-8690, ISSN-0027-8424

Gomez-Cambronero, J. & Keire, P. (1998) Phospholipase D: a novel major player in signal transduction. *Cell Signaling*, Vol. 10, No. 6 (June 1998), pp. 387-397, ISSN: 0898-6568

Harrison, R.A. & Gadella, B.M. (2005) Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology*, Vol. 63, No. 2 (January 2005), pp. 342-351, ISSN: 0093-691X

Hickey, K.D. & Buhr, M.M.(2011) Lipid bilayer composition affects transmembrane protein orientation and function. *Journal of Lipids, Vol.* 2011;2011:208457, ISSN: 2090303

Janmey, P.A. (1998) The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiological Review, Vol.* 78, No. 3 (July 1998), pp. 763-778, ISSN: 0031-9333

Jeong, H., Tombor, B., Albert, R., Oltvai, Z.N. & Barabási, A.L. (2000) The large-scale organization of metabolic networks. *Nature, Vol.* 407, No. 6804 (October 2000), pp. 651-654, ISSN : 0028-0836

Kotwicka, M., Jendraszak, M. & Jedrzejczak, P. (2011) Phosphatidylserine membrane translocation in human spermatozoa: topography in membrane domains and relation to cell vitality. *Journal of Membranes Biology, Vol.* 240, No. 3 (April 2011), pp. 165-170, ISSN: 1432-1424

Krausz, C. (2011) Male infertility: pathogenesis and clinical diagnosis. *Best Practice & Research Clinical Endocrinology & Metabolism,* Vol. 25, No. 2 (April 2011), pp. 271-285, ISSN: 1521-690X

Mattioli, M., Barboni, B., Lucidi, P. & Seren, E. (1996) Identification of capacitation in boar spermatozoa by chlortetracycline staining. *Theriogenolog, Vol.* 45, No. 2 (December 1996), pp. 373-381, ISSN: 0093-691X

Müller, P., Pomorski, T., Porwoli, S., Tauber, R. & Herrmann, A. (1996) Transverse movement of spin-labeled phospholipids in the plasma membrane of a hepatocytic cell line (HepG2): implications for biliary lipid secretion. *Hepatology, Vol.* 24, No. 6 (December 1996), pp. 1497–1503, ISSN: 0270-9139

Naz, R.K. & Rajesh, P.B. (2004) Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reproductive Biology and Endocrinology, Vol.* 2 (November 2004), p. 75, ISSN: 1477-7827

Nixon, B. & Aitken, R.J. (2009) The biological significance of detergent-resistant membranes in spermatozoa. *Journal of Reproductive Immunology, Vol.* 83, No. 1-2 (December 2009), pp 8-13, ISSN: 0165-0378

O'Rand, M.G. & Fisher, S.J. (1987). Localization of zona pellucida binding sites on rabbit spermatozoa and induction of the acrosome reaction by solubilized zonae. *Developmental Biology*, Vol.119, No. 2 (Febuary 1987), pp. 551-559, ISSN: 0012-1606

Perry, R.L., Naeeni, M., Barratt, C.L., Warren, M.A. & Cooke, I.D. (1995) A time course study of capacitation and the acrosome reaction in human spermatozoa using a revised chlortetracycline pattern classification. *Fertility and Sterility,* Vol. 64, No. 1 (July 1995), pp. 150-159, ISSN: 0015-0282

Saling, P.M. & Storey, B.T. (1979) Mouse gamete interactions during fertilization in vitro. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. *Journal of Cell Biology,* Vol. 83, No. 3 (December 1979), pp. 544-555, ISSN:0021-9525

Shafrir, Y., ben-Avraham, D. & Forgacs, G. (2000) Trafficking and signaling through the cytoskeleton: a specific mechanism. *Journal of Cell Science*, Vol. 113, No. 15 (August 2000), pp. 2747-2757, ISSN 0021-9533

Storey BT (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. *International Journal of Developmental Biology*, Vol. 52, No. 5-6, pp. 427-437, ISSN: 0214-6282

Suarez, S.S. (2008) Control of hyperactivation in sperm. *Human Reproduction Update*. Vol. 14, No. 6 (June 2008), pp. 647-657, ISSN: 1355-4786

Urner, F. & Sakkas, D. (2003) Protein phosphorylation in mammalian spermatozoa. *Reproduction*, Vol. 125, No. 1 (January 2003), pp. 17–26, ISSN: 1470-1626

Venditti, J.J. &. Bean, B.S. (2009) Stabilization of membrane-associated alpha-L-fucosidase by the human sperm equatorial segment. *International Journal of Andrology, Vol*. 32, No. 5 (March 2009), pp. 556-562, ISSN: 0105-6263

Visconti, P.E. (2009) Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of National Academy of Science USA,* Vol. 105, No. 52 (December 2009), pp. 66766-66768, ISSN-0027-8424

Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P. & Kopf, G.S. (1995) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development,* Vol. 121, No. 4 (April 1995), pp. 1129-1137, ISSN: 1011-6370

Wagner, A. (2000) Robustness against mutations in genetic networks of yeast. *Nature Genetics*, Vol. 24, No. 4 (April 2000), pp. 355-361, ISSN : 1061-4036

Wennemuth, G., Babcock, B.F. & Hille, B. (2003) Calcium Clearance Mechanisms of Mouse Sperm. *Journal of General Physiology*, Vol. 122, No. 1 (July 2003), pp. 115–128, ISSN: 0022-1295

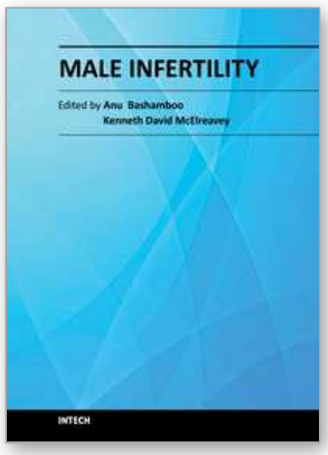
WHO (1986). Consultation on the zona-free hamster oocyte penetration test and the diagnosis of male fertility. *International Journal of Andrology* (Suppl. 6). ISSN: 0105-6263

WHO (2010) *Laboratory manual for the examination and processing of human semen* - 5th ed., pp. 246-152 ISBN 978 92 4 154778 9

Yanagimachi, R. (1969) In vitro capacitation of hamster spermatozoa by follicular fluid. *Journal of Reproduction and Fertility, Vol.* 18, No. 2 (March 1969), pp. 275-286, ISSN: 1470-1626

Yanagimachi, R. (1994). Mammalian fertilization. *In*: Knobil E, Neill JD editors. *The physiology of reproduction (2nd edition).* New York, USA: Raven Press; pp.189–317, ISBN: 0-7817-0086-8

Yook, S.H., Jeong,H & Barabási A.L. (2002) Modeling the internet's large-scale topology *Proceedings of National Academy of Science U S A, Vol.* 99, No. 21 (October 2002), pp. 13382-13386, ISSN-0027-8424



Male Infertility

Edited by Dr. Anu Bashamboo

ISBN 978-953-51-0562-6
Hard cover, 194 pages
Publisher InTech
Published online 20, April, 2012
Published in print edition April, 2012

Male infertility is a multifaceted disease where genetic, epigenetic and environmental factors all contribute to the development of the phenotype. In recent years, there has been an increasing concern about a decline in reproductive health, paralleled by an increase in demand for infertility treatments. This calls for a detailed and thorough understanding of normal and aberrant testicular function and the environmental influences on the establishment and integrity of the male germ cell. This is crucial for understanding the complex pathophysiology of male infertility and eventual success of Assisted Reproductive Technologies.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Nicola Bernabò, Mauro Mattioli and Barbara Barboni (2012). A Systems Biology Approach to Understanding Male Infertility, Male Infertility, Dr. Anu Bashamboo (Ed.), ISBN: 978-953-51-0562-6, InTech, Available from: http://www.intechopen.com/books/male-infertility/a-systems-biology-approach-to-understanding-male-infertility

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen