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# Membrane Dynamics of Spermatozoa during Capacitation: New Insight in Germ Cells Signalling

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Additional information is available at the end of the chapter

## Abstract

The study of germline stem cells and of germline cells has deep implications for the understanding of fertility, development and cancer. Nowadays, we are experiencing the very fascinating challenge of application of –*OMICS* technologies to this issue, which is opening new and unexpected horizons in virtually all the branches of biology. Here, we carried out a review of signalling systems involved in maturation of male germ cells and in the process that leads them to become fully fertile. In particular, we discuss the control mechanisms involved in capacitation and acrosome reaction that act at membrane level. Indeed, spermatozoa membranes play key roles in determining the achievement of fertility: they are the interface with the surrounding environment, they locate the signal transduction systems and they are active in recognizing and binding the oocyte. In addition, we discuss the effect of several compounds that could exert a negative effect on reproductive activity, by interfering with the endocrine axis, the so-called endocrine disruptors.

**Keywords:** germline stem cells, spermatozoa, membrane, signalling, fertility

## 1. Introduction

In recent years, the scientific interest for germline stem cells (GSCs) has enormously grown. They are the cells devoted to the genome transmission to future generations; thus the study of their biology has fundamental implication for understanding of basics of fertilization, embryo development and fertility as well as of stem cells biology and cancer. Nowadays, we are facing with a revolution in biological science, due to the adoption of high-throughput technologies, the

so-called –*OMICS*, that are able to provide a huge quantity of new data on cell physiological and pathological processes. Although the knowledge of complex phenomena, such as the regulatory mechanisms involved in both germ cell (GC) specification and the maintenance of the germline in adults, is rapidly increasing unfortunately, some aspects of GSCs and of germ cells are still poorly understood. For instance, the biology of male GCs and their signalling machinery still poses important open questions to be answered, as proven by the high incidence of male infertility cases in which it is impossible to reach a diagnosis (unexplained infertility of male origin).

Here, we carried out a review of current information about male GCs biology, with particular respect to their signalling systems located at membrane level.

## 2. Germline stem cells and hypothalamus-pituitary-testis axis (HPTA)

One of the key events during embryogenesis is the development of germline stem cells, able to originate mature gametes, sperm or oocytes, becoming responsible for transmitting genetic information from generation to generation. In most mammals, such as mice, the germ cell lineage is determined in the early post-implantation embryo, at approximately 3 weeks after fertilization (E17, embryonic day 17). GSCs specification from somatic lineage occurs by appropriate signals from pluripotent embryonic cells (epigenetic mode): a few epiblast cells become competent in response to bone morphogenetic protein (BMP) and wingless-related integration site family of proteins (WNTs) signals produced by extraembryonic ectoderm (ExE) and primitive endoderm (VE). Among the signals responsible for the induction of the germ cell fate, it is possible to distinguish Bmp4 and Bmp8b produced by the ExE, as well as the signal transducers Smad1, 4 and 5. In addition, Bmp2 arisen from the VE seems to improve the role of Bmp4 to ensure the correct production of GSC. These signals induce the expression of the crucial complex of GSC fate, such as BLIMP1 (also known as PRDM1), PRDM14 and AP2$\gamma$. These last three factors are able to up-regulate some germ cell genes (such as Stella) and pluripotency genes (OCT4, NANOG and SOX2) and to repress somatic genes (Hoxb1, Hoxa1, Evx1 and Lim1). Soon after specification, GSC migrates through the hindgut and dorsal mesentery, and finally colonizes the genital ridges until E11.5.

During this migratory phase, a special epigenetic remodelling takes place and it includes DNA demethylation, changes of histone modifications, X-chromosome reactivation and genomic imprint erasure. At E13.5, in the gonads, they initiate the sex differentiation either towards a spermatogenic (male) or to an oogenic (female) lineage development [1, 2].

As regards the spermatogenic development, male germline stem cells (also called spermatogonial stem cells, SSCs) enter into mitotic quiescence until the end of foetal development. Soon after birth, SSCs resume active mitotic proliferation at the basement membrane of the seminiferous tubules. Furthermore, SSCs are able to balance self-renewing divisions and differentiating divisions. This delicate balance is also maintained from the complex paracrine dialogue with surrounding somatic microenvironment (stem cell niche) consisting of an ensemble of Sertoli, Leydig, peritubular myoid and vascular cells. This stem cell niche is important not only for self-renewal but also for maintaining stem cells, regulating multipotency, asymmetric cell division and migration from niches for differentiation [3].

During early embryo development, the progression of the testis occurs in a gonadotropin-independent manner and is stimulated by the high levels of AMH (anti-Müllerian hormone) produced by Sertoli cells, so being responsible of the differentiation towards male sexual development of the gonads, with the regression of Müllerian ducts. However, during the second trimester of pregnancy and after birth, the follicle-stimulating hormone (FSH) stimulates the proliferation of Sertoli cells so increasing the secretion of AMH and inhibin B, which will act as a negative regulator of the FSH production. At the same time, Leydig cells, regulated by human chorionic gonadotropin (hCG), secrete androgens, which will be responsible of the acquisition and maintaining of some male sexual characters. The pituitary gland initiates producing luteinizing hormone (LH), which is down-regulated by the testosterone secreted by Leydig cells. During this foetal period, the hypothalamus regulates the secretion of gonadotropin-releasing hormone (GnRH), and so stimulating the pituitary to produce LH and FSH, with a higher balance of LH. At the end of pregnancy, the level of these hormones will decrease due to the direct action of oestrogens produced by the placenta, whereas the levels of testosterone will increase as much as in adult life. After birth, the levels of gonadotropins as well as those of testosterone and AMH decrease to a minimum, reaching the maximum levels 3 months after birth and decreasing again from the sixth month until the puberty.

Throughout the childhood, the HPTA axis allows the proliferation of Sertoli and germ cells, but they remain immature. After this period and around the sixth month, a period of quiescence takes place where Leydig cells are missed, thereby decreasing the levels of testosterone. On the other hand, Sertoli cells still remain active and produce AMH at high levels, typical of 'prepubertal' testis.

Once arrived at puberty, the HPTA starts up again and the male sexual characteristics are developed, acquiring the reproductive capacity. Of high importance is the increase on the levels of gonadotropins and because of that the proliferation of Sertoli cells and the rise of testis volume. The high levels of LH produce the differentiation of Leydig cells as well as the maturation of Sertoli cells, the emergence of blood-testis barrier and a lowering of AMH levels. At this point, germ cells enter meiosis, concluding the spermatogenesis [4]. The most primitive SSCs (A-single cells, $A_s$) are capable to divide normally forming two new $A_s$ cells (self-renewing division) or with an incomplete cytokinesis forming a pair (A-paired, $A_{pr}$) of cells connected by an intercellular bridge (differentiating division). Then, the $A_{pr}$ cells continue to divide and generate chains of 4, 8, 16 and (sometimes) 32 cells (aligned cells, $A_{al}$). Recent studies show that the $A_s$ production by cell division is rather rare and the majority of $A_s$ results from the fragmentation of $A_{pr}$ and $A_{al}$ spermatogonia. From longer chains of $A_{al}$ spermatogonia (8, 16 and 32), classes of spermatogonia progressively differentiated ($A_1$, $A_2$, $A_3$, $A_4$, In (intermediate) and B) are produced. In comparison with the undifferentiated spermatogonia, those already differentiated divide synchronously and show the presence of heterochromatin. In most animal species, spermatogonia B are the last in suffering mitosis, producing two primary spermatocytes that progressively enter meiosis after the foetal life through all the stages of prophase I (leptotene, zygotene, pachytene and diplotene) and undergo a double meiotic division: the first originates two secondary spermatocytes diploid (2n), while the second leads to the formation of four haploid spermatids. Finally, spermatids undergo a series of cytoplasmic and nuclear changes, known as spermiogenesis. During this last phase, some important processes take place, as the DNA compaction, the loss of cytoplasm and the acrosome and flagellar formation, so leading to the release of mature spermatozoa in the lumen of the seminiferous tubule [5].

The whole process of spermatogenesis is strongly regulated by the hypothalamus-pituitary-testis axis, in such a way that the proper functioning of this axis will be responsible for the quantity and quality of the spermatozoa produced. It is important to note the reliance on the correct running of the endocrine system as well, since it will also influence the reproductive system by releasing the right amount of hormones that will allow the success of the process, as will be described later. Before explaining the endocrine control of the process, the development of this axis will be described from the beginning of the embryo life.

The hypothalamus-pituitary-testis axis initiates its function in the hypothalamus, which along with the hypophysis is the main regulator centre of the reproductive function. The hypothalamus develops from the forebrain, which along with the midbrain and hindbrain is one of the three expansions derived from the neural tube during the brain development of the embryo. After 34 days of conception, it is possible to distinguish inside the diencephalon (originated from the forebrain), a little cavity called third ventricle, whose progress will form the thalamus and it in turn will originate the hypothalamus and the epithalamus. It is located above the pituitary gland and below the thalamus, and it is surrounded by the optic chiasm, lamina terminalis, commissure rostrally, cerebral peduncle and the interpeduncular fossa caudally. The hypothalamus could be equally anatomically divided in three regions, each one with a determined cluster of neurons that carry out a specific function: periventricular zone (with periventricular, suprachiasmatic, paraventricular and arcuate nucleus), medial zone (medial preoptic, anterior hypothalamic, dorsomedial, ventromedial, premammillary, mammillary and posterior hypothalamic nucleus) and lateral zone (lateral preoptic, lateral hypothalamic and supraoptic nucleus).

The hypothalamus is highly connected with the rest of the brain through a really extensive number of fibres whose connections are complex and intricate. Therefore, this region is a key point where nerve signals that came from the central nervous system by afferent fibres will be decoded and transformed into hormonal messages (neuronal hormones) which will arrive by portal circulation to the pituitary gland. It, in turn, will release peptide hormones as a response. The hormone that functionally connects hypothalamus to pituitary gland is known as gonadotropin-releasing hormone (GnRH). It has been identified and described by the Nobel Laureates Roger Guillemin and Andrew V. Schally in 1977 and is constituted by 10 amino acids:

pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$

GnRH is produced from a 92-amino acid preprohormone in the preoptic area of the hypothalamus by GnRH neurons that originate in the nose and migrate into the brain, where they are scattered throughout the medial septum and hypothalamus and connected by dendrite long over 1 mm. They are regulated by several different afferent neurons and by different neurotransmitters such as norepinephrine, GABA and glutamate.

One of the main regulators of GnRH secretion is Kisspeptin, which acts together with oestrogen, as demonstrated by the finding that Kisspeptin-producing neurons also express oestrogen receptor alpha.

Once secreted in portal bloodstream at the median eminence, GnRH binds its specific receptor, the gonadotropin-releasing hormone receptor (GnRHR), a seven-transmembrane G-protein-coupled receptor, in gonadotrope cells. Here, it activates the beta isoform of phosphoinositide phospholipase C, leading to the calcium mobilization from intracellular stores and to the activation of protein kinase C. Finally, as a result of GnRH stimulation, the pituitary cells synthesize and secrete the gonadotropins LH and FSH.

GnRH activity is deeply connected with the regulation of reproductive activity, thus its concentration is very low during childhood, and strongly increases at puberty. During the adulthood, it regulates several biological processes involved in maintaining of fertility and, in particular, in male it sustains the spermatogenesis via FSH and LH.

The pituitary gland is the second component of the axis involved on the regulation of spermatogenesis. It is an endocrine gland located below the hypothalamus, whose main function is to produce various hormones responsible for the homeostasis of the individual. In this endocrine tissue, it is possible to identify three zones anatomically and embryologically different: adenohypophysis or anterior pituitary, intermediate lobe (present in some species or fused with the anterior pituitary in other species) and neurohypophysis or posterior pituitary [6]. Each zone has its own particular role and cell types with an essential function of hormones secretion, among which should be highlighted the importance of luteinizing hormone and follicle-stimulating hormone, both produced by gonadotrophs cells in the anterior pituitary [7].

LH is a heterodimeric glycoprotein composed by $\alpha$- and $\beta$-subunits (similar to FSH, hCG and to thyroid-stimulating hormone (TSH)). The $\alpha$-subunits contains 92 amino acids in human and 96 amino acids in almost all other vertebrates and is identical to that of LH, FSH, TSH and hCG. The $\beta$-subunit has 120 amino acids and confers its specific biologic to the protein, by interacting with the receptor (LHR). The half-life of LH is about 20 min.

In female, LH is involved in the control of ovarian activity and, mainly, of ovulation. In male, it acts on Leydig cells, stimulating the production of testosterone. This hormone, in turn, regulates the expression of the enzyme 17$\beta$-hydroxysteroid dehydrogenase, thus converting androstenedione, produced by the gonads, to testosterone [9] that exerts both endocrine and paracrine activities and then is involved in controlling spermatogenesis.

LH is released in response to a delicate feed-back that involves all the structures of hypothalamus-pituitary-testis axis. When blood concentration of T is lowering, GnRH is released by the hypothalamus, thus stimulating the LH release by pituitary gland. As the level of T increases, it inhibits the release of GnRH and LH consequently.

At the same time, androgens (T, DHT) inhibit monoamine oxidase (MAO) in pineal, leading to the increase in melatonin concentrations. Through a melatonin-induced increase of inhibiting factors (GnIH), the levels of LH and FSH are reduced. T can also be aromatized into oestradiol (E2) to inhibit LH.

FSH is a 35.5-kDa glycoprotein heterodimer, and as LH it consists of two polypeptide units, alpha and beta.

The beta subunit of 111 amino acids (FSH-β) is responsible for the binding with the follicle-stimulating hormone receptor (FSHR). The sugar portion of the hormone is covalently bonded to asparagine and is composed of N-acetylgalactosamine, mannose, N-acetylglucosamine, galactose and sialic acid.

FSH is involved in the control of several functions, such as development, growth, pubertal maturation and reproduction. In both *males* and *females*, it stimulates the maturation of germ cells and, in males, it induces Sertoli cells to secrete androgen-binding proteins (ABPs) and stimulates primary spermatocytes to undergo the first division of meiosis, to form secondary spermatocytes.

Although many aspects of the spermatogenesis remain unclear, the complexity of the process and the necessity of a major regulation, which integrates all the components aforementioned and others that are still being investigated, are obvious. More research is needed in order to conclude the unknown aspects of the whole process that entail the creation of these indispensable cells for the perpetuation of life.

## 3. Membrane-signalling systems in male germ cells

Once the maturing spermatozoa are released in the lumen of tubuli seminiferi, they progress within rete testis and reach the epididymis. Here, their membrane composition changes [8]. Overall, the lipid remodelling of sperm membrane during the epididymis involves the interaction of male germ cells with epididymal cells, fluid, and with 50–800-nm spherical vesicles present in epididymal lumen, the epididymosomes. They are secreted by principal cells of the epididymis and are involved in the exchange of several kinds of molecules with the sperm membrane [9, 10]. As stated by Rejraji '… it does not seem too farfetched to imagine that epididymosomes (and aposomes in general) could exchange lipids and protein materials with sperm cells, contributing to the formation of structures such as rafts in sperm cells membrane' [11]. In keeping with this idea, in mouse model, it has been found that the epididymosomes membranes are more fluid in the head of the epididymis and that their fluidity gradually decreases in the cauda, while the fluidity of sperm membrane increases as the spermatozoa progress along the epididymal duct [11]. More in detail, it has been found that during the epididymal transit the phosphatidylethanolamine (PE):phosphatidylcholine (PC) ratio does not change, while the concentration of sphingomyelin (SM) increases from 20.9% in caput epididymis to over 29% in cauda epididymis. The cholesterol:phospholipids remain constant and the relative amount of polyunsaturated fatty acids (PUFAs) markedly increase, particularly for 22:5 n-6 and 22:6 n-3. Importantly, the cholesterol concentration changes both in absolute and in relative terms: cholesterol 10–15 mol/spermatozoon: caput 6.9 ± 1.4; cauda 2.4 ± 0.4; cholesterol/phospholipid ratio: caput 0.24 ± 0.04; cauda: 0.289 ± 0.07 [11].

At the end of the epididymal maturation, the spermatozoa membranes have their composition fixed. Human spermatozoa membranes are characterized by high concentrations of ether-linked lipids, of unsaturated fatty acyl groups such as docosahexaenoyl (22:6 chains), and of sphingomyelin. Also present is sulphogalactosylglycerolipid or seminolipid, a spermatozoa-specific lipid,

which has been demonstrated to be involved in human gametes interaction, and immediately after ejaculation the ration cholesterol/phospholipid is around 1. Ether-linked lipids are abundant in sperm plasma membranes (PM): glycerophospholipids contain either one alkyl ether group at position sn-1 of glycerol (plasmalogens), or one (at position sn-1) or two alkyl ether groups. Choline and ethanolamine plasmalogens will be involved in modulation of membrane fluidity during capacitation. Indeed, they will act conferring a more densely packed structure to the membranes, compared with diacylglycerophospholipids. Polyunsaturated ethanolamine plasmalogens will take part in the process of membrane fusion, and choline plasmalogens contribute to form non-diffusible membrane regions that confer stability to the membranes.

Immediately after ejaculation, mammalian spermatozoa are virtually unable to fertilize; indeed, they gain their full fertilizing ability only after they reside in female genital tract for hours to weeks, depending on the species, where they complete a process of biochemical and functional maturation known as capacitation. This process has been described for the first time in the early 1950s [12–14] and has been intensively studied by several groups in human and in different animal models. To date, it is widely accepted that it implies deep changes in metabolism of male germ cells and that it involves virtually their whole biochemical machinery. In this context, sperm membrane plays a key role for important and peculiar reasons. As first, sperm membranes are the interface of male gametes with external environment. Male gametes, during spermatogenesis and before  ejaculation, male gametes are exposed to  different environments (testis, epididymis, male ducts) characterized by the presence of very different chemical components. Each of these factors is able to carry out complex interactions with sperm cells, thus modulating their function. In particular, the interaction with female genital tract is able to drive the process of capacitation by activating or inhibiting specific signal transduction pathways. Then, spermatozoa virtually have no cytosol, thus a great amount of the molecules involved in signalling pathways are localized at the cell membranes level. Consequently, these structures are well organized and highly dynamical, and their architecture and chemical composition change markedly during the process acquisition of fertilizing competence. Finally, the physiological end point of capacitation is the onset of acrosome reaction (AR), which consists in the fusion of outer acrosome membrane (OAM) with the plasma membrane in the anterior of sperm head. In other words, the fertilization is made possible by a fine regulation of inhibiting and activating factor acting at membrane level, able to promote the increase in fusogenicity (i.e. the ability to fuse each other) of PM and OAM avoiding, at the same time, their premature loss.

The pivotal importance of membrane changes during capacitation is the reason why this process has attracted the attention of researchers since its discovery. In particular, on one hand, it could be involved in determining pathological conditions that lead to hypofertility or infertility and, on the other one hand, the control of membranes composition and behaviour during capacitation could have important implication for improving the cryopreservation strategies of male gametes in human and in veterinary andrology.

### 3.1. Spermatozoa membrane dynamics

The data from several groups, obtained in different animal models (particularly boar), converge in describing a multi-step process [15] that leads to the functional maturation of membranes.

Immediately after ejaculation, the spermatozoa are exposed to a gradually increasing concentration of bicarbonate. This ion acts as an activating factor, stimulating a protein kinase A (PKA)-mediated pathway. In detail, $HCO_3^-$ activates a soluble adenylate cyclase (sAC), leading to an increase in the production of cAMP, which in turn activates PKA [16]. This last event represents an important step because it promotes the activation of several enzymes that are involved in lipid translocation across specific domains of sperm PM (particularly located in the anterior area of sperm head). Here, the segregation of specific classes of lipids in the inner or outer leaflet of plasma membrane [17, 18] has been described. The aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine are specifically concentrated in the inner leaflet, while choline phospholipids sphingomyelin and phosphatidylcholine in the outer one. This asymmetry is established and actively maintained by the action of translocating enzymes. Two of them (aminophospholipid translocase, also known as flippase, and floppase) are ATPases, thus working against gradient using ATP as energy source and transferring PS and PE from the outer to the inner lipid leaflet or vice versa, respectively. They are constitutively active in mature spermatozoa and are responsible for maintaining the physiological asymmetry of membranes. A third enzyme, the scramblase, acts as a bi-directional carrier with low specificity for specific classes of lipids, simply moving in both directions (inward and outward) across the membrane following the concentration gradient (it does not require ATP), thus reducing phospholipid asymmetry ('lipid scrambling'). This last event represents a pivotal biological end point because it is believed to be mandatory to allow the cholesterol relocalization [19] and consequent extraction from sperm membrane [20] (**Figure 1**).

Recently, it has been proposed that the capacitation-dependent lipid remodelling of sperm membrane and the apoptotic pathway could be interconnected. In particular, the generation of reactive oxygen species (ROS), particularly peroxynitrite, which takes place during capacitation, could facilitate the removal of cholesterol from PM [21], thus increasing its fluidity, cause a change in the pattern of protein tyrosine phosphorylation and increase the cAMP production [22, 23]. If the fertilization does not occur, the accumulation of ROS could activate the apoptosis via the intrinsic pathway [24]. In a computational biology study, our group implemented this hypothesis, speculating that the spermatozoa, during their journey to reach the fertilizing ability, could pass through an intermediate condition, from which they could reach very different fates: fertilizing spermatozoa, apoptotic spermatozoa, dead spermatozoa. In other words, in earlier stages, it is possible to hypothesize that capacitation and apoptosis are partially overlapping, proceeding parallel, rather than in series [25].

In addition, in spermatozoa, as it occurs in other eukaryotic cell types, specialized micro- and macro-areas known as micro-domains have been described. They can be experimentally isolated by using detergents, such as 0.1% Triton X-100, at 4°C in a discontinuous density gradient, as detergent-resistant membrane (DRM). DRM is organized in a 'lipid-ordered' phase ($L_o$ phase) because of their chemical composition: they are rich in cholesterol, sphingomyelin, gangliosides, phospholipids with saturated long-chain acyl chains, and proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins, caveolin and flotillin. DRM is surrounded by a more fluid portion of membrane organized in a 'liquid-disordered phase' membrane ($L_d$ phase), characterized by higher concentration of unsaturated fatty acids. In this context, the cholesterol plays a pivotal role; indeed its hydroxyl group interacts with the
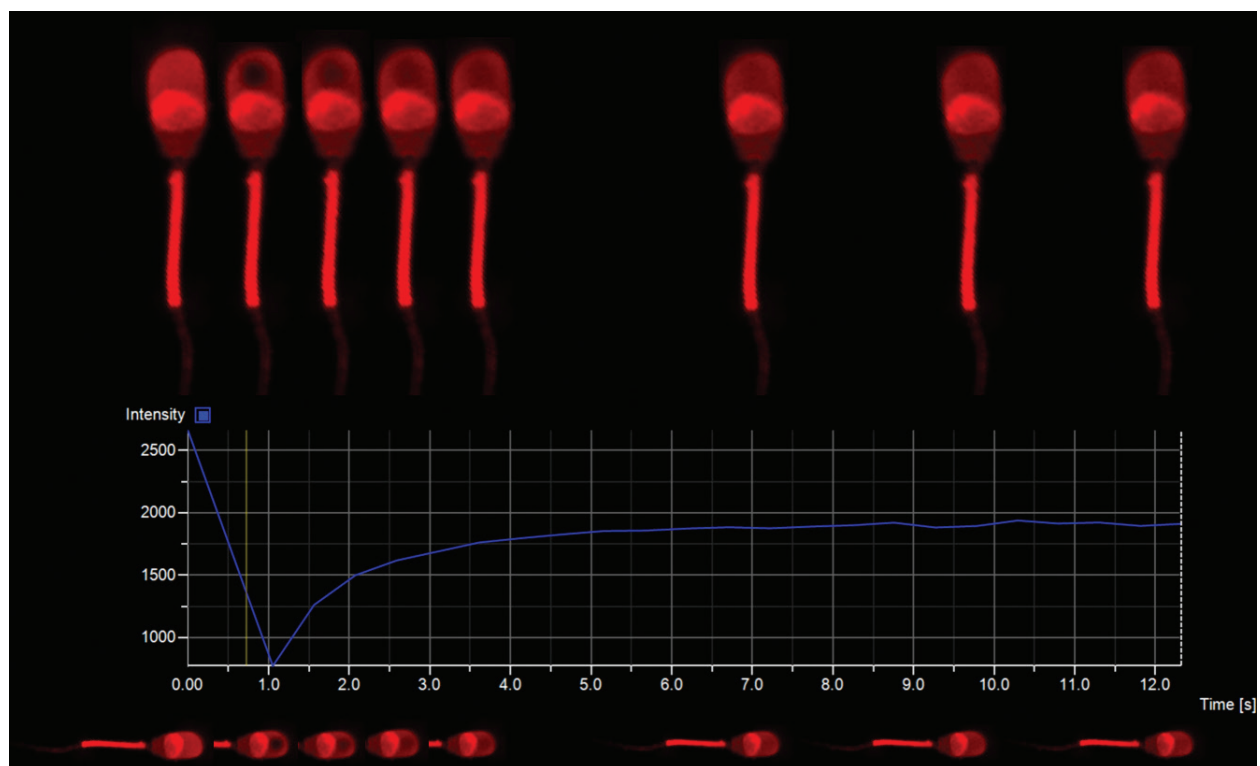
**Figure 1.** Confocal images of spermatozoa stained with DilC12 probe during a fluorescence recovery after photobleaching (FRAP) experiment, in which it is possible to measure the diffusion coefficient of the probe to assess membrane fluidity.

polar head of phospholipids and sphingolipids, while the steroid and hydrocarbon chains are embedded in the membrane, alongside the nonpolar fatty-acid chain of the other lipids. Consequently, it modulates the physical-chemical proprieties of cell membranes, depending on their composition. In unsaturated fatty acid-rich areas of membrane, cholesterol increases membrane packing, reducing membrane fluidity. On the contrary, when cholesterol intercalates in a microenvironment rich in saturated fatty acids it promotes the relaxation of membrane structure, thus increasing membrane permeability and fusogenicity [18].

DRMs organization undergoes evident modifications, with important physiological consequences on the function of male germ cells. Cross in human spermatozoa described the heterogeneous composition of DRMs and found that the two major raft components, GM1 and CD59, displayed a partial sterol loss-dependent shift to the non-raft domain during capacitation [26]. Miranda et al. demonstrated that LR markers (CAV-2, flotillin 1, flotillin 2 and GM3) changed their immunofluorescence pattern during sperm incubation under capacitating conditions and that these changes are correlated with the occurrence of AR [27]. Watanabe and Kondoh showed that in GFP-labelled sperm, treated with compounds for promoting the acrosome reaction, EGFP-GPI was released from the sperm surface and that ganglioside GM1 relocalizes over sperm head [28]. This leads to hypothesize that GPI-anchored protein release could be associated with reorganization of lipid rafts and with the onset of AR. These data became more interesting when considering that it has been described the capacitation-dependent relocalization in DRMs of proteins known to be involved in signal transduction and in sperm-egg interaction and binding.

For instance, it has been found that cannabinoid receptor type 1 (CB1) and transient receptor potential cation channel subfamily V member 1 (TrpV1) receptors are localized in DRMs at sperm head level. Immediately after ejaculation, CB1 localizes in high-density membrane fractions while, after exposure of spermatozoa to bicarbonate, it redistributes in DRMs. Noteworthy, during this translocation, the glycosylated from of CB1 receptor reaches about 50% of the total molecule. This change suggests that these receptors could play an important role in signalling pathways involved in capacitation [29, 30]. TRPV1 concentration in DRMs increases after exposure to bicarbonate. Since it has been demonstrated that this endocannabinoid-endovanilloid receptor is actively involved in regulation of important biological processes (control of transmembrane potential, regulation of intracellular calcium concentration and actin polymerization) [31, 32], it is possible to hypothesize that its redistribution could have important physiological consequences in controlling the acquisition of fertilizing ability by male germ cells (**Figure 2**).

Similarly, in DRMs, several proteins belonging to the Soluble NSF Attachment Protein REceptor (SNARE) family are present, which are known to be involved in the control of membranes fusion, such as R-SNAREs like synaptobrevin (VAMP) and Q-SNAREs like syntaxin, the $Ca^{2+}$ sensor protein synaptotagmin and the ATPase NSF.

It is very interesting to report that in sperm cells, a protein known to be mainly responsible for the formation of caveolae, caveolin 1 (Cav-1), could be involved in DRMs dynamics without forming caveolae. Travis described the localization of caveolin in rat sperm in cholesterol-enriched areas [33]. This finding led to hypothesizing that this protein could be involved in membrane fusion and, ultimately, AR. Gamboa and Ramalho-Santos in an
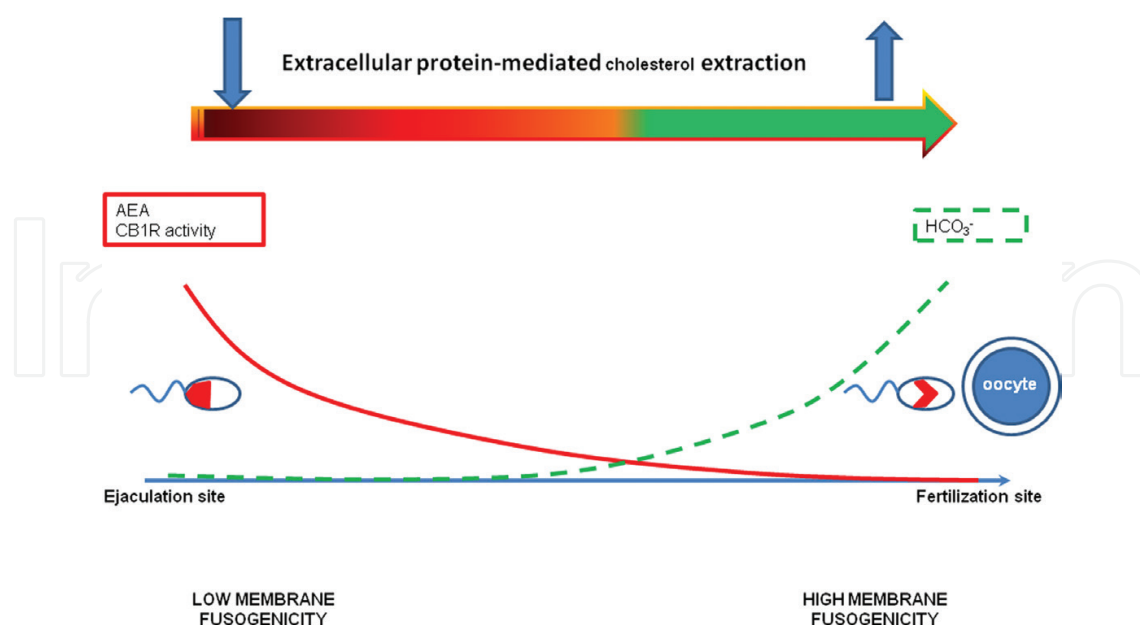


**Figure 2.** Effect of endocannabinoids and bicarbonate gradients on sperm membrane physico-chemical properties. AEA concentration and CB1R-binding activity decrease (dark gray line) while the bicarbonate concentration increases (light gary dot line). In parallel, the localization of CB1R changes (from Pattern A to Pattern B) and the extracellular protein-mediated cholesterol extraction increases. As a consequence, the membranes became more fusogenic. From Ref. [30].

immunocytochemistry experiment found that anti-Cav-1 antibodies display a strong immunopositivity in acrosomal region and in equatorial segment of the sperm head. Botto et al. (2010) demonstrated that the amount of Cav-1 significantly increases in the insoluble membrane fraction in spermatozoa incubated in vitro under capacitating conditions (from 35 and 20% to 60 and 70%, respectively) when compared with freshly ejaculated sperm cells [34]. More recently, Baltiérrez-Hoyos et al. confirmed the idea that CAV-1 could be involved in biochemical machinery that controls capacitation and the AR. They proposed that it interacts with CDC42, which plays a central role in acrosomal exocytosis through the activation of SNARE proteins and actin polymerization; in particular, they suggested that CDC42 activation is favoured by the disruption of the CAV1–CDC42 interaction [35].

### 3.2. Membrane fusion and acrosome reaction

The biological end-point of capacitation is the ability to interact with oocyte, undergoing AR. Ultimately, its first step is the fusion of PM and OAM, which is made possible by the lipid remodelling that has occurred during capacitation. The ability of membrane to fuse each other depends on the physical-chemical characteristics of membranes themselves. Indeed, the ability to form 3D structure and the attraction/repulsion between the two membranes vary with the lipid composition of bilayers, and the formation of nucleation points is favoured by the presence of specific families of proteins (fusion proteins). As it is known, from a physical point of view, this event is the result of the coordinated and regulated interplay among various interfacial forces, namely hydration repulsion, hydrophobic attraction and van der Waals forces.

*Hydration repulsion*: Two hydrated bilayers undergo strong repulsion as they approach each other. This repulsion (hydration repulsion) is due to the water molecules that hydrate the bilayers and is defined as the work required for removing the water molecules bound to hydrophilic molecules exposed to the outside of the bilayer, such as the polar heads of lipids.

The potential $V_R$ is given by

$$V_R = C_R^{[-\frac{z}{\lambda R}]} \tag{1}$$

where $C_R$ (>0) is a measure of the hydration interaction energy for hydrophilic molecules of the given system, $\lambda_R$ is a characteristic length scale of hydration repulsion, and $z$ is the distance of separation.

*Hydrophobic attraction*: Hydrophobic force is active attracting two hydrophobic groups in polar media (usually water). In biological membranes, the attractive force between long hydrocarbon chains of lipids represents the main responsible for hydrophobic attraction. The magnitude of this force depends on the hydrophobicity of the interacting molecules and on the distance among them (it decreases approximately exponentially with the distance). This force is the long-ranged and the strongest among all the physical interactions operating between biological surfaces, and it is thought to be involved in folding and stabilization of proteins and macromolecular complexes.

The potential $V_A$ is given by

$$V_A = C_A \left[ -\frac{z}{\lambda A} \right] \tag{2}$$

where $C_A$ (<0) is a measure of the hydrophobic interaction energy for the given system, $\lambda_A$ is a characteristic length scale of hydrophobic attraction, and $z$ is the distance of separation.

*van der Waals forces*: These forces are due to the dipole-dipole interactions (induced/permanent) between the molecules present in membranes. Indeed, as molecules come closer, this attractive force increases due to the ordering of these dipoles.

van der Waals interaction potential $V_{V DW}$ is given by

$$V_{V DW} = \frac{H}{12 \pi} \left( \frac{1}{z^2} - \frac{2}{(z + 2D)^2} + \frac{1}{(z + 2D)^2} \right) \tag{3}$$

where $H$ is the Hamaker constant, $D$ and $z$ are the bilayer thickness and the distance of separation, respectively.

The balancing among these forces, as the membranes approach, drives their fusion. In this process, different steps have been identified. First, when the two lipid bilayers became closer, they are weakly attracted by van der Waals forces (that contribute minimally to the evolution of the system) and are subjected by the strong repulsive forces of hydration repulsion. Then, the hydrophobic tails of lipids are exposed to the aqueous phase surrounding them, giving rise to a very strong hydrophobic attraction (which overcomes the repulsive force).

It is worth noting that the lipid remodelling that occurs during capacitation has important implication on the ability of membranes to fuse (membrane fusogenicity). In particular, the chemistry of lipids has a key role in controlling the fusogenicity of membranes. The more the lipid head is polar, the more strongly it binds water and the greater is the hydrophilic repulsion force. On the contrary, the more the lipid acyl chain is longer, the greater is the hydrophobic attraction force. In addition, small polar heads and unsaturation points facilitate the formation of 3D geometries, which is a necessary precondition for membranes fusion.

During capacitation, membranes experience some important changes, which altogether concur in increasing membrane fusogenicity. The lipid scrambling allows the increase of phosphatidylethanolamine, which is characterized by a small slightly polar head, concentration and the increase in unsaturated and PUFA relative concentration (by the cholesterol depletion) in the outer leaflet of PM.

Consequently, at the end of capacitation sperm membranes are fusogenic enough to be able to fuse, but remain unfused waiting for the activator stimulus (the ZP proteins). When the oocyte is met and the spermatozoa interact with ZP, the fusion starts, thanks to the formation of nucleation points, that is, of limited areas where the thermodynamic obstacle of the charges present on membrane surface is overcome. As already told, fusion proteins are the key element of nucleation. In human and animal spermatozoa, the most important fusion proteins are thought to be the SNARE. They are a protein superfamily with more than 60 members that can be divided in two different categories: *vesicle* or *v-SNAREs*, embedded in membranes of transport vesicles, and *target* or *t-SNAREs*, located in the membranes of target

compartments. Based on the aminoacidic sequence, they can also be divided in R-SNAREs (arginine-containing SNAREs) or Q-SNAREs (glutamine-containing SNAREs). The SNARE domain is constituted by heptad repeats of 60 amino acids forming a coiled coil.

The mechanism by which SNAREs are involved in membrane fusion is called 'SNARE hypothesis'. This model has been developed in neurons, in which in response to $Ca^{2+}$ influx, synaptic vesicles of neurotransmitter fuse with the membrane at the presynaptic level. The result of vesicle and cell membrane fusion is the release of neurotransmitter into the synaptic cleft. Three different proteins belonging to the SNARE family are involved in this exocytotic event. Vesicle-associated membrane protein (VAMP)-2, located in the vesicular membrane, synaptosome-associated protein (SNAP)-25, which contains two SNARE domains and a region of palmitoylated cysteines, and syntaxin 1A, located in the plasma membrane. At the time of membrane fusion, the coiled-coil-forming domains of syntaxin, SNAP-25 and VAMP form a complex, resistant to sodium dodecyl sulphate (SDS) denaturation, protease digestion and clostridial neurotoxin cleavage, heat stable up to ~90°C. The core of this complex is formed by a long (12-nm), twisted, parallel four-helix bundle: two helices are contributed by SNAP-25 and the others are from VAMP-2 and from syntaxin 1A, respectively. The coiled bundle is 16 layers deep and a layer near the middle, the ionic central layer (the 'zero layer'), contains three glutamines and one arginine (from VAMP-2).

From the data on neuronal SNAREs, a general model has been hypothesized [36, 37]. In the first step, v-SNAREs, on vesicle membrane, form a highly stable trans-complex with t-SNAREs, on cell membrane. The formation of SNARE complex allows the membrane fusion, acting as nucleation point (minimal fusion machinery hypothesis). Interestingly, it is thought that SNAREs have a complex role that go beyond the merely mechanical action. Indeed, they cause the membrane dehydration, thus removing this thermodynamic barrier to fusion, and exert a force on membrane, allowing the formation of a fusion intermediate. After the fusion is completed, SNAREs form a cis-SNARE complex.

The SNARE model of fusion has been proposed also in male [38]. Interestingly, since in spermatozoa AR is a terminal event that occurs only once in cell life, different from what happens in secreting cells where the complex could be disassembled and recycled for further rounds of fusion, here the SNAREs are associated to form ternary cis-complex unstable and insensitive to neurotoxins. When AR takes place, calmodulin is activated by calcium intracellular concentration peak [39] and promotes the activation of RAB3A, which in turn allows the cis-SNAREs disassembly by SF/a-SNAP. Then, monomeric SNAREs form trans-complexes, causing the irreversible docking of the acrosome to the PM. When $Ca^{2+}$ is released from the acrosome through inositol 1,4,5 trisphosphate-sensitive $Ca^{2+}$ channels, the final steps of membrane fusion take place, with the formation of trans-complexes.

Interestingly, in stallion spermatozoa, it has been found that SNARE protein colocalizes with Cav-1 and that fertility seems to be related with the percentage spermatozoa immunopositive for synaptotagmin (a calcium sensor), NSF (a SNARE complex disassembler) and caveolin-1 (a signalling pathways organizer) [34].

# 4. Cytoskeleton dynamics

During the capacitation, as already told, the membranes fluidity and fusogenicity markedly increase, thus they become more and more instable. This condition on one hand is a prerequisite mandatory to achieve membrane fusion, but on the other one, it could cause the loss of acrosome integrity, with irreversible consequences on fertilizing ability of the gamete. This is the reason why a mechanism that acts as a controller of membrane fusion has been evolved, in sperm head. In this context, the key role is played by actin cytoskeleton. Actin is a 42-kDa protein with a diameter of 4–7 nm present in virtually all the tissues. It has a globular structure, composed by two distinct domains (one larger and one smaller) separated by a cleft, which represents the 'ATPase fold', the centre of enzymatic catalysis that binds ATP and $Mg^{2+}$ and hydrolyses the former to ADP plus phosphate. The domains are separated in subdomains: the smaller domain is composed by subdomain I (lower position, residues 1–32, 70–144 and 338–374) and subdomain II (upper position, residues 33–69). The larger domain is also divided in subdomain III (lower, residues 145–180 and 270–337) and subdomain IV (higher, residues 181–269). What is very important, for the functional characterization of actin polymerization, is that the exposed areas of subdomains I and III form the 'barbed' ends, while the exposed areas of domains II and IV form the 'pointed' ends. The two ends show different affinity for other actin molecules, thus allowing a controlled growth of actin filaments. Indeed, when actin binds ATP forms a stable monomer. Three or more monomers, binding each other, form oligomers that act as nucleation point for the growth of F-actin polymer. Interestingly, unlike other biologically relevant polymers, the monomers of actin are assembled to form filaments by weaker bonds, due to the lateral bonds with neighbouring monomers, which contribute to the stabilization of F-actin. In addition, several proteins are involved in favouring actin polymerization, stabilization and de-polymerization, giving rise to a dynamic process that allows a finely regulated participation of cytoskeleton at a myriad of biological processes.

In spermatozoa, during capacitation G-actin present in sperm head and tail undergoes polymerization under the control of a network of signals. In particular, recent observations suggest that PKA activates Src to inactivate by phosphorylation PIP2-bound gelsolin [40]. Gelsolin is an 82-kD protein with six homologous subdomains (S1–S6), each is composed of a five-stranded β-sheet, flanked by two α-helices. It is one of the members of the actin-severing gelsolin/villin superfamily and acts as a binding protein that regulates actin filament assembly and disassembly. In particular, gelsolin activity is under calcium control and binds to the barbed ends of actin filaments, preventing monomer exchange (end-blocking or capping). In addition, it can promote nucleation (the assembly of monomers into filaments), as well as sever existing filaments. PIP2 is a cofactor for PLD activation stimulated by PKCα, that leads to phosphatidylcholine hydrolysis and production of phosphatidic acid, PA [41, 42]. PA, in turn, activates the polymerization of G-actin to form F-actin. Thus, the activation of PLD and the prevention of F-actin dispersion by inhibiting gelsolin allow F-actin formation. F-actin in the head acts as a diaphragm between PM and OAM, thus preventing immature acrosome reaction. At the AR, the fast peak of intracellular calcium concentration caused the rapid
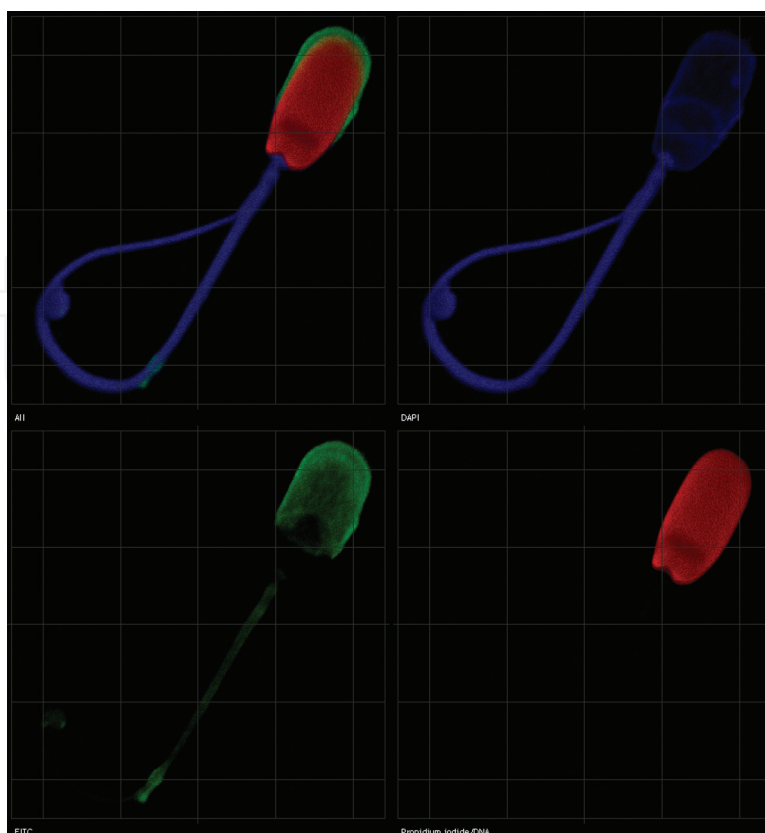
**Figure 3.** Confocal images showing nucleus (upper right panel), acrosome (lower left panel) and actin cytoskeleton (lower right panel).

destruction of this network, allowing membrane fusion. In the tail, F-actin is thought to play a role in regulating sperm motility including HA motility [40] (**Figure 3**).

Interestingly, actin cytoskeleton has been found to be involved in DRM's stability and relocalization in several cellular models. In spermatozoa, it was observed to move out of the DRM fractions in capacitated sperm [43] and that its polymerization and changes in F-actin structure or orientation during capacitation could be responsible for the loss of association with DRMs [43].

In addition, it has been supposed that F-actin formation during capacitation could have other roles, not merely mechanic, being involved in coordinating the spermatozoa-signalling systems [32]. This suggestion is in keeping with newly emerging evidences that in different cellular systems the cytoskeleton exerts a key role in signal transduction. Indeed, it has been proposed that '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' [44]. This hypothesis is confirmed and strengthened by the finding that in a 20-μm-diameter generic cell the plasma membrane surface area is of about 700 μm², while the total surface area of a typical concentration of 10 mg/ml F-actin is 47,000 μm² [44] and that the diffusion of signal molecules along cytoskeleton could be a reliable alternative way of intracellular trafficking.

## 5. GSCs, spermatozoa and endocrine disruptors

The signalling systems that lead GSCs through their road to the differentiation and the achievement of fertilizing ability are very delicate. Any perturbation could cause important negative effects of male fertility. Recently, important international agencies have documented an alarming decrease in human fertility [45, 46]. Although there are yet no conclusive certainties about this phenomenon during the years, different factors have been proposed to be involved in the accumulation of risk factors for infertility and for male infertility. These factors could be either related to social changes as well as to lifestyle [47, 48], such as smoke of tobacco [49–51] and marijuana [52–54], alcohol [55, 56], medications [57] and caffeine [58], but also to the environmental pollution, such as pesticides, solvents [59, 60], electromagnetic fields (EMFs) [61–63] and compounds able to interfere with the endocrine control of biological functions.

One of the most important environmental factors that negatively influence the reproductive health is the exposition to endocrine disruptors, which act altering normal endocrine hormone signalling at the receptor and at the signal transduction level. Exposition to these substances can promote dysfunction in the physiology and epigenetic transgenerational inheritance of disease, affecting also to primordial germ cells. There is a huge number of compounds considered as endocrine disruptors, as, for example, bisphenol A (BPA), vinclozolin (VCZ), dichlorodiphenyltrichloroethane (DDT), methoxychlor, phthalates, genistein, diethylstilbestrol (DES), N,N-diethyl-meta-toluamide (DEET), 2,3,7,8-tetrachlorodibenzo[p]dioxin (TCDD) or jet fuel (JT8).

The study of these disruptors has acquired a higher importance since it has been elucidated that in addition to the direct effects of exposure on an individual these endocrine disruptor compounds (EDCs) are capable of producing also molecular alterations by epigenetic mechanisms, thereby transmitting these changes to the following generations. During the last few years, some researchers have focused their attention on the study of these epigenetic mechanisms, and certain of them will be discussed in order to better understand the *modus operandi* of the participant EDC.

First of all, it is important to note the effects of the widely used bisphenol A, where it is possible to find in many common objects used every day as food containers, feeding bottles or other plastic materials. Some researchers [64] have demonstrated the negative effects of BPA on the expression of pre- and early-meiotic germ cell marker genes and also on somatic cell markers. For example, BPA up-regulates some genes with key roles in germ cell differentiation, as *Stra8* (meiotic entry gene), *Dazl* (required for induction of *Stra8* and initiation of meiosis), *Dmrt1* (gonad-specific transcription factor), *Sycp3* and *Dmc1* (meiosis-specific proteins), modifying also the expression of specific somatic cell markers as *Sox9*, *Fgf9*, *Foxl2* and *Wnt4*, and GSC markers as *Oct4*, *Prdm14* and *Blimp1*.

In relation to plastic materials, the toxic effects of phthalates, plasticizers used to confer flexibility and transparency to these plastic containers, as the di-(2-ethylhexyl) phthalate (DEHP) and the dibutyl phthalate (DBP), whose embryonic exposure was discovered to produce specific changes on the germ cell line [65–67], should also be exposed.

Another EDC that should be mentioned is the fungicide vinclozolin (3-(3, 5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione), extensively used in the wine industry and whose metabolites act as antagonists of the androgen receptor (AR)-binding ligand. Many studies [68–70] have been carried out to elucidate the effects of this fungicide, showing how the embryonic exposition to this EDC induces transgenerational defects in spermatogenesis and in sperm viability. Recently, the presence of transgenerational changes in some miRNAs whose target genes are *Lin28/let-7/Blimp1* was discovered, which are involved in the specification of GSCs in mice (also called PGCs, primordial germ cells) [71]. Some phytoestrogens have been also studied by some of the authors, as the isoflavonoids genistein and daidzein, although different results have been obtained by researchers [68, 72] and the mechanism of action and epigenetic modifications should be accurately elucidated yet.

There are many other EDCs commonly used in agriculture as the insecticide dichlorodiphe-nyltrichloroethane, which has been defined by researchers [73] as promoter of sperm epi-mutations and differential DNA methylation regions (DMRs) causing transgenerational transmission of obesity. As a substitute of DDT, the pesticide methoxychlor has been used, which is also investigated by some researchers [66, 74] because of the transgenerational defects in sperm induced after embryonic exposure, which confers to the active metabolites the capacity of altering the activity of oestrogens and androgens by a receptor-binding mecha-nism. Moreover, related to the environmental field it is possible to find the herbicide dioxin (2,3,7,8-tetrachlorodibenzo[p]dioxin), the pesticide permethrin and the insect-repellent N,N-diethyl-meta-toluamide, also studied by researchers [75–77], who concluded their promotion of epigenetic transgenerational inheritance of adult-onset disease.

After the emergence of the idea that maternal exposition during pregnancy to some drugs and chemical compounds was able to produce changes on individual later in life, tributyltin (TBT), an environmental compound able to produce obesity, was studied because of its criti-cal modifications on adipogenesis [78]. In addition to tributyltin, also hydrocarbon mixture of jet fuel was proved to produce epigenetic transgenerational inheritance of disease. This type of hydrocarbon, used by the military, resulted really toxic for the immune system and a promoter of epimutations in sperm in some generations after exposition.

In conclusion, many EDCs have been investigated during the last years. Some of the most important (because of their wide use) have been exposed, but more research is still necessary in order to better understand the new compounds appearing with the new forms of life.

## 6. Future directions

Nowadays, the extensive adoption of sophisticated biological approaches, such as those based on high-throughput technologies and on –*OMICS*, is providing scientists a huge amount of information. Virtually each month new papers are published about proteomic or genomic and epigenomic analysis of male and female germ cells. This, from one side, could have important positive consequences on our knowledge and on the understanding of human and animal

fertility biochemistry and molecular biology, while from the other side poses new problems. The most important one is the so-called big data challenge.

As claimed by authoritative scientists [79, 80], the reductionist paradigm that 'the ultimate aim of the modern movement in biology is to explain all biology in terms of physics and chemistry' [81] is today inadequate to explain complex biological phenomena, as fertility is. The switch from a single molecule-oriented reductionist approach to the whole system-oriented holistic approach (characteristic of systems biology) requires the adoption of mathematical formalisms used in studying complexity. For instance, our group has recently developed a
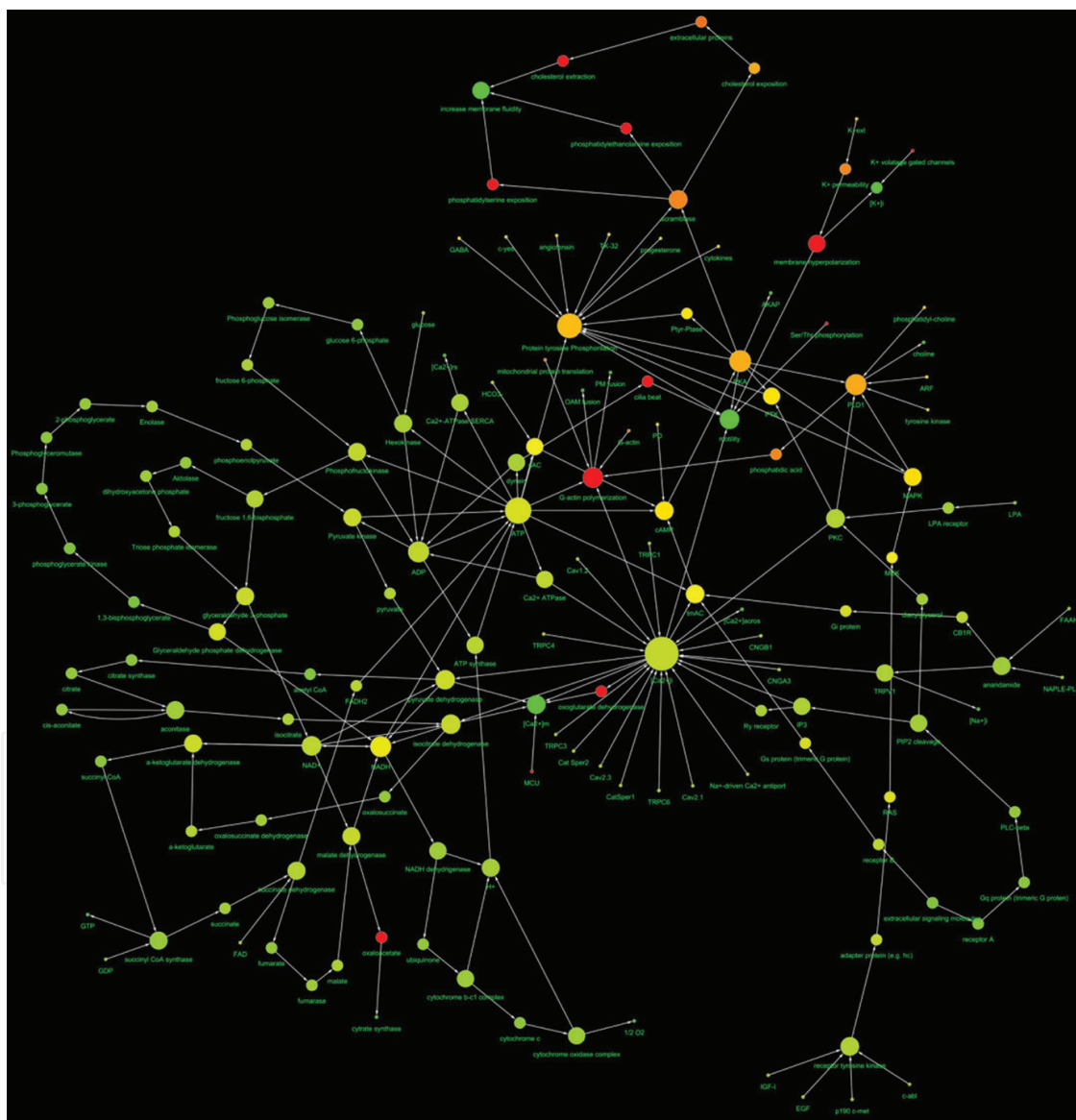


**Figure 4.** Diagram showing the structure of the capacitation network. The nodes diameter is proportional to the number of links; the gray scale varies depending on the network centrality. The direction of arrows represents the direction of the interaction (from the source to the target). The spatial network arrangement was obtained by using the Cytoscape Spring-embedded Layout (see the text for explanation). From Ref. [83].

biological network-based computational modelling approach, useful to describe and to study the events involved in epididymal maturation of spermatozoa [82], as well as sperm capacitation and acrosome reaction [32, 83–85]. In particular, the molecules involved in these events are represented as nodes linked by their reciprocal interactions, thus originating a network. The analysis of network topology could provide very important information about the architecture of the system and could offer the possibility to take biologically relevant inferences (**Figure 4**).

More in general, in our opinion, one of the most promising directions that are emerging in this fascinating field of research is the adoption of mathematical and computational modelling methods. Obviously, it requires a high degree of interconnection among different disciplines (biology, biochemistry, molecular biology, medical and clinical sciences, computer science, systems science, physics, mathematics and statistics) and it poses new challenges related to the data analysis, data storage and security, data property and data sharing as well as to the availability of computational facilities and resources. But potentially it opens very new and unexpected perspectives on biology of germ cells either in physiological and pathological conditions and could be useful in studying infertility of unexplained origin.

# 7. Conclusion

The study of GSCs and of CGs is a very fascinating branch of biology that could give information important for basics and applied science and that could contribute in the understanding of infertility causes.

Much has been done and much still remains to be done, here we conclude with the WHO statement '*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*' [86].

# Acknowledgements

# Abbreviations

| WNTs | Wingless-related integration site family of proteins |
| Smad1 4 5 | Small mother against decapentaplegic 1, 4, 5, proteins |

| | |
|---|---|
| BLIMP1 | A protein that in humans is encoded by the *PRDM1* gene |
| PRDM1 | PR domain zinc finger protein 1 |
| AP2γ | Activator protein 2γ |
| OCT4 | Octamer-binding transcription factor 4 |
| NANOG | A transcription factor encoded by the *NANOG* gene |
| SOX2 | SRY (sex-determining region Y)-box 2 |
| Stella alias DPPA3 | Developmental pluripotency-associated protein 3 |
| Hoxb1 | A transcription factor involved in controlling the body plan of an embryo along the cranio-caudal axis |
| Hoxa1 | A transcription factor involved in controlling the body plan of an embryo along the cranio-caudal axis |
| Evx1 | Even-Skipped Homeobox 1 |
| Lim1 | Transcription factor in mice that is involved in the control of head structures formation |
| DNA | Deoxyribonucleic acid |
| FSH | Follicle-stimulating hormone |
| LH | Luteinizing hormone |
| GnRH | Gonadotropin-releasing hormone |
| HPTA | Hypothalamic-pituitary-testicular axis |
| TSH | Thyroid-stimulating hormone |
| TSHR | Thyroid-stimulating hormone receptor |
| T | Testosterone |
| DHT | Dihydrotestosterone |
| cAMP | Cyclic adenosine monophosphate |
| ROS | Reactive oxygen species |
| EGFP | Enhanced green fluorescent protein |
| CAV2 | Caveolin 2 |
| GM1 | Monosialotetrahexosylganglioside |
| CDC42 | Cell division control protein 42 homologue |
| SNARE | Soluble NSF Attachment Protein) Receptor |
| RAB3A | Ras-related protein Rab-3 |

| NSF | N-ethylmaleimide-sensitive factor |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PLD | Phospholipase D |
| PKC$\alpha$ | Protein kinase C alpha |

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