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Effects of antioxidants, reduced glutathione and melatonin, on in vitro boar sperm capacitation and acrosome exocytosis.

MARTINA ROCCO

Ph.D Thesis

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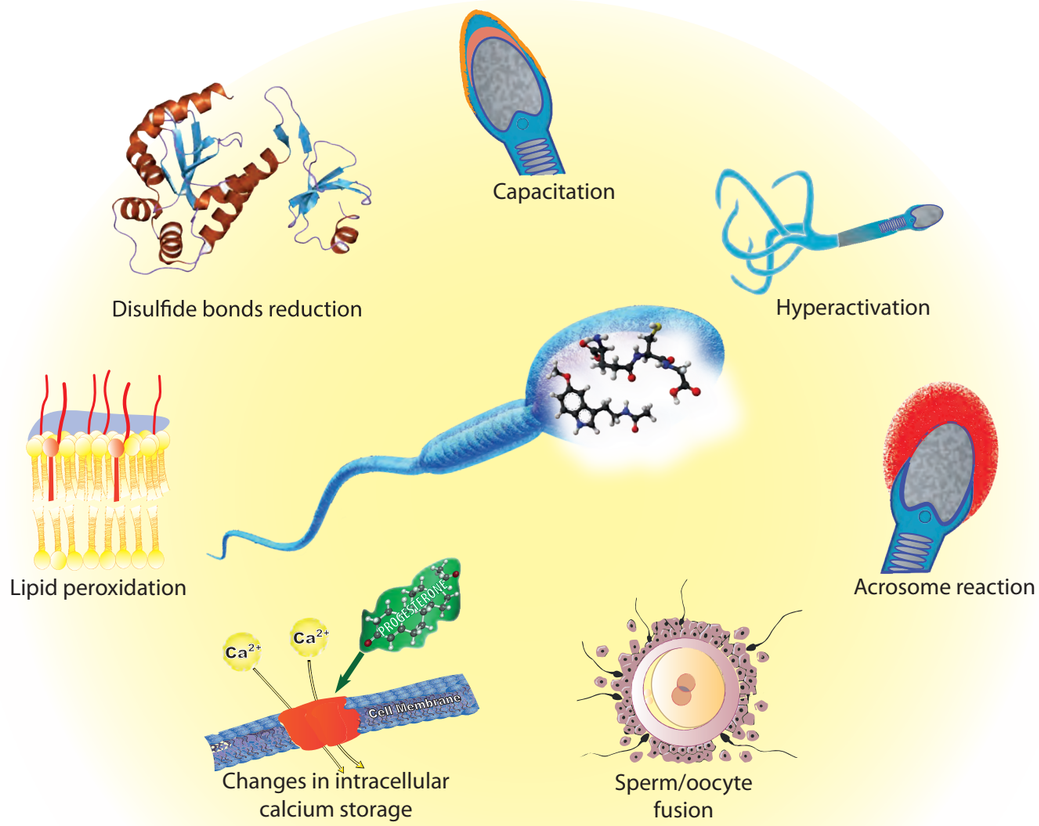

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To my sister

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Abstract

The assisted reproductive technologies allow investigators to discern how mammalian sperm achieve capacitation through a sequence of biochemical modifications that lead to the establishment of fully fertilizing ability. In this way, *in vitro* studies on boar spermatozoa incubated in a specifically designed *in vitro* capacitation medium (CM) for 4h show the whole of structural and functional changes that boar sperm suffers during the achievement of the capacitation status. These changes comprise modifications in parameters like sperm motility, membrane lipid disorders, mitochondrial membrane potential, intracellular calcium levels, tyrosine phosphorylation levels of protein and their new arrangement, and increasing of intracellular levels of free cysteine radicals. The addition of antioxidants such as reduced glutathione or melatonin to the medium prevented or modified the majority of capacitation-like changes. The aim of this thesis was to study the effects of two known antioxidant agents, reduced glutathione (GSH) and melatonin, on the achievement of boar sperm *in vitro* capacitation (IVC) and subsequent *in vitro,* progesterone-induced acrosome exocytosis (IVAE). For this purpose, in the first study the CM was supplemented with three different concentrations of GSH. Results shown that GSH-sensitive mechanisms had important effects on the achievement of a feasible boar sperm IVC and subsequent, progesterone-induced IVAE. This is concluded by the fact that several important markers of the achievement of boar sperm IVC like motion parameters, changes of sperm mebrane fluidity and the overal tyrosine phosphorylation status are altered by GSH. In the second study, melatonin was added to the CM in three separate concentrations. Supplementation of melatonin resulted in a noticeable negative effect on motility, which was concomitant with a very intense increase in the percentage of agglutination between cells in a dose–dependent manner. Meanwhile, melatonin seems to have a positive effect on superoxids and peroxids levels, and also seems to stabilize the levels of free-cysteine radicals depending on its concentration in the extender. In

conclusion, our researchs would suggest that boar sperm capacitation is linked to an increase of free cysteine residues and intracellular reactive oxygen species levels. Furthermore, these increases seem to be instrumental in the launching of subsequent IVAE. Thus, the launching of a mild oxidative stress status might be one of the factors related with the establishment of a feasible IVC and subsequent IVAE. In this way, antioxidants like GSH and melatonin seem to have an effect on ionic and redox mechanisms, altering some biochemical and fisiologic process, and compensing the damageous effects provoked by an excessive exposition of the cells to ROS compounds.

Riassunto

Le tecniche di riproduzione in vitro, permettono di investigare riguardo i meccanismi biochimici connessi con la capacitazione spermatica, e la successiva fecondazione. A tal proposito, studi in vitro su spermatozoi di suino incubati per 4 ore in uno specifico medium capacitante (CM), rendono possibile effettuare un'analisi approfondita dei cambiamenti strutturali e funzionali che si verificano a livello spermatico durante il raggiungimento dello status capacitato. Tra i parametri interessati dai suddetti cambiamenti ritroviamo: la motilità spermatica, disordini della membrana lipidica, cambiamenti nel potenziale di membrana mitocondriale, variazioni nei livelli di calcio intracellulari, differenti livelli di fosforilazione delle tirosine proteiche e una nuova ridistribuzione delle stesse, ed infine un incremento dei livelli intracellulari dei radicali liberi di cisteina. L'aggiunta al medium di antiossidanti come, il glutatione ridotto (GSH) o la melatonina sembra intervengano nella prevenzione e/o modificazione della maggior parte dei cambiamenti biochimici associati alla capacitazione. Scopo della tesi è stato di studiare gli effetti di questi agenti antiossidanti, sul raggiungimento della capacitazione in vitro (IVC) degli spermatozoi di suino e sulla successiva esocitosi a livello acrosomiale indotta dall'aggiunta di progesterone in vitro (IVAE). Nel primo studio il medium capacitante è stato integrato con GSH a tre differenti concentrazioni. I risultati ottenuti in vitro hanno mostrato la presenza di meccanismi GSH-sensibili che sembrano interferiscano in maniera importante nel raggiungimento di uno status capacitato, e della successiva IVAE indotta dal progestorene. Infatti la maggioria dei parametri usati come marcatori principali della capacitazione spermatica suina quali, motilità spermatica, cambi nella fluidità delle membrane spermatiche e lo stato di fosforilazione delle tirosine vengono alterati dalla presenza nel medium di GSH.

Il secondo studio invece, è improntato sull'aggiunta di melatonina al medium capacitante, in tre distinte concentrazioni. L'aggiunta di melatonina ha fatto registare un notevole calo nei valori di motilità spermatica, concomitanti con un netto incremento nella percentuale di agglutinazione tra gli spermatozoi, e un'intensità di questi effetti variabile in maniera dose-specifica. Viceversa l'aggiunta di melatonina al medium ha abbassato la concentrazione di perossidi e superossidi presenti a livello intracellulare, nonchè stabilizzato i livelli di radicali liberi delle cisteine sempre con una modalità d'azione dose-effetto. Entrambe, le ricerche hanno dimostrato che la capacitazione produce un incremento dei radicali liberi di cisteina e dei livelli intracellulari di radicali liberi relativi allo stress ossidativo. Entro determinati livelli questi incrementi sembrano tra l'altro rivelarsi fondamentali nell'innesco del successivo IVAE. La presenza di un leggero stress ossidativo viene considerato indispensabile al fine di innescare la capacitazione spermatica e la successiva esocitosi acrosomiale. Inoltre, antiossidanti come il GSH e la melatonina sembra vengano implicati nei meccanismi redox e ionici a livello cellulare, con l'alterazione di alcuni processi fisiologici e biochimici, e che compensino gli effetti dannosi risultanti da un'eccessiva esposizione delle cellule ad alte concentrazioni di radicali liberi quali, superossidi e perossidi intracellulari (ROS: $O_2\text{-}^{\bullet}$ e H_2O_2).

Resumen

Las tecnologías de reproducción asistida permiten a los investigadores identificar cómo el esperma de los mamíferos consigue la capacitación a través de una secuencia de modificaciones bioquímicas que permiten a la célula conseguir la habilidad de fertilizar. De esta manera, los estudios *in vitro* hechos sobre espermatozoides de cerdo incubados en un medio de capacitación específico (CM) durante 4h muestran el conjunto de cambios funcionales y estructurales que el esperma de cerdo sufre durante la consecución del estado de capacitación. Estos cambios comportan modificaciones en parámetros como: motilidad de los espermatozoides, trastornos de los lípidos de la membrana, potencial de la membrana mitocondrial, niveles de calcio intracelulares, niveles de fosforilación de las tirosinas en la proteína y su nueva reorganización, y aumento de los niveles intracelulares de los radicales libres de cisteína. El hecho de agregar antioxidantes como la glutathión reducida o melatonina al medio parece prevenir y/o modificar la mayoría de cambios conectados con la capacitación. El objetivo de esta tesis era estudiar los efectos de dos agentes antioxidantes, la glutathión reducida (GSH) y la melatonina, en el logro de la capacitación *in vitro* del esperma de cerdo (IVC) y la subsiguiente exocitosis acrosomal *in vitro* (IVAE), inducida a través de la progesterona. Para este propósito, en el primer estudio, el CM se suplementó con tres concrentraciones distintas de GSH. Los resultados muestran que los mecanismos sensibiles al GSH tuvieron efectos importantes en la consecución del estado de capacitación en el esperma de cerdo, y en la siguiente IVAE inducida por la progesterona. A esta conclusión se llega por el hecho que marcadores importantes de la capacitación en el esperma de cerdo como los parámetros de movimiento, los cambios de fluidez en la membrana del esperma y el estado en general de la fosforilación de la tirosina se ven alterados por el GSH. En el segundo estudio, al CM se añadió la melatonina en tres concentraciones distintas. La suplementación de melatonina dio como resultado un destacable efecto negativo en la motilidad que estuvo

asociada a un intenso aumento en el porcentaje de aglutinación entre células de una manera dosis-dependiente. Mientras tanto, la melatonina parece tener un efecto positivo en los niveles de superóxidos y peróxidos, y también parece estabilizar los niveles de radicales libres de cisteína dependiendo de su concentración en el medio. En conclusión, nuestras investigaciones sugerirían que la capacitación del esperma de cerdo va unida al aumento de los residuos libres de cisteína y de los niveles intracelulares de especies reactivas de oxígeno. Por otra parte, estos aumentos parecen ser el instrumento para el lanzamiento de la sucesiva IVAE. Así, la presencia de un ligero estado oxidativo podría ser uno de los factores relacionados con la activación de la capacitación y de la siguiente exocitosis acrosomal. De esta manera, los antioxidantes como GSH y la melatonina parecen tener efectos en los mecanismos iónicos y redox; alterando algunos procesos bioquímicos y fisiológicos y compensando los efectos dañinos provocados por una excesiva exposición de las células a los radicales libres del oxígeno (peróxidos y superoxidos: $O_2^{-\bullet}$ y H_2O_2).

Resum

Les tecnologies de reproducció assistida permeten als investigadors identificar com l'esperma dels mamífers aconsegueix la capacitació mitjançant una seqüència de modificacions bioquímiques que permeten a la cèl·lula assolir l'habilitat de fertilitzar. D'aquesta manera, els estudis *in vitro* fets sobre espermatozoides de porc incubats en un medi de capacitació específic (CM) durant 4h mostren el conjunt de canvis funcionals i estructurals que l'esperma de porc pateix durant la consecució de l'estat de capacitació. Aquests canvis comporten modificacions en paràmetres com: motilitat dels espermatozoides, trastorns dels lípids de la membrana, potencial de la membrana mitocondrial, nivells de calci intracel·lulars, nivells de fosforilació de la tirosina a la proteïna i la seva nova reorganització, i augment dels nivells intracel·lulars dels radicals lliures de cisteïna. El fet d'agregar antioxidants com la glutatió reduïda o la melatonina al medi sembla prevenir i/o modificar la majoria de canvis connectats amb la capacitació. L'objectiu d'aquesta tesi era estudiar els efectes de dos agents antioxidants, la glutatió reduïda (GSH) i la melatonina, a l'assoliment de la capacitació *in vitro* de l'esperma de porc (IVC) i la subsegüent exocitosi acrosomal *in vitro* (IVAE), induïda a través de la progesterona. Per aquest propòsit, en el primer estudi, el CM es va suplementar amb tres concentracions diferents de GSH. Els resultats mostren que els mecanismes sensibles al GSH van tenir efectes importants en la consecució de l'estat de capacitació a l'esperma de porc i a la següent IVAE induïda per la progesterona. A aquesta conclusió s'hi arriba pel fet que marcadors importants de la capacitació a l'esperma de porc com els paràmetres de moviment, els canvis de fluïdesa a la membrana de l'esperma i l'estat en general de la fosforilació de la tirosina es veuen alterats pel GSH. En el segon estudi, al CM s'hi va afegir la melatonina en tres concentracions diferents. La sumplementació de melatonina va donar com a resultat un destacable efecte negatiu en la motilitat que va estar associada a un intens augment en el

porcentatge de aglutinació entre cèl·lules d'una manera dosi-dependent. Mentrestant, la melatonina sembla tenir un efecte positiu en els nivells de superòxids i peròxids, i també sembla estabilitzar els nivells de radicals lliures de cisteïna depenent de la seva concentració al medi. En conclusió, les nostres investigacion suggeririen que la capacitació de l'esperma de porc va unida a l'augment dels residus lliures de cisteïna i dels nivells intracel·lulars d'espècies reactives d'oxígen. Per altra banda, aquests augments semblen ser l'instrument pel llançament de la successiva IVAE. Així, la presència d'un lleuger estat oxidatiu podria ser un dels factors relacionats amb l'activació de la capacitació i de la següent exocitosi acrosomal. D'aquesta manera, els antioxidants com el GSH i la melatonina semblen tenir efectes en els mecanismes iònics i redox; alterant alguns processos bioquímics i fisiològics i compensant els efectes danyosos provocats per una excessiva exposició de les cèl·lules als radicals lliures de l'oxigen (peròxids i superòxids: $O_2\text{-}^{\bullet}$ i H_2O_2).

PART 1. Introduction

Introduction

1. Pig reproduction: male genital tract

The main principal organs of the boar genital system are scrotum, testicles, epididymis, accessory glands and penis.

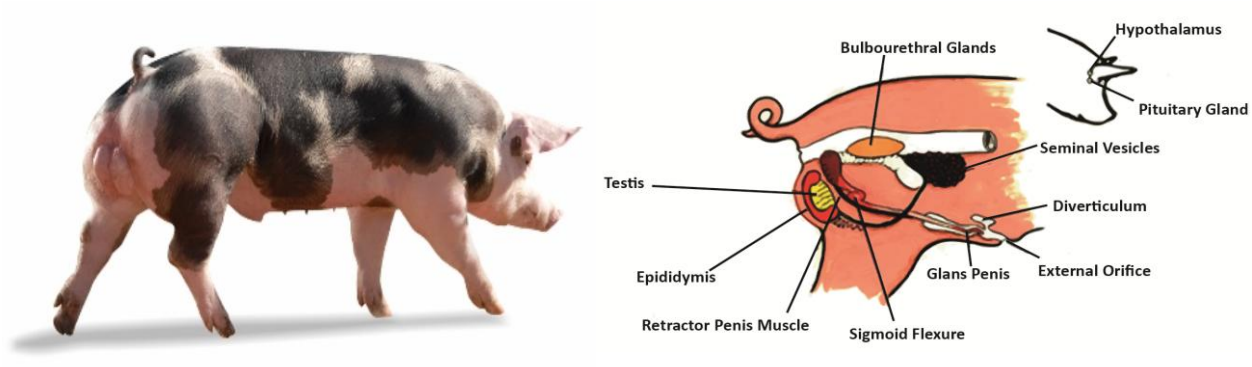


Fig. 1: Pietrain pig - General representation of genital apparatus of boar (*Singleton, 1997*).

Scrotum

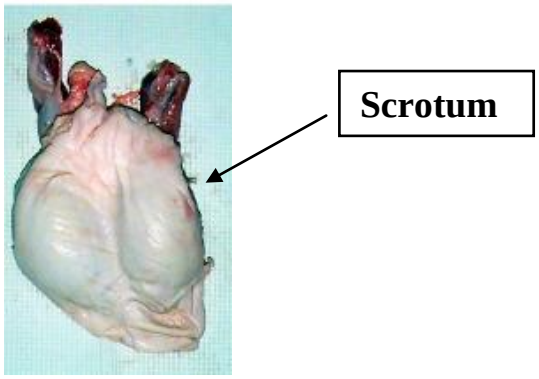


Fig. 1.2: Boar genital apparatus – scrotum (Dwane Davis picture).

The scrotum is a sac of skin characterized for its thin thickness that is present in the male reproductive system of mammals. The scrotum is divided into two compartments and each one contains one of the two testes. It is continuous with the skin of the lower abdomen and is located directly behind the penis and in front of the anus. The scrotal wall is a thin layer of skin lined with smooth muscle tissue (*dartos fascia*). The skin contains more pigment than that of surrounding areas and has many sebaceous (oil-producing) glands and sweat glands, as well as some hair. The two compartments of the scrotum are distinguished externally by a middle ridge called the raphe. Internally, the raphe connects to a muscular partition, the septum, which serves to divide the scrotum into its two areas (https://global.britannica.com/science/scrotum).

Testicle

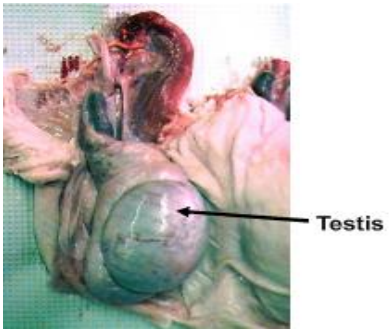


Fig. 1.3: Boar genital apparatus - testicle (Dwane Davis picture).

Testicles have two main functions. The first function is the formation of the male germinal cell, the sperm. The second function is acting as an endocrine gland by secreting into the whole body hormones that are important for the regulation of the reproductive function, like androgens. The testicle is composed by a series of thin tubules, called seminiferous tubules (ST). These tubules originate and terminate in the *rete testis* located in the central portion of the testis.

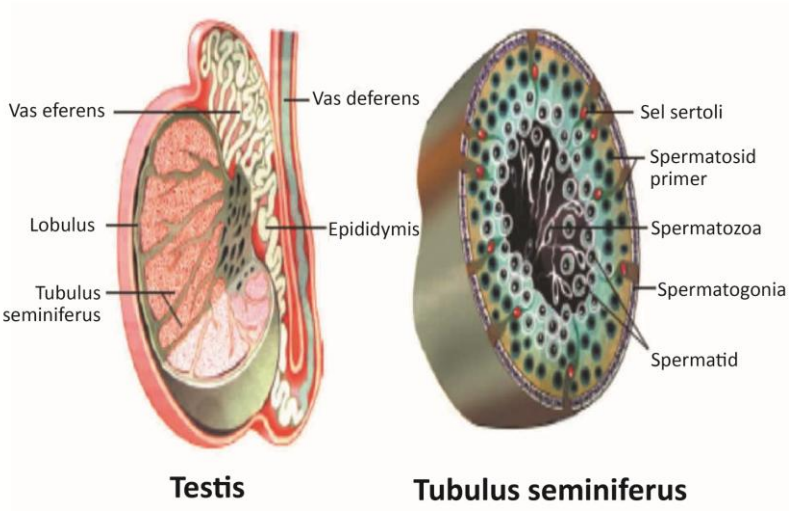


Fig. 1.4: A horizontal section through the scrotum showing the internal organization of the testes.

The surface of the testis is covered by a serous membrane resulting from the peritoneum (vaginal tunic) and by a fibrous capsule (tunica albuginea) which contains smooth muscle cells. When these cells contract, there is an increase in pressure in ST which facilitates the transfer of sperm, still immobile at this stage, towards the *rete testis* and subsequently epididymis. Finally sperm acquire motility after their transit through the epididymis (Sjaastad et al., 2013).

Epididymis

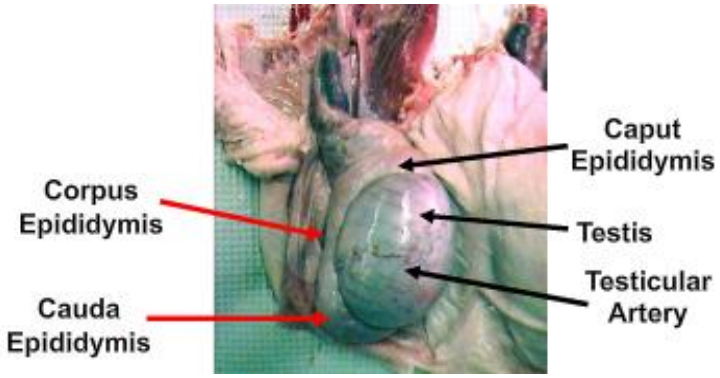


Fig. 1.5: Boar genital apparatus – testis with highlighted epididymis position (Dwane Davis picture).

Epididymes are two crescent-shaped organs that are adjacent to the testis in manner that each testis-epididymis formation can be considered as a unified functional system. Epididymis can be divided in three gross morphological regions, namely head (*caput epididymis*), body (*corpus epididymis*) and tail (*cauda epididymis*). The main structure of epìdidymis is a long and unique convoluted tubule connected to the testis via the efferent ducts and finished by joining the *vas deferens* in its caudal part (Guyonnet et al., 2009). Both *vas deferens* are muscular tubes that undergo peristaltic contractions during ejaculation, propelling the spermatozoa from epididymis to the urethra (Frandson et al., 2009).

Accesory glands

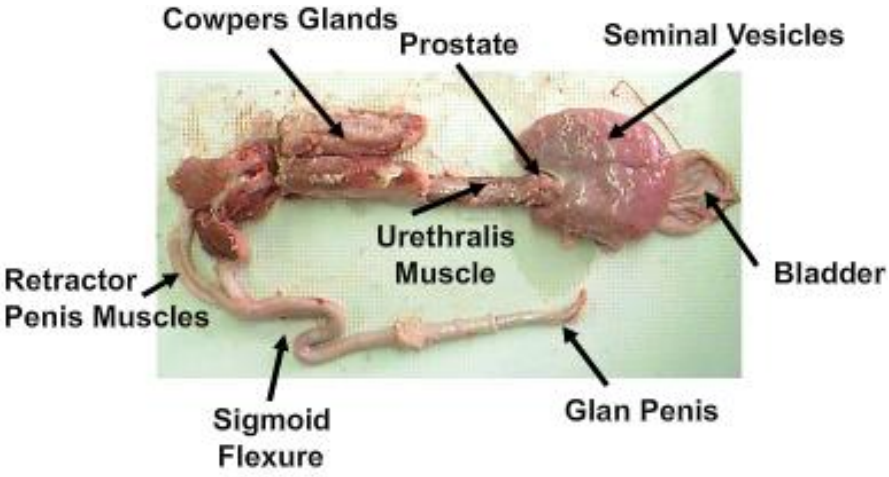


Fig. 1.6: Boar reproductive tract (Dwane Davis picture).

Boar has three main accessory glands, namely the bulbourethral glands, also named Cowper's glands, the seminal vesicles and the prostate (Badia, 2003). All of them are exocrine glands that release their secretion into the lumen of the urethra by means an androgen-dependent secretory activity (Bonet et al., 2013).The main role of these secretions are to form the ejaculate together with the sperm-rich fraction originated in testes. Thus, the final volume of the boar ejaculate is composed by a 55%-75% of secretions from prostate and urethra, 10%–25% of secretions from the Cowper's glands and 15%–20% of secretions from seminal vesicles. Only 2%–5% of the ejaculate volume comes from the caudal region of the epididymis, containing it sperm and epididymal/testicular fluid (Dyce et al., 1999; Badia, 2003; Badia et al., 2005, 2006).

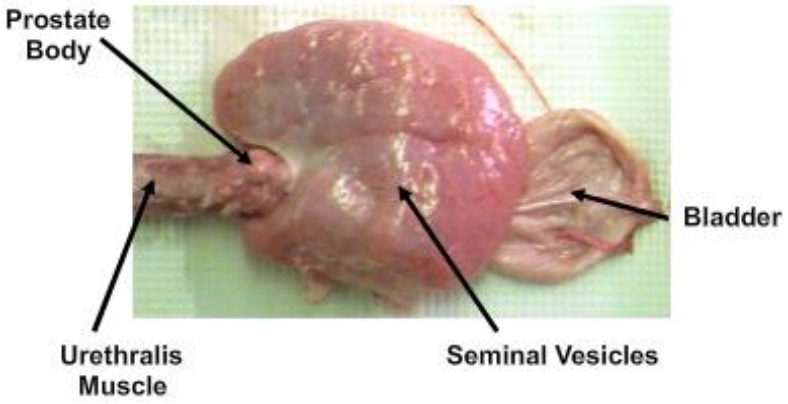


Fig. 1.7: Boar genital apparatus – seminal vesicles; prostate body; bulbourethral glands; bladder (Dwane Davis picture).

Penis

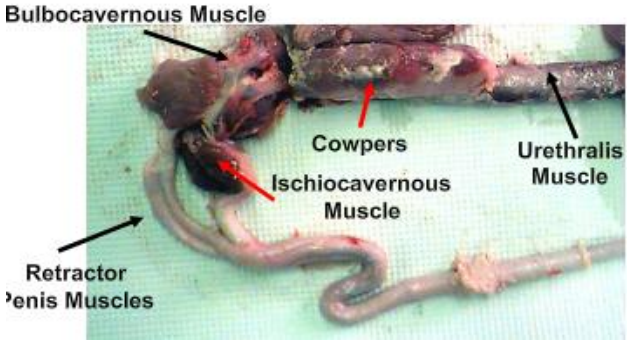


Fig. 1.8: Boar genital apparatus – penis (Dwane Davis picture).

The penis is the male organ of copulation. Under an anatomical point of view, the boar penis is fibro-elastic, with cavernous bodies of reduced size. This implies that erection was mainly due to the relaxation of the retractor penis muscle located at the sigmoid flexure. The boar penis

measures 45 cm-50 cm, with a diameter of 1.5-2 cm even during erection. The gland has a typical corkscrew-shaped shape, which aids to fix the whole peneal structure fits into the corkscrew-shaped cervix canal of the sow (Hollandbeck et al., 1964).

2. Boar Sperm morphology

The mammalian mature spermatozoon is a uniflagellar and motile sperm cell. Spermatozoa are the cellular component of ejaculates, being their primary function to fertilize an oocyte and thus, create a new organism composed half by the sperm-gene and half by the gene load from the oocyte.

The mature boar spermatozoon is an elongated cell of about 43–45 μm in full length (Briz, 1994; Holt et al., 2010). The cell is composed by two major distinguishable regions, the head and the tail, separated by a short linking segment called the connecting piece or neck.

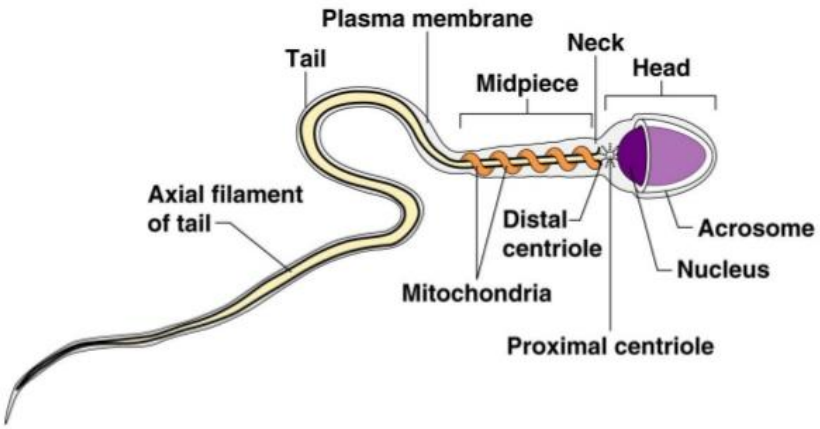


Fig. 2: Structure of mature sperm cell (http://www.slideshare.net/SSpencer53/ch16appt-reproductive).

The boar head sperm is a bilaterally flat structure of about 7µm long designated to interact with the oocyte. The two major components of the sperm head are the nucleus and the acrosome. The nucleus constitutes the major part of the sperm head. The nuclear structure is electrodense due to its very rigid nucleo-proteinic composition. Surrounding the antero-lateral area of the nucleus is the sac-like acrosome, a membrane-bound vesicle that forms a cap that covers approximately 80 % of its length. The acrosome has an inner membrane that overlays the nuclear envelope. This inner membrane is merged at the posterior margins of the acrosome with an outer membrane that is lying directly beneath the plasmalemma. Enclosed inside both acrosomal membranes there is a narrow space, the acrosomal matrix, filled with amorphous material distributed homogeneously and mostly corresponding to densely packed hydrolytic enzymes. The part of the head containing the acrosome is called the acrosomal region (Briz and Fabrega, 2013).

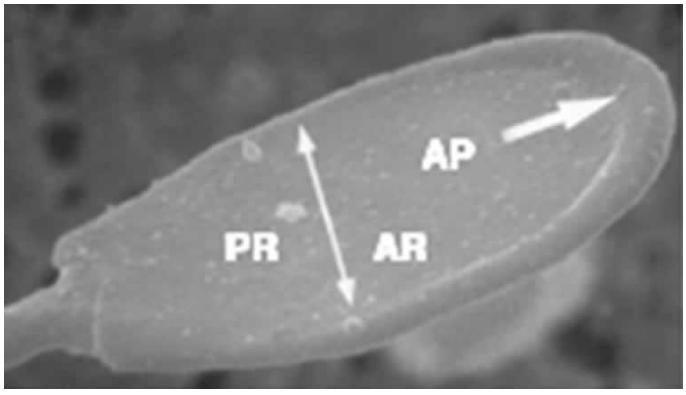


Fig. 2.1: Detail of the head of a boar spermatozoon. Note the great development of the acrosomal protuberance (AP) which extends down to the boundary of the acrosomal region (→) (×9,500). Acrosomal region (AR); postacrosomal region (PR). From: Briz and Fabrega, 2013.

The tail has a filamentous and cylindrical shape and can be subdivided into three major regions, namely the midpiece, the principal piece and the terminal piece. The midpiece is 9 μm in length and 0.7 μm in diameter. The most prominent feature of the midpiece is the presence of mitochondria, organelles strongly involved in the control of many sperm functions, as well as in the generation of part of the energy needed to maintain the overall sperm function (Flesch et al., 2000; Rodríguez-Gil and Bonet, 2016).

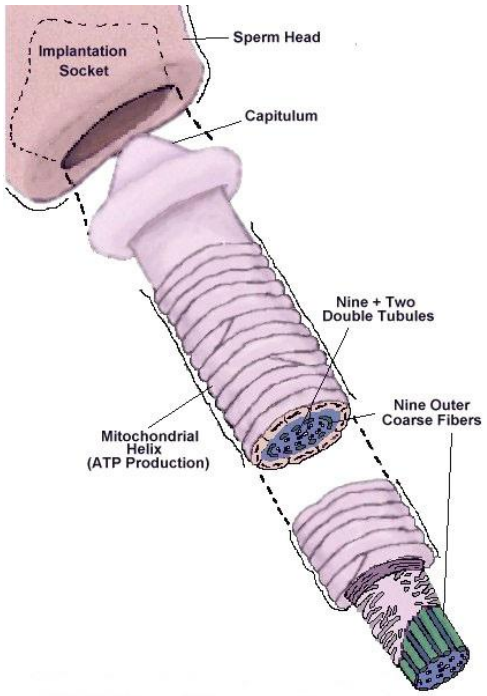


Fig. 2.2: Representation of neck and midpiece sperm structures. From: http://animalsciences.missouri.edu/reprod/Notes/sperm/tail.htm

The principal piece is the longest segment of the spermatozoon tail, extending from the annulus or Jensen's ring to the proximal end of the terminal piece. The principal piece is characterized by the presence of a complex fibrillar structure that maintains the proper motion characteristics

that needs sperm to advance into the female genital tract. The most important parts of this complex structure are the fibrous sheath, the outer dense fibres, the axoneme, and the Jensen's ring.

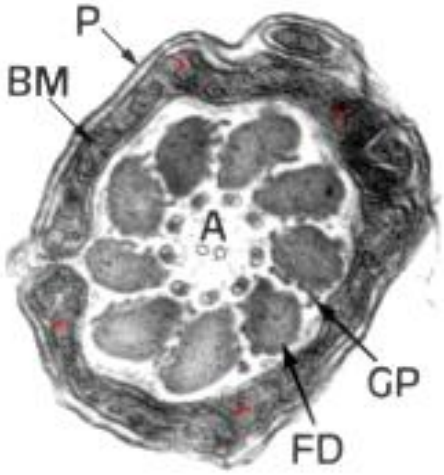


Fig. 2.3: Ultrastructural image of boar sperm mitochondria. The low development of inner crests is noticeable (asterisks). BM: inner mitochondrial membrane. P: cell membrane. A: axoneme. FD: dense fibres. GP: peripheral granules. From: Bonet et al., 2000.

The terminal piece is the last and shorter segment of the spermatozoon tail and has no accessory cytoskeletal structures, consisting only of the axoneme enclosed by the plasmalemma (Briz and Fabrega, 2013).

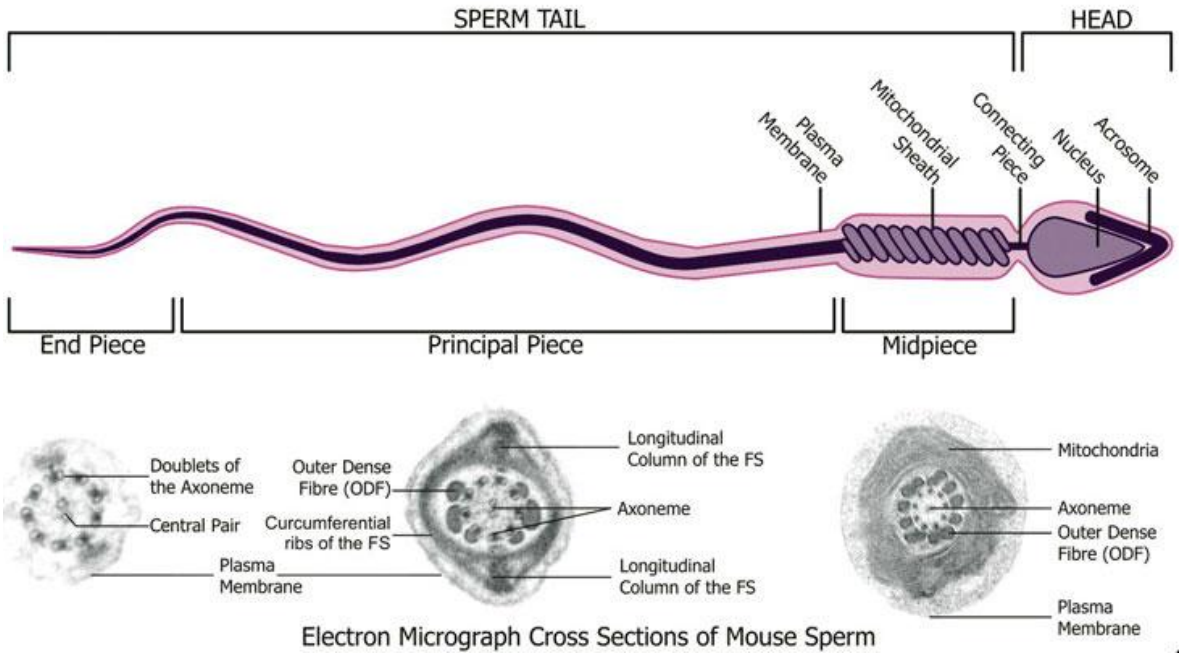


Fig. 2.4: Cross-sections of boar sperm tail and whole diagram of mouse spermatozoa. From: Claire et al., 2009.

3. Sperm journey through female genital tract: processes and molecules involved.

Spermatozoa undergo several important steps throughout their life from spermatogenesis to fusion with the oocyte. During their migration through the female genital tract, spermatozoa undergo a series of tightly controlled biochemical and membranous changes. These changes are globally known with the term *capacitation*, enabling sperm to reach and bind to the zona pellucida, undergo acrosome reaction, penetrate the egg vestments and finally fuse with the oocyte (Austin, 1952; Talukdar et al., 2015). In an *in vivo* fertilization, only a few sperm cells reach the ampulla or the site of fertilization (Sakkas et al., 2015). In boar reproduction it is estimated that from 37.5 billion of spermatozoon which are released into the reproductive tract after ejaculation, only 5000 will be able to reach the site of fertilization (Avilés et al., 2015). Thus, the number of fully capacitated sperm that can reach their final goal is actually very low.

Capacitation is a phenomenon which appears to be controlled by a complex crosstalk involving a myriad of separate molecular signalling pathways (de Lamirande et al., 1997; Fraser, 2010). Historically, Austin (1951) in rat and Chang (1951) in rabbit were the first scientists who described the capacitation process. Following these pioneering studies, capacitation has been reproduced in many other species both in *in vivo* and in *in vitro* conditions. Despite this, many of the factors and molecular pathways involved in its regulation remain unknown or only poorly known. In summary, after a long journey accompanied by several modifications occurring to sperm cells, the spermatozoa become able to meet the oocyte in the specific site of the oviduct named ampulla, and fertilization can takes place. The first step of this complex journey is the semen deposition into the female genital tract during the ejaculation process. Depending on the species, sperm are deposited in different sections of the female tract. Thus, in a large number of mammals, semen is ejaculated into the

anterior vagina during coitus (e.g. primates, cows, sheep, rabbits, dogs and cats). In other species sperm are placed into the uterus (e.g. sows) or directly spurted into the uterus (horses and many rodents; see Coy et al., 2012). In pig reproduction, this positioning in the uterus permits sperm to bypass the vaginal barrier and makes them available to the Fallopian tubes more easily (Hunter, 1981). Nevertheless, sperm meet numerous impediments during their journey, so to maintain their fertility sperm cells need to employ their limited resources and/or exploit those from the female tract (Suarez and Pacey, 2006; Mortimer et al., 2013). For this reason one of the most important parameter to value semen quality is progressive motility. Sperm motility and in particular its progressive swimming pattern, is essential in species with vaginal or cervical deposition to pass through the cervical mucus. Hence, spermatozoa with poor motility and, concomitantly, with abnormal morphology are filtered out during this passage. This is thought to be the first form of sperm selection (Sakkas et al., 2015). However, this phenomenon is less evident in species like pig, in which sperm selection starts into the uterus. This could explain the low motion characteristics that boar sperm show when compared with species with vaginal/cervical deposition like horses, dogs, bulls, or mice (Rodriguez-Gil and Bonet, 2016). Thus, after crossed all anatomical and physiological barriers found in cervix and/or uterus, spermatozoa are able to reach oviducts.

Under physiological conditions, oviducts play an important role in sperm capacitation (Immler, 2008). In fact, previously selected sperm will reach the bottom of the oviduct (isthmus) where they are stored until the moment of ovulation. This sperm storage is known as the oviductal sperm reservoir. Sperm placed inside this reservoir maintains viability for variable time lapses, depending on the species, and avoids a premature capacitation remaining in this physiological state until the time of ovulation (Brüssow et al., 2006). This break in the development of the capacitation process is needs to reach the final goal of fertilization.

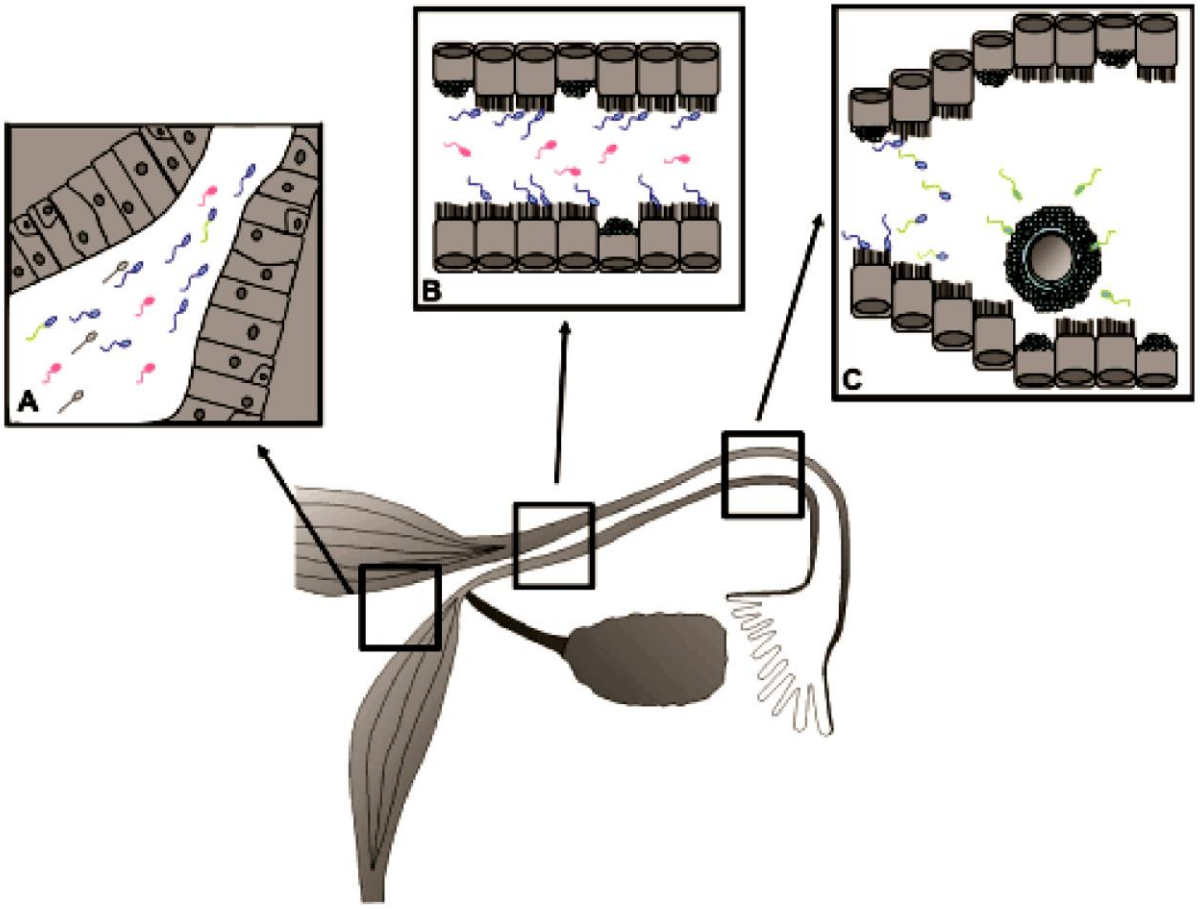


Fig. 3: Capacitation process. A) After ejaculation, a heterogeneous population of sperm reaches the female reproductive tract. B) Only a few sperm achieves the oviduct and forms the Sperm Reservoir (SR) in the caudal portion of the isthmus. C) During peri-ovulatory time, sperm release from the SR and those who complete a correct sperm capacitation are able to contact with the oocyte and fertilize it. Different colours indicate distinct types of sperm: dead (grey), damaged (red), normal (blue), hyperactivated (green-blue) and successfully capacitated (green). From: López-Úbeda and Matás, 2015.

4. Chronological events of sperm capacitation

Capacitation process is a phenomenon that starts at the same time of ejaculation and continues following a precise chronological succession of events that occurring during all the journey of sperm into the female genital tract. These events often are regrouped, in order to their timing, in two subsequent steps (Visconti, 2009):

First step, fast capacitation events: A very early event in sperm capacitation is the activation of sperm motility. In fact, altough sperm stored in the cauda epididymis consume oxygen at a high rate, they are immotile (Visconti, 2009). The contact of spermatozoa with seminal fluid is a trigger point for activation of vigorous flagellum movement. This activation is related with the high levels of HCO_3^- and Ca^{2+} that are present not only in the seminal plasma, but also in the secretions of the female genital tract. Specifically, the HCO_3^- will induce the activation of the sperm transmembrane adenylyl cyclase SACY, which, in turn, will increase intracellular levels of cAMP, activating thus the cAMP-dependent protein kinase A activated (PKA). Finally, the PKA will be responsible for launching a very complex phosphorylation single cascade that affects a myriad of sperm proteins (Salicioni et al., 2007). Among these signals, several of these PKA-activated cascades will be responsible for the activation of sperm motion immediately after ejaculation. Other will initiate other processes that will lead to subsequent molecular changes in sperm function.

Second step, slow sperm capacitation: As a consequence of the PKA-mediated activation of a large number of separate molecular signalling pathways a complex long-term series of changes in sperm function are launched. The complete details of these long-term changes, mainly developed during the stay of sperm into the oviductal reservoir, are not completely elucidated. Despite this, investigators have a reasonable knowledge of these processes thanks to the development of *in vitro* induction of capacitation. To promote the inset of capacitation,

spermatozoa need to be incubated in specific media with different composition depending on the species. Generally, an *in vitro* capacitation medium had to include some sort of energy source, a protein component that usually is bovine serum albumin (BSA), HCO_3^-, and both calcium and sodium salts (Saravia et al., 2007; Ded et al., 2010). Starting from this basis, each species will have some specific requirements to yield a feasible capacitation in this *in vitro* environment. It is established that each species has a precise equilibrium between glycolysis and mitochondrial oxidative pathways. In fact, in species such as boars and mice, this equilibrium is greatly unbalanced in favor of glycolysis, which is the vastly majority energy-obtaining pathway in the presence of sugars such as glucose (Rodriguez-Gil and Bonet, 2016). This implies that the source of energy that must be added to the capacitation medium can vary from glucose in species like the boar to lactate in dog (Rodriguez-Gil and Bonet, 2016). Regarding the role of the other basic components of the capacitation medium, it is well known that the presence of BSA, HCO_3 and Ca^{2+} acting through the launching of the capacitation mechanisms started after the activation of the PKA. Thus, the BSA stimulates the replacement of the membrane cholesterol with other cholesterol-binding compounds such as β-cyclodextrins. Meanwhile, both the HCO_3^- and Ca^{2+} salts are involved in the regulation of SACY and in the consequent increase in cAMP levels and PKA activation (Salicioni et al., 2007). At this respect, it is noteworthy that the increase of intracellular calcium is indispensable for the launching of capacitation as well as for the induction of the acrosome reaction in previously capacitated sperm. Sperm can acquires cytoplasmic calcium thanks to the presence in plasma membrane, and in particular in the principal piece of the flagellum, of Ca^{2+} channels called CatSper proteins (CatSper 1 to 4) (Kirichok et al., 2006, Qi et al., 2007). In this way, the combined presence of BSA, HCO_3 and Ca^{2+} is indispensable not only for launching, but also to develop the capacitation process, being able these compounds to maintain and develop all of the protein phosphorylation cascades that starts as a consequence

of the activation of the PKA. In fact, the absence of BSA, HCO_3 or Ca^{2+} prevents both the overall protein tyrosine phosphorylation and the subsequent full capacitation status (Salicioni et al., 2007; Visconti, 2009). However, the importance of each of these modulators in controlling the protein phosphorylation cascades is different among species. For example, tyrosine phosphorylation in human sperm during sperm capacitation requires the presence of BSA, and HCO_3^-,but no Ca^{2+} (Muratori et al., 2010).

Meanwhile, in the case of stallion tyrosine phosphorylation requires only HCO_3^- but no BSA or Ca^{2+} (González-Fernández et al., 2012). Another event that shows a characteristic pattern for each species is represented by different times of responsiveness to BSA, HCO_3^- and Ca^{2+} in the inset of tyrosine phosphorylation; these differences are observed in pig, mouse, dog and bull. Thus, whereas in spermatozoa of species like boar and mice the full establishment of the tyrosine phosphorylation cascade after its induction appears at about 1 hour after the addition of HCO_3^- (Visconti et al., 1995; Gadella and Van Gestel, 2004), in other species like dog the same phenomenon occurs after 90 minutes of the bicarbonate induction (Petrunkina et al., 2003; 2004) and even in bull sperm where the same event takes place only after 4 hours of the addition of heparin (Galantino-Homer et al., 1997). Regarding the precise location of the capacitation-induced increase of tyrosine phosphorylation, the flagellum seems to be the main placement (Naz et al., 1991; Carrera et al., 1996; Mahony et al., 1999; Si and Okuno, 1999; Lewis and Aitken, 2001; Urner et al., 2001), although the acrosomal area also shows an evident increase in the overall protein tyrosine phosphorylation during capacitation, especially in boar (Petrunkina et al., 2001).

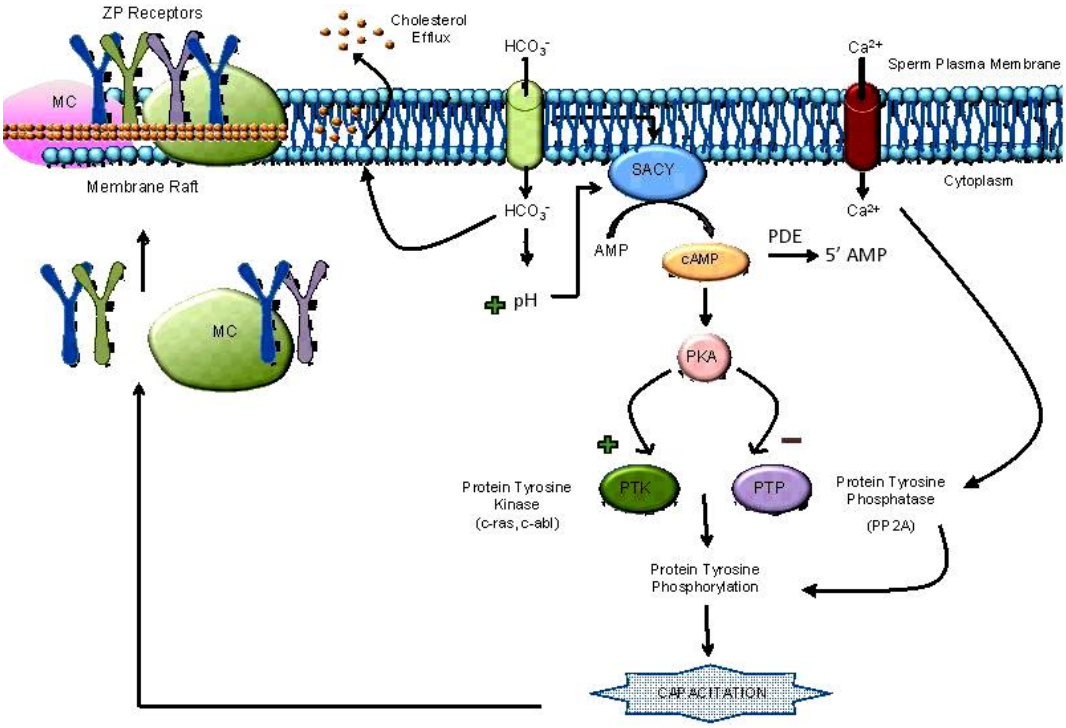


Fig. 4: Sequence of mammalian sperm capacitation events. From: Redgrove, Aitken and Nixon, 2012.

Another of the functional consequences of capacitation is the development of a distinct motility/kinetic pattern that is called hyperactivation. The hyperactivated motion pattern is characterized by a very vigorous motility pattern with high amplitude and asymmetrical flagellar beating (Yanagimachi, 1970). It has been suggested that the mechanical thrust due to hyperactive motility is vital for spermatozoa to penetrate the zona pellucida of the oocyte (Yanagimachi, 1969; Stauss et al., 1995; Bedford, 1998). In this way, hyperactivation is observed as the typical swimming pattern shown by most sperm retrieved from the oviductal ampulla at the time of fertilization (Suarez and Ho, 2003). As seen in mice, but not yet in boar

spermatozoa, these changes occur in the oviduct in *in vivo* conditions (Rodriguez-Martinez, 2007). Notwithstanding, hyperactivation can be also observed in *in vitro* conditions after a proper stimulation. It is known that the progesterone released from the ovary and secreted by oviduct epithelium at ovulation time give rise to increment of sperm intracellular $[Ca^{2+}]i$, which in turn induce turning swimming with asymmetric flagellar bending (Yoshida and Yoshida, 2011; Hunter, 2012). Thus, progesterone can be utilized in many species as a feasible inductor of the hyperactivated motion pattern.

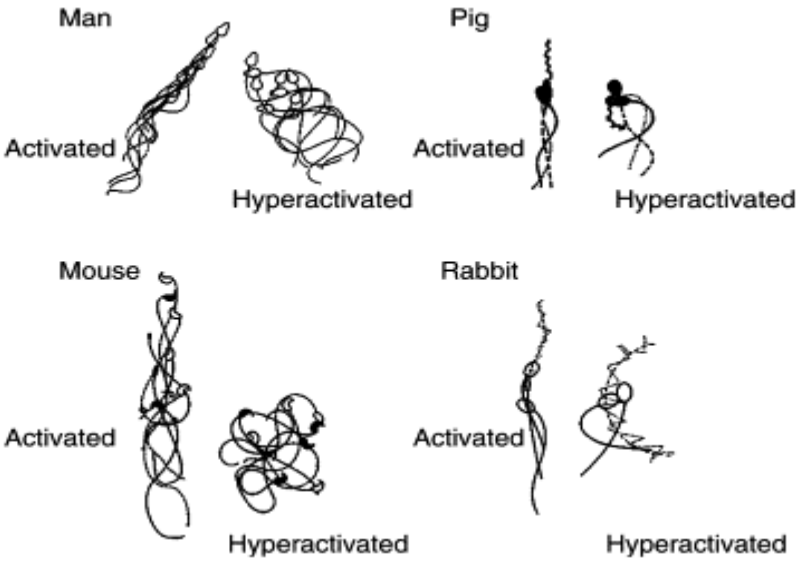


Fig. 4.1: Progressive and hyperactivated motility of mammalian sperm. Motility patterns of mammalian sperm are displayed in time-lapse drawings. The hyperactivated mode consists of transitions to deeper and less symmetrical flagellar bending and results in a path of the sperm head that is less linear than that of activated state motility. From: Florman and Fissore, 2015.

In summary, the most important changes underwent by spermatozoa after their contact with the oviductal fluid and hence with the capacitation modulators contained in this fluid are

modifications of lipid membrane, the loss of plasma membrane cholesterol, the activation of the cAMP/PKA-modulated pathways, the increase of Ca^{2+} uptake, which leads to a concomitant increase in the intracellular pH, the subsequent hyperpolarisation of cell membranes and the increase in the overall protein tyrosine phosphorylation pattern (Aitken & Nixon, 2013). In this way, it is evident the existence of a strong oviductal influence that facilitates closeness of sperm to the oocyte (Hunter & Nichol, 1986; Rodriguez-Martinez et al., 2001; Rodriguez-Martinez, 2007; Coy et al., 2010; Zumoffen et al., 2010; Kumaresan et al., 2012).

5. Acrosome reaction

The sperm capacitation process is absolutely required as a previous step in order to acquire the ability to undergo acrosome reaction in response to physiological stimuli. As mentioned above, the changes of the capacitation in the molecular organization of the sperm plasmalemma make it sensitive to the presence of zona pellucida protein ZP3. The union ZP3-outer acrosome membrane proteins combined with the stimulus of external factors like progesterone launches the acrosome reaction (AR), a phenomenon necessary for sperm cells to achieve gamete fusion (Ferramosca and Zara, 2014). Properly, the AR is the result of the fusion of the sperm plasma membrane with the outer acrosomal membrane. The acrosome content, especially in terms of hydrolytic enzymes, will be released, leaving the inner acrosomal membrane as the sperm surface membrane that will be in direct contact with the oocyte surface. The place where AR begins in mammalian sperm has been a controversial topic. On one side of the debate some researchers think that the AR takes place while the sperm advance through the *cumulus*, while other scientists think that AR occurs on the surface of the ZP (Coy et al., 2012). It should be noted that no one has ever followed a single spermatozoon from the beginning of the AR until

the end of fertilization, so the exact place where fertilizing spermatozoa begin their AR and what triggers inset the AR remain to be determined (Yanagimachi, 2011). Regarding on the myriad of chemical and physical agents reported to induce *in vitro* acrosome reaction, we known the existence of natural inducers, such as the zona pellucida proteins (ZP) and progesterone, or artificial inducers, like heparin and calcium ionophores (Cheng et al., 1998). In fact, the progesterone secreted by the *cumulus* cells that surround oocytes is one of the most important factors, if not the most, which stimulates *in vivo* AR (Chang and Suarez, 2010). Notwithstanding, progesterone has a double function. The first function is being a potent chemo-attractants, whereas the second function is acting as a stimulator of the *in vitro* acrosome exocitosys (IVAE). It is noteworthy that the progesterone-induced AR occurs only when sperm are kept in close contact with the oocyte, but not before although sperm are in previous contact with progesterone presents in the oviductal fluid. A reason that could explain this delay would probably be the differences in progesterone concentration. Thus, oviductal fluid has low concentrations of progesterone that are not able to induce the AR (Publicover et al., 2008). These low concentrations of progesterone could act instead as a chemo-attractant driving the sperm towards the oocyte. Afterwards, progesterone concentrations at the space surrounding the oocyte are much higher. These higher concentrations of progesterone would ultimately be responsible for the induction of AR in the close proximity of the oocyte (Coy et al., 2012).

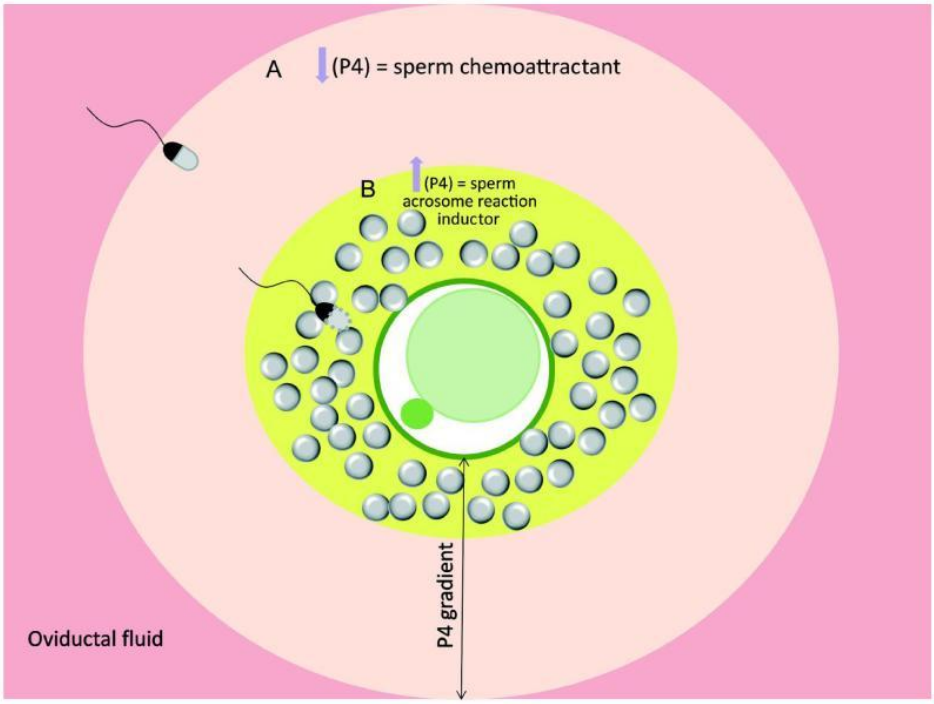


Fig. 5: Changes in effects of Progesterone (P4) concentrations, on sperm cells, during their rute towards oocyte. Zone A: low concentrations of P4 acting as chemo-attractant. Zone B: high P4 levels produced by *cumulus* cells cause acrosome reaction. From: Coy et al, 2012.

Regarding the sperm–ZP interaction, the first topic is that this interaction is a crucial point to achieve fertilization. The interaction between ZP proteins and spermatozoa induces the activation of a specific sperm phospholipase through a mechanism involving the action of a G protein. This activation induces in turn the subsequent activation of the sperm of adenylyl cyclase, which, in turn, will increase cAMP levels. These events ultimately lead to an alkalization of intracellular pH with the subsequent depolarization of sperm membrane. This last event is fundamental because it will open the Ca^{2+} channels that permit the entry of a flux of Ca^{2+} ions. These ions will in turn activate sperm phospholipases C and A_2 (PLC and PLA_2),

which, through the corresponding molecular cascade will finally activate the Protein Kinase C (PKC) (Flesh and Gadella, 2000). This activated PKC will migrate toward the membrane. This process of migration will finally initiate the fusion of sperm plasma membrane with the outer acrosomal membrane, leading thus to the final exocytosis corresponding to the AR (Doherty et al., 1995).

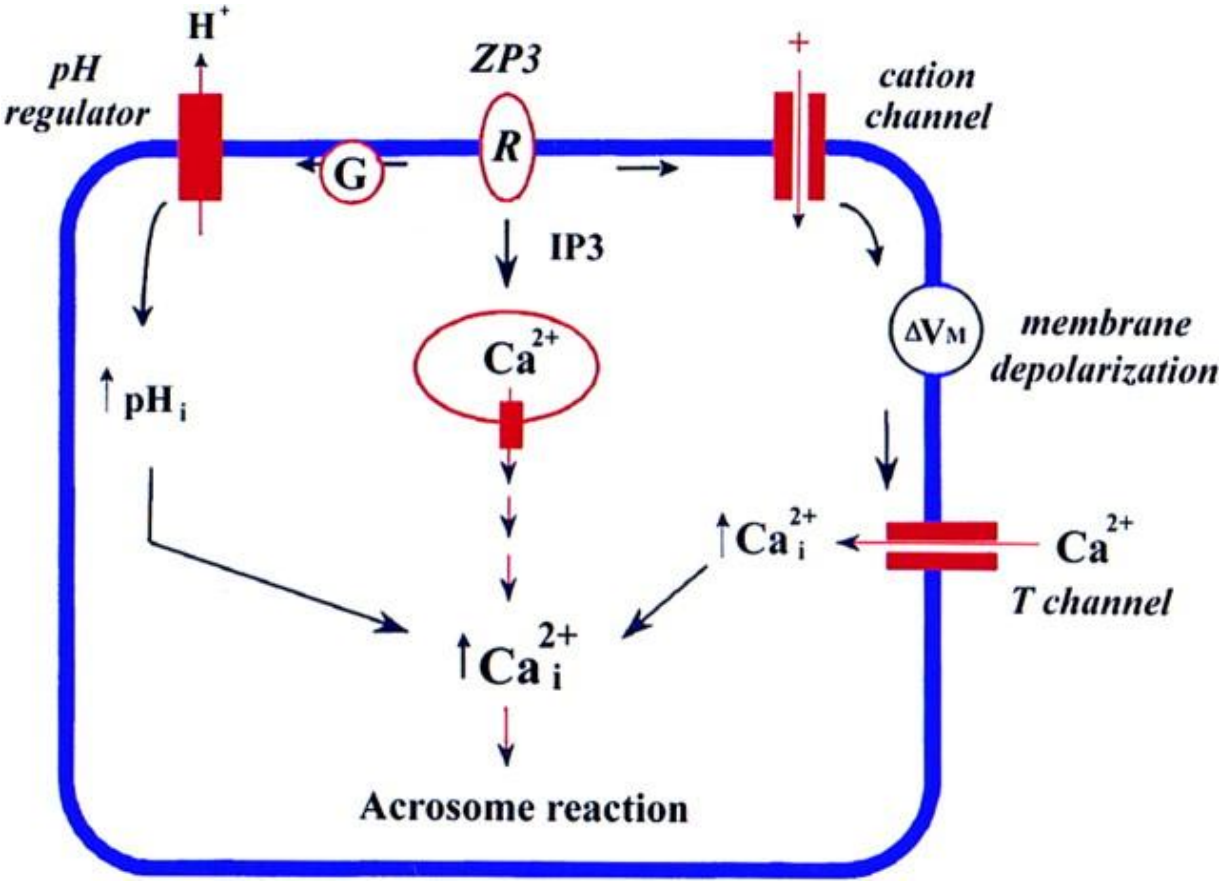


Fig. 5.1: Acrosome reaction (AR) triggers point: molecular events occurring after sperm- ZP protein interaction. From: Wassarman, 1999.

Part 2. Sperm Analisis

6. **Sperm analysis**

Possible fluctuations in seminal quality are associated with factors such as breed (Rijsselaere et al., 2007), age (Stone et al., 2013), seasonality (Chemineau et al., 2008; Zhang et al., 2013), temperature (Thonneau et al., 1998), photoperiod (Mazzarri et al., 1970; Kozdrowski and Dubiel, 2004) and other factors of different etiology (Petroccelli et al., 2015). Taking this into account, the definition of the precise quality of each ejaculate through an accurate analysis is of the utmost importance. Nowadays, to evaluate semen quality we can consider visual and physiological parameters. Until recent years, subjective microscope evaluation represented the only manner to predict a semen quality. However, this visual exam gave to us only a general and diffuse idea about the quality of the ejaculate and its hypothetic fertilizing potential. In this way, the addition of more precise techniques to evaluate semen quality is mandatory to optimize semen analysis. An important group of test that would be included in the routine semen analysis involves the determination of the sperm metabolic function. However, to accomplish that it is indispensable to know sperm cell physiology. This implies that microscope analysis cannot be the only system by which semen might be analyzed. Thus, besides the mandatory previous macroscopic evaluation of the ejaculate, the main parameters included in an optimal sperm quality analysis can be classified into two sections: microscope-linked parameters and physiology and metabolism parameters that cannot be only evaluated through microscope. Microscope-evaluated parameters would comprise concentration, sperm motility, sperm morphology, plasmatic membrane integrity, DNA fragmentation and osmotic resistance; while non-microscope evaluated parameters would be those related with aspects such as nuclear and acrosome structure, membrane fluidity, membrane sheath state, intracellular reactive oxygen species (ROS) levels, which comprise the evaluation of both peroxides and superoxides, and several enzymes activity (Bonet et al., 2012). It is evident how, all these assessments must be carried out using a large number of replicates because of the high

level of variability within and between individual samples and the subjectivity of some assays (Woelders, 1991).

6.1 Assessment of macroscopic characteristics of the ejaculate:

In boar semen, the temperature of the freshly collected ejaculate is about 36ºC±2°C, while its pH is usually between 6.85 and 7.9. Moreover osmolarity is variable, but always around 300 mOsm/Kg (Martín Rillo, 1982). The colour of the sperm-rich fraction is milky-white, although there may be colour changes due to separate alterations like contamination with urine, blood or prepucial secretion (Sancho, 2002).

6.2 Sperm volume and concentration

The total average volume of boar sperm-rich fraction of the ejaculate is about 250 mL, with variations between 50 mL and 400 mL. This volume represents the highest observed among males of the more common domestic animals (Frunză et al., 2008). The number of sperm per ejaculate varies between races and boars due to the action of a multitude of factors such as environment, temperature, nutrition, diseases, frequency of ejaculation and photoperiod (Sancho et al., 2004). Usually, a boar ejaculate contains a sperm concentration from $300x10^6$ sperm/mL to $600x10^6$ sperm/mL (Casas et al., 2010). To evaluate concentration we can utilize several methods like hemocytometry, colorimetry and true spectrophotometry. The hemocytomety allows practitioners to determine the number of immobilized spermatozoa that are present in a known volume. This technique uses a commercial cell counting chamber such as those from Neubauer, Thoma or Burker (Christensen et al., 2005). Related with these chambers there is also the Mackler chamber, which allows practitioners to count concentration and motility at the same time through the application of an informatics system called Computer Assisted Sperm Analysis (CASA; Roca et al., 2011). On the other hand, colorimetric and

spectrophotometric methods are based on the percentage of transmitted light that is trapped by a diluted semen sample (Sancho and Vilagran, 2013). However, although colorimetric and spectrophotometric methods can usually be faster, more sensitive and more objective than the hemocytometric ones, all of them are superseded by flow cytometry. In fact, at this time, flow cytometry is the most precise and sensitive method to evaluate concentration, since practitioners can count with this system a much higher number of fluorochrome-marked cells (more than 10,000) with a much higher precision. Flow cytometry, however, has a high cost, limiting thus its use to large laboratories uniquely devoted to research or analysis (Lu et al., 2006).

6.3 Sperm Motility:

Motility is recognized to be an important characteristic in predicting the fertilizing potential of the ejaculate, although its real importance in explaining the *in vivo* fertilizing ability of an ejaculate is not clear in species like boar. Thus, an adequate motility would be required for sperm to reach the fertilization site and penetrate the oocyte, although this parameter would not be the only needed to achieve this feat (Rodríguez, 2012). The classical method to evaluate motility is the direct, visual estimation of the parameter through an optic microscope. This system is highly subjective, especially in species like pig in which semen motion parameters are low and irregular. Thus, at this moment the use of a CASA instrument is mandatory. Actually, the development of CASA systems has enabled objective and accurate tracking of sperm movement. Modern CASA systems can capture images from 20 to more than 1,000 sperm at 50–60 frames per second, and also provides morphological measurements in addition to comprehensive motion analyses (reviewed in Amann & Waberski, 2014). The CASA measures sperm movement by establishing a centroid for each spermatozoon and evaluate cell motion based on centroid trajectory (Amann & Waberski, 2014). However, *in vitro* measures

may not reflect how the sperm moves within the female tract (Werner et al., 2007; Humphries et al., 2008). Within the female tract, sperm are spatially restricted and are therefore likely to be influenced by physical constraints such as wall effects (Humphries et al., 2008). Furthermore, natural fluids within the female tract will alter sperm movement (Curtis & Benner, 1991). Again, the information obtained through the CASA system are still subjected to the effects of external factors such as sample preparation or the type of chamber used for analysis (Tejerina et al., 2009). In this manner, the standardization of the procedure is necessary for the proper use of the system (Tejerina et al., 2009). Despite these shortcomings, CASA systems represent the best technique to evaluate sperm motility now. Motion parameters that are typically derived using automated CASA systems provide information about the velocity, linearity and lateral displacement of sperm heads as they progress along their trajectories (Holt et al., 2007). These parameters have been described many times previously (see for example: Katz and Davis, 1987; Mortimer et al., 1988; Davis and Katz, 1989, 1992, 1993a, 1993b; Kay and Robertson, 1998). Here are reported a brief description of some of these following King (King et al., 2000):

Total motility: percentage of sperm with a total velocity greater than 10µm/s.

Curvilinear velocity (VCL): distance travelled by the spermatozoa along its actual path as a function of time. It is expressed in µm/s.

Average path velocity (VAP): distance travelled by the spermatozoa along its average path as a function of time. It is expressed in µm/s.

Straight-line velocity (VSL): velocity over the straight-line distance between the beginning and the end of the sperm track. It is expressed in µm/s.

Linearity (LIN): the straight-line distance divided by the incremental deviations along the actual path. Then, it is also the percentage relationship between the curvilinear and straight-line velocity. It is expressed in %.

Head beat-cross frequency (BCF): the frequency with which the undulatory movement of the sperm head crosses the average path in function of time. It is expressed in Hz.

Straightness (STR): the percentage relationship between the curvilinear and the average path velocity. It is expressed in %.

Wobbleness (WOB): the percentage relationship between the average path and curvilinear velocity. It is expressed in %.

Amplitude of lateral head displacement (ALH): shift that makes the head in its curvilinear trajectory of the one part and the other part of the trajectory average or linear. It is expressed in µm.

Progressive motility: the percentage of motile sperm that show an STR greater than 80%.

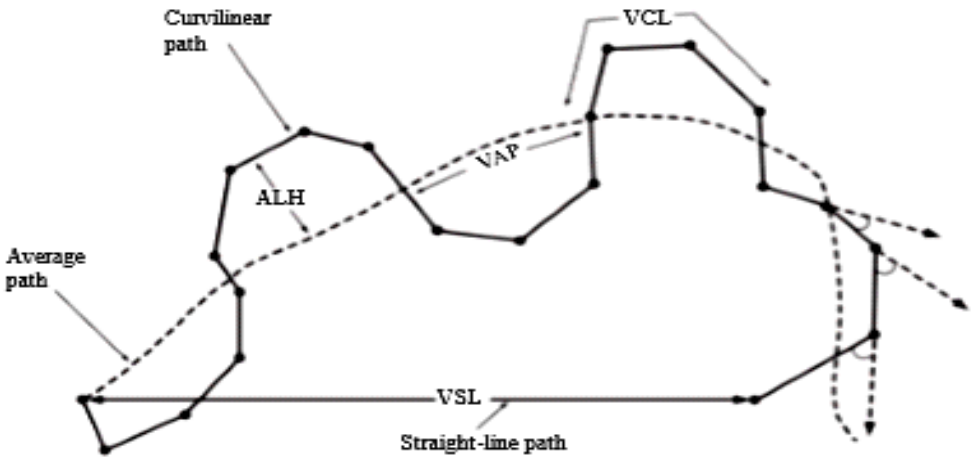


Fig. 6: Scheme of different velocities and parameters of sperm movement measured by CASA system. From: http//www.mibio.org/en/metrics-used

As suggested by Garcia (Garcia et al., 2005), capacitated boar spermatozoa have a species-specific motility pattern. Despite this, there are common features among species. In this way, as a general rule, capacitated sperm show an increase in VAP values and a decrease of LIN percentage. It's noteworthy that in an ejaculate from a healthy individual, a minimal rate of total motility of 60% would be the minimum requirement to be considered fertile (Donadeu, 2004; Broekhuijse et al., 2012).

6.4 Sperm morphology:

Some shape abnormalities of spermatozoa (AS) can be a result of pathological processes that affect testicles and the epididymis tract, whereas others AS can have a gene basis or, indeed, may be caused by unsuitable rearing conditions (Čeřovský et al., 2005). There is an abundant literature centred in the research of this field. This is logical, since AS is known to be an

important marker to assess semen quality (Leidl et al., 1971; Lyczynski and Pawlak, 1974; Blom and Andersen, 1975; Gamčík et al., 1976; Blom, 1977; Stemmler et al., 1982; Wekerle, 1982; Falkenberg et al., 1984; Yoshida and Kojima, 1989; Waberski et al., 1990, 1994; Itoh and Toyama, 1995; Itoh et al., 1996; Věžník et al., 2000; Louda et al., 2001; Corcuera et al., 2002; Gadea, 2002). Following the criteria of Lyczynski and Pawlak (1974) or Bach (Bach et al., 1982) the use of semen containing more than 25% of AS is not recommended for insemination. Other authors established even more restrictive conditions. Thus, Remmen and Tielen (1976) limited the AS content to 20%, whereas Gibson (1983) gave the limit up to 15% for both primary changes and secondary ones. Usually, a morphological examination of the boar ejaculate comprises a qualitative and also a quantitative classification of normal and abnormal sperm morphologies. This classification can be assessed by optical microscopy through the use of simple staining techniques such as eosin/nigrosin, Trypan Blue, Giemsa, Papanicolaou or Diff-Quick (Foxcroft et al., 2008). Currently, a contrast phase microscope can provide better resolution without requiring staining the samples and, moreover, it can be coupled to a CASA system (Sancho and Vilagran, 2013). Sperm abnormalities have traditionally been classified by location of the defect (head, midpiece, tail), or by its origin (primary: testis; secondary: epididymis; tertiary: accessory glands/post-ejaculation; see: Menon et al., 2011). Primary sperm abnormalities are due to disturbance of spermatogenesis by congenital or hereditary factors, high ambient temperature, scrotal insulation or diseases (Adeniji et al., 2010). The most important primary abnormalities are those related with head malformations, although several tail alterations are also included in this group. Since the sperm head contains the genetic material and other key effectors of fertilization, most abnormalities of head are associated with a significant impairment of fertility (Wilmington, 1981; Soderquist et al., 1991). Proximal and distal cytoplasmic droplets are considered as secondary abnormalities. The cytoplasmic droplet shows a diameter of around 1.5µm and is rich in membranous

vescicles that increase as the droplet displaces through the midpiece (Bonet et al., 2013). The presence of the cytoplasmic droplet on ejaculated sperm can be used as an indicator of sperm maturation (Gomez et al., 1996; Keating et al., 1997; Amann et al., 2000; Thundathil et al., 2001; Bassols, 2006). Morphological anomalies acquired by inappropriate handling of semen sample (transport, storage temperature, doses dilution etc.) are considered as tertiary abnormalities, being the most common severe morphological alterations of tail (Adeniji et al., 2010).

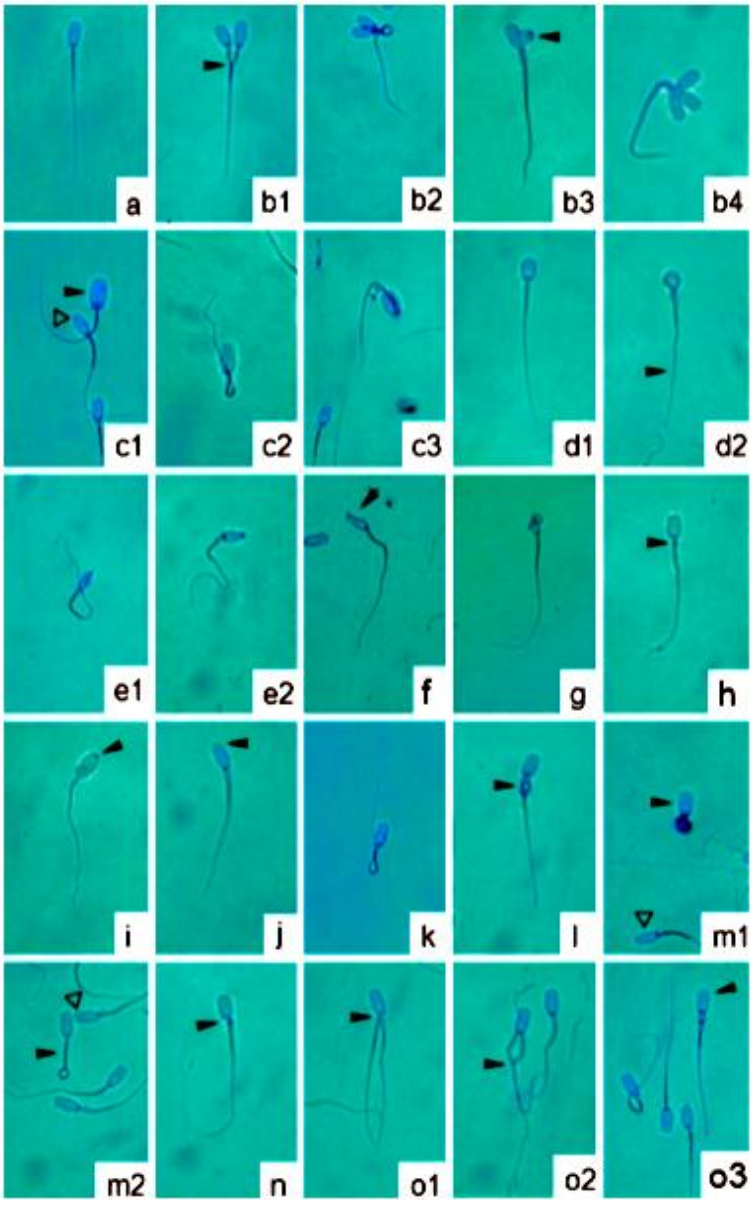


Fig. 6.1: Sperm malformations of the ejaculate of post-pubertal boars with spontaneous unilateral abdominal cryptorchidism on the right side (Quick Panoptic Method of QCA). a)

Mature spermatozoon. b1) Bicephalic spermatozoon with two tails fused from the distal end of the midpiece (arrow). b2) Spermatozoon with two elongated heads, and two tails totally fused and folded at the midpiece. b3) Spermatozoon with two heads, one of them aberrant (arrow), and two totally fused tails. b4) Tricephalic spermatozoon with three totally fused tails. c1) Macrocephalic spermatozoon (arrow); note the presence of an elongated head spermatozoon (triangle). c2) Macrocephalic spermatozoon with folded tail at the midpiece. c3) Macrocephalic spermatozoon with folded tail at the connecting piece. d1) Roundish head spermatozoon. d2) Round head spermatozoon; note the corksrew defect affecting the principal piece (arrow). e1, e2) Pointed head spermatozoon. f) Deformed head spermatozoon due to a cephalic break (arrow). g) Spermatozoon with a transversely folded head. h) Spermatozoon with crater defect and proximal droplet (arrow). i) Macrocephalic spermatozoon with knobbed acrosome (arrow). j) Spermatozoon with acrosomal vacuolization (arrow). k) Spermatozoon with folded tail at the Jensen's ring. l) Macrocephalic spermatozoon with mitochondrial swelling (arrow). m1) Macrocephalic spermatozoon with folded tail; note the presence of a spermatozoon with crater defect and abaxial tail (triangle). m2) Spermatozoon with coiled tail at the terminal and principal pieces (arrow); note the presence of a spermatozoon with acrosomal vacuolization (triangle). n) Immature spermatozoon with proximal droplet and abaxial tail (arrow). o1) Spermatozoon with two unfused tails abaxially attached (arrow). o2) Spermatozoon with two tails fused from the principal piece (arrow). o3) Macrocephalic spermatozoon with vacuolated acrosome (arrow) and two fused tails. ×500. From Pinart et al., 1998.

6.5 Sperm Agglutination:

Sperm agglutination is observed when a spermatozoon binds to another spermatozoon by head-to-head or tail-to-tail contact. Under standard conditions agglutination does not occur. In fact, agglutination can be an infertility cause (Sancho and Vilagran, 2013). It is well known that

bivalent and trivalent cations present in boar seminal plasma as well as long storage and immunological factors or bacterial contamination can induce sperm agglutination (Yeste et al., 2008; Bussalleu et al., 2011). In this manner, the presence of sperm agglutination can suggest an improper handling of the sample.

6.6 Osmotic resistance of sperm:

Boar spermatozoa are sensitive to osmotic changes in the environment and the ability to respond to osmotic stress is related to cell functionality. Thus, an osmotic shock affects the diffusion of phospholipids in the bilayer membrane of spermatozoa (Christova et al., 2002). This implies that the sperm response to environmental osmotic changes can be a good indicator of its physiological status (Yeste et al., 2010). The sperm osmotic response involves complex metabolic pathways, such as ion channels related to the Na^+/K^+-dependent ATPase activity or the Na^+/K^+-dependent antiporter one (Medrano et al., 2006). In boars, an isotonic solution is established between 300 and at 400 mOsm Kg^{-1} (Petrunkina and Topfer-Petersen, 2000; Petrunkina et al., 2000; Fraser et al., 2001). Several tests have been developed to measure the response of spermatozoa to osmotic changes as a parameter of semen quality. These methods are the hypo-osmotic swelling test (HOST), the hyper-osmotic resistance test (HRT) and the osmotic resistance test (ORT), all are based on the sperm tolerance to osmolarity change of medium. The most used test in boar specie is ORT. The ORT consists in checking the capacity of sperm to resist a hypoosmotic shock by challenging sperm to a hypoosmotic medium (≤150 mOsm/Kg). The evaluation is achievable with a contrast phase microscope or by optical microscopy linked to an eosin-nigrosin stain of the sample (Rodriguez- Gil et al., 1994).

6.7 Sperm Plasma Membrane Integrity (Sperm Viability):

Although sperm membrane integrity is often confused with terms like “viability”, “live” and “dead”, this nomenclature is not scientifically correct. Even though is true that the rupture of the membrane leads to the death of the spermatozoon, almost all dyes and fluorochromes that strictly provide information on the status of the membrane are wrongly believed to be the only indicators of sperm viability. Thus, the most correct speaking about viability would have reference to the physiological status of cells but not to their live/dead status. Porcine ejaculates should contain 85% of spermatozoa with intact membranes to be considered of good quality (Pinart et al., 1999; Sancho, 2002). Evaluation of sperm membrane integrity is based on a percentage of viable sperm after the application of a stain exclusion assay (Partyka et al., 2012). At this moment there are in use separate staining techniques. The specific application of them would depend of factors like the utilized microscope. Thus, with a conventional optical microscope, the most used viability stains are aniline-eosin, eosin-nigrosin and eosin-fast green. These techniques allow practitioners to evaluate at the same time membrane integrity and sperm morphology. The optic microscopy-used techniques are based on that viable sperm are non-permeable to stains while membrane of non viable cells permit to be penetrate by eosin, turning thus to a reddish colour. The other complementary stains, namely aniline, nigrosin or fast green, offer a contrast background facilitating the detection of viable, non stained cells. Without doubts these represent the more economic and feasible techniques, although they are not the more precise ones.

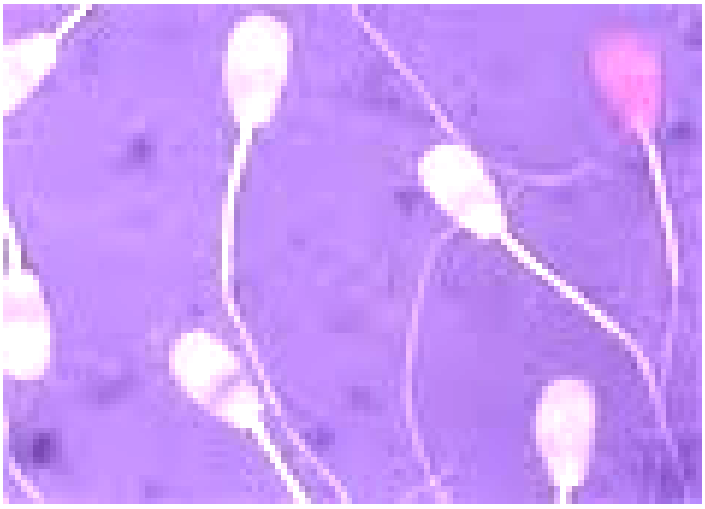


Fig. 6.2: example of eosin- nigrosin stain in boar sperm. From http://www.thepigsite.com/articles/2596/quality-control-of-extended-boar-semen.

Viability can be more precisely evaluated through the use of fluorochromes, but this evaluation can be conducted only by fluorescence microscopy or flow cytometry. At this moment, flow cytometry is the best method for its repeatability, accuracy of reliable results and for the number of sperm that are counted in a short time. The double staining with SYBR-14 and propidium iodide (PI) is the most commonly used for the evaluation of viability. In this technique, the viable spermatozoa emit a green fluorescence for SYBR-14 and non-viable ones a red for PI after argon laser excitation (Yeste et al., 2009).

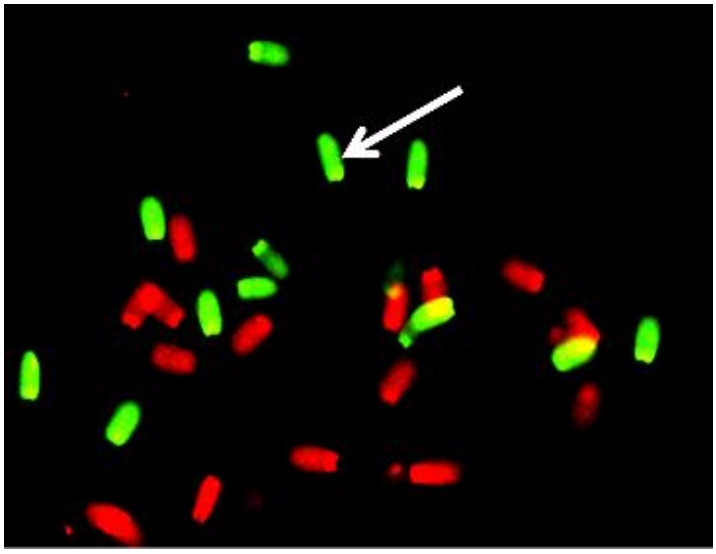


Fig. 6.3: boar semen viability determined using the SYBR/PI staining method. Arrows indicate viable cells which appear green while dead cells appear red in colour. From: Pilane et al., 2016.

6.8 Acrosome Integrity:

An intact acrosome is necessary for the penetration of the oocyte zona pellucid. For this reason its integrity is considered fundamental for the good fertilizing capacity of sperm (Jiménez et al., 2002). Acrosomal integrity can be measured by a number of methods, but the most commonly used method is with a plant lectin labelled by a fluorescent probe (Vázquez et al., 2005). These compounds are very important components of the invertebrate and plants immune system. Each lectin has its specific glycolitic fraction to link. For this reason, sperm surface glycoproteins specifically linked to a precise fluorochrome-marked lectin to determine with precision the location of a specific glucidic fraction linked to determinate proteins of the cell membrane. Use of fluorescent probes makes necessary the analysis by fluorescent microscope or flow cytometry. Probes principally used in andrology research are *Pisum Sativum* agglutinin

(PSA) and *Arachis Hypogaea* agglutinin (PNA). Since lectins are not fluorescent by themselves, they need to be labelled with a fluorochrome (Graham, 2001). The PSA is a lectin from the pea plant that binds to α-mannose and α-galactose moieties of the acrosomal matrix. Since PSA cannot penetrate an intact acrosomal membrane, only acrosome-reacted or damaged spermatozoa will stain. The PNA is a lectin from the peanut plant that binds to β-galactose moieties associated with the outer acrosomal membrane of fixed spermatozoa, indicating acrosome-intact cells (Gillan et al., 2005). Both PSA and PNA when bound to the flurochrome fluorescein isothiocyanate to form the conjugated lectin form, named either PSA-FITC or PNA-FITC, mark damaged sperm acrosome in yellow-green (Celeghini et al., 2007). Lectin binding to sperm samples was examined by fluorescence microscopy to determine the location of sperm-bound fluorescence and the proportion of labelled spermatozoa in the total population (Nikolaeva et al., 1998). Detection can be done on living sperm: the absence of fluorescence is indicative for an intact acrosome, and fluorescence over the anterior sperm head and equatorial regions is indicative for acrosome disruption or acrosome reaction (Marti et al., 2000, Silva and Gadella, 2006).

Meanwhile, the establishment of the capacitation status is also linked with changes in the distribution of sugar residues on glycoproteines of the sperm membrane, as shown by Benoff (1997). As indicate by Diekman (2003), the membrane surface of sperm is coated with a thick layer of glycans, *i.e.*, the sperm glycocalyx, which is the first interface between sperm and the environment. For example, the thickness of sperm glycocalyx of guinea boar spermatozoa varies from 20–60 nm on different sections of the cell (Bearer and Friend, 1990). It is estimated that sperm glycocalyx is comprised of 50-to-150 different glycoconjugates, being these glycoconjugates heterogeneously distributed from head to tail of sperm (Schröter et al., 1999). Although the roles of sperm glycocalyx have not been fully illustrated, it is well known that glycans participate in important events such as sperm maturation, sperm protection during

transportation in female reproductive tract, acrosome reaction, and the final sperm-egg recognition (Diekman, 2003; Schröter et al., 1999). For example, several studies have indicated the existence of a correlation between subtle changes in the sperm glycocalyx and the production of viable spermatozoa, which subsequently helps to determine fertility (Gabriel et al., 1994). Furthermore, modifications in the distribution and/or amount of carbohydrate residues on the top of sperm plasma membrane could be important for the regulation of progressive motility during the achievement of the capacitation status (Gomez-Torres et al., 2012). Thus, the evaluation of the exact distribution of sperm membrane glycoconjugates would be an important asserts to determine the sperm function status, especially in terms to achieve capacitation, but also to ascertain other function aspects like sperm cryo-damage. As in case of the analysis of acrosome integrity, the main technique used to determine changes in sperm membrane glycoconjugates involves again the use of lectins. The validation of the usefulness of a lectin in order to determine specific changes of sperm function, *i.e.*, the achievement of a full capacitation status or the starting of the acrosome reaction, is commonly carried out by using fluorescent dye-conjugated lectins that are then analyzed by fluorescence microscope. Taking into account the heterogeneous distribution of glycoconjugates on the sperm surface, each lectin will mark a different membrane domain. As a consequence, each lectin will underline separate regions of sperm cell membrane. Thanks to fluorescence microscope, either epi-fluorescence or, more efficiently, 3-D confocal microscope, the observation of changes in the distribution of the carbohydrate residues during capacitation process is easily determined. In fact, the use of confocal microscope ionstead the more commonly utilized epifluorescence is even more important in cells like sperm, which have a typical shape with marked asymmetry in its frontal-back part (Marek et al., 2011). In fact while 2D analysis with optical microscope gives mainly information about horizontal plane of the cell, the confocal microscope is able to acquire 3D shape of the cell surface using 3D

reconstruction of horizontal cross-sections (Marek et al., 2011). The most common lectins used are PNA and PSA which bind α-mannosil residues and α-glucosyl ones, respectively. Specifically, PNA binds as its specific sugar methyl a-D-galactopyranoside, which exhibits a weak reaction over the entire surface of the caput epididymal spermatozoa; the PSA presents a most abundant staining at level of the acrosomal region. There are, of course, other lectins of interest in the analysis of sperm function. Thus, the *Triticum vulgaris* lectin, also known as wheat germ agglutinin (WGA), has as its specific sugar linkage the N-acetyl-p(1-4)-D-glucosaminide, showing an homogenously distribution over the acrosomal region. Another used lectin is the *Solanum lycopersicum* one (STL) that shows it greatest affinity for N-acetylglucosamine (GlcNAc) residues (Xin et al., 2014). All of these lectins can be utilized as markers of the evolution of sperm function during processes that the achievement of the capacitation status or the sperm cryo-resistance.

6.9 Determination of mitochondrial membrane potential (Δψm):

The maintenance of the sperm fertilizing potential depends on the integrity and functionality of the different cellular structures (Celeghini et al., 2007). So it is necessary to evaluate mitochondrial membrane potential (Δψm). This parameter can be assessed using flow cytometry, which can be accomplished with JC-1 (5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolcarbocyanine iodide; Ramio-Lluch et al., 2012), Mitotraker green FM (Bonet et al., 2012) or Rhodamine 123 (Fraser et al., 2001) in association with PI and a DNA stain (Garner et al., 1997; Martinez- Pastor et al., 2010) to evaluate at the same moment sperm viability and mitochondrial functionality (Flores et al., 2010). These cationic and lipophilic fluorochromes can pass through plasmatic membrane to be accumulated into the mitochondrial matrix with a negative charge. Mitochondrial activity provokes oxidation of fluorochromes with an increment in light intensity emitted by the same, instead in JC-1 is produced a change

in wavelength emitted, that follow the oxidation turn from green emission to an orange- red light. Thus we can resume an increment in mitochondrial activity as a raise in oxidation level of fluorochromes. In this way has been saw as, mitochondrial activity is correlated with motility and fertility parameters of human and dog sperm (Bonet et al., 2012).

6.10 Oxidative Stress Evaluation by flow cytometry: Superoxide /Oxide Levels in Sperm Cells:

Oxidative stress (OS) threatens mammalian sperm survival, impairing critical functions such as motility, membrane integrity and even fertilizing ability (Aitken et al., 2014). Specifically, boar sperm are particularly sensitive to OS due to the high proportion of polyunsaturated fatty acids present in the membrane, which can be oxidized leading to lipid peroxidation (Browners et al., 2005). The OS is a status related to increased cellular damage triggered by oxygen and oxygen-derived free radicals known as ROS (Agarwal et al., 2014). The ROS compounds have at least one unpaired electron, being the most known peroxides (H_2O_2) and superoxides ($O_2^{-\bullet}$). They are oxidizing agents generated as byproducts from the metabolism of oxygen. Due to the unpaired electron in the outer shell, they form highly reactive molecules (Miranda et al., 2010; Henkel et al., 2011). Spermatozoa are equipped with antioxidant defence mechanisms that likely quench ROS, thereby protecting gonadal cells and mature spermatozoa from oxidative damage (Henkel et al., 2011). However, when production of ROS exceeds the antioxidant capacity of spermatozoa, cells are unable to restore the damage induced by OS because they lack enough strong cytoplasmic-enzyme repair systems (Saleh and Agarwal, 2002). As mentioned earlier, OS results from an imbalance between ROS production and the intracellular/extracellular antioxidants system used by sperm. The detection of ROS can be carried out by reagents that accumulate intracellularly and become fluorescent upon oxidation. Dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE) are used for

measurement of intracellular H_2O_2 and $O_2^{-\bullet}$, respectively, by flow cytometry. The advantages of using flow cytometry for the measurement of intracellular ROS in ejaculated mammals spermatozoa have been reported recently in boar (Guthrie et al., 2006), human (Mahfouz et al., 2008) and dog (Kim et al., 2010). The mechanism of action of H2DCFDA consists in penetrating into the cell through its plasma membrane. Afterwards, the compound is retained after intracellular esterases cleave the acetate groups, and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (green fluorescence at 504 nm; see Guthrie & Welch, 2006). On the other hand, dihydroethidium (DHE) reacts with the $O_2^{-\bullet}$ anion forming the yellow (567 nm of wavelenght) 2-hydroxyethidium (Zhao et al., 2005). The simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa either with PI or with the commercial YO-PRO®-1 stain. Thus, the ROS analysis through cytometry allows practitioners to remove the non-viable population from the analysis.

6.11 Changes of plasma membrane fluidity compatible with capacitation:

The underlying pathways that characterize the process of capacitation of boar spermatozoa are still largely unknown. As spermatozoa must undergo major membrane modifications at fertilization (viz. exocytosis of acrosome and fusion with the egg), it has long been surmised that capacitation involves some form of membrane destabilization. In fact; much evidence has been acquired in the last years that highlight the appearance of specific changes in the surface architecture and membrane function during the achievement of the capacitation status (Bedford, 1994; Cohen-Dayan et al., 1994; Eisenbach, 1994). In the boar, data shown as capacitation results in dramatic changes in the sperm plasma membrane including, increased membrane lipid disorder and phospholipid catabolism of sperm (Gadella and Harrison, 2000). Using merocyanine fluorochrome (M-540) coupled with YO-PRO®-1, to detect non-viable spermatozoa, it's possible by a flow cytometric analysis to evaluate molecular modifications of

the membrane structure. The M-540 is a fluoroscent impermeable lipophilic dye that has been shown to stain cell membranes more intensely if the membrane lipids are in a high disorder status (Williamson et al., 1983; Langner and Hui, 1993). The M-540 has been widely used to detect changes in membrane lipid packaging in several cell types (Aussel et al., 1993; Mower et al., 1994). The application of the M-540/YO-PRO®-1 technique renders two separate sperm populations. The first population is characterized by a cell membrane with low fluidity, which indicates a highly ordered membrane structure. Meanwhile, the other population has high membrane fluidity linked to a greater membrane disorder. This increase in lipid membrane disorder is commonly associated with capacitating and/or acrosome reacted sperm (Januskauskas et al., 2005; Purdy, 2008; Puigmulé et al., 2011).

6.12 Intracellular calcium levels as capacitation markers:

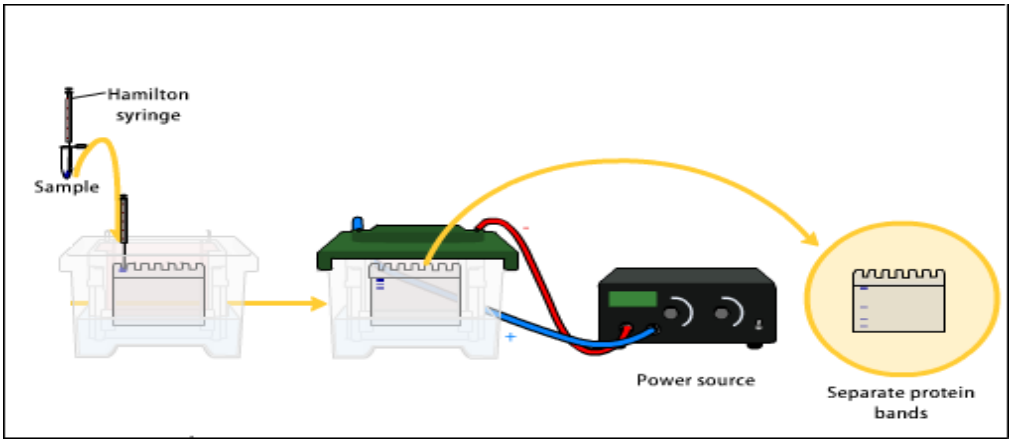
Calcium metabolism plays a key role in mammalian spermatozoa. A considerable body of evidence indicates that calcium ion is responsible for a wide range of cellular processes and is generally considered the ubiquitous second messenger (Berridge et al., 1993). Calcium ion has been shown to be a primary determinant of sperm cell function, including sperm capacitation, hyperactivated motility, chemotaxis and acrosome reaction (Publicover et al., 2007; Costello et al., 2009; Aitken & Nixon, 2013). Pathways by which calcium exerts these important regulatory and modulatory effects vary, since the Ca^{2+} ion can exert its effects through a wide array of separate transductional signalling pathways (Yeste et al., 2015). Functional studies in mammalian sperm have also documented the presence of voltage-gated Ca^{2+} channels, such as CatSper, that controls the entry of positively charged calcium ions into sperm cells, which is essential for sperm hyperactivation and male fertility (Kirichok et al., 2006; Lishko et al., 2011; Shukla et al., 2012). In the case of mammalian spermatozoa, intracellular calcium has been reported to be mainly stored at either the sperm head or connecting/midpiece (Costello et

al., 2009). These separate calcium stores suggest that the role of head and midpiece, mitochondria-related calcium stores may be different (Yeste et al., 2015). In this manner, while head calcium could be involved in the modulation of progesterone-induced acrosome exocytosis, midpiece calcium could be more closely related to mitochondria-modulated processes, such as sperm motility, mitochondria-based energy production and early capacitation steps (Yeste et al., 2015). These separate calcium deposits can be determined through the utilization of two separate calcium markers, Fluo-3 and Rhod-5N, which stain calcium deposits with different physico-chemical characteristics. The Fluo-3 stain has a very high affinity for calcium, although it is unable to cross polarized cell membranes (Takahashi et al., 1999). On the contrary, Rhod-5N specifically labels calcium stored in deposits surrounded by polarized membranes, and its calcium-affinity is lower than that of Fluo-3 (Takahashi et al., 1999). Fluo-3/ PI staining protocol was described for first time by Harrison et al. (1993) and successively modified by Kadirvel et al. (2009); while Rhod-5N/ YO-PRO1 labelling was performed in mammalian spermatozoa adapting the protocol edited for somatic cells (Hayato et al., 2011; De la Fuente et al., 2012). Fluorescent changes are detected by using a fluorescent microscope or better by flow cytometer assessment as saw for others parameters.

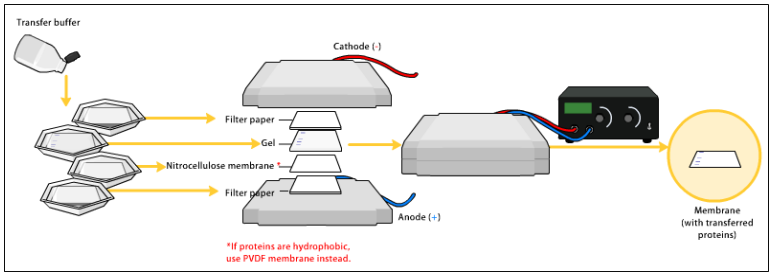
6.13 Detection of tyrosine-phosphorylated proteins by Western blot analysis:

The Western blotting technique made possible the feasible separation and identification of specific proteins relying on the electrophoretic separation of sperm proteins according to their molecular weight and their subsequent identification through immunological means. Providing the use of a reliable, sensitive and non radioactive, Western blotting found from its first implementation a large diffusion. In this way, this technique has been recognized as the best method for the detection of tyrosine-phosphorilated substrates, allowing a precise validation and quantification of these residues. The Western blotting technique has four main steps. The

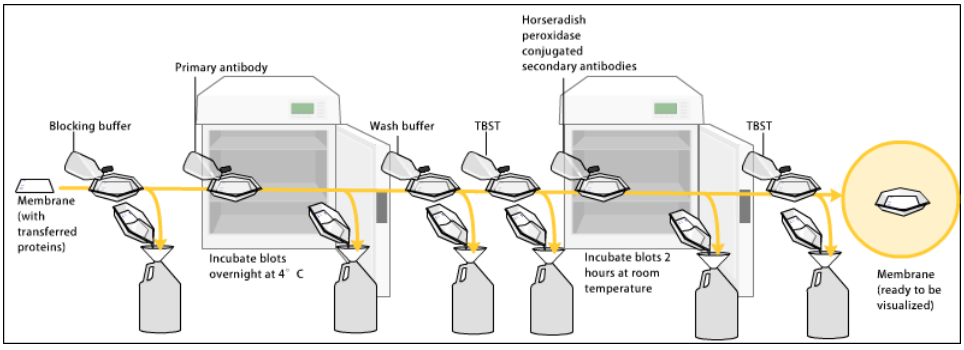
first step, as indicated above, consists in the electrophoretic separation of the sperm proteins from a mixed extract. The separation is based on the molecular weight of each protein. The second step consists in the transference of the separated proteins to a nitrocellulose membrane in which the obtained electrophoretic pattern of proteins is dully reproduced. The third step is based on the specific recognition of phosphorylated tyrosine residues on a wide variety of proteins through the utilization of a specific anti-phosphotyrosine primary antibody. The intensity and position of the antibody-protein union will be then highlighted through the incubation of the membrane with a chemiluminescence-linked secondary antibody that has linking affinity for the used primary antibody. Finally, the fourth and last step consists in a revealing process in which the chemiluminescence linked to the secondary antibody is visualized. In case of phosphotyrosine residues, the revealed membrane will show a specific bands pattern that will be linked with the precise function status of the analyzed sample. Furthermore, not only the position of each band, but also their thickness will be important, since this thickness will be proportional to the amount of phosphorylation that the protein/s present in this band has. Finally, in order to normalize putative variations in the total protein content of each sample, that will have as a consequence art factual changes in the intensity of the bands pattern, the same membranes will be analyzed by the same method to detect the amount of a protein known to maintain its amount stable during all of the sperm life. The most used of these proteins are the α-tubulin and the β-tubulin, although others like the glucose 6-phosphate dehydrogenase can be used for the same aim.



a) electrophoretic separation



b) trasference



c) Incubation with primary antibody d) and revelation

Fig. 6.4: Representation of Western blotting technique in principal four steps: a) electrophoresis separation of protein by molecular weight; b) transference by gel with separated proteins to a nitrocellulose membrane; c) recognition of phosphorylated tyrosine residues incubating with a primary antiphosphotyrosine primary antibody, and d) revelation of membrane with chemiluminescence method.

6.14 Detection of phosphotyrosine residues location in boar sperm through immunocytochemistry:

As indicated in the Introduction section, the involvement of the cAMP–PKA pathway in sperm capacitation (Visconti et al., 1997, Visconti & Kopf, 1998) and acrosome reaction (Breitbart & Naor, 1999, Garde & Roldan, 2000) is the main responsible for the launching of a myriad of processes involving protein phosphorylation (Aitken et al., 1995; Burks et al., 1995; Baldi et al., 1996; Visconti & Kopf, 1998; Visconti et al., 2002; Naz & Rajesh 2004; Liguori et al., 2005; O'Flaherty et al., 2006). Protein phosphorylation is a post-translational modification of proteins that allows the cell to control vital cellular processes such as transduction of extracellular signals and intracellular transport (Ullrich & Schlessinger, 1990; Johnson & Barford, 1993, Johnson & O'Reilly, 1996). In most species studied, sperm tyrosine-phosphorylated proteins are mainly located at the flagellum during the course of capacitation and fertilization (Carrera et al., 1996; Leclerc et al., 1997; Mahony & Gwathmey, 1999; Si & Okuno, 1999; Lewis & Aitken, 2001; Urner et al., 2001; Petrunkina et al., 2003; Pommer et al., 2003; Sakkas et al., 2003). Concomitantly, the increase of protein tyrosine phosphorylation in the principal piece has been correlated with the acquisition of hyperactivated motility (Nassar et al., 1999; Si & Okuno, 1999; Petrunkina et al., 2003). In addition, a capacitation-related redistribution of phosphotyrosine residues to the acrosome has been reported in boars (Petrunkina et al., 2001; Tardif et al., 2001), bulls (Cormier & Bailey, 2003) and buffalo (Roy

& Atreja, 2008), whereas specific phosphotyrosine proteins localized over the acrosomal region have been postulated to be involved in the zona pellucida interaction and/or fusion events (Leyton & Saling, 1989; Naz et al., 1991; Ficarro et al., 2003; Dube et al., 2005). The most reliable technique at this moment to attain the localization of tyrosine-phosphorylated proteins and their modifications according to the functional state of boar spermatozoa is the indirect immunocytochemistry analysis linked to evaluation through microscope. This technique exploits the linking of phosphoresidues to specific sperm domains. For this purpose, a primary antibody-secondary antibody system similar to that described for the Western blotting is used, but utilizing a fluorochrome system instead to a chemiluminescent one to reveal the final result. This system allows practitioners differentiate separate surface distribution patterns of phosphotyrosinated protein on sperm cell according with its physiological state. The results obtained with this method are highly specific. In fact the specificity of sperm specific domain with primary-secondary antibody complex is confirmed by samples that were processed only with the secondary antibody, which doesn't show any fluorescent signal through microscopy.

6.15 Evaluation of free-cysteine residues in sperm nucleoproteins:

Disulfide bonds are universally present between proteins and also between proteins and other macromolecules like nucleic acids. These bonds build a connection between two cysteine aminoacids and their thiol groups. Thus, disulfide bonds are specific of cysteine amino acid groups that are capable to form this bridge, which has this general structure:

R–**S**–**S**–R'

Disulfide bonds represent the strongest and most important globular structure responsible for the stabilization of the tertiary and quaternary structure of proteins. For this reason, these bonds

are the main responsible for holding proteins in their respective conformations and, consequently, to maintain the specific protein folding and stability. Likewise, as indicated above, disulfide bonds represents a bridge between nucleoproteins and DNA (Brewer et al., 2003; Dorigo et al., 2004; Balhorn R., 2007), and alterations in this type of protein-DNA linking can compromise many processes, including DNA replication. In fact, a better stability is considered useful to maintain sperm with a more compact chromatin core increasing their hydro dynamicity and their ability to move faster. This stability, in turn, will increase the possibility to fertilize the oocyte. It is well known, however, that mechanisms such as osmotic oxidative stress induced by osmotic disturbances are able to destroy disulfide bonds in a whole series of cellular structures (Cumming et al., 2004; Yang et al., 2007). This is important, since osmotic stress together with OS is one of the most important mechanism causing alterations in sperm cells, including structural alterations in cellular membranes and mitochondria volume and shape, as well as the rupture of the peri-mitochondrial and head actin network (Hinshaw et al., 1986; Silvia and Gadella, 2006; Correa et al., 2007). Hence, the evaluation of the amount of free cysteine levels in head and tail sperm through a spectrophotometric method is possible. This technique allows practitioners to determine changes in sperm function. In this manner, it is noteworthy that variations in free cysteine levels will be indicative of concomitant changes in aspects like the sperm nucleoprotein structure stability. This is interesting, since changes in the number of intact disulfide bonds have been linked to processes such as cryoresistance and even the achievement of a feasible *in vitro* capacitation (Yeste et al., 2013; 2014; Estrada et al., 2014). The main used technique to determine free cysteine radicals in cell extracts is the 2,2'-dithiodipyridine one, developed by Brocklehurst et al. (1979). This is a spectrophotometric analysis performed at a wavelenght of 343 nm that utilizes known concentrations of L-cysteine to create the appropriate external calibration curve.

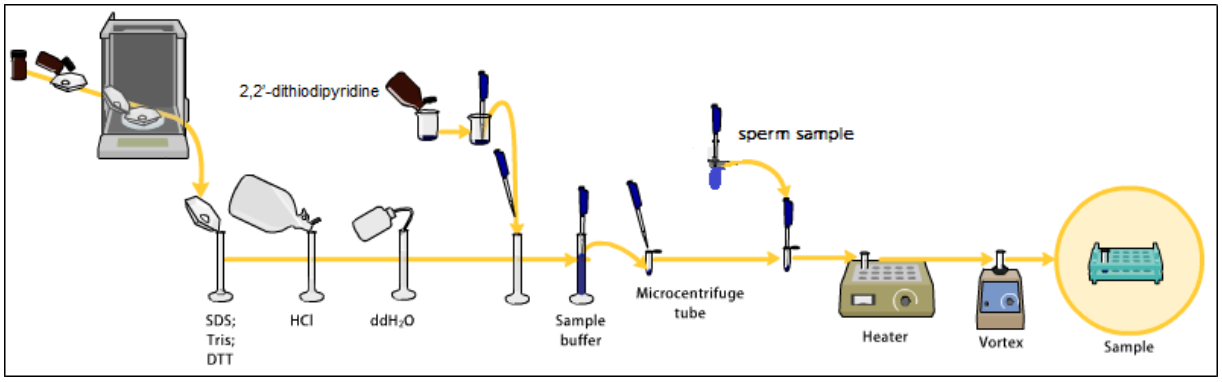


Fig. 6.5: Schematic procedure used for the evaluation of free-cysteine residues in sperm cells nucleoproteins. After the preparation with 2,2'-dithiodipyridine reagent, the samples are incubated for 30 minutes at 37°C, and analyzed with a spectrophotometric instrument at a vawelenght of 343nm.

6.16 Evaluation of DNA fragmentation (SCD test adapted for boar sperm):

Changes in chromatin structure and nuclear DNA integrity of spermatozoa have been associated with infertility in several mammalian species, including pigs (Evenson et al., 1994; Rybar et al., 2004). The SCD test is a rapid, simple, and sensitive test for measuring differential chromatin disorganization in sperm cells (Dugum et al., 2011), and it is proven to be effective for evaluating nuclear DNA fragmentation in boar spermatozoa (Fraser et al., 2010). In fact, using a commercial sperm chromatin dispersion test (SCDt) there is a specifically kit designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain). This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this technique strongly correlated with those obtained with other tests, like the neutral comet assay (Enciso et al., 2006). As suggested in manufacturer's instructions after the treatment of spermatozoa in vials containing agarose (vials included in the

commercial kit), treated sperm samples being charged on slides, and stained with propidium iodide (PI); after are observed under an epifluorescence microscope at 1000× magnification. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only either a small halo or not halo at all.

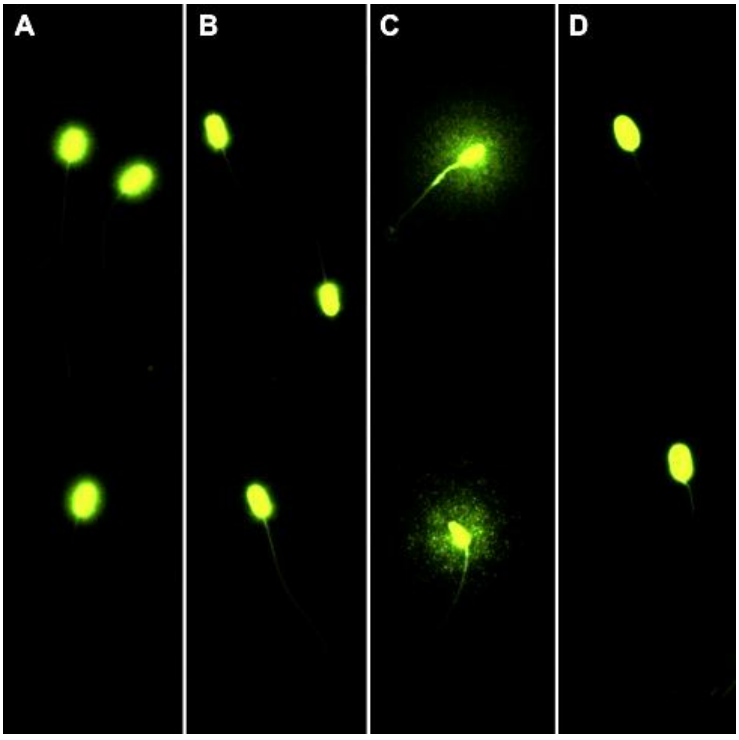


Fig. 6.6: Scoring criteria for assessing sperm nuclear DNA integrity after running the Sperm-Sus-Halomax test in thawed and incubated boar spermatozoa. (A) Compact and clearly visible halo around the sperm head (normal halo), (B) sperm with small halo around the sperm head (small halo), (C) large and scattered DNA halo around the sperm head (large scattered halo), and (D) no halo around the sperm head (absent halo). From: Alkmin et al., 2013.

Part 3. Research Works

Main aim of this thesis:

The main objective of this thesis was the evaluation of the effects of glutathione and melatonin, as antioxidants, on the achievement of boar sperm *in vitro* capacitation (IVC) and subsequent *in vitro*, progesterone-induced acrosome exocytosis (IVAE).

These studies were carried out through the evaluation of several *in vitro* capacitation markers and other sperm quality parameters such as percentages of sperm viability and total motility, true progesterone-induced acrosome exocytosis, acrosome-status by observing lectin pattern, membrane lipid disorder, DNA fragmentation, intracellular levels of peroxides and superoxides, tyrosine phosphorylation of sperm protein and its distribution, and free-cysteine residues in both head and tail sperm extracts.

7. Glutathione

7.1 General function, roles and localization:

Glutathione, a tripeptide thiol (γ-glutamylcysteinylglycine), is the major non-protein sulphydryl compound in mammalian cells that is known to have numerous biological functions (Luberda, 2005). Glutathione exists in two forms: the reduced form (GSH) and the oxidized form (GSSG). Cellular glutathione plays a key role in many biological processes, including the synthesis of proteins and DNA and the transport of amino acids, but notably, it plays a key role in protecting cells against oxidation. Thus, it has been described that the sulphydryl groups of glutathione confer protection against cell damage by oxidants, electrophiles and free radicals (reviewed by Irvine, 1996). The protective action of glutathione against reactive oxygen species (ROS) is facilitated by the interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase (Luberda, 2005). As in other cells, glutathione has been reported also in mammalian sperm cells, (Alvarez and Storey, 1989; Ochsendorf et al., 1998), including boar sperm (Li, 1975) in species-specific concentrations. In fact, previous researches showed that mouse sperm cell represents, into mammalian species, the spermatic cell with the highest concentration of GSH values (90 nM GSH/10^8 cells; Alvarez and Storey, 1989), compared with GSH content founded in ejaculated boar semen (3,84 ± 0,21 nM/10^8 cells; Gadea et al., 2004) and in other mammalian species including human sperm (3,49± 0,87 nM GSH/10^8 cells; Ochsendorf et al., 1998), bull sperm (2,73 ± 0,42 nM GSH/10^8 cells; Agrawal et al., 1988) rabbit sperm (< 0.1 nmol /10^8 cells; Alvarez and Storey, 1989) and ram sperm (0.45 ± 0.14 nmol /10^8 cells; Li, 1975). The basic function of GSH in mammalian semen is related to its interactions with other systems as a preventive mechanism against ROS. This scavenging function of GSH helps to counteract the effects of oxidative stress in sperm cells, which could result in lipid peroxidation of

plasmalemma, irreversible loss of motility, leakage of intracellular enzymes and damage of the chromatin (Aitken, 1999; Luberda, 2001). GSH is synthesized in the γ-glutamyl cycle, and the three amino acids comprising glutathione are glutamate, cysteine, and glycine (Emmett, 2014). The main synthesis pathway is an ATP-dependent, two step process. Briefly, the enzyme γ-glutamylcysteine synthetase (also called γ-glutamylcysteine ligase) catalyzes the unique γ-glutamyl linkage between glutamate and cysteine. Next, glutathione synthetase catalyzes the addition of glycine to the γ-glutamylcysteine (via the more usual α-amide linkage) to form glutathione. Each of these synthetic reactions requires energy provided by the hydrolysis of ATP (Emmett, 2014).

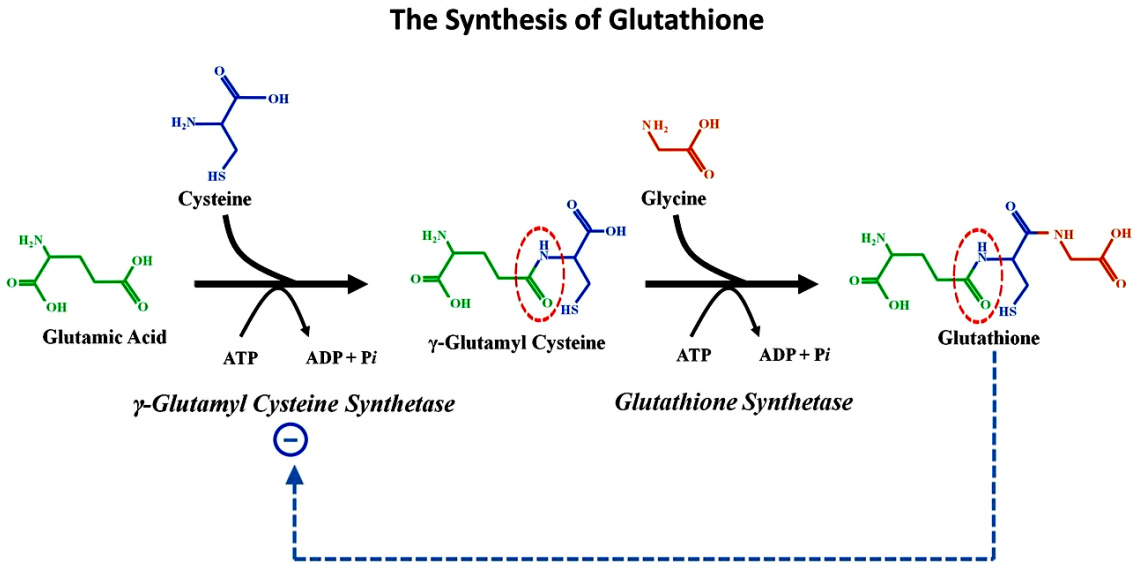


Fig. 7: Glutathione synthesis pathway: γ-glutamyl cycle. First step, starting from glutamic acid and cysteine and via gamma glutamylcysteine synthetase enzyme (GCL), is formed gamma glutamylcysteine. Successively glycine via the enzyme glutathione synthetase is

added to the C-terminal of gamma-glutamylcysteine which leads to glutathione production. From: Emmett, 2014.

Actually, the γ-glutamyl cycle is a larger metabolic one that consists of six enzymatic reactions, the pair of glutathione synthesizing reactions and four degradation steps (Orlowsky and Meister, 1970). This metabolic cycle requires the input of energy, derived from the hydrolysis of three ATP molecules. The net result of each turn of this cycle is the active transport of one amino acid molecule into the cell (Emmett, 2014). Principal mechanism chaired by glutathione is bind to and reduce (i.e., donates electrons) many molecules. These reducing reactions are of great importance for the detoxification of reactive oxygen species, drugs, toxins, and other oxidizing molecules and are also key reducing steps in a variety of normal metabolic synthetic pathways (Franco et al., 2007). In addition, glutathione has a multitude of other vital metabolic functions: it is an important cellular reservoir of the sulfur-containing amino acid cysteine, it is a major cell signalling molecule involved in the regulation of apoptosis, cell cycling, and immunity and it participates in active trans cellular amino acid transport (Franco et al., 2007; Forman et al., 2009). These reducing reactions are catalyzed by glutathione peroxidase, it reduces H_2O_2 to H_2O. Glutathione disulfide thus formed is converted back to glutathione by glutathione reductase. This "glutathione cycle" has been demonstrated to be an effective, intracellular defense mechanism against a variety of oxidant stresses (Kosower et al., 1971).

7.2 How glutathione interacts in spermatozoa's mechanisms:

During the achievement of boar sperm *in vitro* capacitation (IVC) and subsequent *in vitro*, progesterone-induced acrosome exocytosis (IVAE), sperm cells run the risk of generating and being exposed to supra-physiological level of ROS (du Plessis et al., 2008). The imbalance between the production of reactive oxygen species (ROS) and a biological systems ability to

readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress (Agarwal et al., 2003) and, the main destructive aspects of oxidative stress are the production of ROS, which include free radicals and peroxides. The production of ROS by sperm is a normal physiological process that at physiological levels are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm (Bucak et al., 2010). Moreover, a sustained and increased amount of $\cdot O_2^-$ is one of the first steps required by the spermatozoa for induction and development of hyperactivation and capacitation (de Lamirande and Cagnon, 1995), the two main processes that are necessary to ensure sperm fertilization. However, an imbalance between ROS generation and scavenging activity is detrimental to the sperm and has been associated with male infertility as supported by substantial scientific researches (Agarwal et al., 2005). In this way, as said above, GSH is the substrate of glutathione peroxidase (GPX) and plays a prominent role in the body's antioxidant defences (Donnelly et al., 2000). GSH exists in the sperm of several mammalian species, mouse, ram, boar, rabbit, including human beings (Li, 1975; Alvarez and Storey, 1989). Its scavenging function helps to counteract the effects of oxidative stress in sperm cells, which could result in lipid peroxidation of plasmalemma, irreversible loss of motility, leakage of intracellular enzymes and damage of the chromatin (Aitken, 1999; Luberda, 2001). Moreover, GSH would induce a concomitant lowering effect on mitochondrial activity, since mitochondria are the main location in which ROS are produced in sperm (Murphy, 2009). Furthermore, as showed by another research, GSH has a role as protective agent of disulfide bonds (Chatterjee et al., 2001; Jacob et al., 2003), and as showed by Yeste and his co-workers (Yeste et al., 2013), alterations of the nucleoprotein structure that are concomitant with a significant increase in the number of broken head sperm disulfide bonds are been counteracted by the addition of GSH to *in vitro* capacitating medium.

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Effects of reduced glutathione on i*n vitro* boar sperm capacitation and acrosome exocytosis

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Running title: reduced glutathione on boar sperm capacitation

Abstract

Incubation of boar sperm in a specifically designed *in vitro* capacitation medium for 4h significantly increased intracellular levels of free cysteine radicals. This increase was concomitant with capacitation-like changes of sperm motility, membrane lipid disorder, mitochondrial membrane potential, intracellular calcium levels, tyrosine phosphorylation levels of protein P32 and localization of tyrosine phosphorylated proteins. The addition of reduced glutathione to the medium prevented the majority of capacitation-like changes, except tyrosine phosphorylation of P32. Furthermore, reduced glutathione caused a rapid drop of total sperm motility, which was maintained during all of the incubation period. The addition of reduced glutathione at the beginning of the experimental incubation affected the launching of progesterone-induced *in vitro* acrosome exocytosis. The addition of reduced glutathione together with progesterone did not affect acrosome exocytosis launching, but counteracted the drop of Fluo3-marked intracellular calcium in control capacitated cells following the peak observed immediately after progesterone addition. Although the addition of reduced glutathione together with progesterone induced a drop in total sperm motility, the extent of this decrease was lower than that observed when it was added at the beginning of the experiment. Our results suggest that boar sperm capacitation is related with an increase of free cysteine residues and intracellular reactive oxygen species levels, which could play a role in the achievement of capacitation status. Furthermore, phenomena like sperm motility and capacitation-linked changes in membrane lipid disorder and intracellular calcium dynamics seem to be partially controlled by glutathione-sensitive mechanisms.

Keywords: spermatozoa, phosphorylation, free-cysteine residues, *in vitro* capacitation, acrosome exocytosis

Introduction

One of the most important processes that undergo mammalian sperm after ejaculation is capacitation. This process, which leads to the achievement of fully sperm fertilizing ability, is achieved inside the female reproductive tract. This characteristic hampers the study of capacitation in *in situ* conditions. However, with the advent of assisted reproductive technologies through *in vitro* studies, a large amount of information has been gathered on how sperm achieve capacitation through a sequence of biochemical modifications that lead to the establishment of fully fertilizing ability. These modifications involve the activation of several signalling pathways, the increase of intracellular messengers such as cAMP and Ca^{2+}, the reorganization of plasma proteins and membrane lipids and changes in motility patterns. Finally, the destabilization of acrosomal membrane linked with its fusion with sperm cell membranes increases the sperm ability to bind to the oocyte zona pellucida (see Kątska-Książkiewicz, 2007; Visconti, 2009, as reviews).

Recently, with the findings regarding the use of antioxidants agents in the freezing extenders for boar semen, other research horizons have been opened up to elucidate the effects of these substances on sperm metabolism. The cryopreservation process induces strong changes in sperm function. Some of these changes, such as modifications in motion parameters and in the lipidic membrane structure resemble those observed during sperm capacitation (Cormier et al., 1997; Bailey et al., 2000). The similarity of these effects led to several authors to call this phenomenon as cryocapacitation (Bailey et al., 2000). However, not only do these procedures present important differences, but cryocapacitation process greatly impairs fertilizing ability of frozen-thawed boar semen (Talukdar et al., 2015). Thus, any system that allows users of frozen-thawed semen to minimize cryocapacitation-mimiking alterations would be very useful to optimize the utilization of frozen-thawed boar semen for artificial insemination (AI). In this way, the addition of reduced glutathione (GSH) to the freezing medium has shown excellent

in vivo fertility results when applied to boar semen (Estrada et al., 2014). Several of the improving effects that the utilization of GSH has on boar semen freezing are linked to the prevention of some of the cryocapacitation–related alterations, as changes in sperm motility (Yeste et al., 2013; Estrada et al., 2014; Estrada et al., 2015). It is noteworthy that GSH is the most abundant thiol in cells and is considered of vital importance, amongst other functions, for the maintenance of the intracellular redox balance (Jacob et al., 2003). Taking this into account, results obtained in boar frozen-thawed sperm suggest that GSH and hence the precise oxidative status of boar sperm could play a role in the modulation of sperm capacitation process. However, this hypothesis remains to be elucidated.

Regarding the effects of GSH on boar semen cryopreservation, it has been reported that the addition of GSH to freezing and thawing media counteracts the cryopreservation-linked alterations on the integrity of sperm disulfide bridges between proteins (Chatterjee et al., 2001), reduces ROS levels and increases sperm motility and sperm fertilizing ability (Gadea et al., 2004, 2005). The GSH also seems to maintain a stable nucleoprotein structure (Yeste et al., 2013; 2014), and thereby, depending on their concentration in the extender, this set of cell properties could interfere the sperm capacitation, which is a process when occur a range of synchronized events.

Taking into account all of the aforementioned, the main aim of this study was to evaluate the effects of GSH on the achievement of boar sperm *in vitro* capacitation (IVC) and subsequent *in vitro*, progesterone-induced acrosome exocytosis (IVAE). This was carried out through the evaluation of several *in vitro* capacitation markers and other sperm quality parameters such as percentages of sperm viability and total motility, true progesterone-induced acrosome exocytosis, membrane lipid disorder, DNA fragmentation, intracellular levels of peroxides and superoxides, tyrosine phosphorylation of sperm protein, and specifically P32, and free-cysteine residues in both head and tail sperm extracts.

MATERIALS AND METHODS

Seminal samples

A total of 62 ejaculates collected from 35 healthy Pietrain boars aged between two and three years, were used in this study. These animals were housed in climate-controlled buildings (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with an adjusted diet and provided with water *ad libitum*. Sperm-rich fractions were collected manually using the hand-gloved method, diluted to a final sperm concentration of 2×10^7 spermatozoa/mL in a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain), and cooled to 16ºC–17 °C. Diluted semen was then distributed in 90-ml commercial AI doses. The resulting 90-mL doses were placed in a thermal packaging container at 16ºC for approximately 45 min, which was the time required to arrive at our laboratory.

Ethical approval

The utilized ejaculates were initially dedicated for artificial insemination purposes, and we just bought them for our experimental purposes. In this way, we didn't need any specific ethical approval to perform this work, since there were no animals directly manipulated during the carried out experiments. Despite all the aforementioned, the experimental protocol was approved by the Ethics Committee of our institution. This ethics committee was known as "Bioethics Commission of the Autonomous University of Barcelona" (Bellaterra, Cerdanyola del Vallès, Spain).

Experimental design and *in vitro* capacitation and subsequent acrosome exocytosis procedures

In all cases, each ejaculate was divided into five aliquots in accordance with the experimental treatments. In each aliquot, the following sperm parameters were evaluated: sperm viability, sperm motility, acrosome integrity, membrane lipid disorder, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), intracellular calcium levels, DNA fragmentation, free-cysteine residues, tyrosine phosphorylation of P32 and sperm localization of overall protein tyrosine phosphorylation. The work was separated in two separate experiments. In the first one, we tested the effects how the addition of GSH to the capacitation medium (CM) at the start of the incubation affected the achievement of a feasible IVC. The second experimental design was conducted by addition of GSH after the achievement of the ICV status and simultaneously to the induction of progesterone-modulated IVAE, in order to determine the putative effects of GSH in the achievement of IVAE in fully *in vitro* capacitated boar sperm. Thus, in both experimental designs there were 5 separate experimental points: a positive control (C+) in which cells were incubated in CM containing bicarbonate and bovine serum albumin (BSA), three different GSH treatments in which cells were resuspended in CM that was supplemented either at the 0h or after 4h of incubation time with increasing concentrations of GSH ($C_{10}H_{17}N_3O_6S$; GSH, Sigma-Aldrich®, St Louis, MO, USA),and a negative control (C-), which the sperm were re-diluted in a non-capacitating medium (NCM) without GSH, $NaHCO_3$ and BSA.

For the experimental design conducted to determine the effects of GSH from the 0h time of incubation onwards, 50 mL of each ejaculate was utilized through division between all five treatments. For this purpose, the 50-mL aliquot was firstly centrifuged at 600*g* for 10 min at 16ºC and initially resuspended at a final concentration of $20\text{-}30\times10^6$ sperm/mL in NCM. The NCM was composed by a 20-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

(Hepes) buffer (pH 7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM $NaHPO_4$, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (osmolarity: 287mOsm/Kg±6mOsm/Kg) After this resuspension, 40 mL were separated and added with. 5 mg⁄mL BSA and 37.6 mM $NaHCO_3$ in order to modify the NCM to the CM (pH=7.4; osmolarity=304mOsm/Kg±5mOsm/Kg). The pH of both NCM and CM was adjusted to the same value of 7.4 through fine adjustment with high-concentration solutions of NaOH and HCl until the required pH was acquired. The remaining 10 mL-aliquot was lent to be incubated in NCM to obtain the C- experimental point. Subsequently, the 40-mL CM suspension cell was further divided in 4 10-mL aliquots. One of these 10-mL aliquots was lent without further addition of any substance, whereas the other three 10-mL CM-aliquots were added with GSH to final concentrations of 1mM, 2mM and 5mM, respectively. All 5 10-mL aliquots were incubated for 4 h at 38.5ºC in a 5% CO_2 atmosphere, as described in Ramió et al. (2008). Sperm aliquots were taken at 0 hours and 4 hours of incubation to perform the appropriate analysis. After 4h of incubation, cells were subjected to the induction of progesterone-activated "in vitro" acrosome exocytosis as described before (Jiménez et al., 2003; Wu et al., 2006). For this purpose, 10 µg/mL progesterone was added to boar sperm previously incubated in the capacitation medium for 4 h at 38.5ºC in a 5% CO_2 atmosphere. After thorough mixing, the sperm was further incubated for an additional 1h at 38.5 ºC in a 5% CO_2 atmosphere. During this subsequent incubation time, aliquots were taken at 1 min, 5 min and 60 min after the addition of progesterone to perform the required analyses.

Samples utilized for the second experimental design conducted to determine the effect of GSH on IVAE of previously capacitated samples were treated in a similar way to that described above. The main difference was that in the GSH experimental points, GSH was added only after 4h of incubation in the CM and simultaneously to progesterone. The utilized

GSH concentrations were also of 1mM, 2mM and 5mM. Finally, in this second experimental design, aliquots were also taken after 0h and 4h of incubation in CM and after 1min, 5min and 60 min of the simultaneous addition of progesterone and GSH.

Analysis of parameters indicative of the achievement of both in vitro capacitation and in vitro acrosome exocytosis

As indicated above, parameters determined in order to analyse the achievement of both IVC and IVAE were the computer-assisted (CASA) motility analysis, the spectrophotometric determination of the free-cysteine residues in both head and tail sperm extracts, microscopic counting of the percentage of DNA fragmentation, flow cytometry analysis of sperm viability, acrosome integrity, membrane lipid disorder, ROS levels, mitochondrial membrane potential (MMP) and intracellular calcium levels from both head and midpiece location and finally the immunological detection of phosphorylation levels of protein tyrosine residues, with an especial emphasis in the P32 protein as a specific capacitation marker of boar sperm (Bravo et al., 2005).

Sperm motility analysis

Sperm motility analysis was performed by utilizing a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10 x 0.30 PLAN objective lens, Olympus-Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 ms. Five to six separate fields were taken for each replicate, and five replicates were run per sample and treatment. The obtained sperm motility descriptors were described following Ramió et al. (2008). Settings taken into account for all of the utilized motility parameters were as following:

Range of particles area: 10–80 μm^2.

Connectivity: a minimum of 11 images for all parameters, but a minimal of 10 images for only the mean amplitude of lateral head displacement (ALH).

Parameter ranges:

Curvilinear velocity (VCL): 1–500 μm⁄s.

Mean velocity (VAP): 1–500 μm⁄s.

Straightness coefficient (STR): 10–98%.

Linear coefficient (LIN): 10–98%.

Mean amplitude of lateral head displacement (ALH): 0–100 μm.

Frequency of head displacement (BCF): 0–100 Hz.

In this procedure, samples were previously warmed at 37ºC for 5 min in a water bath, and 5 μl aliquots of these samples were then placed onto a warmed (37ºC) slide and covered with a 22 × 22 mm coverslip. Finally, total motility was defined as the percentage of spermatozoa that showed a VAP >10 μm⁄s.

Evaluation of free-cysteine residues in sperm proteins from the subhead area

The determination of free cysteine radicals in sperm head and tail proteins as an indirect measure of disrupted disulfide bridges within nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. (2011). This technique determines free thiol groups. Briefly, samples were centrifuged at 600*g* and 16ºC for 10 minutes and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycholate, 1mM benzamidine, 10 μg/mL leupeptin, 0.5mM phenylmethylsulfonyl fluoride, and 1mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). Obtained homogenates were centrifuged at 850*g* for 20 minutes at 4ºC. The resultant supernatants were reserved to measure the free-cysteine in sperm tail proteins and

the pellets were resuspended in 300 µL of Tris buffer to measure the free-cysteine in sperm head proteins. The presence of both tails in supernatants and heads in pellets were determined by a previous observation under the optical microscope. This observation determined that the percentage of tails in supernatants and heads in pellets were of above of 85% of the total content in both cases (data not shown).

The levels of free cysteine radicals in both tails and heads fractions were determined by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulfide; Sigma-Aldrich) as described by Brocklehurst et al. (1979). With this purpose, 10-µL aliquots of resuspended, isolated sperm heads were added to 990 mL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. The same process were repeated with 10-µL aliquots of supernatants that were added to 990 mL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. Aliquots of 10 µL of cysteine standards from 0.1mM to 5mM (Sigma-Aldrich) were also added to 990 mL of 0.4mM 2,2'-dithiodipyridine for evaluation. In all cases, mixtures were incubated at 37ºC for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 340 nm. The results obtained were normalized through a parallel determination of the total protein content of samples by the Bradford method (1976), using a commercial kit (Quick Start Bradford Protein Assay; BioRad, Hercules, CA, USA). Five replicates per sample and treatment were evaluated, and the corresponding mean ± SEM was calculated.

Evaluation of DNA fragmentation

DNA fragmentation was assessed using a commercial sperm chromatin dispersion test (SCDt) kit specifically designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain) and following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this

technique strongly correlated with those obtained with other tests, like the neutral comet assay (Enciso et al., 2006).

Briefly, the lysing buffer included in the commercial kit was tempered to 22°C and vials containing low-melting agarose were incubated at 100°C for 5 min in a water bath. Vials were then left in another water bath at 37°C for 5 min to equilibrate the agarose temperature. Twenty-five µL of each sperm sample (at a final concentration of 10^7 spermatozoa/mL) were added to a vial prior to mixing it thoroughly. One drop of 25 µL containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid air-bubble formation.

Slides were placed on a cooled plate within a fridge and left at 4°C for 5 min. The coverslip was then removed and 50 µL of lysis solution per slide were added. An incubation step at 22°C for 5 min was performed, prior to washing for 5 min with miliQ® water. The slides were subsequently dehydrated by three steps of 2 min each with aqueous dilutions of 70% (v:v) ethanol, 90% (v:v) ethanol and 100% (v:v) ethanol. Finally, sperm samples were stained with propidium iodide (PI, 2.5 µg/mL; Molecular Probes®, Eugene, OR, USA) and mounted in DABCO anti-fading medium (DABCOTM anti-fading medium; Sigma-Aldrich®). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 1000× magnification. Three counts of 250 spermatozoa each using three different slides were carried out per sample and treatment, prior to calculating the corresponding mean±SEM. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only either an small halo or not halo at all.

Flow cytometric analysis

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC; Lee et al., 2008). These analyses

were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), sperm membrane lipid disorder, acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, M540/YO-PRO®-1, PNA-FITC/PI, H2DFCDA/PI, HE/YO-PRO®-1, JC-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSCTM cytometer (Beckman Coulter, Fullerton, CA, USA; Serial Number AL300087, Technical specification at https://www.beckmancoulter.com/wsrportal/ajax/downloadDocument/721742AD.pdf?autonomyId=TP_DOC_32032&documentName=721742AD.pdf). This instrument, which had not been altered in the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the singleline visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab QuantaTM SC cytometer employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10 µm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. In this system, the optical characteristics for these filters were as follows: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band pass filter: 525 nm, detection width 505– 545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm); and FL3

(red fluorescence): long pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, YO-PRO®-1, H2DFCDA and Fluo 3), whereas FL3 was used to detect red fluorescence (M-540, HE, PI and Rhod 5).

Sheath flow rate was set at 4.17 µL/min in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE vs. SS dot plots) for a minimum of 10000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter>12 µm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described below, compensation was used to minimize spill-over of the fluorescence into a different channel.

Information on the events was collected in List-mode Data files (LMD), and these generated files were then analysed using Cell Lab Quanta_SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyse the cytometric histograms. In both PNA-FITC/PI, H2DFCDA/PI and HE/YO-PRO®-1 assessments, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina et al. (2010). Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± SEM.

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen, Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma).

Cytometric determination of sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner & Johnson (1995). Briefly, sperm samples were incubated at 38°C for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 µM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, whereas PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: (i) viable green-stained spermatozoa ($SYBR\text{-}14^{+}$/ PI^{-}); (ii) non-viable red-stained spermatozoa ($SYBR\text{-}14^{-}/PI^{+}$); and (iii) non-viable spermatozoa that were stained both Green and red ($SYBR\text{-}14^{+}/PI^{+}$). Non sperm particles (debris) were found in the $SYBR\text{-}14^{-}/PI^{-}$ quadrant.

Single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Cytometric analysis of true acrosome exocytosis

True acrosome exocytosis was determined through co-staining of sperm with ethidium homodimer (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1) and peanut agglutinin (from *Arachis hypogaea*) conjugated with fluorescein isothiocyanate (FITC-PNA). This protocol was originally described by Cooper and Yeung (1998) and has been adapted to boar spermatozoa in our laboratory. The utilization of this protocol was due to the fact that it has a higher specificity for the detection of true acrosome exocytosis than that previously utilized in our studies (see Yeste et al., 2015, as an example). Briefly, samples were incubated with EthD-1 (final concentration: 2.5 $\mu g \cdot mL^{-1}$) at 38°C for 5 min in the dark. Following this step, samples were washed by centrifugation at 2000*g* for 30 seconds and then resuspended with PBS containing 4 $mg \cdot mL^{-1}$ bovine serum albumin (BSA) to remove free dye. Thereafter, samples were again centrifuged and then fixed and permeabilized by adding 100 µL of ice-cold methanol (100%) for 30 seconds. Methanol was removed by centrifugation at

2000*g* for 30 seconds and resuspension with PBS containing 4 $mg \cdot mL^{-1}$ BSA. The resulting pellet was resuspended in 250 µL BTS. Following this step, 0,8 µL PNA-FITC (final concentration: 2.5 µM) were added and samples were incubated at room temperature in the dark for 15 min. Next, samples were washed twice with PBS at 2000*g* for 30 seconds finally were resuspended in BTS.

After this assessment, four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC^{+}/EthD-1^{-}); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC^{-}/EthD-1^{-}); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC^{+}/EthD-1^{+}); (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC^{-}/EthD-1^{+}). Fluorescence of EthD-1 was detected through FL-3, while that of PNA-FITC was detected through FL-1.

Determination of sperm membrane lipid disorder compatible with capacitation

Lipid disorder of boar sperm membrane was evaluated by Merocyanine-540 (M540) and YO-PRO-1 co-staining, following a modified procedure from Rathi et al. (2001). Briefly, spermatozoa were stained with M540 (final concentration: 400 µM) and YO-PRO®1 (final concentration: 40 µM) and incubated for 10 min at 38 °C in the dark. Red fluorescence from M540 was collected through FL-3 and green fluorescence from YO-PRO®1 was collected through FL-1. A total of four sperm populations were observed in flow cytometry dot plots: (i) viable spermatozoa with low membrane lipid disorder (M540^{-}/YO-PRO-1^{-}); (ii) viable spermatozoa with high membrane lipid disorder (M540^{+}/YOPRO-1^{-}); (iii) non-viable spermatozoa with low membrane lipid disorder (M540^{-}/YO-PRO-1^{+}) and (iv) non-viable spermatozoa with high membrane lipid disorder (M540^{+}/YO-PRO-1^{+}). In this test, data were not compensated.

Determination of mitochondrial membrane potential

The MMP was analyzed by applying the method described in Gillan et al. (2005). For this purpose, samples were incubated with the fluorochrome 5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) to a final concentration of 0.3µM at 38ºC for 10 min in the dark. Afterwards, samples were processed through the flux cytometer. There emission filters FL1 and FL2 were utilized to differentiate two subpopulations. The first subpopulation was formed y sperm with high MMP, which presented JC-1 aggregates with and orange staining. The second subpopulation was composed by cells with low MMP, which presented JC-1 monomers with a green staining. Data were not compensated.

Assessment of intracellular superoxide and peroxide levels

Intracellular superoxide ($O_2^{-\bullet}$) and peroxide (H_2O_2) levels were determined using two different oxidation-sensitive fluorescent probes: hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed by containing the spermatozoa either with PI or with YO-PRO®-1.

In the case of superoxides, samples were stained with HE (final concentration: 4 µM) and YO PRO®-1 (final concentration: 40 µM) and incubated at 25°C for 40 minutes in the dark (Guthrie & Welch, 2006). Hydroethidine is freely permeable to cells and is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of ethidium (E^+) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means±SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E^+).

In the case of peroxides, spermatozoa were stained with H2DCFDA at a final concentration of 200 µM and PI at a final concentration of 10 µM, and incubated at 25°C for 60 minutes in the dark. H2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie & Welch, 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3.

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

Intracellular calcium levels

Intracellular calcium of spermatozoa was determined by using two separate, specific stains, Rhod-5N-AM (Rhod 5) and Fluo-3-AM (Fluo 3). The Rhod 5 labelling was determined as in Yeste et al. (2015), whereas Fluo 3 marking was analyzed as in Harrison et al. (1993) and modified as in Kadiervel et al. (2009). The evaluation of intracellular Rhod 5-marked calcium, which is mainly located at the boar sperm head (Yeste et al., 2015), was performed together with a simultaneous staining with YO-PRO®1 in order to evaluate sperm membrane integrity. With this purpose, boar spermatozoa were stained with Rhod-5N-AM (final concentration: 5µM) and YO-PRO®1 (final concentration: 25nM) and incubated at 38ºC for 10 min. The FL-3 detector was used for Rhod-5N-AM fluorescence, whereas that of YO-PRO®1 was collected through the FL-1 detector. Following this procedure, a total of 4 sperm populations were identified: i. viable spermatozoa with low levels of intracellular calcium (Rhod 5-/YO-PRO1-); ii. viable spermatozoa with high levels of intracellular calcium (Rhod 5+/YO-PRO-1+); iii. non-viable spermatozoa with low levels of intracellular calcium (Rhod 5-/YO-PRO-1+), and iv. non-viable spermatozoa with high levels of intracellular calcium (Rhod5+/YO-PRO-1+). Fluorescence from Rhod-5N-AM was compensated for through the FL1-channel (3.16%).

In boar sperm, Fluo 3 marked intracellular calcium is mainly located at the sperm midpiece (Yeste et al., 2015). In our method, sperm were simultaneously stained with Fluo 3 and PI in order to evaluate sperm membrane integrity. For this purpose, sperm samples were incubated for 10 min at 38ºC with Fluo-3-AM (final concentration: 1µM) and PI (final concentration: 12µM). The FL-1 detector was used for collecting the fluorescence of Fluo-3-AM and the FL-3 detector was utilised to determine the PI-fluorescence. Again, a total of 4 sperm populations were identified: i. viable spermatozoa with low levels of intracellular calcium (Fluo 3-/PI-); ii. viable spermatozoa with high levels of intracellular calcium (Fluo 3+/PI-); iii. non-viable spermatozoa with low levels of intracellular calcium (Fluo 3-/PI+), and iv. non-viable spermatozoa with high levels of intracellular calcium (Fluo 3+/PI+). Unstained and single-stained samples were used for setting the EV-gain, FL-1 and FL-3 PMT voltages and for compensating Fluo-3-AM spill over into the FL3-channel (2.45%) and PI spill-over into the FL1-channel (28.72%).

Western blot assay

A total of seven ejaculates were selected for the Western blot assay. For this purpose, 1 mL-aliquot belonging to each experimental point was centrifuged at 1000*g* for 30 seconds and pellets were stored at -80 °C until the beginning of the assay. When stated, pellets were then resuspended and sonicated in 300 µL of ice-cold lysis buffer (pH 7.4) containing 50mM Tris-HCl, 1mM EDTA, 10mM EGTA, 25mM dithiothreitol, 1,5 % (v:v) Triton® X-100, 1mM phenylmethanesulfonyl fluoride, 10 µg/mL leupeptin, 1mM ortovanadate and 1mM benzamidine. After 30 minutes in ice, the homogenized suspensions were centrifuged at 4°C at 10000 rpm for 20 min and total protein amount in supernatants was calculated through the Bradford method (1976) using a commercial kit (Bio-Rad Laboratories; Fremont, CA, USA). Afterwards, samples were added to a loading buffer (1:5; v:v) containing 250mM Tris-HCl

(pH 6.8), 50mM dithiothreitol, 10 % (w:v) SDS, 0.5 % (v:v) bromophenol blue and 50% (v:v) glycerol and stored at -20°C until their subsequent assay.

To perform the Western blot, samples were loaded into 0.75 mm gels containing 10% acrylamide (w:v) to perform SDS-PAGE (Laemmli, 1970). After running the gels at constant voltage (180 V), the activated proteins of gels were transferred to an immuno-blot low-fluorescence polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in 7 min using the Trans-Blot® Turbo Transfer System (Bio-Rad) with Trans-Blot® Turbo Midi Transfer Packs. Membranes were subsequently submerged for 60 min in blocking solution consisted in a Tris-buffered saline solution added with 5% (w:v) BSA and 0.1% (v:v) Tween-20. After this time, membranes were then submerged in blocking solution containing the appropriate concentration of the utilized primary antibody. Membranes were incubated with the antibody at 4°C for a minimum of 8h. The utilized primary antibody were a mouse monoclonal clone PY20 anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich; St. Louis, Missouri, USA) and a mouse monoclonal anti-β-tubulin (ref. T5201; Sigma-Aldrich; St. Louis, Missouri, USA). In both cases, the utilized dilution was of 1:1000 (v:v). The β-tubulin was used as an internal standard to normalize the volume of protein bands, stripping and reprobing membranes when necessary.

After three washes, the immunoreaction was tested for 60 minutes using a horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse secondary antibody (Dako; Glostrup, Denmark) at a dilution of 1:5000 (v:v) in blocking solution.

After six washes, the reaction was developed for 2 minutes with a chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology®, Dallas, Texas, USA) according to the manufacter's instructions and the membranes were revealed. Prestained protein standards with a molecular mass range of approximately 250–10

kDa were used. Finally, the image analysis system ImageJ 1.49 (National Institute of Health, USA) was used to quantify the changes in intensity of various bands.

Detection of phosphotyrosine residues location in boar sperm through inmunocytochemistry

The treatments tested in this analysis were both C+ and C- and just a concentration of reduced glutathione, namely, 2mM GSH. A total of seven ejaculates were selected for the immunocytochemistry assay.

Immunocytochemistry of phosphotyrosine in boar sperm was carried out as following: Aliquots of 200 µL were centrifuged at 1000*g* for 30 s and pellets were fixed through of the resuspension in 400 µL of 4% (w:v) paraformaldehyde in PBS. Sperm samples were spread onto poly-lysined (Poly-L-lysine solution 1 % w⁄v in H_2O; Sigma, St Louis, MO, USA) microscope slides and then were left to air-dry. After this, samples were permeabilized by incubation for 10 min at room temperature in a standard phosphate-buffered solution (PBS; pH 7.4) added with 0.3% (v:v) Triton® X-100. Afterwards, slides were washed three times with PBS and they were then blocked through incubation with PBS including 0.1% (v:v) Tween-20® and 5% (w:v) BSA for 60 min at 20ºC. Incubation with the specific mouse monoclonal clone PY20 anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich; St. Louis, Missouri, USA) as in Medrano et al. (2006) at a final dilution of 1:200 (v:v) in PBS was carried out a minimum of 8h at 4ºC. After incubation, the sperm was washed thoroughly three time with PBS at 4ºC and incubated with the corresponding Alexa Fluor® 488 donkey anti-mouse secondary antibody to a final dilution of 1:500 (v:v). Slides were gently washed three time with PBS at 4ºC and were then incubated with 10 µL of a commercial solution of 4.6-diamidino-2-phenylindole hydrochloride (DAPI, 125 ng/ml; Vysis Inc., Downers Grove, IL, USA) as both a nuclear stain and an anti-fade mounting solution. After being covered, the slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with

colourless nail polish, and slides were stored at 4ºC in the dark until their microscopic observation. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik, Heidelberg, Germany) adapted to an inverted LeitzDMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo lens (Leitz, Wetzlar, Germany). The light source was an argon⁄krypton laser. Successive confocal slices of images (image thickness: 0.5 µm) were integrated to perform three-dimensional spermatozoa images, which were further stored as TIFF-format images.

Statistical evaluation of results

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 2.0; SPSS Inc.; Chicago, Illinois, USA), and data from all assessments are presented as the mean ± standard error of the mean (SEM). Data were first tested for normality and variance homogeneity through Shapiro-Wilk and Levene tests, respectively. When required, data (x) were transformed using the arcsine square root (arcsin Öx) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media) and the intrasubject factor was the incubation time (i.e. 0h, 4h, 4h and 1 min, 4h and 5 min, 4h and 60 min). In all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were calculated using Sidak's test.

When no transformation remedied the normality, non-parametric procedures were used with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as non-parametric alternatives to repeated measures ANOVA. In all statistical analyses, the minimal level of significance was set at $P<0.05$.

RESULTS

Effects of GSH on the sperm viability and acrosome integrity during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Incubation of sperm cells in both CM and NCM showed a progressive drop on viability, although this dynamics was much more accentuated in the case of cells incubated in the NCM (Figs. 1A,B). The addition of GSH in the CM at 0h of incubation had no remarkable effect on viability, although there were a noticeable decrease of this parameter in cells incubated in CM in the presence of 1mM GSH after the addition of progesterone (60.5%±3.8% in control CM cells after 60 min of progesterone addition vs. 54.2%±3.3% in 1mM GSH at the same incubation time, see Fig. 1A). The addition of GSH with progesterone together after 4h of incubation in CM did not affect the percentage of viability under any circumstances (Fig. 1B).

Values of TAE in sperm incubated in CM were maintained very low after 4h of incubation. The addition of progesterone induced a progressive and continuous increase in TAE levels, which reached maximum values after 60 min of the addition of the hormone (59.0%±2.9%, see Figs. 1C,D). This phenomenon was not observed in cells incubated in the NCM (Figs. 1C,D). The addition of GSH at 0h of incubation counteracted the progesterone-induced increase of TAE after the progesterone induction in a concentration-dependent manner, reaching the maximal counteracting effects at GSH concentrations of 5 mM (46.1%±2.0%, see Fig. 1C). The addition of GSH together with progesterone after 4h of incubation in CM did not significantly modify the TAE dynamics observed in the spermatozoa incubated in the CM (Fig. 1D).

Effects of GSH on the sperm motility during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Incubation of boar sperm in the control CM induced a progressive decrease in the percentage of total motility, which reached values of 55.1%±2.9% after 4h of incubation (Figs. 1E,F).

This decrease was related with a noticeable, albeit not evaluated, increase in the number of agglutinated sperm (data not shown). It is noteworthy that sperm incubated in the control NCM showed a much lower total motility values with no visually noticeable increase in sperm agglutination, and these lower values were maintained, and even worsened, during all of the incubation time. (Figs. 1E,F). The addition of GSH at time 0 caused an immediate, concentration-dependent drop of total motility, which was maintained during all of the experiment (Fig. 1E). The GSH-induced decrease of total motility was not seemingly related with any noticeable increase in the number of agglutinated sperm (data not shown). Likewise, the addition of GSH with progesterone together also caused a drop of total motility, although this effect was only statistically significant ($P<0.05$) after 60 min of incubation with progesterone and GSH together (Fig. 1F).

Several of the obtained results of motility parameters for all treatments are presented in Tables 1-3. The incubation in the control CM induced a progressive, time-dependent increase in VCL, VAP, LIN and STR. This effect was concomitant with a decrease of mean ALH. All of these changes are similar to those previously published in the same conditions (García-Herreros et al., 2005; Yeste et al., 2013; Yeste et al., 2015), and were assumed to be concomitant with the achievement of a feasible IVC status. The addition of progesterone after 4h of incubation in CM induced a rapid increase of VCL, VAP and mean ALH that was concomitant with a decrease of LIN and STR. These changes were practically abolished after 60 min of progesterone addition (Tables 1-3). The addition of GSH at the time 0h of incubation had, in an overall view, slight effects of boar sperm motility parameters dynamics (Tables 1-3).Meanwhile, the addition of GSH after 4h of incubation in CM with progesterone together increased VCL and BCF values whereas counteracted the progesterone-induced effects observed on LIN and STR, although only at the higher concentrations of GSH of 2mM and specially 5mM (Tables 1-3).

Effects of GSH on the free-cysteine residues in both head and tail extracts and DNA fragmentation during the achievement of both *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

The incubation of sperm in the CM caused a progressive increase in the levels of free cysteine in the sperm head (from 3.0 nmol/μg protein±0.2 nmol/μg protein at 0h of incubation to 17.4 nmol/μg protein±1.9 nmol/μg protein after 4h of incubation), appearing a gradual decline after the induction of the acrosome exocytosis (Figs. 2A,B). The addition of GSH at the start of the incubation inhibited this increase, whereas the addition of GSH and progesterone together after 4h of incubation in CM exacerbated the observed decrease in sperm incubated in the CM alone (Figs. 2A,B). Similarly, incubation of boar sperm in the CM induced a parallel increase in the levels of free cysteine in the sperm tail, although this increase was maintained after induction of the acrosome exocytosis (Figs. 2C,D). The addition of GSH at the 0h time in the CM inhibited this increase, while adding with progesterone does not cause a marked effect relative to that observed in the CM (Figs. 2C,D).

In all cases, the percentage of boar sperm that showed signs of DNA fragmentation was very low. Only a very slight increase was observed in cells incubated in NCM at the end of the experimental procedure (Figs. 2E,F). The addition of GSH both at 0h of incubation and together with progesterone after 4h of incubation in the CM did not cause any significant effect on this parameter (Figs. 2E,F).

Effects of GSH on the sperm membrane lipid disorder compatible with capacitation during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Incubation of boar sperm in the CM was concomitant with a noticeable and significant ($P<0.05$) increase in the percentage of viable cells with capacitation-like changes in membrane fluidity (from 8.3%±1.9% at 0h of incubation to 52.1%±4.5% after 4h of

incubation, see Figs. 3A,B). The subsequent addition of progesterone cursed with a progressive decrease of this percentage, reaching values of 37.8%±3.6% after 60 min of further incubation (Figs. 3A,B). The addition of GSH at 0h of incubation significantly ($P<0.05$) diminished the observed increase in the percentage of viable cells with capacitation-like membrane fluidity changes, reaching the maximal counteracting effects at GSH concentrations of 2 mM and 5 mM (Fig. 3A). On the contrary, the addition of GSH after 4h of incubation together with progesterone did not significantly modify the dynamics of the percentage of viable spermatozoa with capacitation-like membrane fluidity changes after the IVAE induction (Fig. 3B).

Effects of GSH on the mitochondrial membrane potential during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Incubation of sperm in C+ for 4h induced a steady increase in the percentage of cells with high MMP, which went from 23.0%±3.8% at 0h of incubation to 54.6%±4.9 after 4h of incubation (Figs. 3C,D). The addition of progesterone induced a further increase in this percentage, reaching peak values of 70.9%±6.1% after 1 min of the IVAE induction. Afterwards, the percentage of high MMP sperm decreased, reaching values of 57.7%±5.2% after 60 min of the progesterone addition (Figs. 3C,D). The addition of GSH at all of the tested concentrations at 0h of incubation in the CM counteracted the increase in the percentage of high-MMP cells observed after 4h of incubation in CM as well as the further, progesterone-linked increase of this parameter (Fig. 3C). Likewise, the addition of GSH after 4h of incubation in CM together with progesterone prevented the progesterone-induced peak of the high MMP cells percentage observed 1 min after the progesterone addition, but only at GSH concentrations of 2mM and 5mM (Fig. 3D).

Effects of GSH on the intracellular superoxide levels during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

The percentage of viable sperm with high intracellular superoxide levels significantly ($P<0.05$) increased after 4h of incubation in CM (from 11.6%±1.3% at 0h of incubation to 17.2%±1.9% after 4h of incubation, see Figs. 4A,B). The subsequent addition of progesterone induced a temporary decrease in this percentage which, again raised after 5 min of the addition of the hormone, reaching maximal values after 60 min of incubation (Figs. 4A,B). The addition of GSH at 0h of incubation counteracted the observed increase in the percentage of sperm with high superoxide levels at all of the tested concentrations (Fig. 4A). This counteracting effect of GSH was progressively diminishing after the addition of progesterone, observing no effects after 60 min of incubation with the hormone (Fig. 4A). The addition of GSH after 4h of incubation simultaneously with progesterone did not induce any significant effect in the percentage of viable cells with high superoxide levels (Fig. 4B).

Effects of GSH on the intracellular peroxide levels during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

The percentage of viable sperm with high intracellular peroxide levels significantly ($P<0.05$) increased after 4h of incubation in CM from 2.4%±0.5% at 0h of incubation to 18.6%±2.0% after 4h of incubation (Figs. 4C,D). This percentage was roughly maintained after the addition of progesterone, observing only a slight decrease to 16.5%±1.7% 60 min after progesterone addition (Figs. 4C,D). The addition of GSH at 0h of incubation diminished the observed increase in the percentage of cells with high peroxide levels (Fig. 4C). Furthermore, the addition of GSH after 4h of incubation in CM together with progesterone induced a rapid and significant ($P<0.05$) decrease of the percentage of viable sperm with high peroxide levels, with a more marked effect at GSH concentrations of 5mM (Fig. 4D).

Effects of GSH on boar sperm intracellular calcium levels from both head and midpiece location during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

The percentage of viable boar sperm with high intracellular levels of head sperm-placed Rhod 5N-marked calcium (Yeste et al., 2015), progressively increased after incubation in the CM, reaching values of 38.5%±3.9% after 4h of incubation (Figs. 5A,B). The subsequent addition of progesterone induced a fast increase, with values of 65.6%±5.1% after 1 min. Afterwards, there was a rapid decrease in this percentage, with values of about 46.4%±4.7 after 5 min of progesterone addition, that were roughly maintained during all of the determined incubation time (Figs. 5A,B). The addition of GSH in the CM at 0h of incubation only induced a slight decrease in the observed calcium peak after 1 min of the progesterone addition, that was only significant ($P<0.05$) at GSH concentrations of 5 mM (Fig. 5A). Furthermore, the addition of GSH together with progesterone after 4h of incubation in the CM only induced a significant ($P<0.05$) and partial counteraction of the observed calcium peak at GSH concentrations of 5 mM (56.0%±4.9%, see Fig. 5B). Strikingly, GSH at concentrations of 1mM added together with progesterone partially counteracted the decrease in the percentage of viable sperm with high Rhod 5N levels observed after the progesterone-induced peak (see Fig. 5B).

On the other hand, the percentage of viable boar sperm with high mainly midpiece located intracellular Fluo 3-marked calcium levels (Yeste et al., 2015), also progressively increased during the incubation of CM, with values of 14.1%±2.2% after 4h of incubation (Figs. 5C,D). In a similar manner to that observed with the Rhod 5N probe, addition of progesterone induced an intense and rapid increase of the percentage of Fluo 3-marked viable cells, reaching values of 38.2%±3.9% after 1 min of the addition (Figs. 5C,D). Subsequently, this percentage underwent a rapid decrease, with values of 19.1%±2.6% after 5 min of the progesterone addition. This percentage was roughly maintained after 60 min of incubation

(Figs. 5C,D). The addition of GSH at the time 0h of incubation in the CM induced a significant ($P<0.05$) decrease in the percentage of viable Fluo-3 marked cells after 4h of incubation at all of the tested concentrations (Fig. 5C). Furthermore, GSH at all of the determined concentrations also significantly decreased ($P<0.05$) this percentage after 1 min of progesterone addition (Fig. 5C). No other significant effects were observed at further incubation times after progesterone addition. Interestingly, the addition of GSH together with progesterone did not affect the observed Fluo 3 peak after 1 min of incubation (Fig. 5D). Notwithstanding, in these conditions GSH had a counteracting action on the observed decrease of the percentage of viable cells with high observed Fluo 3 observed 5 min and 60 min after the progesterone addition. This GSH action was concentration-dependent, reaching maximal values at GSH concentrations of 5 mM (31.1%±3.6% after 5 min of incubation with 5mM GSH plus progesterone vs. 19.2%%±2.3% in corresponding control cells, see Fig. 5D).

Effects of GSH on boar sperm tyrosine phosphorylation levels of P32 protein during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Images of the P32 tyrosine phosphorylation levels are presented in Fig. 6. The incubation of boar sperm in CM for 4h induced an increase in the phosphotyrosine intensity signal of the P32 protein. This increase was maintained after the addition of progesterone. The addition of GSH at the time 0h of incubation in the CM had no noticeable effects on the P32 phosphotyrosine signal during the first 4h of incubation. However, there was a decrease of P32 phosphotyrosine signal after the progesterone treatment, which was statistically significant ($P<0.05$) after 60 min of post-progesterone addition in the presence of 2mM GSH and after already 5 min of post-progesterone incubation in the presence of 5mM GSH (Fig. 6B). The addition of GSH after 4h of incubation in CM together with progesterone did not show any significant effect on the P32 phosphotyrosine signal. (Fig. 6D).

Effects of GSH on boar sperm tyrosine phosphorylation location patterns during the achievement of *in vitro* capacitation and subsequent progesterone-induced *in vitro* acrosome exocytosis

Boar sperm incubated 0h in the CM showed an overall slight phosphotyrosine signal form all the cell, while the maximal intensity of this signal was located at the equatorial, post-acrosomal segment of the head (Fig. 7). The incubation during 4h in the CM increased the intensity of the post-acrosomal signal. The addition of progesterone after 4h of incubation in CM induced a very noticeable increase of phosphotyrosine signal intensity located at the post-acrosomal area, which was accompanied with a noticeable signal in the entire sperm tail (Fig. 7). This increase of both post-acrosomal and tail intensity phosphotyrosine signal was maintained after 5 min of progesterone addition, although both signals were clearly diminished after 60 min of incubation (Fig. 7). The addition of 2mM GSH at time 0h of incubation in CM counteracted the observed increase in post-acrosomal signal, as well as the appearance of a clear tail signal after the progesterone induction (Fig. 7). On the contrary, the addition of 2mM GSH after 4h of incubation in CM together with progesterone did not modify the IVAE-related phsohpotyrosine signal changes. The only remarkable data was the maintenance of the tail signal after 60 min of the addition of both 2mM GSH and progesterone together after 4h of previous incubation in the CM (Fig. 7).

DISCUSSION

The results showed here indicate that GSH-sensitive mechanisms are important in the achievement of a feasible boar sperm IVC and subsequent, progesterone-induced IVAE. This is concluded by the fact that several important markers of the achievement of boar sperm IVC like motion parameters and changes of sperm membrane fluidity and the overall tyrosine phosphorylation status are altered by GSH. As a consequence, further progesterone-induced IVAE was subsequently altered. The hypothesis that the GSH-induced alteration of IVAE is linked to previous actions of GSH on capacitation-launching mechanisms is suggested by results indicating that GSH was not able to diminish IVAE when added together with progesterone. In fact, addition of GSH together with progesterone had very few effects on the results obtained after the induction of IVAE. In fact, our results suggest that mechanisms prone to be modulated by GSH can be important for the achievement of capacitation status, but are much less important for the achievement of progesterone-induced acrosome exocytosis itself. However, effects of GSH on specific parameters like motility or intracellular calcium levels clearly indicate the existence of a more complex overview for GSH action. This complexity could be linked with the existence of separate and parallel pathways controlling boar sperm IVC/IVAE. In this way, the importance of a proper calcium metabolism during IVC and IVAE is well known. At this respect, it is noteworthy that intracellular calcium of boar sperm is mainly located in two separate storage sites, namely the head and midpiece (Yeste et al., 2015). In fact, the dynamics of both calcium storage places is concomitant during the achievement of IVC and IVAE (Yeste et al., 2015, and results of this work). Strikingly, following our results, GSH has a more profound altering action on midpiece-stored calcium than to that accumulated in the head, with a noticeable lowering of midpiece-stored calcium after 4h of incubation in CM. This lowering is concomitant with the effect of GSH on other capacitation markers like membrane fluidity, as well as with that on

MMP. A similar effect was observed when boar sperm are incubated in a capacitating medium without calcium (Yeste et al., 2015). This could suggest that several of the GSH-induced actions during the achievement of IVC could be linked with the lack of ability of sperm to increase midpiece-located calcium intracellular levels. Notwithstanding, the explanation can not be entirely based in this hypothesis, taking into account motility results. First of all, as indicated in the Results section, the drop in total motility induced by GSH was not seemingly related to an increase in the number of agglutinated sperm, since although we didn't count this percentage there were not any visually appreciable difference in the number of agglutinated sperm when compared control and GSH-treated cells. Thus, GSH effects on motility would be exerted on the sperm motility machinery. Notwithstanding, whereas GSH inhibits motility, the lack of external calcium induced the opposite effect (Yeste et al., 2015). In this way, although effects of GSH on the achievement of IVC can be partially explained by its action on midpiece-accumulated calcium, this action does not fully explain GSH action.

However, calcium-independent mechanisms seem to be also instrumental to explain the observed effects of GSH on boar sperm IVC and IVAE. We don't exactly know what would be these pathways. However, the obtained results can yield some suggestions on this point. Thus, it is noteworthy that the achievement of IVC was accompanied by an increase in the levels of free radicals of cysteines in both sperm head and tail. This increase was concomitant with parallel, if slight, increases of intracellular ROS levels and, as indicated above, overall mitochondrial activity as indicated the JC-1 analysis. All of these phenomena were counteracted by GSH. In the meantime, it is noteworthy to indicate that the presence of the free thiol groups present in the GSH structure did not affect the observed results, since the addition of GSH induced a decrease, and not an artifactual increase, in the value of free radicals of cysteines. This is not surprising, since GSH has a potent anti-ROS action that was concomitant with a role of protective agent of disulfide bonds (Chatterjee et al., 2001; Jacob

et al., 2003). Furthermore, the anti-ROS action of GSH would induce a concomitant lowering effect on mitochondrial activity, since mitochondria are the main location in which ROS are produced in sperm (Murphy, 2009). Meanwhile, the increase of both levels of radicals of cysteine and the mitochondrial activity leading to a subsequent increase of intracellular ROS concentration can be a role in the achievement of IVC by themselves. Centring on free radicals of cysteine, this parameter is a direct indicator of the integrity of disulfide bonds among proteins, including nucleoprotein structures (Reyes et al., 1989; Jager et al., 1990; Perreault, 1990, 1992; Chatterjee et al., 2001; Cheng et al., 2009). It is noteworthy that although the technique utilized here doesn't entirely isolate free cysteine radicals from nucleoproteins form other radicals present in other sperm head and sub-head areas, it is enough efficient to interpret that a very high and significant percentage of the observed free cysteine radicals from head-containing fractions will became from the nucleoprotein structure. Taking this into account, it is well known that the maintenance of a tight nucleoprotein structure in mammalian sperm is partially controlled by the number of disulfide bonds established between nucleoproteins and DNA (Reyes et al., 1989; Jager et al., 1990; Perreault, 1990, 1992; Chatterjee et al., 2001; Cheng et al., 2009). This relationship is especially evident in frozen-thawed boar sperm, in which the freezing-thawing related alterations of the nucleoprotein structure are concomitant with a significant increase in the number of broken head sperm disulfide bonds in a way that is counteracted by the addition of GSH (Yeste et al., 2013). Concomitantly, disulfide bonds are instrumental in the maintenance of the sperm tail structure by involvement of bonds among the separate proteins of flagellum, fibrous sheath and longitudinal columns (Su et al., 2005; Buffone et al., 2012). In this way, we must remind that the exact union strength among the separate components that regulate motility at the sperm tail will have a strong influence in the exact type of motion pattern that sperm achieve at a determined moment (Luconi and Baldi, 2003). Thus, the increase in the number of broken

tail disulfide bonds would results in specific changes in the motion pattern that sperm present, being thus one of the mechanisms that might underlie the appearance of the boar sperm capacitation-specific motion pattern. Taking this hypothesis into account, it would be possible that the protecting effect of GSH on tail disulfide bonds could be one of the mechanisms whereby GSH alters sperm motility in the CM medium, avoiding thus the establishment of capacitation-specific motion patterns.

However, disulfide bonds are not the only point on GSH is acting. As indicated above, IVC was related with a slight increase in ROS levels that can be a consequence of an increased activity of boar sperm mitochondria during the achievement of IVC. Furthermore, this putative increase in mitochondrial activity could be also linked with the assumption of the capacitation-specific motility pattern that can be observed in boar sperm after 4h of incubation in the CM. In fact, a close relationship between mitochondrial activity and boar sperm motility has been already published, although this relationship is not directly related with the overall ATP intracellular levels but with the specific chemiosmosis activity (Ramió-Lluch et al., 2013). In any case, ROS increase is very modest, which would imply that no deleterious effects would be caused by it. On the contrary, these changes in ROS levels could play a role in launching several of the molecular phenomena linked with the achievement of capacitation. In this sense, several reports indicate the ability of ROS to initiate capacitation or to modulate its progression (O'Flaherty, 2015). At this respect, it is noteworthy that ROS can modulate the activity of a myriad of separate protein kinases and phosphatases, like protein kinase C, microtubule-associated proteins kinase, PI3 kinase and several protein phosphatases (Schieven, 1997). Remarkably, our results show that the lowering of ROS levels induced by GSH was concomitant with impairment in the dynamics of the overall sperm protein tyrosine phosphorylation observed during the achievement of IVC. This could be important taking into account that all of these above mentioned protein kinases and phosphatases are involved in

the regulation of sperm capacitation acting on several of the molecular changes affected by the GSH addition such as motility and changes in membrane fluidity (Wright et al., 2009; Corcoran and Cotter, 2013). Nevertheless, the putative regulatory action of ROS on boar sperm IVC would be modest, taking into account the low intracellular ROS levels that boar sperm cells have. In any case, this putative ROS role would not control launching of progesterone-linked IVAE, since the GSH-induced ROS lowering will not affect this process. In any case, more work is needed in order to elucidate the putative role of ROS, and also mitochondria activity, in the launching of boar sperm capacitation.

As indicated above, the addition of GSH had little effect on boar sperm that previously reached the capacitation status and were then led to the progesterone-induced IVAE launching. This is obvious in the majority of determined parameters, with exception of motility, and much more slightly, head sperm free cysteine residues, MMP and intracellular peroxide levels. These results could be the consequence of an overall less sensitive function status of boar capacitated sperm in front of GSH. In fact, after 4h of incubation in CM boar sperm have underwent the majority of changes that have been shown to be sensitive to the GSH action, such as modifications of ROS levels, membrane fluidity and even strength of nucleoprotein structure. These changes showed to be not reversible, since the addition of GSH did not revert them. Under a mechanistic point of view, results suggest that the action mechanisms by which GSH acts on disulfide bonds and even ROS production/mitochondrial activity are inactive or in a no-sensitive status in front GSH during and after the achievement of IVC and subsequent IVAE. This function change could be related with the already described function changes at IVC/IVAE. Thus, a sudden peak of O_2 consumption and ATP synthesis has been described immediately after the launching of progesterone-induced IVAE (Ramió-Lluch et al., 2013). This peak seems to be related with the assumption of a fully coupled status of boar sperm mitochondria, which seem to be uncoupled or with low activity

before and after IVAE (Ramió-Lluch et al., 2013; Yeste et al., 2015). On the other hand, GSH inhibits total motility both when added at 0h of incubation in CM and after the IVAE launching. This results suggest that the mechanism of action of GSH on boar sperm motility were not entirely dependent on variations of both tail sperm free cysteine radicals and intracellular ROS levels. At this moment, we don't know what would be this/these mechanism/s, although one hypothesis would be a mechanism related with the control of potential and hence redox-sensitive enzymes regulating tail contractibility. In this way, proteins like the Na^+/K^+-dependent ATPase is a well known regulator of sperm motility (Koçak-Toker et al., 2002). One of the mechanisms by which the activity of this ATPase is controlled is through changes in redox status (Liu et al., 2012). This could be key point in our case, since the anti-oxidative action of GSH would act on the overall boar sperm redox status, modulating thus activities of proteins like the Na^+/K^+-dependent ATPase. This action, in turn, would modify the overall boar sperm motility. This hypothesis would also explain the fact that the addition of GSH to frozen-thawed boar sperm increases motility of thawed sperm, acting, (Yeste et al., 2013) in a very different manner to that observed in IVC/IVAE conditions. This striking difference, in fact would be a consequence of the existence of very separate overall redox status in fresh samples, frozen-thawed cells and sperm subjected to incubation in the CM medium. However, more work is needed in order to elucidate this point.

In summary, boar sperm IVC is related with a significant increase of both overall broken disulfide bonds and intracellular ROS levels. These phenomena could play a role in the achievement of the capacitation status, although they are not instrumental in the achievement of subsequent progesterone-induced IVAE. Finally, sperm motility seems to be partially controlled by ionic and redox mechanisms, as the incubation with GSH in separate conditions would indicate.

CONTRIBUTIONS OF THE AUTHORS

Rafael Pedroso Betarelli: One of the two main responsible for the laboratory work performed here. Moreover, he contributed to the development of the experimental design.

Martina Rocco: The other main responsible for the laboratory work performed here. Equally, she also contributed to the development of the experimental design.

Marc Yeste: Responsible coordinator for all work involving flux cytometry. Beside this, Dr. Yeste significantly contributed to the development of the experimental design and to the writing and final processing of the manuscript.

Josep Mª. Fernández-Novell: Responsible coordinator for all work involving laser confocal microscopu. Beside this, Dr. Fernández-Novell significantly contributed to the development of the other immunological techniques, the experimental design and the writing and final processing of the manuscript.

Anna Placci: Significant collaboration in the development of laboratory work.

Barbara Azevedo Pereira: Significant collaboration in the development of laboratory work.

Efren Estrada: Significant collaboration in the development of laboratory work.

Alejandro Peña: Significant collaboration in the development of laboratory work.

Márcio Gilberto Zangeronimo: main responsible for the coordination of work carried out by the Brazilian investigation group. He also significantly contributed to the development of the experimental design and the final writing of the manuscript. Likewise, main responsible for the Brazilian side of funding invested in the work.

Joan Enric Rodríguez-Gil: main responsible for the whole experimental design and the overall coordination of the work. He was also responsible for the final version of the manuscript. Likewise, main responsible for the Spanish side of funding invested in the work.

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DECLARATION OF INTERESTS

There is no conflict of interests that could be perceived as prejudicing the impartiality of the reported research.

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Table 1. **Effects of reduced glutathione on curvilinear velocity and mean velocity of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
VCL (μm/s)					
C-	52.4±1.1^{a}	61.8±1.7^{b}	101.6±3.5^{c}	94.0±2.9^{c}	112.2±4.4^{d}
C+	62.6±1.8^{a}	54.7±1.9^{b}	70.5±2.8^{c}	67.5±3.1^{ac}	70.8±5.3^{c}
GSH 1mM	62.6±1.8^{a}	60.1±5.0^{ab}	60.2±4.3^{ab}	56.3±2.8^{b*}	78.0±6.2^{c}
GSH 2mM	62.6±1.8^{a}	37.1±1.5^{b*}	58.0±3.6^{a*}	60.6±4.2^{a}	64.5±4.0^{a}
GSH 5mM	62.6±1.8^{a}	41.7±3.0^{b*}	58.0±5.2^{a*}	47.2±5.6^{b*}	39.0±3.4^{b*}
GSH 1mM+PG	62.6±1.8^{a}	54.7±1.9^{b}	75.1±4.7^{b*}	68.0±4.8^{b}	91.8±6.9^{c*}
GSH 2mM+PG	62.6±1.8^{a}	54.7±1.9^{b}	83.8±5.6^{a}	77.4±5.2^{a}	84.4±6.0^{a}
GSH 5mM5+PG	62.6±1.8^{a}	54.7±1.9^{b}	78.2±5.2^{a}	70.7±5.6^{b*}	86.9±6.4^{b*}
VAP (μm/s)					
C-	23.8±0.9^{a}	47.8±4.9^{b}	47.4±5.0^{b}	55.3±5.9^{b}	78.6±7.0^{c}
C+	31.4±1.0^{a}	32.1±1.1^{a}	38.8±2.2^{b}	39.1±2.3^{b}	44.3±2.7^{b}
GSH 1mM	31.4±1.0^{a}	33.5±3.0^{a}	45.3±5.0^{b}	37.1±2.5^{ab}	40.6±5.0^{ab}
GSH 2mM	31.4±1.0^{a}	22.2±1.4^{b*}	37.5±4.2^{a}	36.9±4.6^{a}	42.7±4.1^{b}
GSH 5mM	31.4±1.0^{a}	23.3±1.8^{b*}	36.9±6.5^{a}	31.4±3.1^{a*}	19.2±2.1^{b*}
GSH 1mM+PG	31,4±1.0^{a}	32.1±1.1^{a}	30.1±1.0^{a}	36.4±2.1^{b}	46.2±3.2^{c}
GSH 2mM+PG	31.4±1.0^{a}	32.1±1.1^{a}	37.9±2.0^{b}	44.9±2.9^{b}	44.5±3.1^{b}
GSH 5mM+PG	31.4±1.0^{a}	32.1±1.1^{a}	45.4±2.8^{b*}	50.7±3.3b^{c*}	59.8±3.9^{c*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses

has been also described in the Material and Methods section. Cells were incubated in a no-capacitating medium (C-) or in a capacitating medium without (C+) or with reduced glutathione at final concentrations of 1mM (GSH 1mM), 2mM (GSH 2mM) and 5mM (GSH 5mM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4h of incubation were added with progesterone and 1mM reduced glutathione (GSH 1mM+PG), progesterone with 2m reduced glutathione (GSH 2mM+PG) and progesterone with 5 mM reduced glutathione (GSH 5mM+PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means±S.E.M for 7 separate experiments.

Table 2. **Effects of reduced glutathione on linearity coefficient and straightness coefficient of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
LIN (%)					
C-	24.0±1.1^{a}	74.1±6.3^{b}	76.6±6.9^{b}	75.7±6.5^{b}	81.6±7.2^{b}
C+	34.2±1.4^{a}	50.1±2.9^{b}	33.9±2.5^{a}	41.8±2.1^{c}	50.5±3.0^{b}
GSH 1mM	34.2±1.4^{a}	37.8±4.8^{a*}	58.9±5.5^{b*}	59.3±5.2^{b*}	37.1±4.0^{a*}
GSH 2mM	34.2±1.4^{a}	38.6±2.0^{b*}	45.6±4.9^{b*}	45.8±4.6^{b}	49.4±2.2^{b}
GSH 5mM	34.2±1.4^{a}	40.1±4.2^{b*}	63.3±6.2^{c*}	37.2±5.6^{b}	43.5±1.4^{b*}
GSH 1mM+PG	34.2±1.4^{a}	50.1±2.9^{b}	40.5±4.4^{b}	39.1±4.6^{b}	58.2±6.0^{c}
GSH 2mM+PG	34.2±1.4^{a}	501±2.9^{b}	39.1±4.6^{b}	42.0±4.9^{b}	60.6±7.8^{c}
GSH 5mM+PG	34.2±1.4^{a}	50.1±2.9^{b}	38.3±5.2^{b}	40.4±4.9^{b}	70.8±7.3^{c*}
STR (%)					
C-	59.6±2.2^{a}	83.0±5.4^{b}	82.8±5.5^{b}	84.4±5.9^{b}	83.9±5.1^{b}
C+	64.9±1.2^{a}	74.2±3.5^{b}	59.9±2.2^{c}	63.3±3.9^{a}	71.6±3.7^{b}
GSH 1mM	64.9±1.2^{a}	45.5±5.6^{b*}	79.1±4.0^{c*}	79.8±4.1^{c*}	56.9±5.5^{b*}
GSH 2mM	64.9±1.2^{a}	61.4±2.3^{a*}	64.4±4.8^{a}	76.0±2.3^{b*}	68.6±4.6^{a}
GSH 5mM	64.9±1.2^{a}	58.7±5.5^{a*}	85.2±3.0^{b*}	65.6±6.2^{a}	75.9±2.5^{a}
GSH 1mM+PG	64.9±1.2^{a}	74.2±3.5^{b}	58.1±2.7^{c}	64.1±4.2^{c}	71.1±4.2^{c}
GSH 2mM+PG	64.9±1.2^{a}	74.2±3.5^{b}	76.5±4.9^{b*}	79.1±5.4^{b*}	74.1±4.8^{b}
GSH 5mM+PG	64.9±1.2^{a}	74.2±3.5^{b}	87.0±6.1^{b*}	87.6±6.7^{b*}	88.3±7.1^{b*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Cells were incubated in a no-capacitating medium (C-) or in a capacitating medium without (C+) or with reduced glutathione at final concentrations of 1mM (GSH 1mM), 2mM (GSH 2mM) and 5mM (GSH 5mM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4h of incubation were added with progesterone and 1mM reduced glutathione (GSH 1mM+PG), progesterone with 2mM reduced glutathione (GSH 2mM+PG) and progesterone with 5 mM reduced glutathione (GSH 5mM+PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means±S.E.M. for 7 separate experiments..

Table 3. **Effects of reduced glutathione on mean amplitude of lateral head displacement and frequency of head displacement of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
ALH (μm)					
C-	3.01±0.09^{a}	2.41±0.15^{b}	2.24±0.16^{b}	3.02±0.20^{c}	4.17±0.35^{d}
C+	3,07±0,08^{a}	2.29±0.12^{b}	2.93±0.13^{ac}	2.64±0.16^{c}	2.71±0.14^{ac}
GSH 1mM	3,07±0,08^{a}	2.14±0.13^{b}	2.00±0.19^{b*}	2.52±4.8^{b}	2.95±0.22^{a}
GSH 2mM	3.07±0.08^{a}	1.96±0.15^{b}	2.18±0.14^{c*}	2.77±0.16^{ad}	2.47±0.06^{d*}
GSH 5mM	3.07±0.08^{a}	1.89±0.10^{b}	1.71±0.19^{b*}	2.56±0.14^{c}	1.81±0.10^{b*}
GSH 1mM+PG	3.07±0.08^{a}	2.29±0.12^{b}	3.12±0.21^{ac}	2.61±0.21^{abc}	2.57±0.29^{abc}
GSH 2mM+PG	3.07±0.08^{a}	2.29±0.12^{b}	2.74±0.19^{ac}	3.36±0.31^{c}	3.01±0.22^{c}
GSH 5mM+PG	3.07±0.08^{a}	2.29±0.12^{b}	3.49±0.26^{c*}	3.28±0.27^{c*}	3.09±0.25^{c}
BCF (Hz)					
C-	6.63±0.10^{a}	10.88±0.41^{b}	11.24±0.45^{b}	11.98±0.63^{b}	14.18±0.74^{c}
C+	7.76±0.13^{a}	8.17±0.23^{a}	7.88±0.24^{a}	8.00±0.22^{a}	7.78±0.24^{a}
GSH 1mM	7.76±0.13^{a}	7.91±0.22^{a}	7.28±0.10^{b*}	10.17±0.37^{c*}	9.25±0.36^{c*}
GSH 2mM	7.76±0.13^{a}	6.49±0.22^{b*}	8.13±0.25^{a}	8.87±0.24^{b*}	7.60±0.24^{a}
GSH 5mM	7.76±0.13^{a}	8.05±0.24^{a}	7.58±0.24^{a}	8.47±0.32^{b}	6.70±0.18^{c*}
GSH 1mM+PG	7.76±0.13^{a}	8.17±0.23^{a}	8.88±0.37^{b*}	10.29±0.49^{b*}	10.86±0.55b*
GSH 2mM+PG	7.76±0.13^{a}	8.17±0.23^{a}	8.63±0.29^{b*}	10.59±0.58^{b*}	10.34±0.47^{b*}
GSH 5mM+PG	7.76±0.13^{a}	8.17±0.23^{a}	11.42±0.38^{b*}	11.68±0.47^{b*}	12.29±0.53^{b*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Cells were incubated in a no-capacitating medium (C-) or in a capacitating medium without (C+) or with reduced glutathione at final concentrations of 1mM (GSH 1mM), 2mM (GSH 2mM) and 5mM (GSH 5mM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4h of incubation were added with progesterone and 1mM reduced glutathione (GSH 1mM+PG), progesterone with 2mM reduced glutathione (GSH 2mM+PG) and progesterone with 5 mM reduced glutathione (GSH 5mM+PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means±S.E.M. for 7 separate experiments.

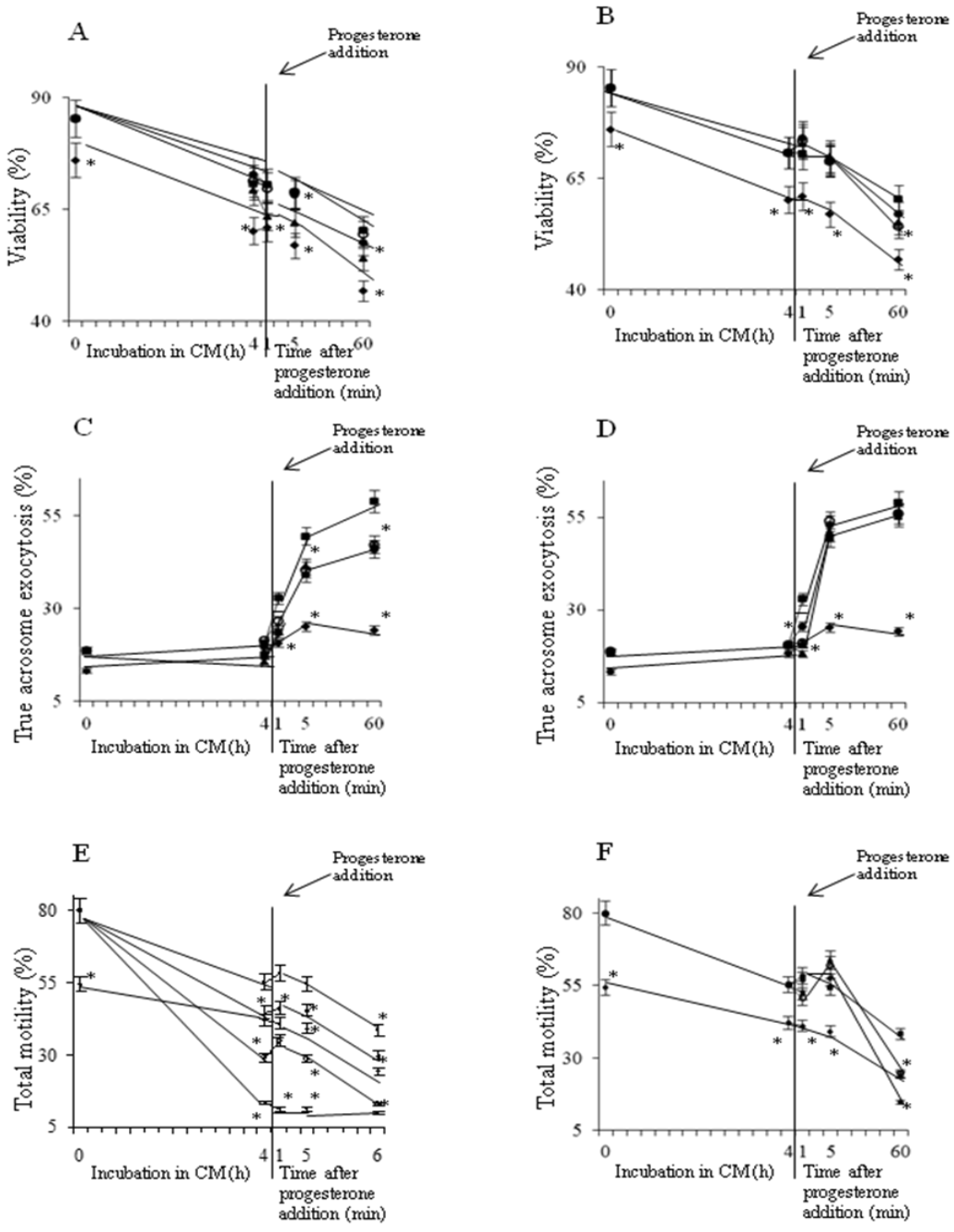

A
Progesterone addition
Viability (%)
90
65
40
0
4
1
5
60
Incubation in CM(h)
Time after progesterone addition (min)
B
Progesterone addition
Viability (%)
90
65
40
0
4
1
5
60
Incubation in CM(h)
Time after progesterone addition (min)
C
Progesterone addition
True acrosome exocytosis (%)
55
30
5
0
4
1
5
60
Incubation in CM(h)
Time after progesterone addition (min)
D
Progesterone addition
True acrosome exocytosis (%)
55
30
5
0
4
1
5
60
Incubation in CM(h)
Time after progesterone addition (min)
E
Progesterone addition
Total motility (%)
80
55
30
5
0
4
1
5
6
Incubation in CM(h)
Time after progesterone addition (min)
F
Progesterone addition
Total motility (%)
80
55
30
5
0
4
1
5
60
Incubation in CM(h)
Time after progesterone addition (min)

Figure 1. **Effects of reduced glutathione on percentages of viability, true acrosome exocytosis and total motility of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**
Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, the determination of percentages of viability (A, B), true acrosome exocytosis (C, D) and total motility (E, F) have been also described in the Material and methods section. A, C, E: Boar spermatozoa were incubated in a no-capacitating medium (◆) or in capacitating medium without (■) or with reduced glutathione at final concentrations of 1mM (▲), 2mM (○) or 5mM (●) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. At the indicated times, aliquots were taken to determine the appropriate parameters. B, D, F: One aliquot of boar sperm was incubated during 4h in a no-capacitating medium (◆). Simultaneously, four more aliquots were incubated for 4h in capacitating medium. After this time, the cells incubated in the no-capacitating medium was added with progesterone, whereas the other aliquots were treated as follows: The first aliquot was added with progesterone alone (■), the second aliquot was added with progesterone and 1mM glutathione (▲), the third aliquot was added with progesterone and 2mM glutathione (○), finally, the fourth aliquot was added with progesterone and 5mM glutathione (●). Subsequently, sperm was incubated during 1 min, 5 min and 60 min and aliquots were taken at these times to determine the appropriate parameters. Results are shown as means±S.E.M. for 7 separate experiments. Asterisks indicate significant differences ($P<0.05$) when compared with the respective time point of cells incubated in a capacitated medium without addition of reduced glutathione at any point.

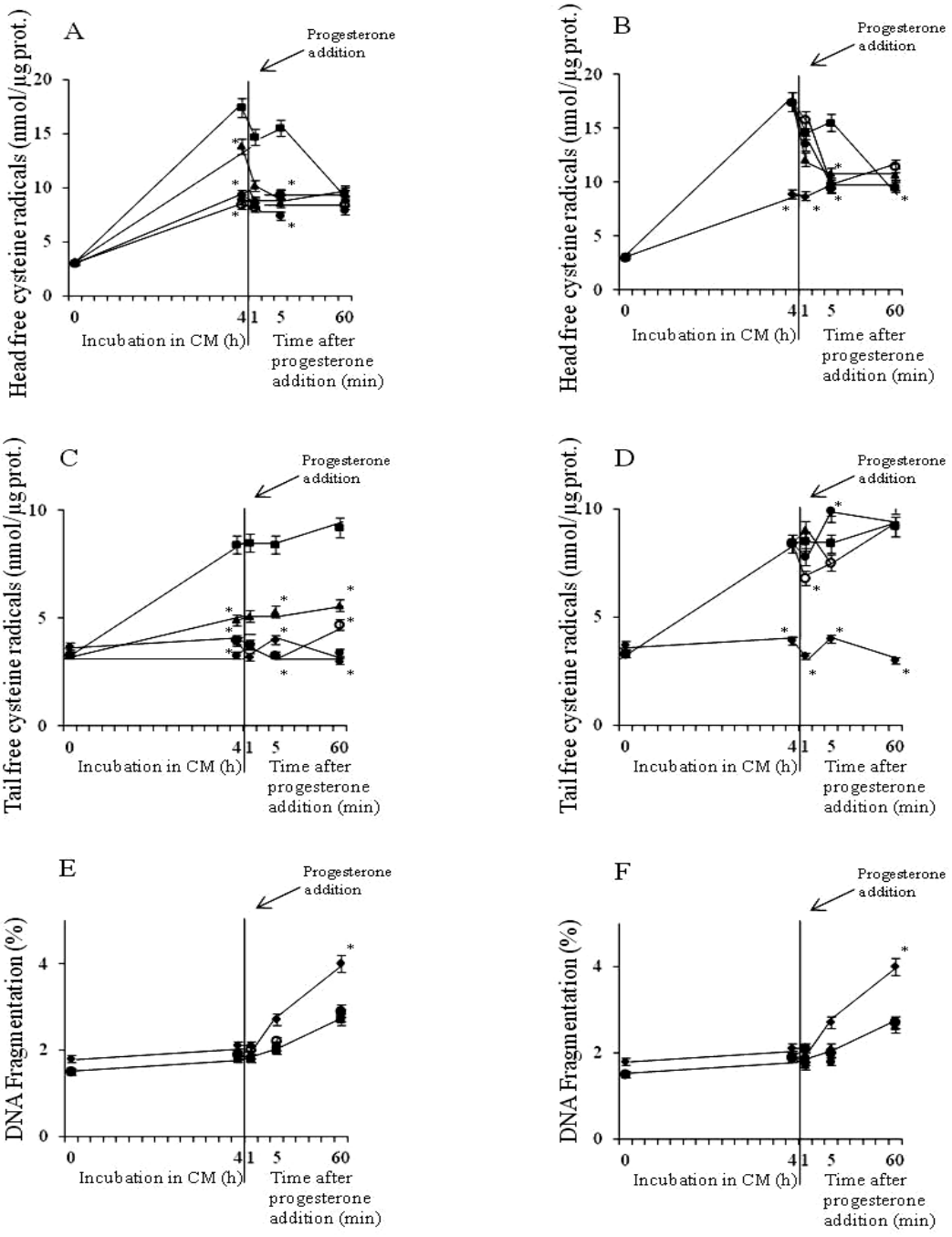
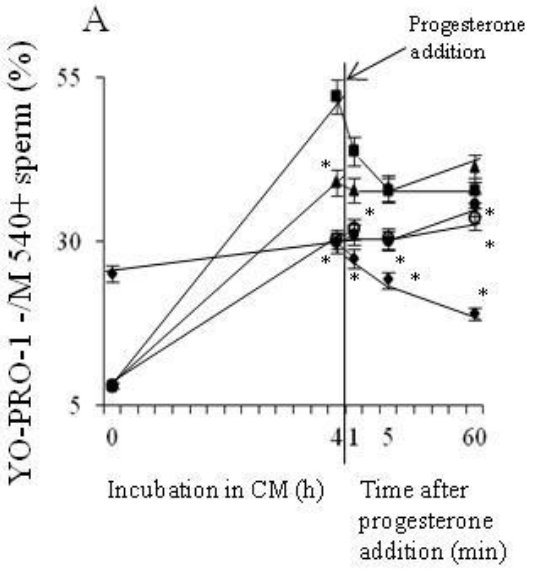
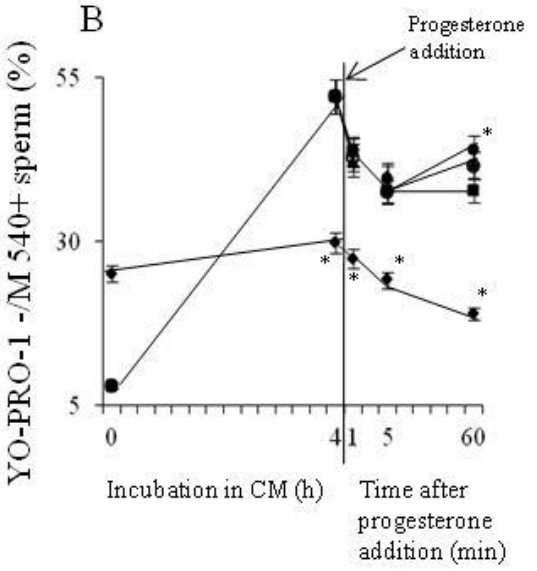
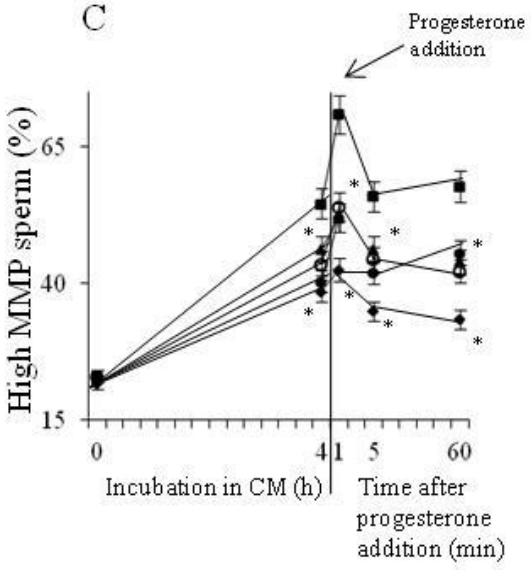

A
B
C
D
E
F
Progesterone addition
Head free cysteine radicals (nmol/µg prot.)
Tail free cysteine radicals (nmol/µg prot.)
DNA Fragmentation (%)
Incubation in CM (h)
Time after progesterone addition (min)
0
4
1
5
60

Figure 2. **Effects of reduced glutathione on free cysteine radical levels from both head and tail extracts and on the percentage of cells with detectable DNA fragmentation of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination techniques of free cysteine radical levels in both head extracts (A, B) and tail ones (C, D), as well as percentage of DNA fragmentation (E, F) have been also described in the Material and methods section. A, C, E: Boar spermatozoa were incubated in a no-capacitating medium (◆) or in capacitating medium without (■) or with reduced glutathione at final concentrations of 1mM (▲), 2mM (○) or 5mM (●) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. At the indicated times, aliquots were taken to determine the appropriate parameters. B, D, F: One aliquot of boar sperm was incubated during 4h in a no-capacitating medium (◆). Simultaneously, four more aliquots were incubated for 4h in capacitating medium. After this time, the cells incubated in the no-capacitating medium was added with progesterone, whereas the other aliquots were treated as follows: The first aliquot was added with progesterone alone (■), the second aliquot was added with progesterone and 1mM glutathione (▲), the third aliquot was added with progesterone and 2mM glutathione (○), finally, the fourth aliquot was added with progesterone and 5mM glutathione (●). Subsequently, sperm was incubated during 1 min, 5 min and 60 min and aliquots were taken at these times to determine the appropriate parameters. Results are shown as means±S.E.M. for 7 separate experiments. Asterisks indicate significant differences ($P<0.05$) when compared with the respective time point of cells incubated in a capacitated medium without addition of reduced glutathione at any point.


A
Progesterone addition
YO-PRO-1 -/M 540+ sperm (%)
55
30
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)


B
Progesterone addition
YO-PRO-1 -/M 540+ sperm (%)
55
30
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)


C
Progesterone addition
High MMP sperm (%)
65
40
15
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

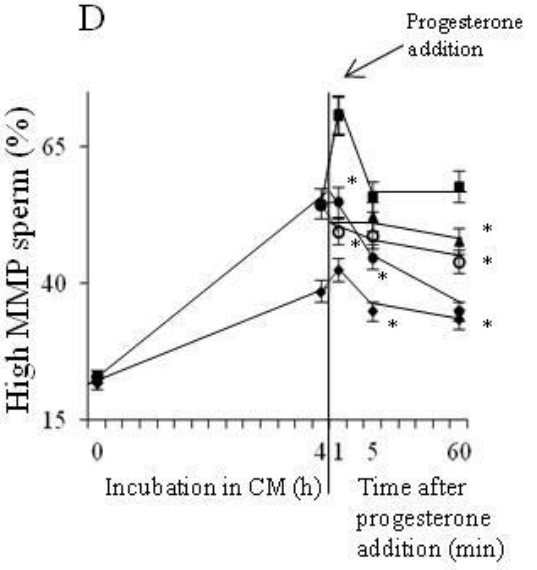

D
Progesterone addition
High MMP sperm (%)
65
40
15
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

Figure 3. **Effects of reduced glutathione on percentages of cells with capacitation-like changes in cell membrane fluidity and those with high mitochondrial membrane potential of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**
Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, both YO-PRO-1/merocyanine 540 (A, B) and JC-1 (C, D) staining determination techniques have been also described in the Material and methods section. A, C: Boar spermatozoa were incubated in a no-capacitating medium (◆) or in capacitating medium without (■) or with reduced glutathione at final concentrations of 1mM (▲), 2mM (○) or 5mM (●) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. At the indicated times, aliquots were taken to determine the appropriate parameters. B, D: One aliquot of boar sperm was incubated during 4h in a no-capacitating medium (◆). Simultaneously, four more aliquots were incubated for 4h in capacitating medium. After this time, the cells incubated in the no-capacitating medium was added with progesterone, whereas the other aliquots were treated as follows: The first aliquot was added with progesterone alone (■), the second aliquot was added with progesterone and 1mM glutathione (▲), the third aliquot was added with progesterone and 2mM glutathione (○), finally, the fourth aliquot was added with progesterone and 5mM glutathione (●). Subsequently, sperm was incubated during 1 min, 5 min and 60 min and aliquots were taken at these times to determine the appropriate parameters. Results are shown as means±S.E.M. for 7 separate experiments. Asterisks indicate significant differences ($P<0.05$) when compared with the respective time point of cells incubated in a capacitated medium without addition of reduced glutathione at any point.

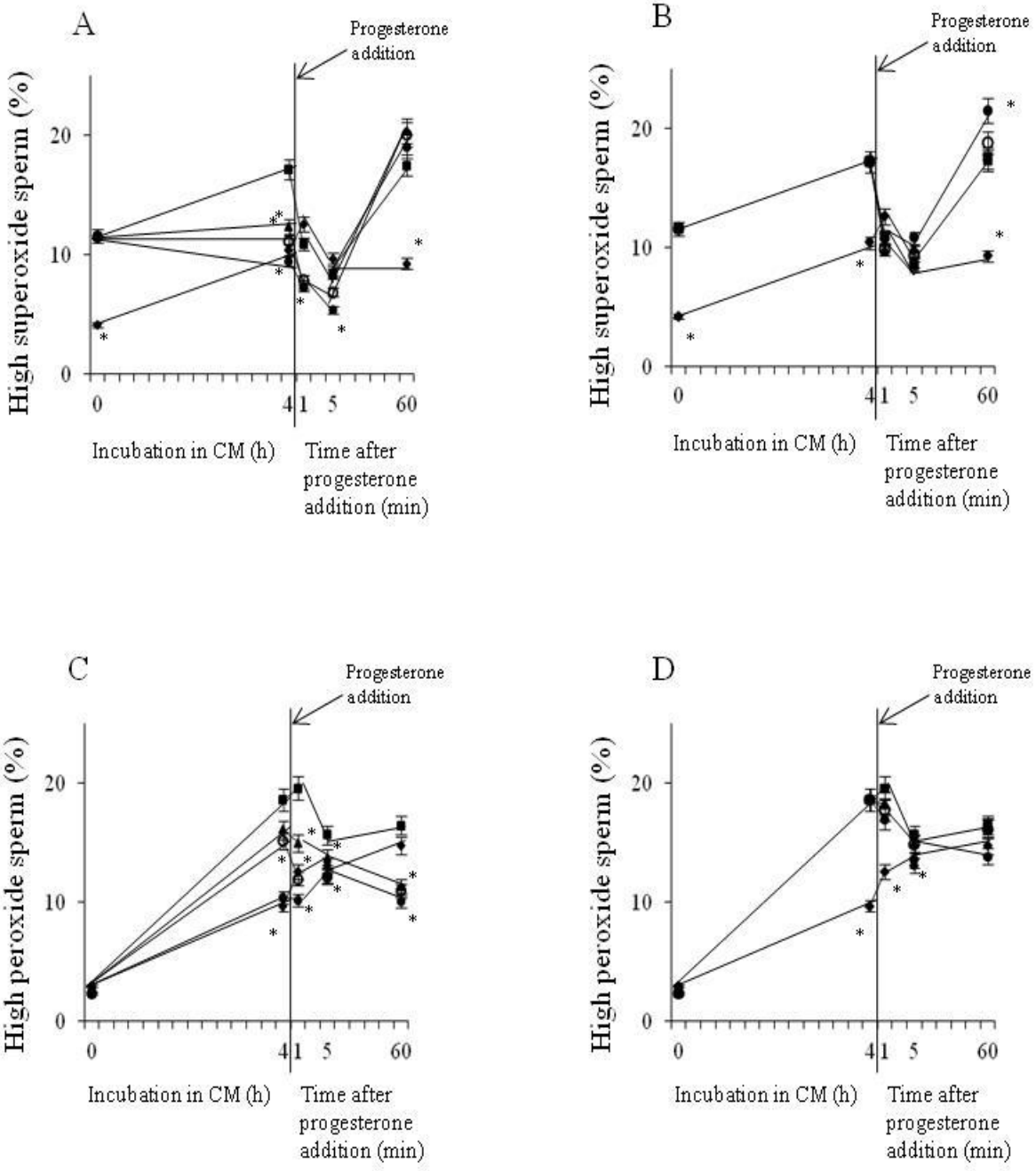
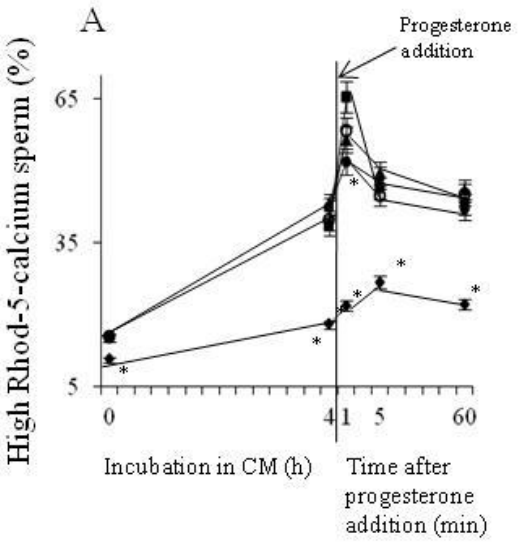
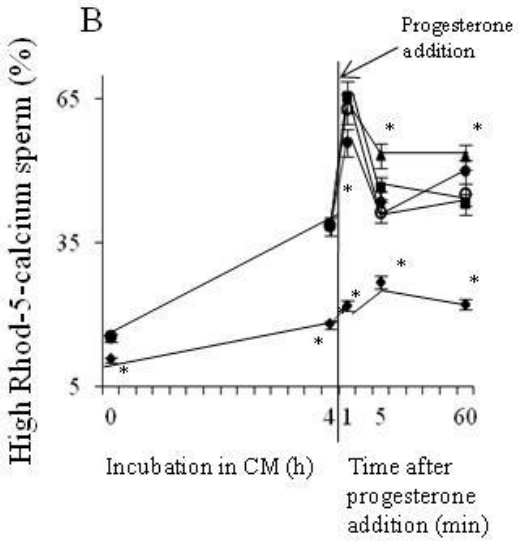
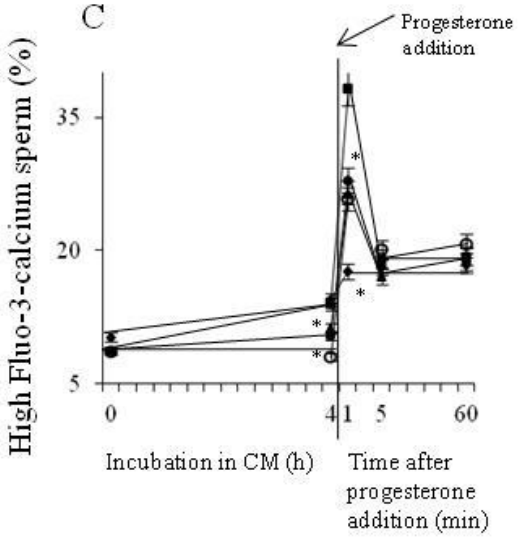

A
Progesterone addition
High superoxide sperm (%)
20
10
0
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)
B
Progesterone addition
High superoxide sperm (%)
20
10
0
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)
C
Progesterone addition
High peroxide sperm (%)
20
10
0
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)
D
Progesterone addition
High peroxide sperm (%)
20
10
0
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

Figure 4. **Effects of reduced glutathione on percentages of cells with high superoxides and high peroxides levels of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**
Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, both superoxides levels (A, B) peroxides levels (C, D) determination techniques have been also described in the Material and methods section. A, C: Boar spermatozoa were incubated in a no-capacitating medium (◆) or in capacitating medium without (■) or with reduced glutathione at final concentrations of 1mM (▲), 2mM (○) or 5mM (●) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. At the indicated times, aliquots were taken to determine the appropriate parameters. B, D: One aliquot of boar sperm was incubated during 4h in a no-capacitating medium (◆). Simultaneously, four more aliquots were incubated for 4h in capacitating medium. After this time, the cells incubated in the no-capacitating medium was added with progesterone, whereas the other aliquots were treated as follows: The first aliquot was added with progesterone alone (■), the second aliquot was added with progesterone and 1mM glutathione (▲), the third aliquot was added with progesterone and 2mM glutathione (○), finally, the fourth aliquot was added with progesterone and 5mM glutathione (●). Subsequently, sperm was incubated during 1 min, 5 min and 60 min and aliquots were taken at these times to determine the appropriate parameters. Results are shown as means±S.E.M. for 7 separate experiments. Asterisks indicate significant differences ($P<0.05$) when compared with the respective time point of cells incubated in a capacitated medium without addition of reduced glutathione at any point.


A
Progesterone addition
High Rhod-5-calcium sperm (%)
65
35
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)


B
Progesterone addition
High Rhod-5-calcium sperm (%)
65
35
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)


C
Progesterone addition
High Fluo-3-calcium sperm (%)
35
20
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

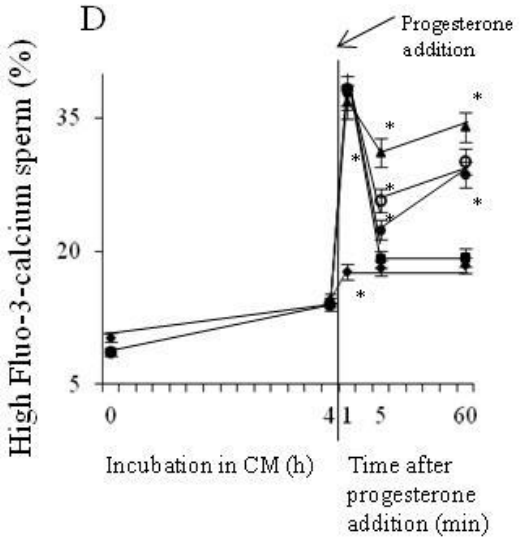

D
Progesterone addition
High Fluo-3-calcium sperm (%)
35
20
5
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

Figure 5. **Effects of reduced glutathione on percentages of cells with high intracellular calcium levels of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**
Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, both Rhod 5 (A, B) and Fluo 3 (C, D) staining determination techniques have been also described in the Material and methods section. A, C: Boar spermatozoa were incubated in a no-capacitating medium (◆) or in capacitating medium without (■) or with reduced glutathione at final concentrations of 1mM (▲), 2mM (○) or 5mM (●) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. At the indicated times, aliquots were taken to determine the appropriate parameters. B, D: One aliquot of boar sperm was incubated during 4h in a no-capacitating medium (◆). Simultaneously, four more aliquots were incubated for 4h in capacitating medium. After this time, the cells incubated in the no-capacitating medium was added with progesterone, whereas the other aliquots were treated as follows: The first aliquot was added with progesterone alone (■), the second aliquot was added with progesterone and 1mM glutathione (▲), the third aliquot was added with progesterone and 2mM glutathione (○), finally, the fourth aliquot was added with progesterone and 5mM glutathione (●). Subsequently, sperm was incubated during 1 min, 5 min and 60 min and aliquots were taken at these times to determine the appropriate parameters. Results are shown as means±S.E.M. for 7 separate experiments. Asterisks indicate significant differences ($P<0.05$) when compared with the respective time point of cells incubated in a capacitated medium without addition of reduced glutathione at any point.

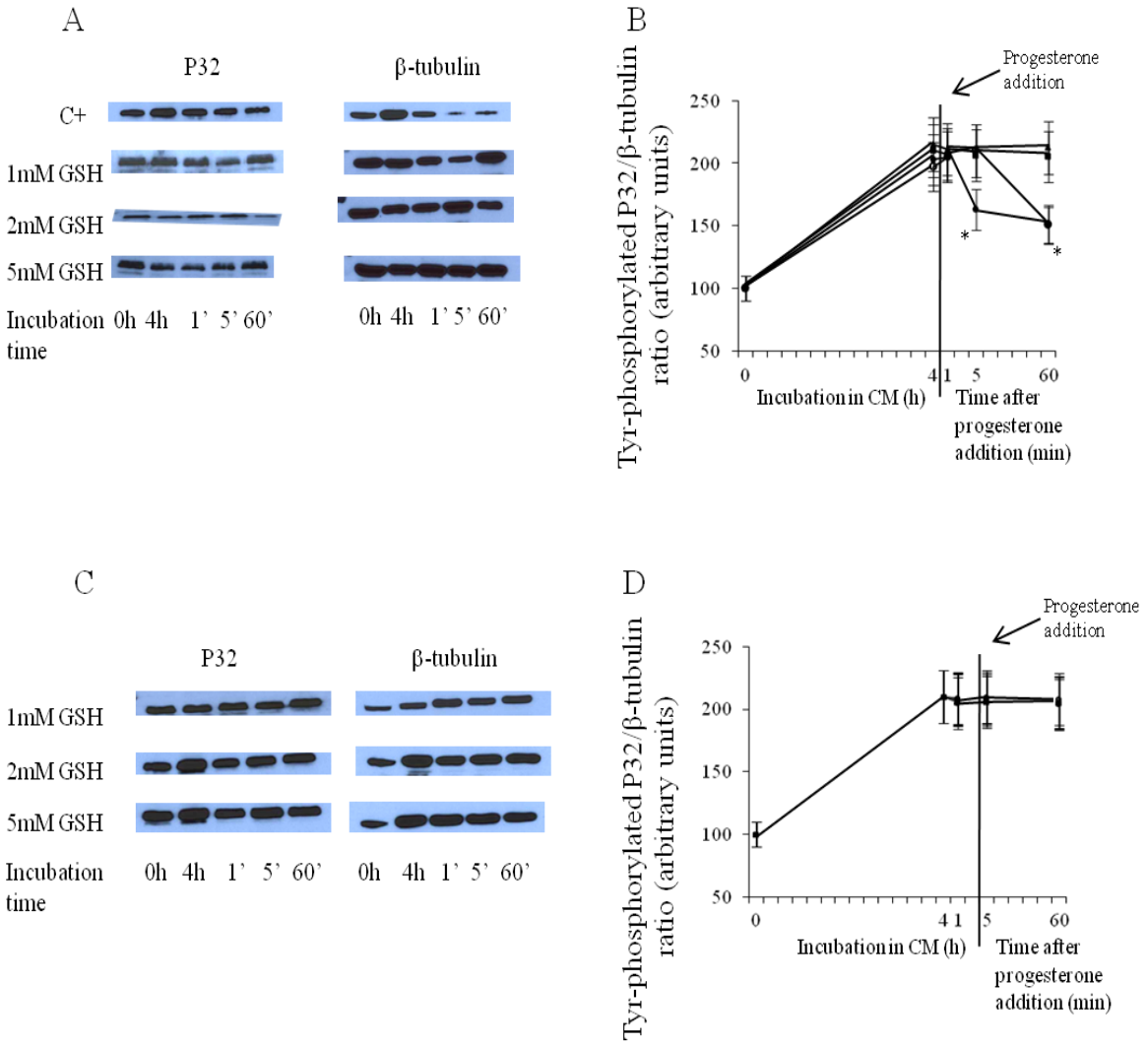

A
P32
β-tubulin
C+
1mM GSH
2mM GSH
5mM GSH
Incubation time
0h 4h 1' 5' 60'
0h 4h 1' 5' 60'
B
Progesterone addition
Tyr-phosphorylated P32/β-tubulin ratio (arbitrary units)
250
200
150
100
50
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)
C
P32
β-tubulin
1mM GSH
2mM GSH
5mM GSH
Incubation time
0h 4h 1' 5' 60'
0h 4h 1' 5' 60'
D
Progesterone addition
Tyr-phosphorylated P32/β-tubulin ratio (arbitrary units)
250
200
150
100
50
0
4
1
5
60
Incubation in CM (h)
Time after progesterone addition (min)

Figure 6

Figure 6. **Effects of reduced glutathione on tyrosine phosphorylation levels of the P32 protein of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, Western blot technique has been also described in the Material and Methods section. A,B: Boar spermatozoa incubated in capacitating medium without (C+) or with reduced glutathione at final concentrations of 1mM (1mM GSH), 2mM (2mM GSH) or 5mM (5mM GSH) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min, 5 min and 60 min. C,D: Boar sperm incubated for 4h in capacitating medium and subsequently added with progesterone and 1mM glutathione (1mM GSH), progesterone and 2mM glutathione (2mM GSH) and progesterone and 5mM glutathione (5mM GSH). A,B,: representative pictures of Western blot against tyrosine-phosphorylated P32 (P32) and β-tubulin (β-tubulin) in aliquots taken at the indicated times B,D: Densitometry results of the P32 tyrosine phosphorylation/β-tubulin intensity ratios, taking the ratio of incubation in the CM at 0h of the point C+ as a basal, arbitrary value of 100. Figure B corresponds to the results yielded from Western blots shown in Figure A, whereas Figure D corresponds to the quantification of blots shown in Figure C. (■): cells without glutathione addition. (▲): sperm added with 1mM glutathione. (○): sperm added with 2mM glutathione. (●): sperm added with 5mM glutathione. Images and results are representative for 7 separate experiments.

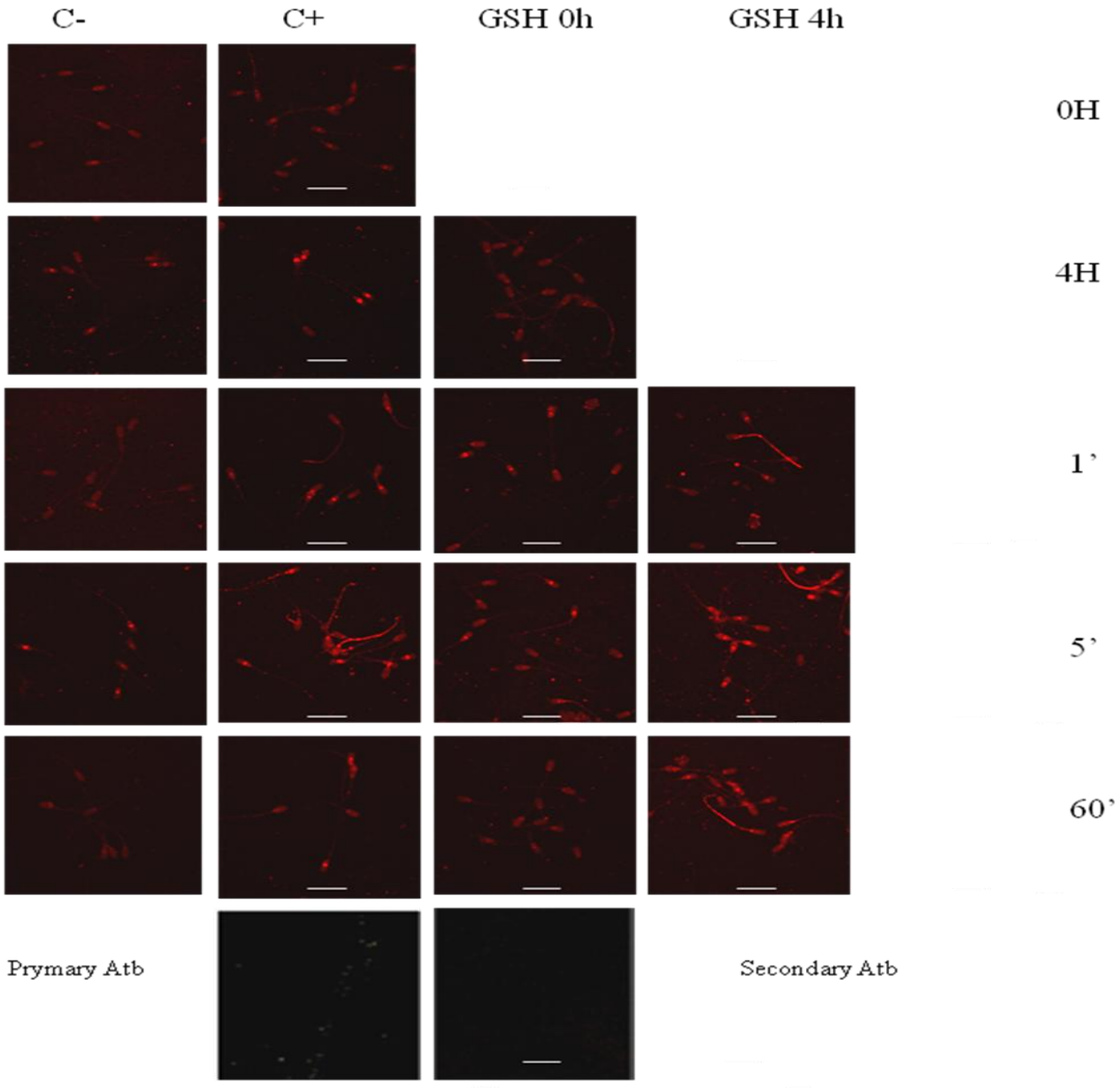


Figure 7

Figure 7. **Effects of reduced glutathione on the location of overall protein tyrosine phosphorylation of boar sperm subjected to *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis.**
Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, the utilized immunocytochemistry technique has been also described in the Material and Methods section. Boar spermatozoa were incubated in no-capacitating medium (C-) or in a capacitating medium without (C+) or with reduced glutathione at final concentration of 2mM (GSH 0h) during 4h. Afterwards, progesterone was added as described in Material and Methods and cells were subsequently incubated during 1 min (1'), 5 min (1') and 60 min (60´). Additionally, another aliquot of boar sperm were incubated for 4h in a capacitating medium and afterwards these cells were added with progesterone and 2mM GSH together (GSH 4h). Subsequently, cells were incubated during 1 min (1'), 5 min (1') and 60 min (60´). In all cases, at the indicated times aliquots were taken to determine their overall protein tyrosine phosphorylation patterns. Figure shows representative images for 7 separate experiments. –Primary Atb: a representative image for a negative control of immunochemistry performed without the addition of the primary antibody. –Secondary Atb: a representative image for a negative control of immunochemistry performed without the addition of the secondary antibody. Bars indicate a size of 15μm.

8. Melatonin

8.1 General function, roles and localization:

Melatonin, in chemical nomenclature *N-acetyl-5-methoxy tryptamine,* has been detected in vegetables, fruits and a variety of herbs. In some plants, especially in flowers and seeds, which are the most vulnerable plant areas to oxidative insults, melatonin concentrations are several orders of magnitude higher than measured in the blood of vertebrates (Manchester et al., 2000; Afreen et al., 2006). Considering the antioxidative activity of melatonin (Tan et al., 1993; 2003) and its presence in plant products that vertebrates consume, melatonin could be also classified as an antioxidant vitamin (Tan et al., 2003). Melatonin in plants not only provides an alternative exogenous source of melatonin for herbivores but also suggests that melatonin may be an important antioxidant in plants which protects them from a hostile environment that includes extreme heat, cold and pollution, all of which generate free radicals (Tan et al., 2000). Regarding mammalians, melatonin was found for the first time in 1950 in bovine pineal tissue (Lerner et al., 1958), showing an intricate relationship with the control of animal reproductive physiology and neuroendocrine physiology (Hoffman and Reiter, 1965; Ashrafi et al., 2011; Cebrián-Pérez et al., 2014).

Melatonin is a small lipophilic indolamine derivated from the amino acid tryptophan. Although it has been considered that the main mammalian organ that synthesizes melatonin is the pineal gland, there are many other organs that can produce melatonin, such as eyes, lymphocytes, bone marrow, skin, and the ovarian follicle (Reiter et al., 2000; Cebrián-Pérez et al., 2014). The general synthesis pathway in the pineal gland is a four-step process. Firstly, tryptophan is converted into 5-hydroxytryptophan by tryptophan-5-hydroxylase (T5M; EC 1.14.16.4), which is transformed to 5-hydroxytryptamine by aromatic-L-amino-acid decarboxylase (AAAD; EC 4.1.1.28), which is subsequently synthesized to N-acetylserotonin by serotoninN-acetyltransferase (SNAT; EC 2.3.1.87). The last step in this biosynthetic pathway is catalysed

by hydroxyindol-O-methyltransferase (ASMT; EC 2.1.1.4), which leads to melatonin production (Boutin et al., 2005). Pineal melatonin is secreted into blood following a circadian rhythm that shows a nocturnal maximums and diurnal basal levels (Tamura et al., 2008; Cebrián-Pérez et al., 2014). The circadian rhythm of melatonin synthesis is managed by the rate limiting enzyme serotonin N-acetyltransferase (NAT) activity. The final result of this secreting pattern is to preview a low concentration during daylight and peaks of activity under dark environment. However, there are other enzymes linked to melatonin processing, like methyltransferase that don't show dependence from light exposition.

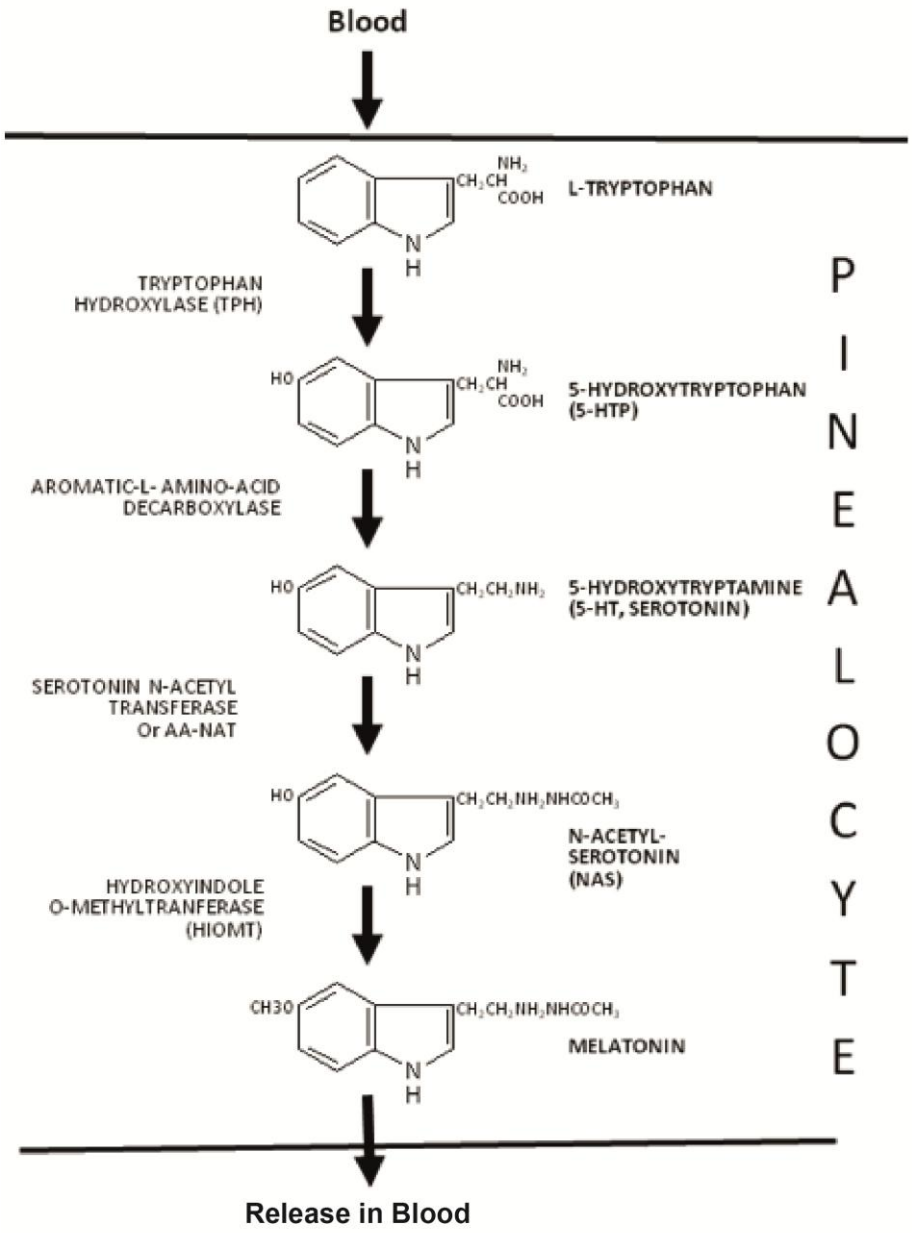


Fig. 8: Biosynthesis of melatonin from tryptophan in mammalian pinealocytes. From: http://photobiology.info.

Moreover, besides its well known role in the control of circadian rhythms and possibly sleep processes in diurnal species (Ashrafi et al., 2011), melatonin acts on other function points like sugar and lipid metabolism, oxidative stress defence acting as a universal reactive oxygen (ROS) or nitrogen (RNS) species scavenger, protection against carcinogenesis (Cebrián-Pérez et al., 2014), immune regulation and additionally, melatonin also supports *in vitro* maturation and fertilization and embryo development in species like pig, rat, sheep and cow (Cebrián-Pérez et al., 2014).

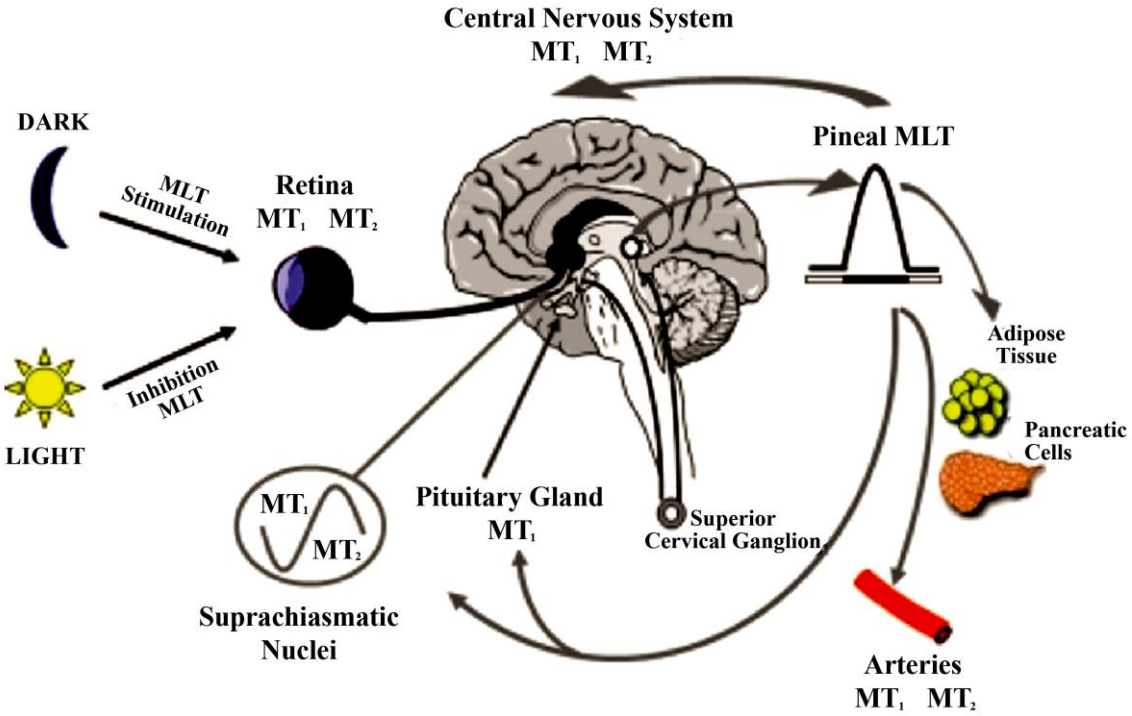


Fig. 8.1: trigger point of melatonin secretion. From: https://sadam001.files.wordpress.com/2014/05/2.png.

To exert its function melatonin needs membrane receptors or nuclear binding site (Tamura et al., 2008). At this moment, three types of receptors have been identified in mammals: MT1, present above all in brain of all vertebrates; MT2, expressed in the retina in all vertebrates and a third receptor called MT3, which is also known to be the enzyme quinone reductase 2 (QR2; Tan et al., 2007). However the melatonin-linked signal transduction cascade induced from the activation of the MT3 receptor has not been identified yet. Meanwhile, the melatonin nuclear binding site corresponds to two nuclear receptors from the retinoic acid receptor family named RZR and ROR. The acronym RZR is for retinoid Z receptor, whereas ROR is for retinoid acid receptor-related orphan receptor (Becker-André et al., 1994; Cebrián-Pérez et al., 2014). Although the exact role of the melatonin-induced activation of these receptors is not well known, there are some evidences that it can play a modulator role in events like cell proliferation and differentiation as well as in cellular homeostasis (Evans, 2005). Otherwise, the melatonin cell membrane MT receptors are G protein-coupled cell surface (GPPCRs) characterized by specific high amino acid sequence homology. These receptors are implicated in the inhibition of the production of cyclic AMP (cAMP; Cebrián-Pérez et al., 2014). In this way, the melatonin activation of the MT-linked pathways would lead to a subsequent inhibition of the PKA (Tamura et al., 2008). Furthermore, each MT receptor plays a specific function in the SNC. Thus, MT1 seems to have an inhibitory function versus melatonin and androgen production (Pintor et al., 2001), whereas MT2 is involved in the circadian rhythm by desensitization at the beginning of the night due to internalization after exposure to melatonin, as with most GPCRs (Evans, 2005). Finally, the molecular pathways activated by the MT3 receptor are not known at this moment. In fact, the only function that has been described for the MT3 receptor is linked to the reduction of the intraocular pressure (Pintor et al., 2001).

8.2 How melatonin can modifies sperm dynamics:

Several studies have shown a direct beneficial effect of melatonin on cells, and several of these studies are been focalized on its effect on spermatic cells. Regarding sperm quality, the *in vitro* treatments conducted on human, ram and pig spermatozoa showed that melatonin can improve the motility and several other quality parameters in these species (Cebrián-Pérez et al., 2014). Concerning the mechanism of action, it has been postulated that could be linked to a reduction of the levels of oxidative stress. In this way, melatonin could act through its free radical scavenging properties after crossing the sperm plasma membrane (Reiter et al., 2000). In this sense, *in vitro* treatment with melatonin has resulted in decreased oxidative cell damage and intracellular levels of ROS and NO (Rao and Gangadharan, 2008; Du Plessis et al., 2010; Jang et al., 2010), reduced membrane lipid peroxidation (Gadella et al., 2008; Du Plessis et al., 2010), apoptosis markers (Casao et al., 2010; Espino et al., 2011) and DNA fragmentation (Sarabia et al., 2009). Another study conducted on ram sperm suggests that melatonin can have a modulatory role during capacitation (Casao et al., 2010). Of course, the identification in ram sperm plasma membrane of MT1 and MT2 melatonin receptors (Evans, 2005), suggests that modulation of capacitation by melatonin is a mechanism mediated through these receptors (Cebrián-Pérez et al., 2014). Differences in melatonin levels seem to be also a part of natural reproduction pattern. In fact, during the breeding season ram seminal plasma has high melatonin levels, which seems play a role in protecting sperm from oxidative damage (Reiter et al., 2000; Drevet, 2006). At the same time, spermatozoa in the oviduct are exposed to low levels of melatonin. These low levels would promote sperm capacitation through a mechanism of action linked to the scavenger of extracellular ROS, taking into account that ROS by themselves can act as potent capacitation modulators (De Lamirande et al., 1984). The effects of melatonin on sperm capacitation are not exclusive to ram. Thus, melatonin is able to promote capacitation in buffalo sperm (Di Francesco et al., 2010). There are no other reports

centred on this point, which could be one of the most important roles for melatonin in the reproductive tract.

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Kev Words:	acrosome reaction, spermatozoa, sperm function

MELATONIN IMPEDES THE LOOSING OF HEAD DISULFIDE BONDS AND INDUCES AGGLUTINATION DURING THE ACHIEVEMENT OF BOAR SPERM *IN VITRO* CAPACITATION AND *IN VITRO* ACROSOME EXOCYTOSIS

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Running Title: Melatonin action on boar sperm *in vitro* capacitation

ABSTRACT

The main aim of this work was to assess the putative effects that melatonin could have in the achievement of boar sperm *in vitro* capacitation (IVC) and subsequent progesterone induced acrosome exocytosis (IVAE). For this purpose, boar sperm samples were subjected to a standard protocol to achieve both IVC and IVAE in the presence or absence of increasing concentrations of melatonin added either at the start of the incubation or together with progesterone in sperm that have previously achieved the capacitates status. Several indicative parameters of the achievement of capacitation were carried out, as well as other complementary determinations such as intracellular levels of peroxides, superoxides, free cysteine levels and cell distribution of determined lectins were also performed. Our results indicate that melatonin did not affect the majority of parameters associated with the achievement of capacitation, as percentages of cells with capacitation-like changes in lipid membrane fluidity or the increase in the tyrosine phosphorylation levels of the P32 protein. Likewise, melatonin did not affect the launching of IVAE both added to pre-capacitated cells and to previously capacitated spermatozoa. However, melatonin counteracted the IVC-concomitant changes in sperm motion parameters, reaching to a complete immobilizing effect at a concentration of 5 µM. This effect was accompanied by a very intense and significant increase in the percentage of agglutinated sperm, which was already high in control samples, capacitated samples (84.7%±8.2% with 5µM melatonin vs. 60.9%±7.5% in control cells, after 4 h of incubation). This agglutination activating effect was not accompanied by highly noticeable changes in the distribution of markings from lectins WGA, STL, PSA and PNA. Furthermore, melatonin had no effect on the dynamics of the sperm rate with high peroxide levels, whereas only induced a slight decrease in the percentage of cells with high superoxide levels when was applied after 4 h of incubation together with progesterone. Notwithstanding,

melatonin did have an intense and significant ($P<0.05$) decreasing action on the head-located intracellular free cysteine levels (4.8 nmol/g protein±0.7 nmol/g protein in cells incubated with 0.5 µM melatonin vs. 17.2 nmol/g protein±2.3 nmol/g protein in control cells after 4 h of incubation), which was only observed in tail-located free cysteine levels when melatonin was added at the 0 h of incubation in the capacitation medium. Taken together, these results would suggest that melatonin acts as a strong modulator of the agglutination phenomena appeared in capacitating conditions with high bicarbonate levels. Furthermore, melatonin effects seem not to be related either with an antioxidant effect or with noticeable changes in the composition and distribution of the sperm glycocalix. Meanwhile, melatonin could act on IVC-related changes in processes controlled by changes in the number and location of disulfide bonds, like motility patterns and stability of the nucleoprotein structure.

Key words: boar sperm, capacitation, acrosome exocytosis, melatonin

INTRODUCTION

In the last years increasing evidence has been accumulated regarding the existence of the complete and functional machinery to sustain a feasible melatonin metabolism in the mammalian reproductive tract from several species (Reiter et al., 2009). Linked to these findings, sperm cells of species like ram, human, hamster, stallion, dog, boar, deer and bull show the presence of melatonin receptors MT1 and MT2 (González-Arto et al., 2016). The presence of both receptors in all of these species is remarkable, since it is inferred that they are present in both seasonal and non-seasonal species. These findings are concomitant with the presence of significant levels of melatonin in the seminal plasma of all of these species (Luboshitzky et al., 2002; Casao et al., 2010; Perez- Patiño et al., 2016), suggesting thus the existence of an active messenger-receptor melatonin pathway system in mammalian spermatozoa. Classically, the main role of melatonin has been linked to the regulation of the physiology of circadian rhythms, among them those linked with the launching and switching of reproductive function (Reiter et al., 2009). However, although in species like ram the semen-linked melatonin content and metabolism could be associated with a circadian modulatory role (Casao et al., 2010), in other species like boar the circadian-like effects seem to be dismissed (González-Arto et al., 2016). Centring on the known effects on sperm function, melatonin seems to have a prominent modulatory role on ram and water buffalo sperm capacitation (Casao et al., 2009; Ashrafi et al., 2013). Furthermore, *in vitro* treatments carried out in human, ram and pig spermatozoa showed that melatonin improves motility and several other quality parameters in these species (Cebrián-Pérez et al., 2014). It has been postulated that the melatonin action mechanism could be linked to a reduction in the levels of oxidative stress, acting through its free radical scavenging properties after crossing the sperm plasma membrane (Reiter et al., 2000). In this sense, *in vitro* treatment with melatonin of

sperm from several species resulted in decreased oxidative damage and intracellular levels of ROS and NO (Rao and Gangadharan, 2008; Du Plessis et al., 2010; Jang et al., 2010), membrane lipid peroxidation (Gadella et al., 2008; Du Plessis et al., 2010), apoptosis markers (Casao et al., 2010; Espino et al., 2011) and DNA fragmentation (Sarabia et al., 2009). Despite all of these data, little is known regarding the possibility that melatonin could exert any effect on sperm function through receptor-linked pathways that were not uniquely related with the anti-oxidative properties of the molecule. This is especially relevant is sperm cells from species like boar, in which ROS production seems not to be prominent when compared with other such as bull or horse (Bilodeau et al., 2000; Yeste et al., 2015a; 2015b). In this way, ROS production or accumulation seems to play a minor role in explaining phenomena like boar sperm cryodamage (Yeste et al., 2015b). Likewise, changes in ROS levels are low during the achievement of *in vitro* boar sperm capacitation (IVC; see Awda et al., 2009), suggesting that ROS play a minor role in the achievement of boar sperm IVC. Taking all these data into account, we can hypothesize that melatonin could affect the sperm function through a receptor-linked mechanism. To prove this hypothesis, the main aim of this study was to determine the effects of melatonin in the achievement of a feasible IVC and subsequent progesterone-induced in vitro acrosome exocytosis (IVAE) in boar spermatozoa. For this purpose, boar spermatozoa were subjected to the IVC/IVAE procedure in the presence of increasing concentrations of melatonin, added either before or after 4 h of incubation in capacitating conditions. Several parameters indicative of the achievement of both IVC and IVAE were subsequently determined, highlighting thus the putative effects of melatonin on both phenomena.

MATERIALS AND METHODS

Seminal samples

A total of 62 ejaculates collected from 35 healthy Pietrain boars aged between two and three years, were used in this study. These animals were housed in climate-controlled commercial farms (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with a commercial adjusted diet and provided with water *ad libitum*. Furthermore, boar housing followed all the ethical guidelines established for the European laws regarding domestic animal and, specifically, pig farm management. Furthermore, and even though it was not required as the authors did not manipulate any boar and only worked with seminal doses donated by the commercial firm, the experimental protocol was approved by the Ethics Committee of our institution. This ethics committee was known as ‘‘Bioethics Commission of the Autonomous University of Barcelona’’ (Bellaterra, Cerdanyola del Vallès, Spain). Utilized samples came from sperm-rich fractions that were obtained through manual collection after applying the conventional hand-gloved method. Samples were immediately diluted after collection in a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain) to a final sperm concentration of 2×10^7 spermatozoa/mL and cooled to 16ºC–17°C. Diluted semen was then distributed in 90-ml commercial AI doses. The resulting 90-mL doses were placed in a thermal packaging container at 16ºC for approximately 45 min, which was the time required to arrive at our laboratory.

Experimental design, *in vitro* capacitation and subsequent acrosome exocytosis procedures

Two separate experimental designs were carried out. In the first experimental procedure, we tested how the addition of increasing concentrations of melatonin to the capacitation medium (CM) before incubation affected the achievement of a feasible IVC. In the second experimental procedure, melatonin was added after 4 h of incubation in CM, an incubation

time that has been previously reported as able to induce IVC in boar spermatozoa (Ramió et al., 2008), together with the IVAE-induced progesterone (Jimenéz et al. 2003; Wu et al. 2006). This second experimental design was performed to evaluate the putative effects of melatonin in the achievement of IVAE in fully *in vitro* capacitated boar sperm. Thus, in both experimental designs there were 5 separate experimental points: a positive control (C+): spermatozoa incubated in CM containing bicarbonate and bovine serum albumin (BSA); three different melatonin treatments in which the cells were resuspended in CM supplemented either at 0 h or after 4 h of incubation with increasing concentrations of melatonin (0.5µM, 1µM, 5µM); and a negative control (C-): spermatozoa re-diluted in a non-capacitating medium (NCM) without melatonin, neither $NaHCO_3$ nor BSA.

In the first experiment, 50 mL of each ejaculate was utilized through division between all five treatments. Thus, aliquots were firstly centrifuged at 600 x*g* for 10 min at 16 ºC and subsequently resuspended at a final concentration of $2\text{-}3\times10^7$ sperm/mL in NCM composed of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM $NaHPO_4$, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (osmolarity: 287 mOsm/Kg ± 6 mOsm/Kg). 40 mL of the resuspended sample were taken separately and 5 mg/mL BSA and 37.6 mM $NaHCO_3$ were added in order to transform the NCM in the CM (pH=7.4; osmolarity: 304 mOsm/Kg ± 5 mOsm/Kg). This implied that the remaining 10 mL-aliquot was taken as the C- experimental point. Subsequently, the 40-mL CM sperm sample was further divided in four separate 10-mL aliquots. One of these 10-mL aliquots was lent without further addition of any substance, whereas the other three CM-aliquots were added with melatonin to final concentrations of 0.5 µM, 1 µM and 5 µM, respectively. Afterwards, all aliquots were incubated for 4 h at 38.5 ºC in a 5% CO_2 atmosphere, as previously described in Ramió et al. (2008). Samples were taken at 0 h and 4 h of incubation to perform the

appropriate analysis. After 4 h of incubation, spermatozoa were subjected to progesterone-induced *in vitro* acrosome exocytosis (IVAE) by addition of 10 mg/mL progesterone following the procedure described in previous reports (Jiménez et al., 2003; Wu et al., 2006).. After thoroughly mixing, sperm samples were further incubated for an additional 1 h at 38.5 ºC in a 5% CO_2 atmosphere. Aliquots were taken at 1 min, 5 min and 60 min after the addition of progesterone to perform the required analyses.

The general procedure for the second experimental design was similar to that described above. The main difference was that in the melatonin-treated experimental points the effector was added only after 4 h of incubation in the CM and simultaneously to progesterone. Melatonin concentrations and sampling procedure were the same as in the first experimental design.

Analysis of parameters indicative of the achievement of *in vitro* capacitation acrosome exocytosis

The parameters determined in order to analyse the achievement of both IVC and IVAE were the computer-assisted (CASA) motility analysis, the spectrophotometric determination of the free-cysteine residues in both head and tail sperm extracts, cytometric analysis of sperm viability, acrosome integrity, membrane lipid disorder, ROS levels, mitochondrial membrane potential (MMP) and intracellular calcium levels in both head and midpiece location, and finally the immunological detection of phosphorylation levels of protein tyrosine residues, with an especial emphasis in the P32 protein as an specific capacitation marker of boar sperm (Bravo et al., 2005).

Sperm motility analysis

Sperm motility analysis was performed by utilizing a commercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field at a

magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10 x 0.30 PLAN objective lens, Olympus-Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image capturing of one photograph every 40 ms. Five to six separate fields were taken for each replicate, and five replicates were run per sample and treatment. The obtained sperm motility descriptors were described following Ramió et al. (2008). Settings taken into account for all of the utilized motility parameters were as following:

Range of particles area: 10–80 μm^2.

Connectivity: a minimum of 11 images for all parameters, but a minimal of 10 images for only the mean amplitude of lateral head displacement (ALH).

Parameter ranges:

Curvilinear velocity (VCL): 1–500 μm⁄s.

Mean velocity (VAP): 1–500 μm⁄s.

Linear coefficient (LIN): 10–98%.

Straightness coefficient (STR): 10–98%.

Mean amplitude of lateral head displacement (ALH): 0–100 μm.

Frequency of head displacement (BCF): 0–100 Hz.

In this procedure, samples were previously warmed at 37 ºC for 5 min in a water bath, and 5 μl aliquots of these samples were then placed onto a warmed (37 ºC) slide and covered with a 22 × 22 mm coverslip. Finally, total motility was defined as the percentage of spermatozoa that showed a VAP >10 μm⁄s.

Evaluation of free-cysteine residues in sperm nucleoproteins

The determination of free cysteine radicals in sperm head and tail proteins as an indirect measure of disrupted disulphide bridges within nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. (2011). Briefly, samples

were centrifuged at 600 x*g* and 16 ºC for 10 min and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycholate, 1 mM benzamidine, 10 µg/mL leupeptin, 0.5mM phenylmethylsulfonyl fluoride, and 1mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). Obtained homogenates were centrifuged at 850 x*g* for 20 min at 4 ºC. The resultant supernatants were reserved to measure the free-cysteine in sperm tail proteins and the pellets were resuspended in 300 µL of Tris buffer to measure the free-cysteine in sperm head proteins. The presence of both tails in supernatants and heads in pellets were determined by a previous observation under optical microscopy. This observation determined that the percentage of tails in supernatants and heads in pellets were of above of 85% of the total content in both cases (data not shown).

The levels of free cysteine radicals in both tails and heads fractions were determined by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulfide; Sigma-Aldrich) as described by Brocklehurst et al. (1979). With this purpose, 10-µL aliquots of resuspended, isolated sperm heads were added to 990 mL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. The same process were repeated with 10-µL aliquots of supernatants that were added to 990 mL of an aqueous solution of 0.4 mM 2,2'-dithiodipyridine. Aliquots of 10 µL of cysteine standards from 0.1mM to 5mM (Sigma-Aldrich) were also added to 990 mL of 0.4 mM 2,2'-dithiodipyridine for evaluation. In all cases, mixtures were incubated at 37ºC for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 340 nm. The results obtained were normalized through a parallel determination of the total protein content of samples by the Bradford method (1976), using a commercial kit (Quick Start Bradford Protein Assay; BioRad, Hercules, CA, USA). Five replicates per sample and treatment were evaluated, and the corresponding mean ± SEM was calculated.

Flow cytometric analysis

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC; Lee et al., 2008). These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), sperm membrane lipid disorder, acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/ mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, M540/YO-PRO®-1, PNA-FITC/PI, H2DFCDA/PI, HE/YO-PRO®-1, JC-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSCTM cytometer (Beckman Coulter, Fullerton, CA, USA; Serial Number AL300087, Technical specification at https://www.beckmancoulter.com/wsrportal/ajax/downloadDocument/721742AD.pdf.) This instrument, which had not been altered in the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the singleline visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab QuantaTM SC cytometer employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10 µm Flow- Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. In this system, the optical characteristics for these filters were as follows: FL1 (green fluorescence): Dichroic/Splitter,

DRLP: 550 nm, band pass filter: 525 nm, detection width 505– 545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm); and FL3 (red fluorescence): long pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, YO-PRO®-1, H2DFCDA and Fluo 3), whereas FL3 was used to detect red fluorescence (M-540, HE, PI and Rhod 5).

Sheath flow rate was set at 4.17 µL/ min in all analyses; while EV and side scatter (SS) were recorded in a linear mode (in EE vs. SS dot plots) for a minimum of 10000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter>12 µm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described below, compensation was used to minimize spill-over of the fluorescence into a different channel.

Information on the events was collected in List-mode Data files (LMD), and these generated files were then analysed using Cell Lab Quanta_SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyse the cytometric histograms. In PNA-FITC/PI, H2DFCDA/PI and HE/YO-PRO®-1 assessment, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina et al. (2010). Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± SEM.

Unless otherwise stated, all fluorochromes used for these analyses were purchased from Molecular Probes® (Invitrogen, Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma).

Cytometric determination of sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner & Johnson (1995). Briefly, sperm samples were incubated at 38°C for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 µM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, whereas PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: (i) viable green-stained spermatozoa ($SYBR\text{-}14^{+}$/ PI^{-}); (ii) non-viable red-stained spermatozoa ($SYBR\text{-}14^{-}/PI^{+}$); and (iii) non-viable spermatozoa that were stained both Green and red ($SYBR\text{-}14^{+}/PI^{+}$). Non sperm particles (debris) were found in the $SYBR\text{-}14^{-}/PI^{-}$ quadrant.

Single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Cytometric analysis of true acrosome exocytosis

True acrosome exocytosis was determined through co-staining of sperm with ethidium homodimer (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1) and *Arachis hypogaea* agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC-PNA). This protocol was originally described by Cooper and Yeung (1998) and has been adapted to boar spermatozoa in our laboratory. The utilization of this protocol was due to the fact that it has a higher specificity for the detection of true acrosome exocytosis than that previously utilized in our studies (see Yeste et al., 2015b, as an example). Briefly, samples were incubated with EthD-1 (final concentration: 2.5 $\mu g \cdot mL^{-1}$) at 38 °C for 5 minutes in the dark. Following this step, samples were washed by centrifugation at 2000 x*g* for 30 s and then resuspended with PBS containing 4 $mg \cdot mL^{-1}$ bovine serum albumin (BSA) to remove free dye. Thereafter, samples were again centrifuged and then fixed and permeabilized by adding 100 µL of ice-cold methanol (100%) for 30 s. Methanol was removed by centrifugation at 2000*g* for 30 s

and resuspension with PBS containing 4 mg·mL^{-1} BSA. The resulting pellet was resuspended in 250 µL BTS. Following this step, 0.8 µL PNA-FITC (final concentration: 2.5 µM) were added and samples were incubated at room temperature in the dark for 15 min. Next, samples were washed twice with PBS at 2000*g* for 30 s finally were resuspended in BTS.

After this assessment, four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC^{+}/EthD-1^{-}); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC^{-}/EthD-1^{-}); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC^{+}/EthD-1^{+}); (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC^{-}/EthD-1^{+}). Fluorescence of EthD-1 was detected through FL-3, while that of PNA-FITC was detected through FL-1.

Determination of sperm membrane lipid disorder compatible with capacitation

Lipid disorder of boar sperm membrane was evaluated by Merocyanine-540 (M540) and YO-PRO-1 co-staining, following a modified procedure from Rathi et al. (2001). Briefly, spermatozoa were stained with M540 (final concentration: 400 µM) and YO-PRO®1 (final concentration: 40 µM) and incubated for 10 min at 38 °C in the dark. Red fluorescence from M540 was collected through FL-3 and green fluorescence from YO-PRO®1 was collected through FL-1. A total of four sperm populations were observed in flow cytometry dot plots: (i) viable spermatozoa with low membrane lipid disorder (M540^{-}/YO-PRO-1^{-}); (ii) viable spermatozoa with high membrane lipid disorder (M540^{+}/YOPRO-1^{-}); (iii) non-viable spermatozoa with low membrane lipid disorder (M540^{-}/YO-PRO-1^{+}) and (iv) non-viable spermatozoa with high membrane lipid disorder (M540^{+}/YO-PRO-1^{+}). In this test, data were not compensated.

Assessment of intracellular superoxide and peroxide levels

Intracellular superoxide ($O_2^{-\bullet}$) and peroxide (H_2O_2) levels were determined using two different oxidation-sensitive fluorescent probes: hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed by containing the spermatozoa either with PI or with YO-PRO®-1.

In the case of superoxides, samples were stained with HE (final concentration: 4 µM) and YO PRO®-1 (final concentration: 40 µM) and incubated at 25°C for 40 min in the dark (Guthrie & Welch, 2006). Hydroethidine is freely permeable to cells and is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of ethidium (E^+) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means ± SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E^+).

In the case of peroxides, spermatozoa were stained with H2DCFDA at a final concentration of 200 µM and PI at a final concentration of 10 µM, and incubated at 25°C for 60 min in the dark. H2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie & Welch, 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3.

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

Western blot assay

A total of seven ejaculates were selected for the Western blot assay. For this purpose, 1 mL-aliquot belonging to each experimental point was centrifuged at 1000 x*g* for 30 s and pellets were stored at -80 °C until the beginning of the assay. When stated, pellets were then resuspended and sonicated in 300 µL of ice-cold lysis buffer (pH 7.4) containing 50mM Tris-HCl, 1mM EDTA, 10mM EGTA, 25mM dithiothreitol, 1,5 % (v:v) Triton® X-100, 1mM phenylmethanesulfonyl fluoride, 10 µg/mL leupeptin, 1mM ortovanadate and 1mM benzamidine. After 30 min in ice, the homogenized suspensions were centrifuged at 4°C at 600 x*g* for 20 min and total protein amount in supernatants was calculated through the Bradford method (1976) using a commercial kit (Bio-Rad Laboratories; Fremont, CA, USA). Afterwards, samples were added to a loading buffer (1:5; v:v) containing 250mM Tris-HCl (pH 6.8), 50mM dithiothreitol, 10 % (w:v) SDS, 0.5 % (v:v) bromophenol blue and 50% (v:v) glicerol and stored at -20 °C until their subsequent assay.

To perform the Western blot, samples were loaded into 0.75 mm gels containing 10% acrylamide (w:v) to perform SDS-PAGE (Laemmli, 1970). After running the gels at constant voltage (180 V), the activated proteins of gels were transferred to an immune-blot low-fluorescence polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in 7 min using the Trans-Blot® Turbo Transfer System (Bio-Rad) with Trans-Blot® Turbo Midi Transfer Packs. Membranes were subsequently submerged for 60 min in blocking solution consisted in a Tris-buffered saline solution added with 5% (w:v) BSA and 0.1% (v:v) Tween-20. After this time, membranes were then submerged in blocking solution containing the appropriate concentration of the utilized primary antibody. Membranes were incubated with the antibody at 4°C for a minimum of 8h. The utilized primary antibodies were a mouse monoclonal clone PY20 anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich; St. Louis, Missouri, USA) and a mouse monoclonal anti-tubulin (ref. T5201; Sigma-Aldrich; St. Louis, Missouri, USA).

In both cases, the utilized dilution was of 1:1000 (v:v). The β-tubulin was used as an internal standard to normalize the volume of protein bands, stripping and reproving membranes when necessary.

After three washes, the immunoreaction was tested for 60 min using a horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse secondary antibody (Dako; Glostrup, Denmark) at a dilution of 1:5000 (v:v) in blocking solution.

After six washes, the reaction was developed for 2 min with a chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology®, Dallas, Texas, USA) according to the manufacter's instructions and the membranes were revealed. Prestained protein standards with a molecular mass range of approximately 250–10 kDa were used. Finally, the image analysis system Image J 1.49 (National Institute of Health, USA) was used to quantify the changes in intensity of various bands.

Detection of lectins distribution through fluorescence microscope

The lectins used in this study were the *Triticum vulgaris* agglutinin (WGA), the *Solanum lycopersicum* lectin (STL), the *Pisum Sativum* agglutinin (PSA) and the *Arachis Hypogaea* agglutinin (PNA). All of them were obtained from Molecular Probes® (Invitrogen, Eugene, OR, USA). Furthermore, all four lectins were FITC-conjugated. Briefly, 400 µL-aliquots of sperm cells at the appropriated incubation times and treatments, were centrifuged at 1000 x*g* for 30 s and the resultant pellets were fixed through of their resuspension in 400 µL of 4% (w:v) paraformaldehyde in PBS. Fixation was conducted for a minimum of 2 h at 4 ºC in the dark. Afterwards, samples were spread onto poly-lysine (1% w:v poly-lysine solution in H_2O; Sigma, St Louis, MO, USA) microscope slides and then were left to air-dry. After this, samples were permeabilized by incubation for 10 min at room temperature in a standard phosphate-buffered solution (PBS; pH 7.4) added with 0.3% (v:v) Triton® X-100. Next, slides were washed three times with PBS and they were then blocked through incubation with

PBS including 0.1% (v:v) Tween-20® and 5% (w:v) BSA for 60 min at 20ºC. After blocking, the cover slip was incubated with the lectin at a different final concentration of: 1:200 (w:v) for WGA and PSA, 1:300 (w:v) for PNA and 1:50 (w:v) for STL. All lectins solutions were performed in PBS, and samples were incubated for 1 h at 20 ºC in a humid chamber. After incubation, slides were further washed three times in PBS for 5 min each time and then mounted with the anti-fading medium Vectashield H-1000 (Vector laboratories, Burlingame CA, USA). After being covered, the slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with colourless nail polish, and slides were stored at 4 ºC in the dark until their microscopic observation. Negative control experiments were performed omitting the lectin. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik, Heidelberg, Germany) adapted to an inverted LeitzDMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo lens (Leitz, Wetzlar, Germany). The light source was an argon⁄krypton laser. Successive confocal slices of images (image thickness: 0.5 µm) were integrated to perform three-dimensional spermatozoa images, which were further stored as TIFF-format images. Each lectin used in this study generated distinct staining patterns that were found in all non-capacitated, capacitated and AR sperm populations, with a different intensity and position of marking.

Statistical evaluation of results

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 2.0; SPSS Inc.; Chicago, Illinois, USA), and data from all assessments are presented as the mean ± standard error of the mean (SEM). Data were first tested for normality and variance homogeneity through Shapiro-Wilk and Levene tests, respectively. When required, data (x) were transformed using the arcsine square root (arcsin Öx) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media) and the intrasubject factor was the

incubation time (i.e. 0h, 4h, 4h 1 min, 4h 5 min, 4h 60 min). In all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were calculated using Sidak's test. When no transformation remedied the normality, non-parametric procedures were used with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as non-parametric alternatives to repeated measures ANOVA. In all statistical analyses, the minimal level of significance was set at $P<0.05$.

RESULTS

Effects of melatonin on viability, true acrosome exocytosis and capacitation-like changes

Incubation of boar spermatozoa in CM for 4 h induced a decline in the percentage of viability, which went from 80.4%±3.7% at 0 h to 67.9%±2.8% after 4 h of incubation (Figure 1). This decline was maintained after the addition of progesterone. Cells incubated in NCM showed an even greater rhythm of decline (Figure 1A,B). The addition of melatonin to the CM either at 0 h of incubation or after 4 h together with progesterone did not significantly modify the observed drop in viability (Figure 1A,B).

Percentages of true acrosome exocytosis were very low in cells incubated in CM during 4 h. The addition of progesterone after this incubation time induced an increase in this percentage, which reached maximal values after 60 min of the progesterone addition (67.4%±2.3%, see Figure 1C,D). This increase was not observed in cells incubated in NCM. The addition of melatonin either at 0 h of incubation or after 4 h together with progesterone did not modify the observed increased in control cells incubated in CM (Figure 1C,D).

Incubation of boar sperm in the CM was concomitant with a progressive, significant ($P<0.05$) increase in the percentage of viable cells with capacitation-like changes in membrane fluidity (from 9.4%±2.6% at 0 h of incubation to 54.3%±4.9% after 4 h of incubation, see Figure 2A,B). The subsequent addition of progesterone was associated with a progressive decrease of this percentage, underwent values of 34.6%±3.0% after 60 min of further incubation (Figure 2A,B). The addition of melatonin both at 0 h and after 4 h of incubation in CM together with progesterone did not have any noticeable effect on the dynamic observed in control cells (Figure 2A,B).

Effects of melatonin on total motility and motion patterns

The percentage of total motility of cells incubated in CM underwent a progressive decline, reaching minimal values of 27.0%±2.5% after 5 h of incubation (60 min after the addition of progesterone, see Figure 2C,D). Incubation of sperm in NCM yielded even worse total motility values, with a complete immobilization at the final incubation time. The addition of melatonin at 0 h of incubation induced an immediate decrease in total motility, which was more evident at the maximal concentration of melatonin utilized (47.2%±3.0% with 5μM melatonin at 0 h of incubation vs. 64.0%±3.9% in control cells, see Fig. 2C). The effect of melatonin was maintained during all of the incubation time and, in fact with 5 μM a complete immobilization after 4 h of incubation in CM was found (Figure 2C). The addition of melatonin after 4 h of incubation together with progesterone also induced a significant ($P<0.05$) decrease in total motility when compared with control cells, reaching values near the complete immobility after 60 min of incubation at both melatonin concentrations of 1 μM and 5 μM (Figure 2D).

Regarding motion parameters, sperm incubated during 4 h in CM showed significant increases of several parameters like VCL, VAP and ALH (Table 1,3). The addition of melatonin at time 0 h significantly ($P<0.05$) decreased VAP values at melatonin concentrations that did not inhibit motility (Table 1). The addition of melatonin together with progesterone after 4 h of incubation in CM induced a decrease in VAP, LIN and STR values after 1 min of the addition of both effectors. A slight further recovery was found with all of the utilized melatonin concentrations excepting of 5 μM in which effects were in an overall overview more intense and more extended in time than with the lower concentrations (Table 1, 2).

Effects of melatonin on agglutination

The incubation in CM induced a notorious percentage of agglutinated sperm, which increased during the incubation, reaching values of 60.9%±7.5% after 4 h of incubation (Figure 3A,B). Agglutinations were of medium size (Complementary Figure 1D,E) and the percentage of agglutinated sperm that showed appreciable tail movement was about 45% after 4 h of incubation (Figure 3C,D). The percentage of agglutinated sperm was roughly maintained afterwards, without great variations after the addition of progesterone (Figure 3A,B and Complementary Figure 1F). However, the percentage of agglutinated sperm with appreciable tail movements showed a transient increment during the first 5 min post-progesterone addition, and afterwards it was gradually decreasing, reaching values of only 17.3%±2.6% after 60 min of progesterone addition (Figure 3C, D). Spermatozoa incubated in NCM did show low percentages of agglutinated sperm (Figure 3A, B and Complementary Figure 1A-C). The addition of melatonin at 0 h of incubation induced an immediate and intense, significant ($P<0.05$) increase in the percentage of agglutinated sperm (56.2%±6.4% with melatonin 5 μM vs. 26.1%±3.8% in control cells, see Figure 3A and Complementary Figure 1G). This increase was further maintained, reaching values of about 80-85%, depending on the melatonin concentration, after 4 h of incubation. At these times, the size of the observed agglutinations was very large, showing the presence of hundreds of sperm in a single agglutination (Complementary Figure 1H). Afterwards, the percentage of agglutinated sperm as well as the size of agglutinations was roughly maintained during all of the experimental period (Figure 3A and Complementary Figure 1I). Regarding the percentage of agglutinated sperm with appreciable tail movements, melatonin induced a significant ($P<0.05$) decrease at all the tested concentrations, reaching minimal values after 4 h of incubation (for example, 12.8%±1.9% in cells incubated with 5 μM melatonin vs. 43.2%±3.2% in control cells

incubated in CM, see Figure 3C). Further addition of progesterone induced a similar dynamics to that observed in control cells incubated in CM, although the intensity of the peak found after the first 5 min post-progesterone addition was much lower, or even practically abolished with 5 μM melatonin (Figure 3C). Finally, the addition of melatonin after 4 h of incubation in CM together with progesterone had not a clear effect either on the percentage of agglutination or in the percentage of agglutinated sperm with tail movements (Figure 3B, D). The only remarkable effect was observed after 60 min of post-progesterone addition in which cells incubated with progesterone and 1 μM or 5 μM melatonin maintained the percentage of agglutinated sperm with tail movements at similar levels to those observed during the first 5 min of post-progesterone incubation (Figure 3D and Complementary Figure 1J).

Effects of melatonin on the peroxide or superoxide concentrations

Incubation of boar sperm in CM induced a slight but significant ($P<0.05$) increase in the percentage of cells with high H_2O_2 intracellular level, which went from 1.6%±0.2% at 0 h of incubation to 5.8%±1.3% after 4 h (Figure 4A,B). This is in contrast with cells incubated in CM in which the increase of this percentage was remarkably greater (9.4%±2.5% after 4 h of incubation, Figure 4A, B). The subsequent addition of progesterone did not significantly modify the percentage of high-H_2O_2 cells in sperm incubated in CM, whereas those incubated in NCM showed a slight, gradual increase, reaching values of 13.6%±2.9% after 60 min of progesterone addition (Figure 4A,B). The addition of melatonin to the CM at 0 h of incubation did not significantly affect the dynamics observed in control cells except with 5μM melatonin in which there was a significant ($P<0.05$) decrease of this percentage after 1 min of the progesterone addition that was not further recovered (Figure 4A). The addition of melatonin together with progesterone after 4 h of incubation in CM did also show a significant ($P<0.05$) decrease of this percentage compared to control cells with both 1 μM and

5 μM melatonin at 1 min post-progesterone addition, although subsequent incubation recovered this effect (Figure 4B).

The percentage of cells with high intracellular $O_2^{-\bullet}$ levels in boar sperm samples incubated in CM went from 1.4%±0.2% at 0 h to 6.2%±1.7% after 4 h of incubation (Figure 4C,D). Subsequent addition of progesterone did not have a remarkable effect on this point, showing only a gradual increase with values of 9.0%±2.8% after 60 min of the progesterone addition (Figure 4C,D). This percentage was significantly greater in cells incubated in NCM, reaching values of 19.8%±4.1% after 60 min of the progesterone addition (Figure 4C,D). The addition of melatonin at any of the tested concentrations both at 0 h and after 4 h of incubation together with progesterone did not have any remarkable effect on the dynamics of the percentage of cells with high $O_2^{-\bullet}$ levels observed in control cells incubated in CM (Figure 4C,D).

Effects of melatonin on the free-cysteine residues in both head and tail extracts of boar spermatozoa

The incubation of boar sperm samples in CM induced a progressive increase in the free cysteine levels from head extracts which went from 3.9 nmol/g protein±0.3 nmol/g protein at 0h of incubation to 17.2 nmol/g protein±2.3 nmol/g protein after 4 h of incubation (Figure 5A,B). This increase was not observed in cells incubated in NCM. Subsequent addition of progesterone did not increase these levels, and there were even a slight gradual decrease with values of 11.3 nmol/g protein±1.7 nmol/g protein 1h after the progesterone addition (Figure 5A,B). The addition of melatonin at 0 h of incubation had a dramatic effect on free cysteine levels from head extract, inducing a practical abolishing of the increase observed in control cells. Thus, as observed in Figure 5A, the addition of 0.5 μM melatonin caused an increase of

these levels after 4 h of incubation to levels of only 4.8 nmol/g protein±0.7 nmol/g protein, whereas higher melatonin concentrations inhibited any increase. This abolishing effect was maintained after the addition of progesterone. When melatonin was added after 4 h of incubation with progesterone, a similar abolishing effect on free cysteine levels from head extract was also observed. In this way, the addition of the lowest concentration of melatonin utilized lowered these levels after only 1 min of the addition to values of 9.4 nmol/g protein±2.0 nmol/g protein, whereas higher concentrations of the effector even had a greater effect.

Similarly to that observed in head extracts, the free cysteine levels from tail extracts underwent a progressive increase during the incubation of cells in CM. Thus, these values went from 3.7 nmol/g protein±0.6 nmol/g protein at 0 h of incubation to 9.3 nmol/g protein±1.9 nmol/g protein after 4 h of incubation (Figure 5C,D). These values were roughly maintained after the addition of progesterone. The addition of melatonin to the CM at 0 h induced a practically complete abolition of this increase at any concentration tested (Figure 5C). On the contrary, the addition of melatonin after 4 h of incubation together with progesterone had no any clear effect on this parameter until 60 min post-progesterone addition, in which the free cysteine levels from tail extracts increased in a melatonin dose-dependent manner (12.8 nmol/g protein±2.4 nmol/g protein in cells treated with 5 μM melatonin vs. 8.9 nmol/g protein±1.7 nmol/g protein in control sperm, see Figure 5C).

Effects of melatonin on tyrosine phosphorylation levels of P32 protein

As expected, incubation of boar sperm in CM during 4 h induced a noticeable increase in the tyrosine phosphorylation levels of the P32 protein, which was roughly maintained after the progesterone addition (Figure 6,7). The addition of melatonin at time 0 h in the CM did not appreciably modified the increase in P32 tyrosine phosphorylation after 4 h of incubation in

CM (Figure 6). Variations in the tyrosine phosphorylation of P32 were only found with 5 μM melatonin, but only after the addition of progesterone and in a non-uniform manner, with a great variability in the observed changes among the performed experiments (Figure 6 and data not shown). Furthermore, the addition of melatonin together with progesterone after 4 h of incubation in CM only showed a noticeable decrease in the tyrosine phosphorylation intensity mark of P32 at 5 min and 60 min post-progesterone addition and with 5 μM melatonin (Figure 7).

Effects of melatonin on spatial location of separate lectins

At the beginning of incubation in CM, boar sperm showed a specific WGA signal located at the head and the whole tail, although the maximal intensity of the signal was observed at the acrosomal edge (Figure 8). Cells incubated in the NCM showed a similar marking, although the acrosomal signal was much less intense. The incubation for 4 h in the CM induced an evident increase in the intensity of the acrosome-located signal that was uniformly distributed through the entire acrosomal structure (Figure 8). These changes were not detected in cells incubated in the NCM for 4 h. The subsequent addition of progesterone to the CM induced further modifications in the acrosome signal of WGA. Thus, after 1 min of progesterone addition there were small changes, with some cells showing a certain degree of loss of the inner lectin signal and other with an irregular acrosome marking. The progesterone-induced changes were more evident after 5 min of its addition, with a much greater number of cells showing a diffuse inner acrosomal signal or an irregular acrosome marking (Figure 8). These patterns were also detected after 60 min post-progesterone addition. The incubation of boar sperm in CM added with 1 μM melatonin from the start of the incubation induced after 4 h of incubation the appearance of a noticeable number of sperm in which the incubation-linked increase of the acrosomal signal observed in control cells was altered. Thus, there were sperm

in which the lectin signal of the triangular post-acrosomal area was totally lost, whereas the majority of cells showed a much more intense acrosome signal than that of control sperm (Figure 8). There were even cells in which the head signal was practically lost at all (Figure 8). Furthermore, the addition of progesterone to sperm incubated for 4 h in melatonin-added CM did not induce evident and immediate changes in the WGA acrosome signal. However, after 60 min of incubation a whole acrosome signal (without markings in the triangular post-acrosomal area) slightly more intense than that observed in control CM cells was found (Figure 8). The addition of 1 μM melatonin to the CM after 4 h of incubation together with progesterone did not induce evident changes in the distribution of the WGA signal (Figure 8).

Regarding the spatial location of the STL signal, it was located in boar sperm at the head and the midpiece at 0 h of incubation in CM (in some cells, STL was evident in the entire tail), with a more prominent intensity signal at the whole acrosome area (Figure 9). The signal in cells incubated in the NCM at 0 h was similar, although there was a noticeable heterogeneity in the distribution of the acrosome marking. The incubation for 4 h in the CM induced the appearance of at least two separate types of STL markings. The first type was formed by cells with and intense and uniform signal through the entire acrosome area. The other type of cells showed a less intense signal, with the greater intensity concentrated at the acrosomal edge (Figure 9). In all types, the midpiece STL signal was much decreased or even totally disappeared. The addition of progesterone after 4 h of incubation induced a rapid loss of the acrosome signal in practically all cells, that was already evident 1 min post-progesterone addition, whereas tail signal prominently increased (Figure 9). The loss and the increase in the heterogeneity of the acrosome signal were maintained afterwards, together with the maintenance of the tail marking. The addition of 1 μM melatonin at the start of the incubation with CM was also accompanied with an increased heterogeneity of the STL acrosome signal

after 4 h of incubation, together with a noticeable marking of the tail (Figure 9). The addition of progesterone also induced a clear loss of STL acrosome signal in those sperm incubated in the presence of melatonin, showing the majority of cells a more intense signal at the acrosome edge. Subsequent incubation times did not clearly modify this loss; although the majority of cells showed a more or less uniform intensity signal on the entire acrosome area until 60 min post-progesterone addition (Figure 9). The addition of 1 μM melatonin to the CM together with progesterone did not clearly affected the observed STL signal in control CM cells, although in contrast to control CM sperm, several cells showed a clearly intense acrosome signal after 1 min of the melatonin-plus-progesterone addition (Figure 9).

The signal linked to the PSA marking of boar sperm at the start of the incubation in CM was observed in the whole cell, although the most intense marking was detected at the entire acrosomal area. In case of cells incubated in NCM, the acrosomal signal was much less intense, with a well-marked acrosome edge (Figure 10). The incubation for 4 h in CM induced an overall increase of the intense signal both at the head and the tail, although the triangular post-acrosomal area was left without PSA signal. The subsequent addition of progesterone induced a rapid loss of the PSA signal linked to acrosome, that was evident in a high percentage of cells as soon as 1 min after the hormone addition, whereas other showed a clear disorganization of the acrosome signal (Figure 10). The presence of separate types of sperm with various degrees of disorganization of the acrosome signal (from an intense signal to its loss), was maintained after 60 min of the progesterone addition. The addition of 1 μM melatonin to the CM at 0 h of incubation did not seem to have a clear effect on the PSA location during the incubation time, even after 4h of the progesterone addition (Figure 10). Likewise, the addition of 1 μM melatonin after 4 h of incubation in CM together with progesterone did not show any clear effect on the PSA distribution signal.

Regarding the location of the PNA signal in boar sperm incubated in CM for 0 h, this lectin was exclusively located at the whole acrosome surface. This distribution did not change after 4 h of incubation either in CM or in NCM (Figure 11). The addition of progesterone did not modify the main location of the PNA signal, although there were cells that showed heterogeneity of the acrosome signal and, surprisingly, there were even sperm with a clear PNA marking located at the entire tail (Figure 11). The addition of progesterone after 4 h of incubation in the CM induced the appearance of a greater heterogeneity of the acrosome signal, starting to appear cells less intensity of marking and others with a faint marking uniquely limited to the acrosome edge associated with an intense signal in the triangular post-acrosomal area (Figure 11). These changes were very rapid, starting at only 1 min post-progesterone addition and melatonin did not have any clear effect on the observed dynamics of the PNA signal, either when added at the time 0h of incubation or after 4h together with progesterone (Figure 11).

DISCUSSION

The results shown in this manuscript unveil a complex overview on the role of melatonin during the achievement of the capacitation status and subsequent sensitization to launch acrosome exocytosis in boar sperm. Thus, whereas on one side melatonin seems not to have a definite effect on the achievement of IVC and further launching of IVAE, on the other side the hormone profoundly affects sperm through promoting sperm agglutination. In this sense, our results seem to indicate that the yielding of capacitation status requires the coordination of a myriad of separate groups of events that could be a common basis in the initial activation of the cAMP-dependent protein kinase PKA (Ickowicz et al., 2012). From the activation of PKA onwards, however, there are a very wide array of PKA-sensitive molecular cascades that regulate specific points of the capacitation process. In many cases, these cascades are activated by separate and specific PKA-activated pathways. For instance, it is well known that PKA can activate the Ca^{2+}-dependent pathway linked to the activation of kinases like PKC through a canonical phosphoinositol modulation system (Ickowicz et al., 2012). Meanwhile at the same time the same PKA activation leads to a subsequent activation of the molecular cascade linked to the $pp60^{sc\text{-}src}$ (SRC) factor (Mitchell et al., 2008). These examples are important, since both of them acts on sperm motility regulation (Ickowicz et al., 2012) although through separate ways. In this way, it is possible that factors like melatonin could act on only one or a very few points of the separate PKA-activated molecular pathways in a manner that could act on only specific points of the whole capacitation process.

Taking into account the effects of melatonin on sperm motility, results seem to show that, in fact, these effects were more likely due to a stimulation of agglutination than a direct action of motion parameters. In fact, the launching of IVC in several species is accompanied with an increase in the percentage of agglutinated sperm ((monkey (Boatman & Bavister, 1984), bull

(Ehrenwald et al., 1990, Lefebvre & Suarez, 1996), pig (Funahashi & Day, 1993)). In this way, sperm agglutination can be caused by phenomena other than the presence of antibodies (Yakirevich et al., 1999) simple cell degeneration (Harayama et al., 1998) as capacitation by itself. Thus, many components of the capacitation media in separate species can activate agglutination through mechanisms that are only poorly understood. In this way, substances like heparin in many species (Boatman and Bavister, 1984; Ehrenwald et al., 1990; Funahashi and Day, 1993; Lefebvre and Suarez, 1996) BSA in horse (Bromfield et al., 2014) or bicarbonate and calcium in boar, bull and monkey (Lindahl & Sjöblom, 1981; Boatman and Bavister, 1984; Harayama et al., 1998; Harayama and Kato, 2002) can act as agglutination-activating factors. In this way, in our conditions melatonin could simply act as an activator of another component of the CM that has a potent agglutinating effect by itself. In fact, our results suggest that in our conditions bicarbonate could be the agglutinating factor that would be potentiated by the addition of melatonin. This hypothesis is based in previous works from our own laboratory, in which boar sperm IVC was achieved in a medium without supplementation of bicarbonate. In these cases, both IVC and subsequent IVAE were achieved but without the presence of significant amounts of sperm agglutination (Ramió et al., 2008; Ramió-Lluch et al., 2011, 2014 and unpublished data). On the contrary, the addition of bicarbonate to the CM, which was the only change that the CM had in this work when compared with previous reports (Yeste et al., 2015b) induced by itself a noticeable percentage of agglutination from the 0h incubation time onwards. The mechanism by which bicarbonate would induce head-to-head sperm agglutination seems to be related with the increase of intracellular cAMP via activation of the bicarbonate-sensitive adenylyl cyclase and the subsequent PKA activation (Harayama and Kato, 2002). At this moment, we cannot determine the exact mechanism of action by which melatonin could potentiate this putatively bicarbonate-mediated effect. Despite this, we could hypothesize a putative melatonin

potentiating action on the cAMP-mediated, bicarbonate induced sperm agglutination in CM. Regardless of this hypothesis; our results also indicate that boar sperm IVC/IVAE could be better conducted in a medium without the addition of bicarbonate. The lack of appreciable levels of sperm agglutination accompanied with the achievement of feasible IVC and further IVAE could indicate, in fact, that the low, endogenously produced bicarbonate after the incubation for 4h in a 5% CO_2 atmosphere is enough to launch the appropriate cAMP-PKA-dependent molecular changes conducting to a feasible capacitated status. Finally, regarding the mechanism/s of action by which melatonin induced sperm agglutination, our results are not conclusive. Despite this, these mechanisms seem not to be related with notorious changes in the glycocalix composition of sperm membrane surface, since results obtained after the incubation with separate lectins did not show any prominent change on this point. Thus, other mechanisms like changes in the ionic strength of the sperm cell membrane could be more adequate to explain the observed agglutinating effects.

Regarding the effects of melatonin on the percentage of sperm with high intracellular ROS, our results indicate that the antioxidant action of melatonin seems not to play a prominent role in the observed effects during IVC and IVAE and, specifically, on the agglutination stimulation. This conclusion could be surprising at a first glance. However, we must remind that, in fact, boar sperm is characterized by a very low ROS production rate, even when subjected to treatments that induces the appearance of high intracellular ROS in other species, such as freezing-thawing (Bilodeau et al., 2000; Yeste et al., 2015a; 2015b). Thus, if high ROS levels are a fairly rare event in boar sperm, it would be logical that any antioxidant effect caused by an effector should very subtle, if detectable. The main cause by which boar sperm don't accumulate high ROS levels in practically no circumstance could be linked to a species-specific mitochondria function. Thus, previous reports pointed out that mitochondrial energy production of boar sperm is very low, even though important to maintain several functions

like motility (Rodríguez-Gil and Bonet, 2015). When centring in the launching of boar sperm IVC and subsequent progesterone-induced IVAE, results also showed that the O_2 consumption rate was maintained low, as well as the overall intracellular ATP levels, during almost all of the procedure (Ramió-Lluch et al., 2014). Even more, when cells were incubated in the presence of olygomicin A, a specific inhibitor of the mitochondrial ATP synthase (Ramió-Lluch et al., 2014), both the O_2 consumption rate and the ATP levels were not diminished (Ramió-Lluch et al., 2013). This lack of effect of olygomicin A on O_2 consumption rate could suggest that in the majority of the lifespan of boar sperm mitochondria would be in an uncoupled status. One consequence of this uncoupled status would be the yielding of a low rate of ROS production due to a sub-stimulation of the electronic chain, which is the most important ROS-synthesizing point (Rodríguez-Gil and Bonet, 2015). Interestingly, the progesterone-modulated launching of IVAE is concomitant with a sudden and intense peak in both O_2 consumption rate and intracellular ATP levels (Balis et al., 1999; Ramió-Lluch et al., 2011), which could be related with a progesterone-concomitant mitochondria coupling. Following this rationale, the moment of the progesterone-concomitant mitochondrial coupling would be associated with a temporary increase in ROS formation, especially in form of peroxides (Tait et al., 2012). This is interesting, since the addition of melatonin together with progesterone induces a decrease in the percentage of high peroxides levels, which would be compatible with the above described hypothesis. Notwithstanding, regardless of the existence of a putative relationship between the progesterone-induced launching of IVAE and the adoption of a temporary mitochondrial coupling status, our results also indicate that peroxides levels seems not to be instrumental in the fully achievement of the progesterone-induced IVAE, since the melatonin-induced decrease when the effector is added together with progesterone did not modify the IVAE dynamics observed in control cells.

Another striking result observed following the incubation of melatonin was the intense drop in the intracellular free cysteine levels from both head and tail extracts. In fact, a noticeable result that can be observed from our results is that the achievement of boar sperm IVC is concomitant with an increase of both head and tail free cysteine levels. This parameter is an indirect marker of the number of broken disulfide bonds present in the cellular extracts, since although not all of the determined free cysteine radicals would be originated after the breaking of disulfide bonds, a significant percentage of them would have this origin (Yeste et al., 2014). We can only hypothesize about the biological significance of this result. In any case, an increase in the number of broken disulfide bonds in boar sperm head would be linked with a loss in the number of linking points among boar sperm DNA and the surrounding nuclear protamines, since disulfide bonds are one of the most important linkage mechanisms between both structures (Yeste et al., 2013). As a consequence, boar sperm achieving IVC would have a nucleoprotein structure less tightly structured than that from sperm at the start of the incubation in the CM. Obviously, this change would not be present in cells incubated with melatonin. Our results also indicated that the melatonin-induced inhibition of the IVC-concomitant increase in head boar sperm free cysteine levels had no effect in the achieving of this status, as well as in the fully accomplishment of the subsequent progesterone-induced IVAE. However, one possibility would be that the maintenance of a proper number of head sperm free cysteine levels and hence of DNA-protamines disulfide bonds, would be of importance in further events of boar sperm biology, such as the proper decondensation of head sperm after oocyte penetration. Further work involving studies of *in vitro* fecundation will be needed in order to elucidate this point. Another matter would be the concomitant increase of tail sperm free cysteine levels during the achievement of the IVC status and its melatonin inhibition. It is well known that disulfide bonds are important in the maintenance of a proper structure in the whole sperm flagellum structure (Ijiri et al., 2014). Specifically,

disulfide bonds linked to a protein linked to the outer dense fiber 1 (Cabrillana et al., 2011) Thus, it would be logical to assume that changes in the tail sperm free cysteine levels would be related with changes in flagellum structure that, in turn, would yield in subtle changes of the precise sperm motion dynamics. In this sense, it has been described that the cleavage of disulfide bonds in mouse hexokinase-I isozyme 1 is related with the initiation of sperm motility (Nakamura et al., 2008). In this manner, it would be logical to suppose that subtle changes in the number and location of disulfide bonds along the flagellum or in flagellum-bond related proteins like sperm hexokinase-1 (Cabrillana et al., 2011; 2016) could be involved in the changes of motion parameters observed during the achievement of IVC. In fact, in our experimental design, melatonin at concentrations below 5µM effectively disrupted the IVC-linked changes of motion parameters like VCL, VAP and ALH in non-agglutinated sperm. Of course, this melatonin effect could be related with other mechanisms like the agglutination activation by itself. Despite this, our results strongly suggest the existence of a regulating mechanism of action linked to changes in the number and location of disulfide bonds as a part of the whole mechanisms involved in the control of sperm motility changes during IVC.

In summary, our results suggest that melatonin can play a significant role in the modulation of the achievement of IVC and subsequent, progesterone-induced IVAE in boar sperm. The main role of melatonin in this fact would be linked to effects on sperm motility, both through stimulation of sperm agglutination and modulation of changes in sperm motion parameters through stabilization of flagellum disulfide bonds, although other roles like modulation of IVC-concomitant changes in nucleoprotein structure tightness through stabilization of head sperm disulfide bonds could be also of importance. In any case, further studies will be needed in order to establish the precise mechanism/s by which melatonin could exert these effects.

AUTHORS' ROLES

Martina Rocco and Rafael Betarelli: Both carried out the majority of the experimental work, collaborating also in the experimental design and the writing of the manuscript.

Anna Placci and Marcella Spinaci: A.P. collaborated with M.R. and R.F. in carrying out the experimental work, whereas M.S. coordinated the work conducted in her lab.

Josep M. Fernández-Novell: The main responsible for the confocal analysis of lectin location.

Teresa Muiño-Blanco, Adriana Casao, José A. Cebrián-Pérez: The working team of the experimental work performed in their laboratory. Furthermore, T.M.- B. and J.A., C.-P. collaborated in the optimization of the experimental design and the writing of the manuscript.

Alejandro Peña: Coordinator of the experimental work carried out at the Autonomous University of Barcelona

Teresa Rigau: collaborated with M.R. and R.F. in carrying out the experimental work conducted at the Autonomous University of Barcelona

Sergi Bonet, MarcYeste: Coordinators of all flux cytometer work carried out in this manuscript. Moreover, M.Y. collaborated in the optimization of the experimental design and in the writing of the manuscript.

Joan E Rodríguez-Gil.: The main coordinator of all of the experimental work carried out in this manuscript. Moreover, he was the responsible for the final design and organization of the experimental design, as well as for the final writing of the manuscript.

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DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1. **Effects of melatonin on curvilinear velocity and mean velocity of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
VCL (µm/s)					
C-	77.02.9^{a*}	39.01.1^{b*}	39.10.9^{b*}	38.51.2^{c*}	N.D.
C+	65.72.1^{a}	72.92.9^{b}	74.13.2^{b}	81.43.7^{b}	81.64.3^{b}
Melatonin 0.5µM	67.52.3^{a}	55.61.5^{b*}	57.91.7^{b*}	57.02.2^{b*}	44.51.2^{c*}
Melatonin 1 µM	56.21.8^{a*}	60.72.5^{a*}	59.12.6^{a*}	61.23.5^{a*}	41.22.8^{b*}
Melatonin 5 µM	62.52.5^{a}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	65.72.1^{a}	72.92.9^{b}	68.22.7^{a}	90.84.7^{c}	76.53.9^{b}
Melatonin 1 µM +PG	65.72.1^{a}	72.92.9^{b}	67.72.6^{a}	89.94.5^{c}	76.84.2^{b}
Melatonin 5 µM +PG	65.72.1^{a}	72.92.9^{b}	71.43.1^{ab}	81.14.3^{c}	66.84.0^{a*}
VAP (µm/s)					
C-	35.51.4^{a*}	25.11.1^{b*}	27.51.6^{b*}	33.60.9^{b*}	N.D.
C+	46.02.4^{a}	62.63.2^{b}	59.82.7^{b}	56.72.8^{b}	56.83.3^{b}
Melatonin 0.5 µM	48.12.0^{a}	44.62.2^{a*}	47.02.7^{a*}	62.63.7^{b}	85.65.4^{c*}
Melatonin 1 µM	39.81.4^{a*}	37.41.2^{a*}	47.22.6^{b*}	67.23.6^{c*}	92.46.1^{d*}
Melatonin 5 µM	39.81.6^{a*}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	46.02.4^{a}	62.63.2^{b}	40.82.0^{a*}	72.24.1^{c*}	49.13.0^{a}
Melatonin 1 µM +PG	46.02.4^{a}	62.63.2^{b}	40.92.0^{a*}	71.93.9^{c*}	50.13.1^{a}
Melatonin 5 µM +PG	46.02.4^{a}	62.63.2^{b}	38.81.8^{c*}	55.43.1^{d}	39.51.7^{c*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Cells were incubated in a no-capacitating medium (C-) or in a capacitating medium without (C+) or with melatonin at final concentrations of 0.5 µM (Melatonin 0.5 µM), 1 µM (Melatonin 1 µM) and 5 µM (Melatonin 5 µM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were

incubated in capacitating medium and, after 4h of incubation were added with progesterone and 0.5 µM melatonin (Melatonin 0.5 µM +PG), progesterone with 1 µM melatonin (Melatonin 1 µM +PG) and progesterone with 5 µM (Melatonin 5 µM +PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means ± S.E.M. for 7 separate experiments.

Table 2. **Effects of melatonin on linearity coefficient and straightness coefficient of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
LIN (%)					
C-	23.61.1^{a*}	35.11.3^{b*}	36.31.6^{b}	33.61.5^{b*}	N.D.
C+	36.21.4^{a}	44.82.4^{b}	39.91.7^{a}	47.92.3^{b}	37.12.0^{a}
Melatonin 0.5 µM	42.01.9^{a}	34.81.8^{b*}	33.51.6^{b}	43.22.2^{a}	33.22.1^{b}
Melatonin 1 µM	36.21.7^{a}	30.82.1^{a*}	33.61.4^{a}	44.92.4^{b}	35.31.8^{a}
Melatonin 5 µM	39.82.2^{a}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	36.21.4^{a}	44.82.4^{b}	26.81.2^{c*}	36.51.8^{a*}	32.71.5^{a}
Melatonin 1 µM +PG	36.21.4^{a}	44.82.4^{b}	25.71.6^{c*}	36.31.9^{a*}	33.21.8^{a}
Melatonin 5 µM +PG	36.21.4^{a}	44.82.4^{b}	26.01.3^{c*}	30.11.9^{c*}	28.21.2^{c*}
STR (%)					
C-	55.82.3^{a*}	48.52.4^{b*}	44.62.0^{b*}	43.61.9^{b*}	N.D.
C+	63.32.4^{a}	70.93.0^{b}	67.72.8^{ab}	71.02.9^{b}	76.23.3^{b}
Melatonin 0.5 µM	64.72.3^{a}	83.93.4^{b*}	67.13.0^{a}	69.03.6^{a}	81.24.5^{b}
Melatonin 1 µM	62.12.4^{a}	52.11.5^{b*}	68.62.5^{ab}	79.13.6^{c}	73.93.1^{b}
Melatonin 5 µM	63.12.4^{a}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	63.32.4^{a}	70.93.0^{b}	60.62.5^{a}	76.13.7^{b}	77.14.4^{b}
Melatonin 1 µM +PG	63.32.4^{a}	70.93.0^{b}	58.11.8^{a}	70.52.6^{b}	76.04.5^{b}
Melatonin 5 µM +PG	63.32.4^{a}	70.93.0^{b}	55.61.6^{b*}	64.61.9^{a*}	64.82.9^{a*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Cells were incubated in a no-

capacitating medium (C-) or in a capacitating medium without (C+) or with melatonin at final concentrations of 0.5 µM (Melatonin 0.5 µM), 1 µM (Melatonin 1 µM) and 5 µM (Melatonin 5 µM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4h of incubation were added with progesterone and 0.5 µM melatonin (Melatonin 0.5 µM +PG), progesterone with 1 µM melatonin (Melatonin 1 µM +PG) and progesterone with 5 µM (Melatonin 5 µM +PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means± S.E.M. for 7 separate experiments.

Table 3. **Effects of melatonin on mean amplitude of lateral head displacement and frequency of head displacement of boar spermatozoa subjected to *in vitro* capacitation and subsequent, progesterone-induced *in vitro* acrosome exocytosis**

Incubation time	0h	4h	1 min	5 min	60 min
ALH (µm)					
C-	2.560.05^{a*}	2.570.09^{a*}	2.180.11^{b*}	2.140.10^{b*}	N.D.
C+	3,800,12^{a}	4.130.13^{b}	4.930.15^{c}	4.640.15^{c}	4.000.11^{b}
Melatonin 0.5 µM	3,310,09^{a*}	2.880.08^{b*}	4.100.12^{c*}	3.630.12^{ac*}	5.130.21^{d*}
Melatonin 1 µM	3.500.14^{a}	2.720.09^{b*}	3.680.08^{a*}	3.490.11^{a*}	3.850.10^{a}
Melatonin 5 µM	3.890.13^{a}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	3,800,12^{a}	4.130.13^{b}	4.820.20^{b}	5.090.20^{b}	3.810.14^{a}
Melatonin 1 µM +PG	3,800,12^{a}	4.130.13^{b}	5,380.25^{c}	4.960.19^{c}	3.090.11^{d*}
Melatonin 5 µM +PG	3,800,12^{a}	4.130.13^{b}	5.180.20^{c}	4.930.18^{c}	2.990.06^{d*}
BCF (Hz)					
C-	2.920.04^{a*}	3.970.26^{b*}	3.880.21^{b*}	4.010.24^{b*}	N.D.
C+	6.680.17^{a}	6.540.19^{a}	7.180.18^{b}	6.280.21^{a}	6.030.19^{c}
Melatonin 0.5 µM	6.730.15^{a}	6.700.16^{a}	7.200.12^{b}	6.970.24^{b*}	6.850.21^{ab*}
Melatonin 1 µM	6.160.11^{a*}	5.950.11^{a*}	6.760.20^{b*}	6.900.24^{b*}	7.200.27^{c*}
Melatonin 5 µM	6.050.10^{a*}	N.D.	N.D.	N.D.	N.D.
Melatonin 0.5 µM +PG	6.680.17^{a}	6.540.19^{a}	6.860.21^{a}	5.460.09^{b*}	4.370.06^{c*}
Melatonin 1 µM +PG	6.680.17^{a}	6.540.19^{a}	7.480.29^{b}	4.980.09^{c*}	3.910.08^{d*}
Melatonin 5 µM +PG	6.680.17^{a}	6.540.19^{ab}	6.440.17^{b*}	4.920.08^{c*}	3.850.07^{d*}

Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Cells were incubated in a no-

capacitating medium (C-) or in a capacitating medium without (C+) or with melatonin at final concentrations of 0.5 µM (Melatonin 0.5 µM), 1 µM (Melatonin 1 µM) and 5 µM (Melatonin 5 µM). Incubation was maintained during 4h and afterwards, progesterone was added as described in the Material and Methods section. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4h of incubation were added with progesterone and 0.5 µM melatonin (Melatonin 0.5 µM +PG), progesterone with 1 µM melatonin (Melatonin 1 µM +PG) and progesterone with 5 µM (Melatonin 5 µM +PG). In all cases, cells were subsequently incubated and aliquots were taken after 1 min, 5 min and 60 min. Separate superscripts indicate significant differences ($P<0.05$) among values in a row. Asterisks indicate significant differences ($P<0.05$) when compared with the C+ value from the same time of incubation. Results are shown as means± S.E.M. for 7 separate experiments.

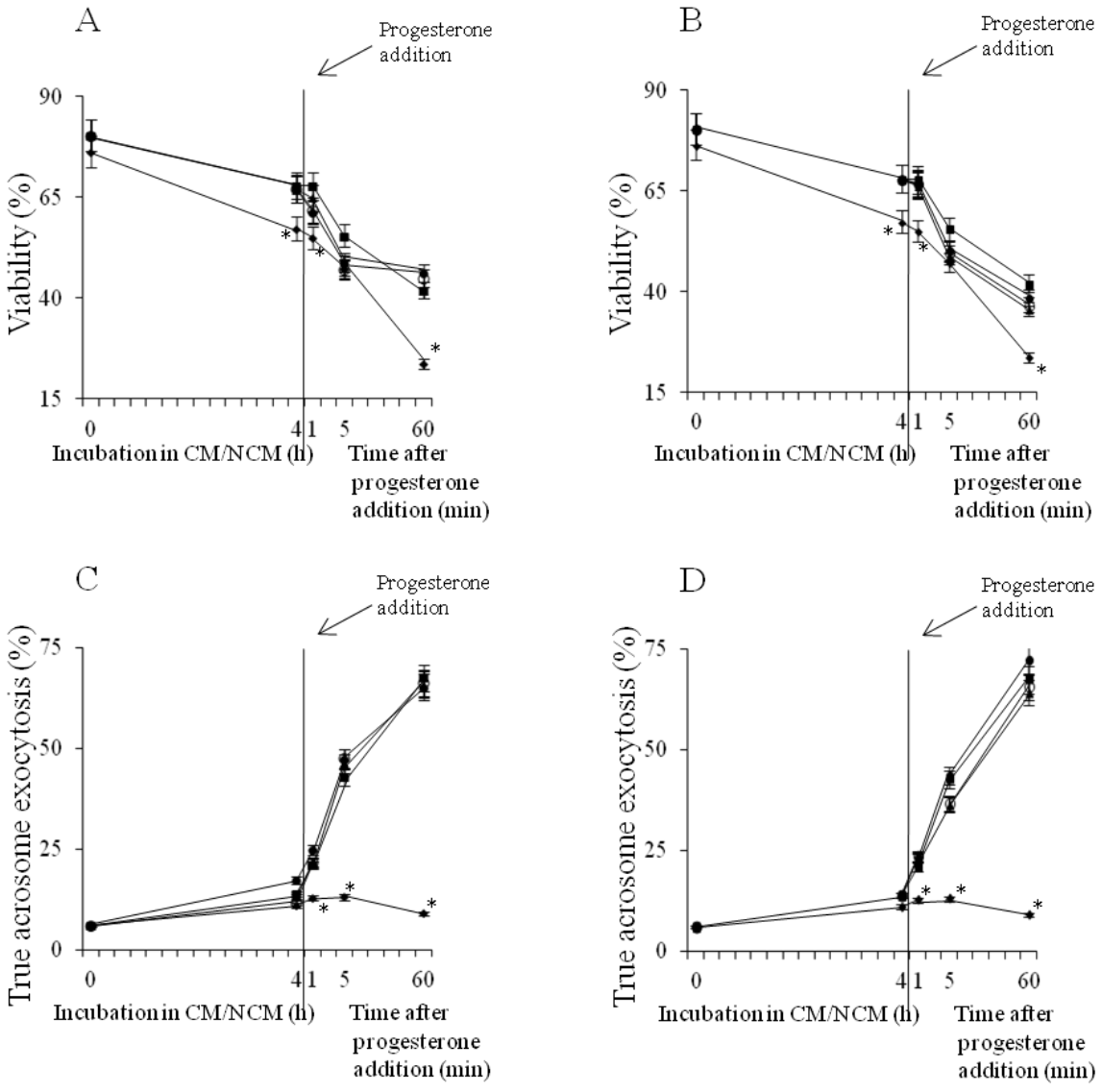


Figure 1. **Effects of melatonin on percentages of viability and true acrosome exocytosis of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of viability. C,D): percentages of true acrosome exocytosis. A,C): results obtained after incubation of sperm in the presence of melatonin from the time 0h of incubation. B,D): results obtained through the addition of melatonin after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ◆: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 0.5µM melatonin.○: spermatozoa incubated in CM in the presence of 1µM melatonin. ●: spermatozoa incubated in CM in the presence of 5µM melatonin. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means± S.E.M. for 6 separate experiments.

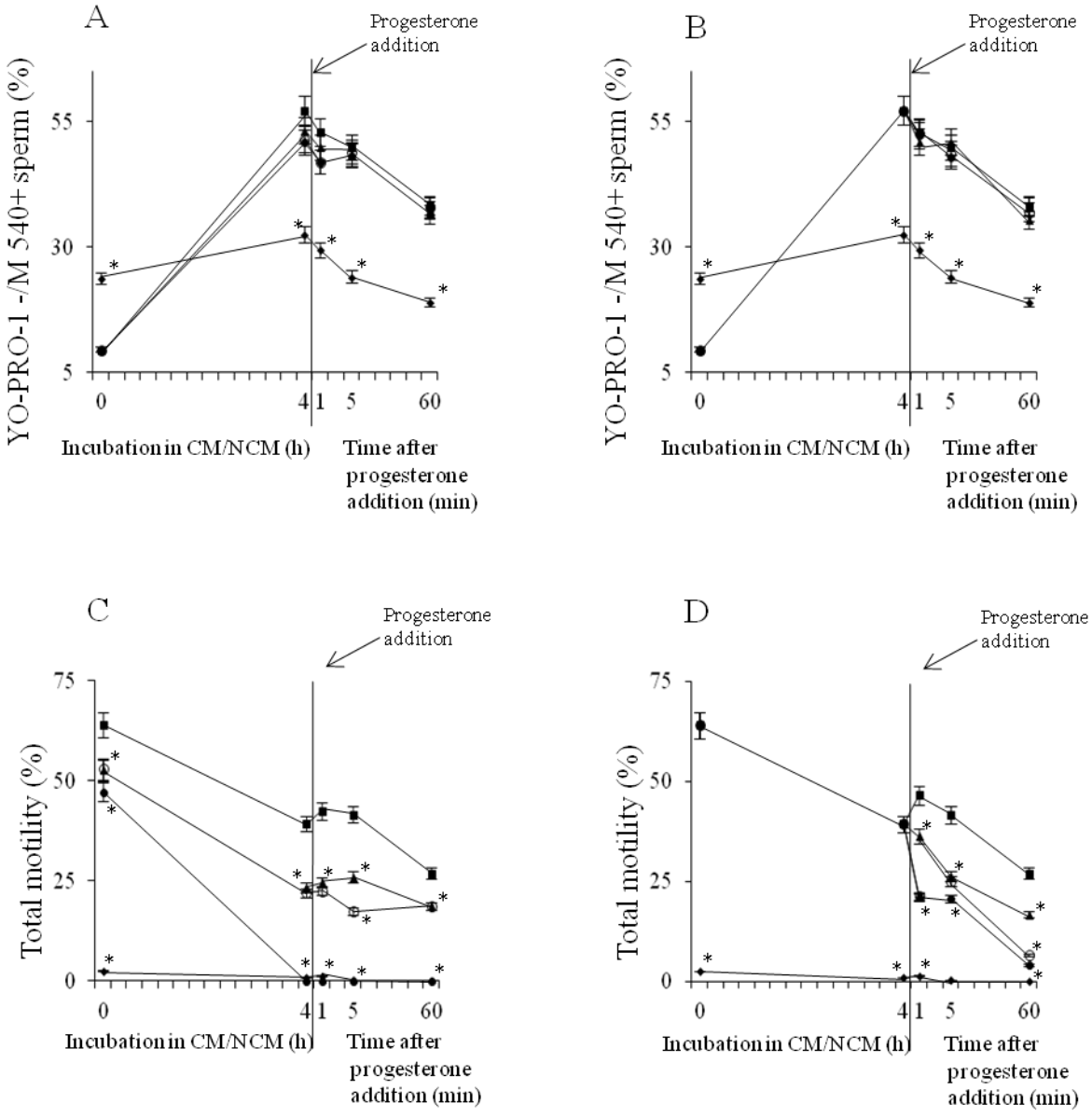


Figure 2. **Effects of melatonin on percentages of sperm with capacitation-like changes in membrane fluidity and total motility of boar sperm subjected to *in vitro* capacitation**

and subsequent progesterone-induced acrosome exocytosis.

Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of changes in membrane fluidity. C,D): percentages of total motility. A,C): results obtained after incubation of sperm in the presence of melatonin from the time 0h of incubation. B,D): results obtained through the addition of melatonin after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ◆: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 0.5µM melatonin.○: spermatozoa incubated in CM in the presence of 1µM melatonin. ●: spermatozoa incubated in CM in the presence of 5µM melatonin. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means± S.E.M. for 6 separate experiments.

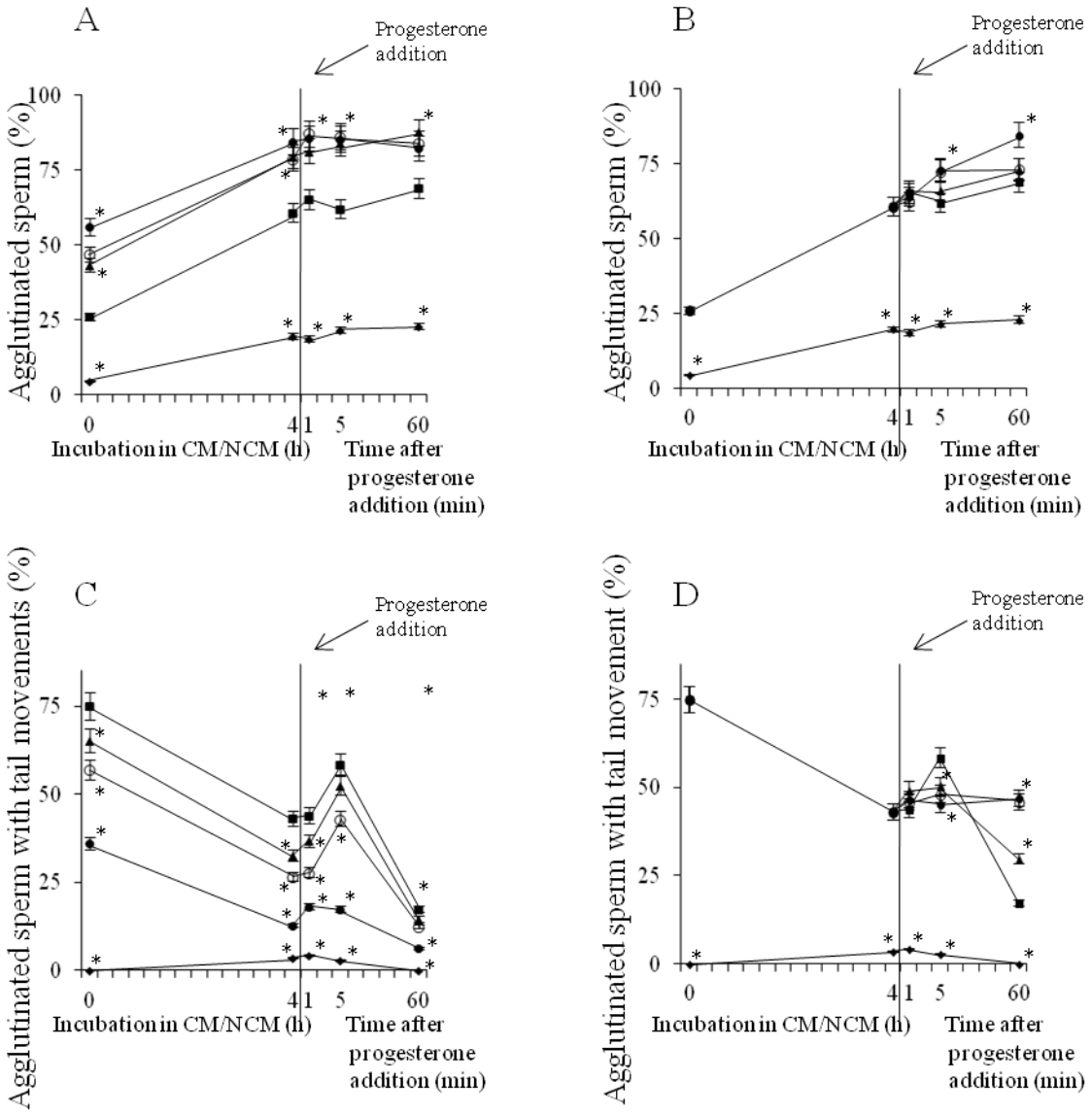


Figure 3. **Effects of melatonin on percentages of agglutinated sperm and agglutinated sperm with noticeable tail movements of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of agglutinated sperm. C,D): percentages of agglutinated sperm with tail movement. A,C): results obtained after incubation of sperm in the presence of melatonin from the time 0h of incubation. B,D): results obtained through the addition of melatonin after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ◆: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 0.5µM melatonin.○: spermatozoa incubated in CM in the presence of 1µM melatonin. ●: spermatozoa incubated in CM in the presence of 5µM melatonin. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means± S.E.M. for 6 separate experiments.

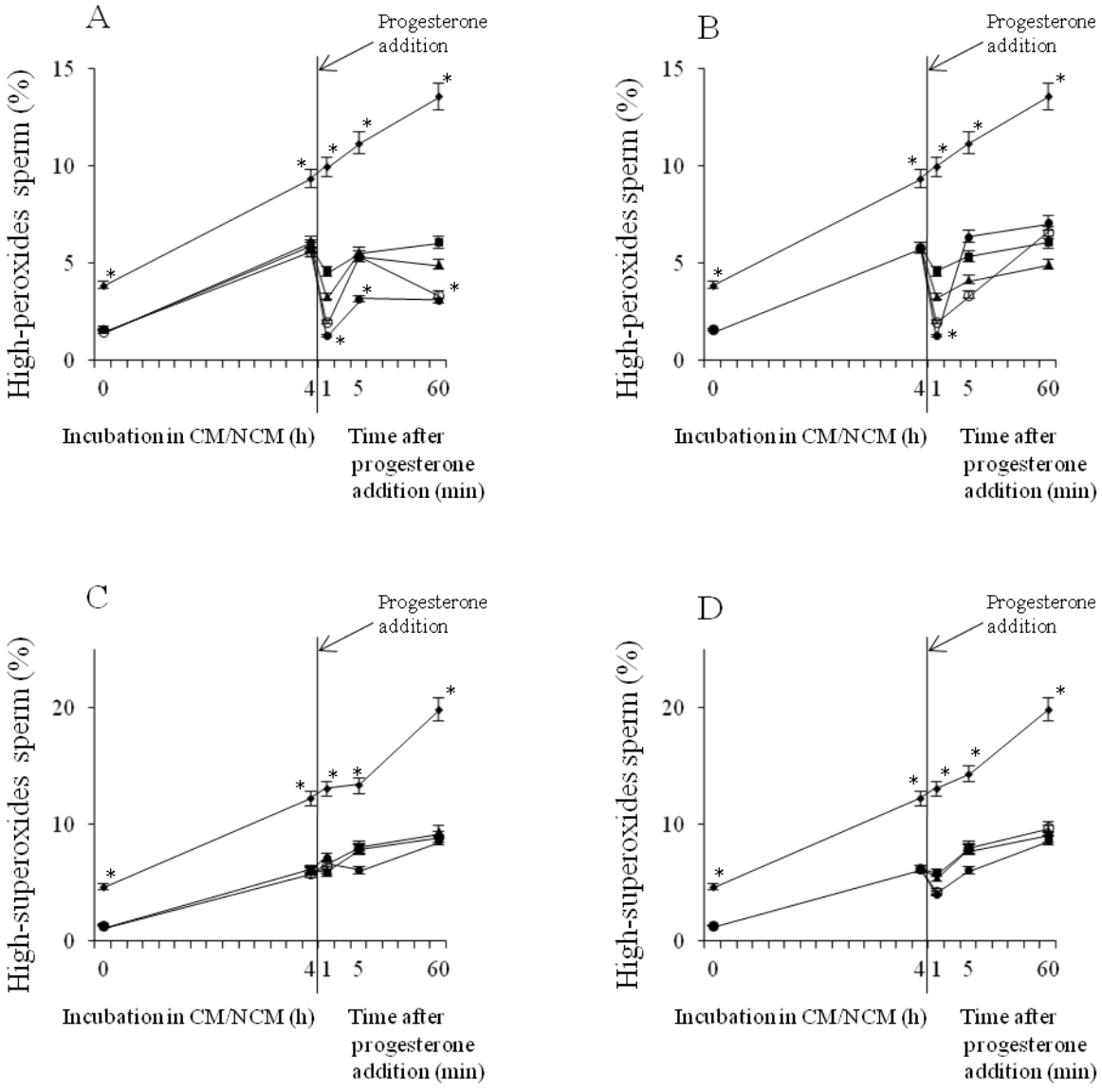


Figure 4. **Effects of melatonin on percentages of cells with high intracellular peroxides and superoxides levels of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of high peroxides. C,D): percentages of high superoxides. A,C): results obtained after incubation of sperm in the presence of melatonin from the time 0h of incubation. B,D): results obtained through the addition of melatonin after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ◆: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 0.5µM melatonin.○: spermatozoa incubated in CM in the presence of 1µM melatonin. ●: spermatozoa incubated in CM in the presence of 5µM melatonin. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means± S.E.M. for 6 separate experiments.

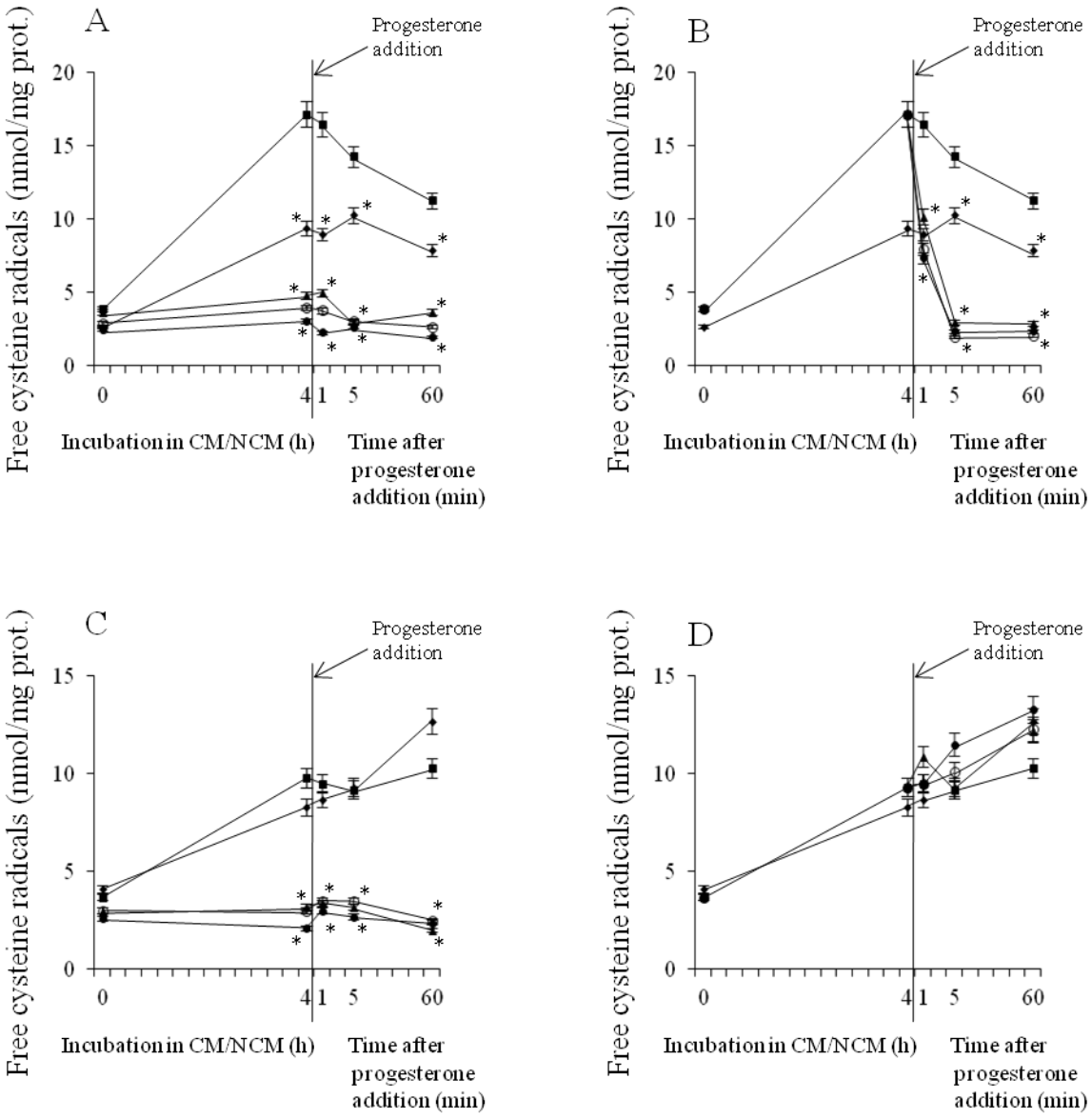


Figure 5. **Effects of melatonin on intracellular free cysteine radicals levels from both heads and tails extracts of boar sperm subjected to *in vitro* capacitation and subsequent**

progesterone-induced acrosome exocytosis.

Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. Sperm were subjected to IVC and further IVAE as described in the Material and Methods section. Likewise, determination techniques of free cysteine radical levels in both head extracts (A, B) and tail ones (C, D) have been also described in the Material and methods section. A,C): results obtained after incubation of sperm in the presence of melatonin from the time 0h of incubation. B,D): results obtained through the addition of melatonin after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ◆: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 0.5µM melatonin.○: spermatozoa incubated in CM in the presence of 1µM melatonin. ●: spermatozoa incubated in CM in the presence of 5µM melatonin. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means± S.E.M. for 6 separate experiments.

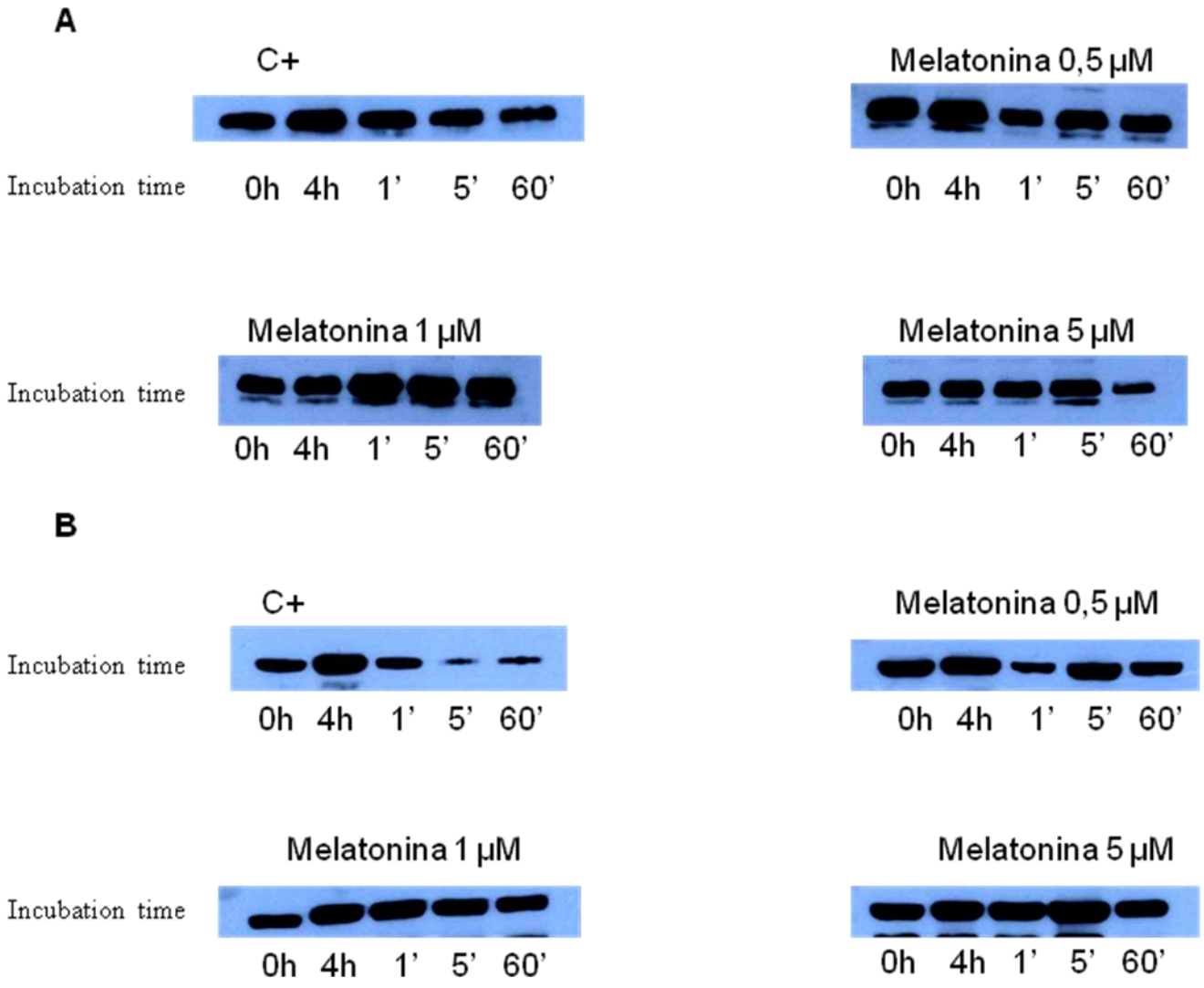


Figure 6. **Effects of melatonin added at the 0h time of incubation on the tyrosine phosphorylation intensity levels of the P32 protein in boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**
Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. A): P32 bands obtained through the Western blot analysis. B): β-tubulin

bands obtained in the corresponding Western blot in which P32 phosphorylation has been previously analyzed. In this case, both P32 and β-tubulin bands are from the same samples. C+: sperm incubated in the CM medium. 0.5µM melatonin: cells incubated in the CM medium and added with 0.5µM melatonin from the 0h time of incubation. 1µM melatonin: cells incubated in the CM medium and added with 1µM melatonin from the 0h time of incubation. 5µM melatonin: cells incubated in the CM medium and added with 5µM melatonin from the 0h time of incubation. 0h,4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Bands shown here are representative for 5 separate experiments.

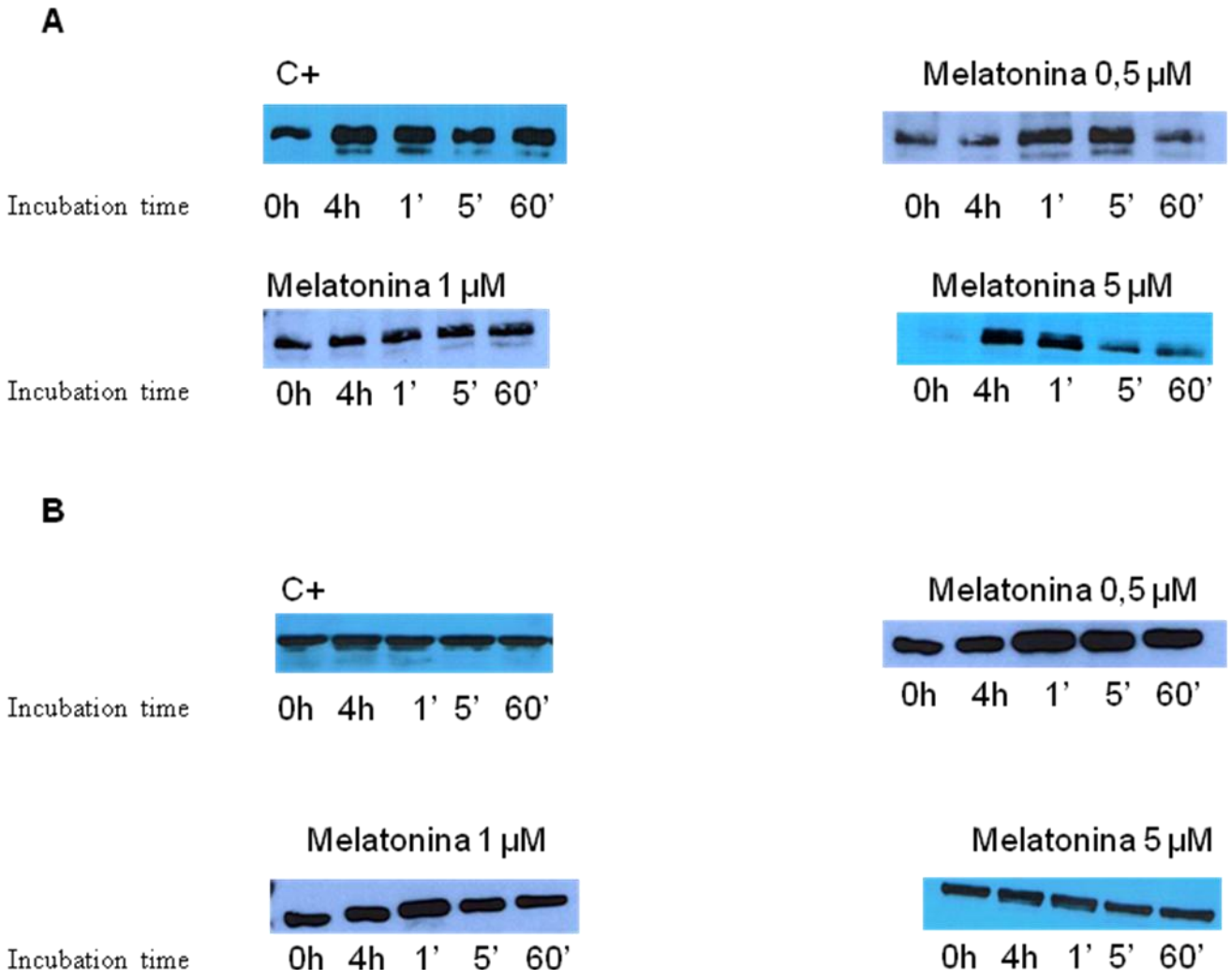


Figure 7. **Effects of melatonin added after 4h of incubation together with progesterone on the tyrosine phosphorylation intensity levels of the P32 protein in boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. A): P32 bands obtained through the Western blot analysis. B): β-tubulin

bands obtained in the corresponding Western blot in which P32 phosphorylation has been previously analyzed. In this case, both P32 and β-tubulin bands are from the same samples. C+: sperm incubated in the CM medium. 0.5μM melatonin: cells incubated in the CM medium and added with 0.5μM melatonin after 4h of incubation together with progesterone. 1μM melatonin: cells incubated in the CM medium and added with 1μM melatonin after 4h of incubation together with progesterone. 5μM melatonin: cells incubated in the CM medium and added with 5μM melatonin after 4h of incubation together with progesterone. 0h, 4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Bands shown here are representative for 5 separate experiments.

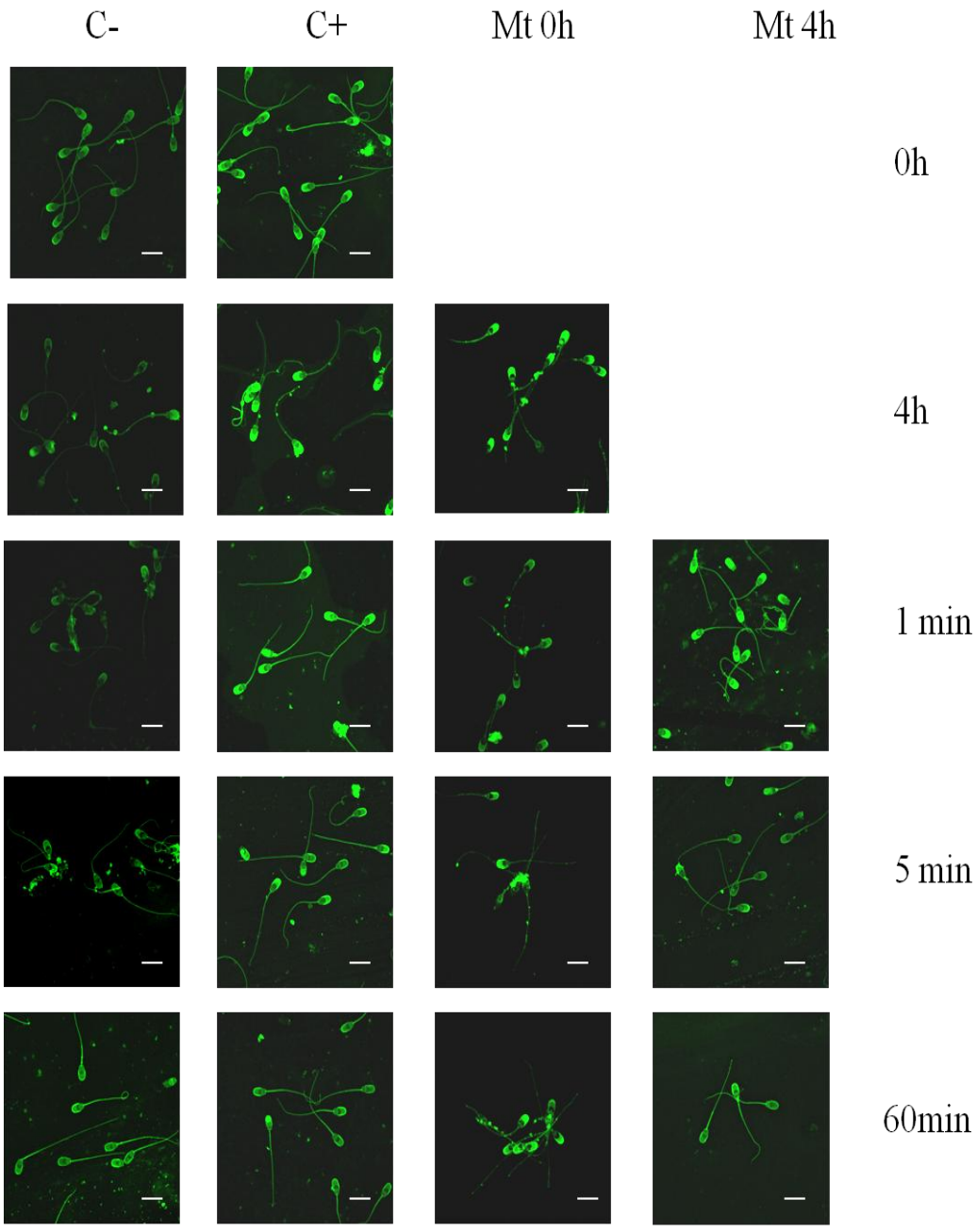

C-
C+
Mt 0h
Mt 4h
0h
4h
1 min
5 min
60min

Figure 8. **Effects of 1μM melatonin on the WGA lectin distribution of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C-: cells incubated in the NCM medium. C+: sperm incubated in the CM medium. Mt 0h: sperm incubated in the CM and added with 1μM melatonin at the time 0h of incubation. Mt 4h: cells incubated in the CM medium and added with 1μM melatonin after 4h of incubation together progesterone. 0h, 4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Figure shows representative images for 5 separate experiments. Bars indicate a size of 20 μm.

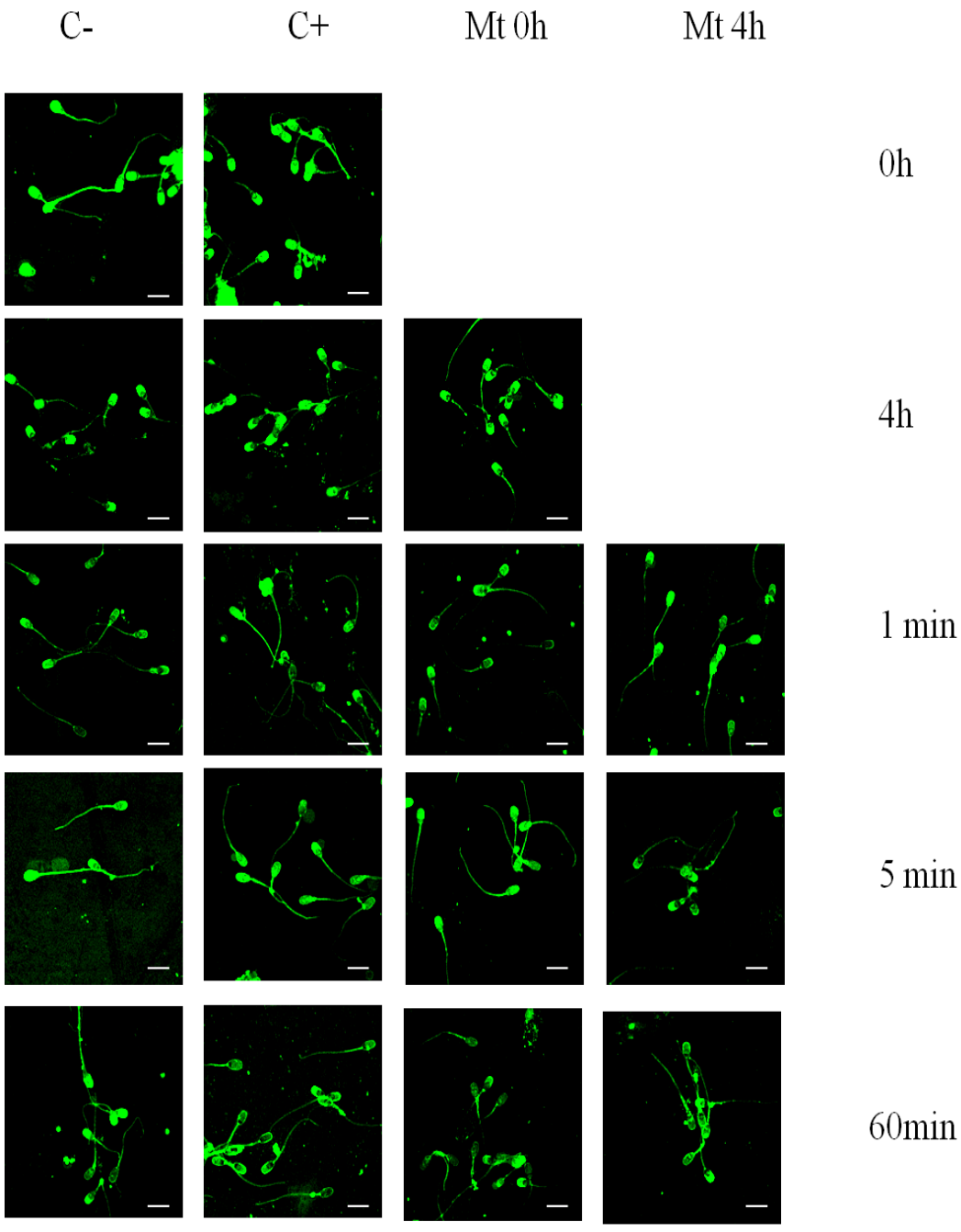

C-
C+
Mt 0h
Mt 4h
0h
4h
1 min
5 min
60min

Figure 9. **Effects of 1µM melatonin on the STL lectin distribution of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C-: cells incubated in the NCM medium. C+: sperm incubated in the CM medium. Mt 0h: sperm incubated in the CM and added with 1µM melatonin at the time 0h of incubation. Mt 4h: cells incubated in the CM medium and added with 1µM melatonin after 4h of incubation together progesterone. 0h, 4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Figure shows representative images for 5 separate experiments. Bars indicate a size of 20 µm.

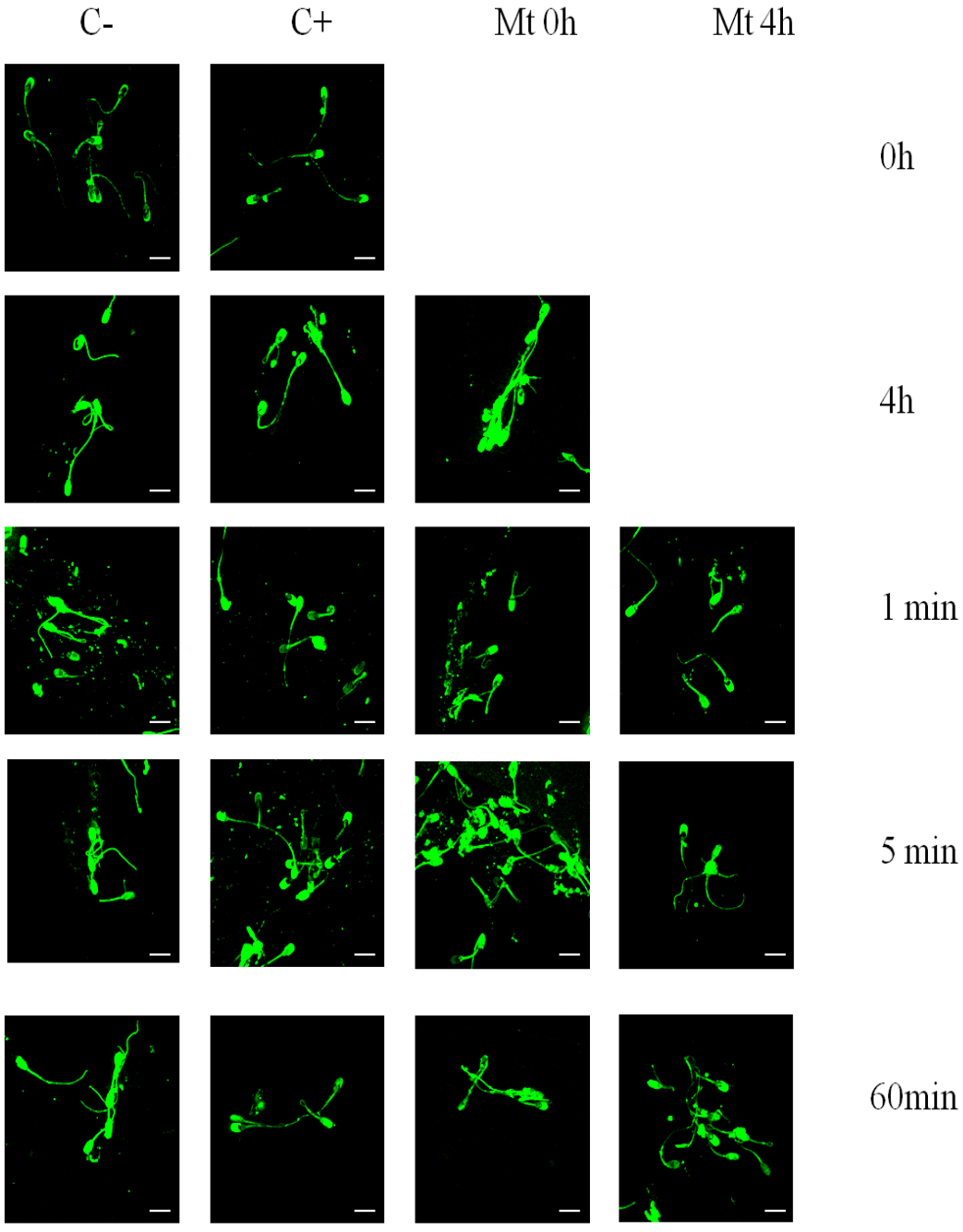

C-
C+
Mt 0h
Mt 4h
0h
4h
1 min
5 min
60min

Figure 10. **Effects of 1µM melatonin on the PSA lectin distribution of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C-: cells incubated in the NCM medium. C+: sperm incubated in the CM medium. Mt 0h: sperm incubated in the CM and added with 1µM melatonin at the time 0h of incubation. Mt 4h: cells incubated in the CM medium and added with 1µM melatonin after 4h of incubation together progesterone. 0h, 4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Figure shows representative images for 5 separate experiments. Bars indicate a size of 20 µm.

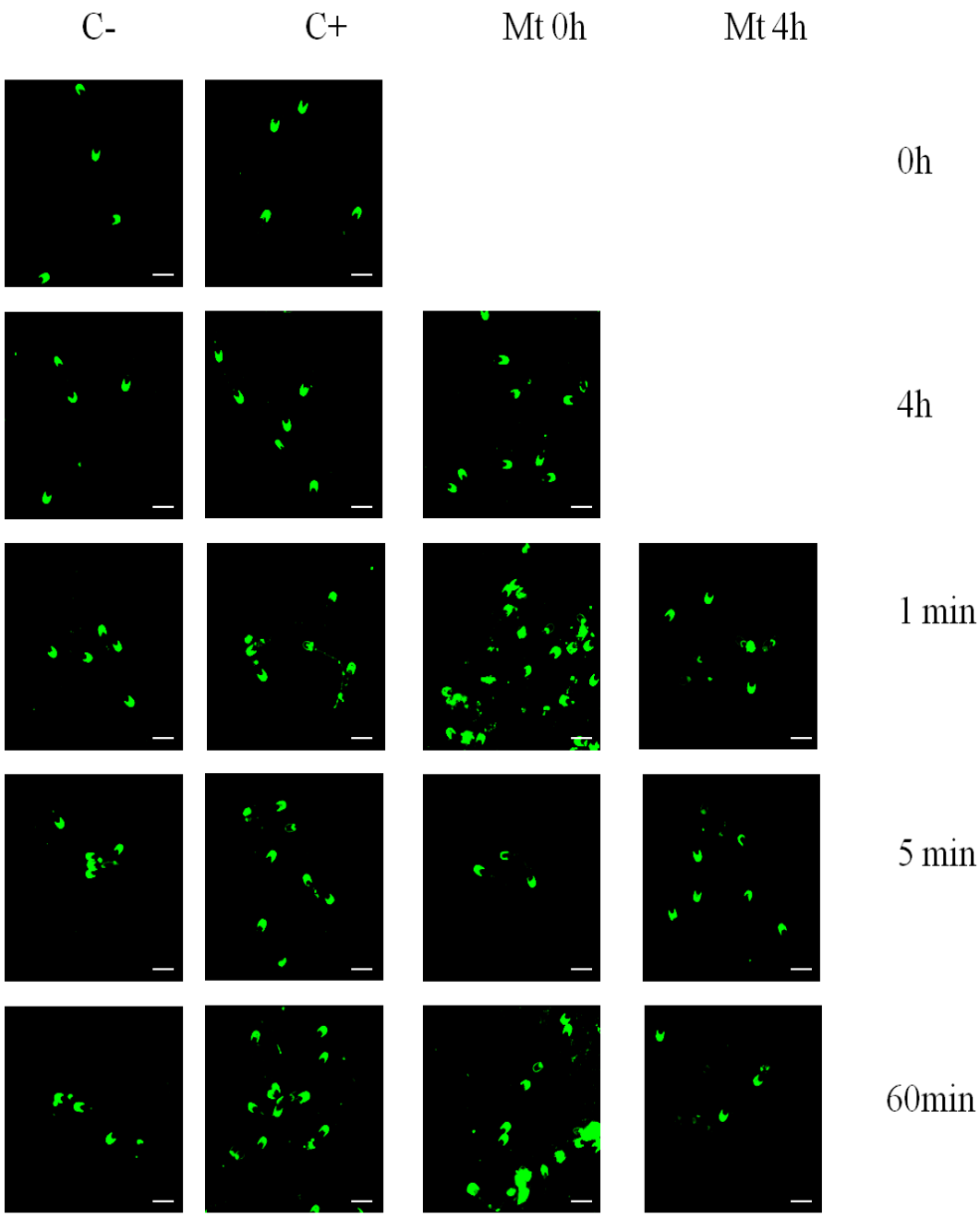
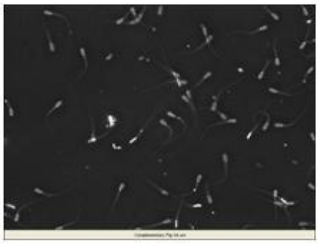

C-
C+
Mt 0h
Mt 4h
0h
4h
1 min
5 min
60min

Figure 11. **Effects of 1µM melatonin on the PNA lectin distribution of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

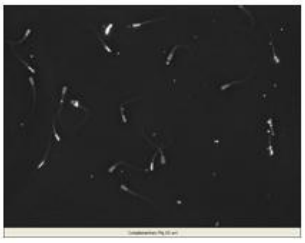
Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C-: cells incubated in the NCM medium. C+: sperm incubated in the CM medium. Mt 0h: sperm incubated in the CM and added with 1µM melatonin at the time 0h of incubation. Mt 4h: cells incubated in the CM medium and added with 1µM melatonin after 4h of incubation together progesterone. 0h, 4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Figure shows representative images for 5 separate experiments. Bars indicate a size of 20 µm.

A



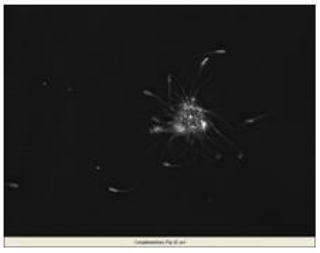
B

C



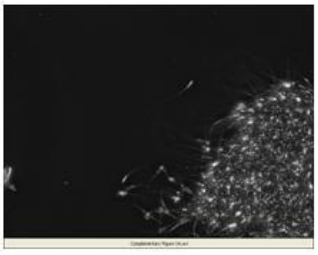
D

E



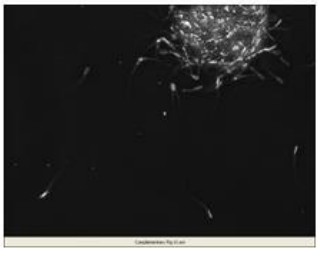
F

G



H

I



J

Complementary Figure 1. **Images showing the effect of 1µM melatonin on the formation of cell agglutinations in boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.**

The CASA analysis and the achievement of both IVC and IVAE have been described in the Material and Methods section. The AVI files shown here are representative for 7 separate experiments. A: general view of sperm incubated in NCM at 0h of incubation. B: sperm incubated in NCM after 4h of incubation. C: general aspect of sperm incubated in NCM after 60 min of progesterone addition subsequently to a previous incubation in NCM for 4h. D: sperm incubated in CM at 0h of incubation. E: sperm incubated in CM after 4h of incubation. F: sperm incubated in CM after 60 min of progesterone addition subsequently to a previous incubation in CM for 4h. G: sperm incubated in CM and added with 1µM melatonin at 0h of incubation. H: General view of sperm incubated in CM and added with 1µM melatonin after 4h of incubation. I: view of sperm incubated in CM and added with 1µM melatonin after 60 min of progesterone addition subsequently to a previous incubation in CM with melatonin for 4h. J: view of sperm incubated in CM for 4h and then added with 1µM melatonin and progesterone together in an AVI clip taken 60 min after the addition of melatonin and progesterone.

Part 4. Conclusions

9. Conclusions

Results yielded in this PhD thesis would contribute to a better comprehension of the molecular mechanisms launched during capacitation and acrosome reaction of boar sperm. Thus, we tested different molecules recognized as antioxidants to observe how mammalian sperm achieve capacitation through a sequence of biochemical modifications that lead to the establishment of fully fertilizing ability.

In particular, the first research showed that reduced glutathione (GSH) caused changes on "*in vitro*" acrosome reaction (IVAE) only when added at the start of the incubation in a capacitating medium (CM). This fact could suggest that GSH effects on IVAE are linked to previous actions of GSH on capacitation-launching mechanisms. Thus, when GSH was added at the start of the incubation, the effector inhibited the observed capacitation-related increase of free cysteine levels in sperm head. Moreover, the addition of both GSH and progesterone together after 4h of incubation in the CM exacerbates the observed decrease in sperm incubated in the CM alone. Similarly, incubation of boar sperm in the CM induced a parallel increase in the levels of free cysteine in the sperm tail, although this increase was maintained after induction of the acrosome exocytosis. These results can be explained taking as a basis the protective role of GSH on disulfide bonds. This protective action would be due to the antioxidant action of GSH, which includes a potent anti-ROS effect. These results are in accordance with previous researches that have demonstrate that levels of intact disulfide bonds have been connected to processes such as cryoresistance and achievement of a feasible *in vitro* capacitation (Chatterjee et al., 2001; Jacob et al., 2003).

The aim of second work was to study effect of melatonin in the achievement of feasible IVAE induced by progesterone in boar spermatozoa. Again, we centred our study on an antioxidant agent, since melatonin acts as a potent free radical scavenger with a great ability to pass trough plasma membrane. The combination of both factors implies that melatonin would effectively counteract the deleterious effects of an excessive intracellular ROS exposition (Reiter et al., 2000). The most evident effect of this study it has been observed on sperm motility. Thus, the addition of 5μM melatonin induced a rapid and intense drop of total sperm motility, mainly due to an increase in the number of agglutinated sperm. In this way, it would be possible to hypothesize that melatonin would to stimulate the launching of capacitation process, since the head-to-head agglutination process is a phenomenon associated with the status of capacitation acquired by sperm cells (Harayama and Kato, 2002). Furthermore, likewise to GSH, melatonin protects sperm disulfide bonds, as indicated by the results obtained through the analysis of intracellular free cysteine levels. In fact, melatonin would be involved in the reduction of free cysteine radicals after the launching of IVAE. In this way, our results seem to suggest that the presence of melatonin provokes a recovery of the disulfide bonds of head sperm, preventing a premature nuclear decondensation process that would be necessary to the sperm after the oocyte penetration.

The overall analysis of the obtained results, thus, seem to indicate that boar sperm progesterone-induced IVAE may be, at least partially modulated through GSH- and melatonin-sensitive mechanisms. These mechanisms would be thus linked to the regulation of the stability of disulfide bonds. In turn, the stability of these bonds would be related with the launching of phenomena like IVAE, since disulfide bonds are linked to the maintenance of proper motility and optimal nuclear sperm condensation status. In this way, the maintenance of proper disulfide bonds levels would be instrumental in launching subsequent optimal oocyte

penetration and further syngamia. Further researches are necessary to fully understand the effect of both GSH and melatonin on the IVAE induction and the possible influences of both effectors on the specific *in vitro* fertilizing ability of boar spermatozoa in order to consider the possibility of utilizing GSH and melatonin to improve the efficiency of *in vitro* fertilization techniques in pig.

10. Appendix

Publications derived from the Doctoral Thesis

The results obtained in the present Doctoral Thesis have been included in the following publica tions:

1. Articles submitted in journals in the science citation index (SCI) of the institute for scientific information (ISI):
    - ✓ Rafael Pedroso Betarelli & **Martina Rocco**; Marc Yeste; Anna Placci; Barbara Azevedo Pereira; Efren Estrada; Alejandro Peña, Márcio Gilberto Zangeronimo and Joan Enric Rodríguez-Gil (2016) Effects of reduced glutathione on in vitro boar sperm capacitation and acrosome exocytosis. Under review (In: *Molecular of Human Reproduction*).

    - ✓ **Rocco M.** & Bettarelli R.P., Placci A., Fernández -Novell J.M., Spinaci M., Muiño -Blanco T., Casao A., Cebrián-Pérez J.A., Peña A., Rigau T., Bonet S., Yeste M., Rodríguez-Gil J.E. (2017) Melatonin impedes the loosing of head disulfide bondsand induces agglutination during the achievement boar sperm *in vitro* capacitation and in vitro acrosome exocytosis. Under review (In: *Molecular of Human Reproduction*).

2. Other articles:

    - ✓ Michele Di Iorio, Angelo Manchisi, **Martina Rocco**, Peter Chrenek, Nicolaia Iaffaldano (2014) Comparison of different extenders on the preservability of rabbit semen stored at 5°C for 72 hours. *Italian Journal of Animal Science*, 13: 710-714. doi:10.4081/ijas.2014.3444

3. Abstracts in journals included in the science citation index (SCI) and communication to congress:

   - ✓ **Rocco M.**, Pedroso Betarelli R., Azevedo Pereira B., Manchisi A., Yeste M., Rodríguez-Gil J.E. 2016. Effects of reduced glutathione upon intracellular peroxide and superoxide levels of boar sperm during in vitro capacitation and progesterone-induced acrosome exocytosis. International Ph.D. Workshop on "Welfare, Biotechnology and Quality of Animal Production", Nitra (Slovak Republic), 8 December, 2016, p.163.

   - ✓ Bettarelli R.P., Silva W.E., **Rocco M.**, Rodríguez-Gil J.E., Resende C.O., Rabelo S.S., Zangeronimo M.G. 2016. Effects of reduced glutathione on the free cysteine radicals and reactive oxygen species levels in boar sperm during the in vitro capacitation.VI International Symposium on Animal Biology of Reproduction (VI ISABR 2016), Campos do Jordão, (Brasil), 6-9 November 2016.

   - ✓ Bettarelli R.P., Rivera del Alamo M., Rigau T., **Rocco M.**, Zangeronimo M.G., Rodríguez-Gil J.E. 2016. Reduced glutathione prevents the observed increases in intracellular levels of both free cysteine radicals and overall reactive oxigen species in boar sperm subjected to "in vitro" capacitation. 20th Annual ESDAR Conference Lisbon (Portugal), 27-29 October 2016.

   - ✓ Ferreira E. R., Pedroso Betarelli R., Rodríguez-Gil J.E., **Rocco M**., Kaiser E., Zangeronimo M. G. 2016. Concentrações intracelulares de espécies reativas de oxigẽnio durante a capacitação in vitro em espermatozoides suínos. XXIX

Congresso de Iniciação Cientifica de UFLA, Universita de Lavras (Brasil), 26-30 September 2016.

- ✓ Iaffaldano N., Di Iorio M., **Rocco M**., Manchisi A., Rosato M.P. (2013) Effects of two different concentration of dimethylsulfoxide on the quality of turkey semen cryopreserved in straws over liquid nitrogen vapor. XX National Congress ASPA Bologna (Italia), 11-13 Giugno 2013, Italian Journal of Animal Science, 12(1):44.

- ✓ Iaffaldano N., Di Iorio M., **Rocco M**., Manchisi A., Rosato M.P., Paventi G., Pizzuto R., Passarella S. 2013. He-Ne laser irradiation effects on cryopreserved ram semen. XX National Congress ASPA Bologna (Italia), 11-13 Giugno 2013, Italian Journal of Animal Science, 12(1):76.

- ✓ Iaffaldano N., Di Iorio M., **Rocco M**., Manchisi A., Rosato M.P. 2013. Cryosurvival of turkey semen at different freezing rates. XX National Congress ASPA Bologna (Italia), 11-13 Giugno 2013, Italian Journal of Animal Science, 12(1):132.

10.1. List of Abbreviations:

Abbreviation	Meaning
A	axoneme
AAAD	aromatic-L-amino-acid decarboxylase
ALH	amplitude of lateral head displacement
AP	acrosomal protuberance
Ar	acrosomal region
AR	acrosome reaction
AS	shape abnormalities of spermatozoa
ASMT	hydroxyindol-O-methyltransferase
ATP	adenosine triphosphate
BCF	head beat-cross frequency
BM	inner mitochondrial membrane
BSA	bovine serum albumin
cAMP	cyclic adenosine monophospahate
CASA	Computer Assisted Sperm Analysis
CM	capacitation medium

DCF	2′,7′-dichlorofluorescein
DCFH-DA	dichlorofluorescin diacetate
DHE	dihydroethidium
DNA	deoxyribonucleic acid
FD	dense fibres
GCL	gamma glutamylcysteine synthetase enzyme
GlcNAc	N-acetylglucosamine
GP	peripheral granules
GPPCRs	G protein-coupled cell surface
GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	glutathione oxidized form
H_2O_2	hydrogen peroxide
HOST	hypo-osmotic swelling test
HRT	hyper-osmotic resistance test
IVAE	*in vitro* progesterone-induced acrosome exocytosis
IVC	*in vitro* capacitation

JC-1	5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolcarbocyanine iodide
LIN	linearity
M-540	merocyanine fluorochrome
NAT	N-acetyltransferase
NO	nitrogen oxide
$O_2^{-\bullet}$	superoxide anion radical
ORT	osmotic resistance test
OS	oxidative stress
P4	progesterone
PI	propidium iodide
PKA	protein kinase A activated
PKC	protein Kinase C
PLA_2	phospholipases A_2
PLC	phospholipases C
PNA	*Arachis Hypogaea* agglutinin
PR	postacrosomal region

PSA	*Pisum Sativum* agglutinin
QR2	enzyme quinone reductase 2
RNS	reactive nitrogen species
ROR	retinoid acid receptor-related orphan receptor
ROS	reactive oxygen species
RZR	retinoid Z receptor
SCDt	sperm chromatin dispersion test
SNAT	serotoninN-acetyltransferase
SNC	central nervous system
SR	sperm reservoir
ST	seminiferous tubules
STL	*Solanum lycopersicum* lectin
STR	straightness
T5M	tryptophan-5-hydroxylase
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity

WGA	wheat germ agglutinin
WOB	wobbleness
ZP3	zona pellucida protein
$\Delta\psi m$	mitochondrial membrane potential

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10.3. List of Images:

Fig 1. General representation of genital apparatus of boar: Singleton W. (1997) Diagram of boar reproductive system, Purdue University. *https://www.extension.purdue.edu*

Fig 1. Pietrain boar: *http:// www.upbgeneticworld.com*

Fig 1.2. -1.8. Particulars of boar reproductive system: Dwane Davis picture: *http://www.powershow.com/view1/58067*

Fig 1.4. Rete testis image: *http://nabilariyantidclovers.blogspot.com.es*

Fig 2. Sperm cell structure: *http://www.slideshare.net/SSpencer53/ch16appt-reproductive*

Fig 2.1. Sperm head: Briz M., Fabrega A. (2013) Boar Reproduction: Fundamentals and New Biotechnological Trends. *Springer Science & Business*.

Fig 2.2. Tail sperm structure:
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Fig 2.3. Mitochondrial structure: Bonet S., Briz M., Pinart E., Sancho S., García-Gil N., Badia E. (2000) Morphology of boar spermatozoa. *J, eds. Insititu d'Estudis Catalans,* Barcelona (Spain), 8-47283-533-2.

Fig 2.4. Sperm tail structure citoskeletal: Claire L., Borg K., Wolski M., Gerard M., Gibbs M., O'Bryan K. (2009) Phenotyping male infertility in the mouse: how to get the most out of a 'non-performer. *Hum. Reprod. Update*, 16(2): 205-24.

Fig 3. Journey of sperm: López-Úbeda R.., Matás C. (2015) An Approach to the Factors Related to Sperm Capacitation Process. *Andrology*, 4: 128.

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Fig 5.1. Acrosome reaction: Wassarman P.M. (1999) Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*, 96: 175– 183.

Fig 6. CASA system: Scheme of different velocities and parameters of sperm movement measured by CASA system. *From: http//www.mibio.org/en/metrics-used*

Fig 6.1. Sperm anomalies: Pinart E., Camps R., Briz M., Bonet S., Egozcue J. (1998) Unilateral spontaneous abdominal cryptorchidism: structural and ultrastructural study of sperm morphology. *Anim Reprod Sci.*, 49: 247–268.

Fig. 6.2. Viability with eosin-nigrosin: example of eosin- nigrosin stain in boar sperm. From: *http://www.thepigsite.com/articles/2596/quality-control-of-extended-boar-semen*

Fig 6.3. Viability with SYBR/ PI: Pilane C., Audrey Bopape M., Helen Mapeka M., Richard Netshirovha T. (2016) Assessment of the Susceptibility of Boar Semen to Oxidative Stress. *Open Journal of Animal Sciences*, 6: 123-130.

Fig 6.4. Representation of Western blotting technique in principal four steps: Paint image.

Fig. 6.5. Schematic procedure used for the evaluation of free-cysteine residues in sperm cells nucleoproteins: Paint image.

Fig 6.6. Halomax imagine: Alkmin D.V., Martinez-Alborcia M.J., Parrilla I., Vazquez J.M., Martinez E.A., Roca J. (2013) The nuclear DNA longevity in cryopreserved boar spermatozoa assessed using the Sperm-Sus-Halomax. *Theriogenology*, 79 (9): 1294–1300.

10.4. Glutathione image reference:

Fig 7. Glutathione synthesis pathway: Emmett M. (2014) Acetaminophen Toxicity and 5-Oxoproline (Pyroglutamic Acid): A Tale of Two Cycles, One an ATP-Depleting Futile Cycle and the Other a Useful Cycle. *Clin. J. Am. Soc. Nephrol.*, 9: 191–200.

10.5. Melatonin images reference:

Fig 8. Biosynthesis of melatonin from tryptophan in mammalian pinealocytes: *http://photobiology.info/Tosini.html*

Fig 8.1. Melatonin secretion: *https://sadam001.files.wordpress.com/2014/05/2.png*