Universitat de Girona

Facultat de Ciències | Memòria del Treball Final de Grau

Títol del treball:

Bibliographic review on the molecular mechanisms involved in epididymal maturation and sperm capacitation

Revisió bibliogràfica sobre els mecanismes moleculars involucrats en la maduració epididimària i la capacitació espermàtica

---

Estudiant: Alejandra Portavella Gramunt | Grau en Biologia

Correu electrònic: alejandraportavella@me.com

Tutor: Elisabeth Pinart Nadal

Empresa / institució: Universitat de Girona

Vistiplau tutor:

Nom del tutor: Elisabeth Pinart Nadal

Empresa / institució: Universitat de Girona

Correu electrònic: elisabeth.pinart@udg.edu

Data de dipòsit de la memòria a secretaria de coordinació: 22 de maig de 2018

RESUM

Els espermatozoides testiculars dels mamífers no tenen la capacitat de moure's ni de fecundar oòcits. Per tal d'adquirir aquestes dues capacitats, han de sotmetre's a dos processos de maduració extratesticular: la maduració epididimària en el tracte reproductor masculí i la capacitació espermàtica en el tracte reproductor femení. Durant la maduració epididimària, els espermatozoides experimenten modificacions estructurals i bioquímiques modulades per la composició del fluid epididimari. Entre aquestes modificacions cal destacar: canvis en la composició de fosfolípids de membrana i en la proporció de colesterol/fosfolípids, augment de la càrrega negativa superficial neta i de la quantitat d'enllaços disulfur, alteració de les proteïnes i els antígens superficials, i migració de la gota citoplasmàtica. Després d'aquest procés, els espermatozoides queden emmagatzemats al cauda epididimari en un estat quiescent fins a la seva ejaculació.

La maduració epididimària i l'ejaculació són esdeveniments clau per a la posterior capacitació, ja que només els espermatozoides ejaculats són capaços de capacitar-se. La capacitació és un procés complex de canvis estructurals i funcionals que es produeixen durant el trànsit a través del tracte reproductor femení i que finalitza quan els espermatozoides travessen la zona pel·lúcida gràcies a la reacció acrosòmica. La capacitació implica modificacions en la distribució de proteïnes superficials de membrana, alteracions en les característiques de la membrana plasmàtica, canvis en l'activitat enzimàtica i modulació de l'expressió dels components intracel·lulars. Tots aquests canvis es produeixen com a conseqüència de l'activació de diverses rutes de senyalització.

Tant la maduració epididimària com la capacitació espermàtica són processos absolutament necessaris per a la generació d'espermatozoides amb capacitat fecundant. És per això que, l'estudi dels mecanismes moleculars que regulen aquests dos processos és molt important. Aquesta revisió pretén compilar tota la informació que s'ha anat acumulant en les últimes dècades sobre els mecanismes moleculars implicats en la maduració epididimària i la capacitació espermàtica i, la comprensió dels mecanismes moleculars mitjançant els quals els espermatozoides adquireixen la capacitat de moure's i fecundar l'oòcit.

RESUMEN

Los espermatozoides testiculares de los mamíferos no tiene la capacidad de moverse ni de fecundar ovocitos. Para adquirir estas habilidades, deben someterse a dos procesos de maduración extratesticular: la maduración epididimaria en el tracto reproductivo masculino y la capacitación espermática en el tracto reproductivo femenino. Durante la maduración epididimaria, los espermatozoides experimentan modificaciones estructurales y bioquímicas moduladas por la composición del líquido epididimal. Entre estas modificaciones destacan: cambios en la composición de los fosfolípidos de membrana y en la relación colesterol/fosfolípidos, aumento en la carga neta superficial negativa y en el número de enlaces disulfuro, alteración de las proteínas y los antígenos de superficie, y migración de la gota citoplasmática. Después de este proceso, los espermatozoides permanecen almacenados en la cauda epididimario en un estado quiescente hasta su eyaculación.

La maduración epididimaria y la eyaculación son eventos clave para la posterior capacitación, ya que solo los espermatozoides eyaculados son capaces de capacitarse. La capacitación es un proceso complejo de cambios estructurales y funcionales que tienen lugar durante el tránsito a través del tracto reproductivo femenino, y se completa cuando los espermatozoides pueden atravesar la zona pelúcida gracias a la reacción acrosómica. La capacitación implica modificaciones en la distribución proteinas de la superficie espermática, alteraciones en las características de la membrana plasmática, cambios en la actividad enzimática y modulación de la expresión de constituyentes intracelulares. Todos estos cambios ocurren como resultado de la activación de varias vías de señalización.

El estudio de los mecanismos moleculares que sustentan la maduración epididimaria y la capacitación espermática son muy importantes ya que ambos procesos son absolutamente necesarios para generar espermatozoides con capacidad fecundante. Esta revisión pretende recopilar toda la información que se ha descubierto en las últimas décadas sobre los mecanismos moleculares implicados en la maduración epididimaria y la capacitación espermática, y comprender los mecanismos moleculares por los cuales los espermatozoides adquieren un movimiento progresivo y la capacidad de fecundar ovocitos.

ABSTRACT

Testicular spermatozoa of all mammalian species are unable to move progressively or fertilize oocytes. To acquire these two abilities, they must undergo two extratesticular maturational processes: epididymal maturation in the male reproductive tract and sperm capacitation in the female reproductive tract. During epididiymal maturation, spermatozoa undergo structural and biochemical modifications which are modulated by the composition of the epididymal fluid. Among these modifications to highlight: changes in membrane phospholipid composition and in cholesterol/phospholipid ratio, increase in net surface negative charge, increases in the number of disulfide bonds, modification, elimination or addition of surface protein, relocalization of surface antigens, and migration of the cytoplasmic droplet. After this process, spermatozoa are stored in the epididymal cauda until ejaculation.

Epididymal maturation and ejaculation are key events for further capacitation, since only ejaculated spermatozoa are capable to undergo this process. Capacitation is a complex process of structural and functional changes that takes place during the transit through the female reproductive tract, and it is completed when spermatozoa are able to penetrate zona pellucida by undergoing the acrosome reaction. This process involves modifications in sperm surface protein distribution, alterations in the plasma membrane characteristics, changes in enzymatic activity and modulation of expression of intracellular constituents. All these changes occur as a result of the activation of signaling pathways cascades.

Both, epididymal maturation and sperm capacitation are absolutely needed in order to generate fertile spermatozoa. This makes the study of the molecular mechanisms underpinning this two processes very important. This review aims to compile all the information that has been discovered in recent decades about the molecular mechanisms of epididymal maturation and sperm capacitation, and to understand the molecular mechanisms by which spermatozoa acquire fertilizing ability and motility.

ACKNOWLEDGES

I would like to thank my tutor, Dr. Elisabeth Pinart, for the time devoted to my work and her great help and guidance during all the process.

CONTENTS

1. INTRODUCTION

After leaving the testis, mammalian spermatozoa are unable to move progressively or fertilize an oocyte. To acquire these two abilities, they must undergo two extratesticular maturational processes: epididymal sperm maturation in the male reproductive tract and capacitation in the female reproductive tract (Fàbrega *et al.*, 2011). These two processes are associated with biochemical changes occurring in different sperm compartments (Gervasi & Visconti, 2016).

Epididymal maturation involves changes in spermatozoa which take place during their transit through the epididymis. These changes give functionality to the spermatozoa and the ability to capacitate in the female reproductive tract (De Jonge, 2005, 2017). The epididymal epithelium secretes proteins, under androgenic stimulation, that interact with the maturing spermatozoa to generate fully functional gametes (Dacheux *et al.*, 2005; Zhou *et al.*, 2018). Epididymal maturation also includes post-translational modifications of endogenous proteins synthesized during spermiogenesis in the testis (Gervasi & Visconti, 2016).

Main molecular modifications that spermatozoa undergo during epididymal maturation are: changes in membrane phospholipid composition and in cholesterol/phospholipid ratio, increases in disulfide bonds and in net surface negative charge, relocalization of surface antigens, modification of surface proteins and, migration of the cytoplasmic droplet (Cooper & Yeung, 2006; Gervasi & Visconti, 2016; De Jonge, 2005, 2017).

Capacitation involves a cascade of controlled biochemical and physiological events that make spermatozoa able to gain hyperactive motility, to adhere to the zona pellucida (ZP), to respond to physiological inducers of the acrosome reaction and, to iniciate the fusion with the oocyte. Such events do not occur synchronously in all spermatozoa (Barbonetti *et al.*, 2008; De Jonge, 2017), and they include: modifications in sperm surface protein distribution, alterations in the plasma membrane characteristics, changes in the enzymatic activities and modulation of the expression of intracellular constituents. The occurence of all these changes is dependent on the activation of signaling pathways cascades (Baldi *et al.*, 2000).

Both, epididymal maturation and sperm capacitation are absolutely needed in order to generate fertile spermatozoa (Fàbrega *et al.*, 2011, 2012). This highlights the relevance of the study of molecular mechanisms underpinning these two processes (Aitken *et al.*, 2007). Despite several studies have been focused on the changes occuring in spermatozoa during epididymal maturation and sperm capacitation, these mechanisms are still not well-kown and much research is still needed to better understand the sequence of events and their impact on sperm fertility.

This review aims to compile all the information that has been compiled in recent decades about the molecular mechanisms of both processes. For this, high impact journals mainly placed on the first quartile (Q1) of the knowledge area from the PubMed have been used.

2. METHODOLOGY

National Center for Biotechnology Information (NCBI) website forms part of the National Library of Medicine and provides acces to biomedical and genomic information. This website contains different bioinformatic portals as: GenBank (information regarding gene sequences), OMIM (information about genetic diseases) and PubMed (base of scientific articles), among others.

This work consists in a bibliographic compilation on the molecular mechanisms involved in epididymal maturation and sperm capacitation in mammalian species. The material used to perform this review has been extracted from the bioinformatic portal PubMed. This portal is a search engine that allows free access to the MEDLINE data base with about 5.000 journals published in more than 70 countries around the world since 1966.

The information of this compilation has been extracted from different articles. Despite, most information has been obtained from Reviews related with epididymal maturation and sperm capacitation, the present work also includes information from specific research articles focused on concrete issues. The words used to find these articles in PubMed were: *testicular spermatozoa, mammalian sperm, spermatogenesis, epididymis*, *epididymal maturation, sperm capacitation* and *acrosome reaction*.

One of the main criteria when looking for a reliable article is the date on which it has been published. For this compilation, articles published from the year 2005 onwards have been considered to have updated information about the topic. However, articles should be as recent as possible because knowledge about this topic changes every day. Another criteria used has been the quartil to which the articles belong, and only those articles published in the first quartile (high impact journals) of the subject area have been selected.

For sections dealing with more general subjects, as the description of the structure and function of the male and female reproductive tract, books of histology and physiology have been used. These books have been provided by the medicine and sciences libraries from the University of Girona and they correspon to the latest edition to ensure that the content is up-to-date.

3. OBJECTIVES

The objectives of this review are:

1. Perform a bibliographic research and compilation of the molecular mechanisms involved in epididymal maturation and sperm capacitation.
2. Define the sequence of events implicated in epididymal maturation and capacitation of mammalian spermatozoa.
3. Understand the molecular mechanisms by which spermatozoa acquire fertilizing ability and motility.

For many decades, several investigations have attempted to clearly define the molecules and processes implicated in epididymal maturation and sperm capacitation. Despite ethical constraits have limited *in vivo* studies however, *in vitro* approaches allow the understanding of epididymal maturation and sperm capacitation in mammalian species (De Jonge, 2005).

4. SPERMATOGENESIS

Male reproductive system consists of the testis, the epididymis, vas deferens, ejaculatory duct, the urethra, accessory glands (the seminal vesicles, the prostate gland and the bulbourethral glands), and the penis (Figure 1). It is responsible for the continous production, nourishment and temporary storage of the haploid male gamete (spermatozoa), and the synthesis and secretion of male sex hormones (androgens) (Kierszenbaum, 2007).

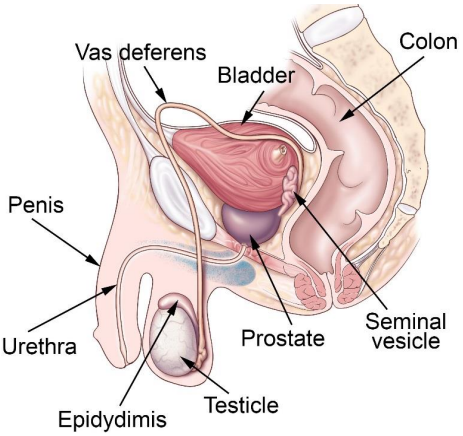

Figure 1. Scheme of the male reproductive system. Cleveland Clinic. (2017). *Male Reproductive System.* Recovered from https://my.clevelandclinic.org/health/articles/9117-male-reproductive-system

The male reproductive functions are controlled by the hypothalamic-pituitary-gonadal axis. The hypothalamus secretes the gonadotropin-realeasing hormone (GnRH) that regulates the release of the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) from the anterior pituitary (Ruwanpura *et al.*, 2010). In the mammalian testis, the main biological activity of LH is to regulate the testosterone production by Leydig cells, which is necessary for spermatogenesis. FSH activates the release of nutrients and growth factors from Sertoli cells, which ensures the survival and proliferation of differentiating spermatogonia (Schulz *et al.*, 2001).

4.1 THE TESTIS

One of the components of the mammalian male reproductive system are the testes (Figure 2). Testes are paired encapsuled organs localted in the scrotum, outside the abdominal cavity. They are covered by a capsule called the tunica albuginea which emits septae from the mediastinum into the testicular mass. In human, septae define 250-300 testicular lobules, each one containing 1-4 seminiferous tubules. The seminiferous tubules run towards the mediastinum where they form the rete testis, a network of convergent tubules that connects the seminiferous tubules with the efferent ducts (Kierszenbaum, 2007).

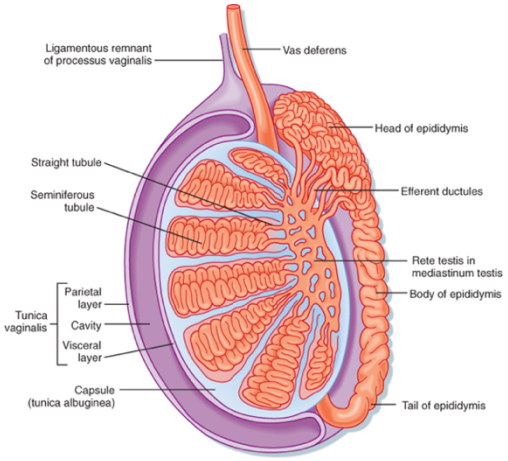

Figure 2. Structure of the mammalian testis. AKB Sir. (2018). *Mammalian Reproductive System*. Recovered from http://www.ibiokaare.com/advance-material/111-mammalian-reproductive-system.html

Testes have two main functions: the production of male germ cells (spermatozoa) through spermatogenesis and the production of steroid hormones (testosterone) assuring the male phenotype and the delivery of differentiated male gametes (Kierszenbaum, 2007; Fayomi & Orwig., 2018)

4.2 SPERMATOGENESIS

Spermatozoa are formed in the seminiferous epithelium and mature during their transit through the epididymis. Seminiferous tubules are formed by a stratified epithelium limited by a basal lamina. The seminiferous epithelium is compered by two cell types: somatic cells (Sertoli cells) and germ cells in different stages of development. Sertoli cells are the major component of the seminiferous epithelium, and they support and nourish the germ cells and take part in the production of the testicular fluid (Kierszenbaum, 2007). The seminiferous epithelium is divided into different sections, each one containing a defined group of germ cell types at particular phases of development, thus ensuring the constant production of spermatozoa (Yadav & Kotaja, 2013).

Spermatogenesis is the process that implies the formation of a spermatozoa from a male germ cell and it consists of different sequential phases: mitotic phase (spermatocytogenesis), meiotic phase, and post-meiotic phase (spermiogenesis) (Figure 3). All spermatogenic cells remain interconnected by intercellular bridges because cytokinesis is incomplete (Kierszenbaum, 2007). The molecular events that take place during spermatogenesis have to be strictly regulated to enable the correct transmission of genetic and epigenetic information to next generations (Yadav & Kotaja, 2013).

- MITOTIC PHASE (SPERMATOCYTOGENESIS)

Mitotic phase starts at puberty when a prespermatogonium derived from a primordial germinal cell undergoes mitotic cell division to give rise to successive generations of spermatogonia, each generation being more differentiated than the preceding one (Kierszenbaum, 2007). The first mitotic division produces two daughter cells: one initiates a spermatogenic cycle (spermatogonia) and the other remains as a stem cell with self-renewal capacity and being able to initiate another spermatogenic cycle (Fayomi & Orwig, 2018). Spermatogonia are diploid cells located on the basement membrane of the seminiferous epithelium and they can undergo a different number of mitotic divisions, from 2 to 5 divisions depending on the testosterone levels, before their differentiation to primary spermatocytes (Pinart *et al.*, 2001).

- MEIOTIC PHASE

After the last mitotic division, spermatogonia differentiate into primary spermatocytes. Primary spermatocytes are diploid cells that undergo two successive meiotic divisions: meiosis I or reductional division, which results in the separation of the homologous chromosomes and in the formation of the secondary spermatocytes, and meiosis II or equatorial division, which results in the separation of the

sister chromatid and in the formation of round spermatids (haploid). Both divisions involve a reduction in germ cell size (Yadav & Kotaja, 2013; Fayomi & Orwig, 2018).

- POST-MEIOTIC PHASE (SPERMIOGENESIS)

Spermiogenesis is a complex and long process of cell differentiation by which round spermatids differentiate into testicular spermatozoa. Once differentiated, testicular are released from the seminiferous epithelium to the lumen by the spermiation process (Yadav & Kotaja, 2013; Fayomi & Orwig, 2018). Throughout this phase, haploid round spermatids undergo morphological, biochemical and physiological changes to form an asymmetric flagelled spermatozoa. During spermiogenesis there is no cell division but there are four major events: 1) development of the flagellum and alignment of mitochondria along the developing flagellum, 2) development of the acrosome, 3) caudally migration of sperm cytoplasm and condensation, 4) condensation of nuclear chromatin, and 5) elongation of the nucleus. There is no significant RNA transcription after spermiogenesis (Kierszenbaum, 2007; Pinart *et al.*, 2001).

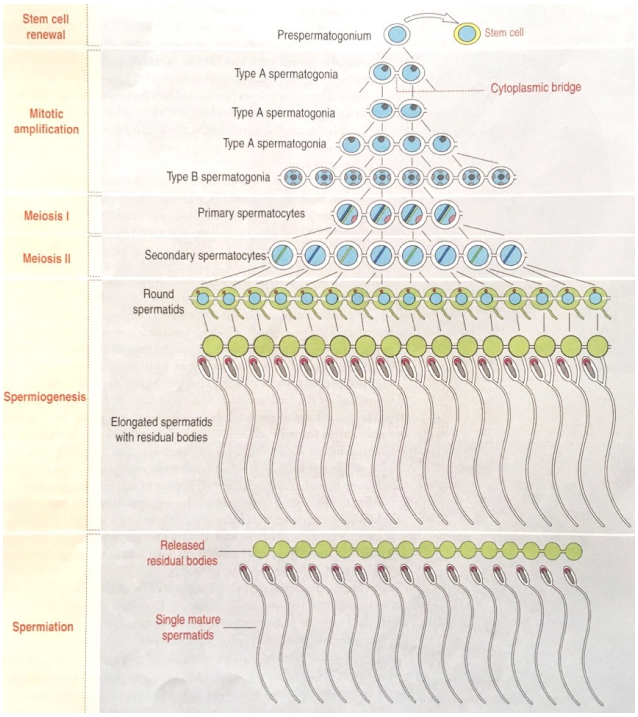

Figure 3. Scheme of spermatogenesis. Figure from (Kierszenbaum, 2007).

4.3 TESTICULAR SPERMATOZOA

Testicular spermatozoa are haploid cells which consist of two parts: head and tail, both connected by a connecting piece and covered by a continuous plasma membrane (Figure 4). The head is composed by the nucleus which is covered by the acrosome on the anterior half. The nucleus is highly condensed and contains a high electron dense chromatin; its volume is lower than the nucleus of the somatic cells (Bonet *et al.*, 2000).

The acrosome contains hydrolytic enzymes which are released as a result of the acrosome reaction to facilitate sperm penetration across the zona pellucida. The tail is divided into three segments: the midpiece, the principal piece and the end piece (Kierszenbaum, 2007).

The connecting piece is a narrow segment containing a pair of centrioles. The distal centriole gives rise to the axoneme (microtubules and associated motor, and linker proteins of the flagellum), whereas and the proximal centriole contributes to the assembly of the connecting piece to the nucleus. The midpiece consists of a helically arranged mitochondrial sheath, the axoneme and nine outer dense fibers. The principal piece consists of the central axoneme surrounded by seven outer dense fibers and a fibrous sheath. Both dense fibers and fibrous sheath contain fibrous proteins that provide a rigid scaffold during microtubular sliding and tail bending during forward motility of the sperm. The annulus is a dense ring located between the midpiece and the principal piece. The end piece is only constituted by the axoneme (Kierszenbaum, 2007).

Testicular spermatozoa also characterizes by the presence of the cytoplasmic droplet located on the connecting piece. Such droplet is a cytoplasmic remnant derived from spermiogenesis.

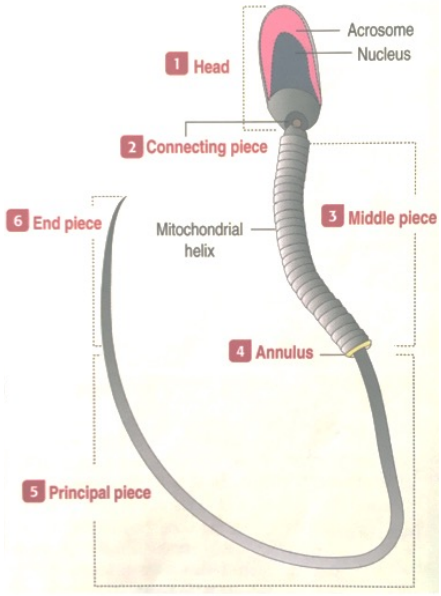

Figure 4. Sperm structure. Figure from (Kierszenbaum, 2007).

5. EPIDIDYMAL MATURATION

5.1 THE EPIDIDYMIS

- ANATOMY OF THE EPIDIDYMIS

The epididymis consists of a single but very convoluted duct located on the posterior or superior border of the testis, between the efferent ducts and the vas deferens. The efferent ducts arise from the rete testis and then become confluent with the epididymal duct. The epididymal duct is generally divided into four morphological regions: the initial segment, caput (head), corpus (body) and cauda (tail), which is continuous with the vas deferens (Figure 5). The epididymal regions can also be subdivided into segments or lobules of coiled tubule (Dacheux *et al.*, 2005; Kierszenbaum, 2007; Zhou *et al.*, 2018).

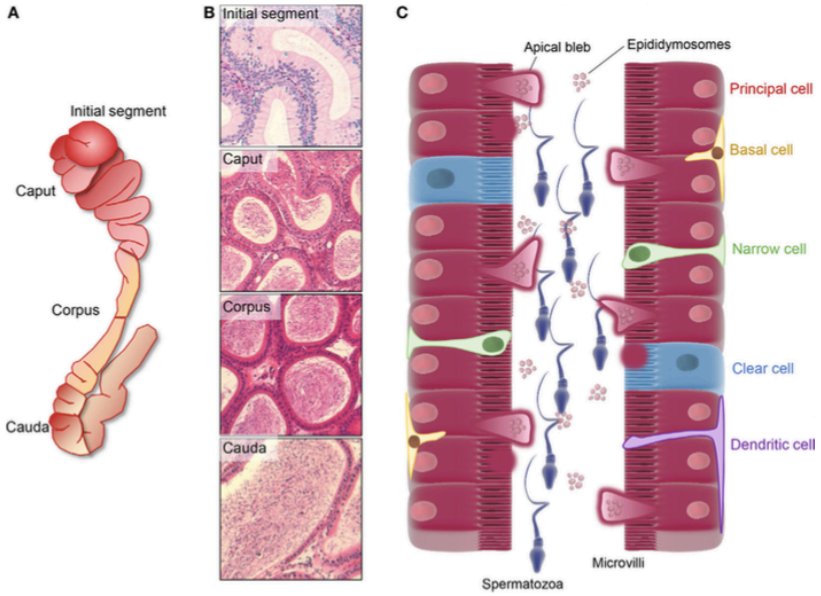

Figure 5. Anatomy and histology of the epididymal regions. Figure from (Zhou *et al.*, 2018).

- HISTOLOGY OF THE EFFERENT DUCTS AND THE EPIDIDYMIS

The epithelium of the efferent ducts is cubic and mono-stratified with two cell types: ciliated cells and absorptive cells (Figure 6). Ciliated cells have cilia and a large amount of mitochondria in the apical area, which provide energy for ciliar movement. Absorptive cells do not have cilia, but they have many microvellosities with a large number of endosomes and lysosomes in the apical region which are implicated in endocytosis of testicular fluid (Zhou *et al.*, 2018). Ciliated cells help the transport of nonmotile spermatozoa towards the epididymis, whereas absorptive cells concentrate the sperm mass as a result of the testicular fluid reabsortion from the lumen. Efferent ducts are limited by a muscular tunica that allows low contraction, thus facilitating sperm transport towards the epididymis (Kierszenbaum, 2007).

The epididymal duct is formed by a pseudostratified columnar epithelium constituted by two cell types: columnar principal cells with stereocilia and basal cells with a pyramidal shape (Zhou *et al.*, 2018) (Figure 6). The height of epithelium varies with respect to the epididymal segment, it is taller in caput and shorter in cauda. Epididymal regions also differ in lumen diameter, which is narrow in the caput and wider in the cauda (Briz *et al.*, 1995). The muscular tunica is formed by smooth muscle cells and it becomes progressively thicker from caput to cauda. This tunica helps to make the sperm move forward so in most mammalian species it usually reaches the deferent duct. In humans, the sperm transit takes 10-15 days (Kierszenbaum, 2007).

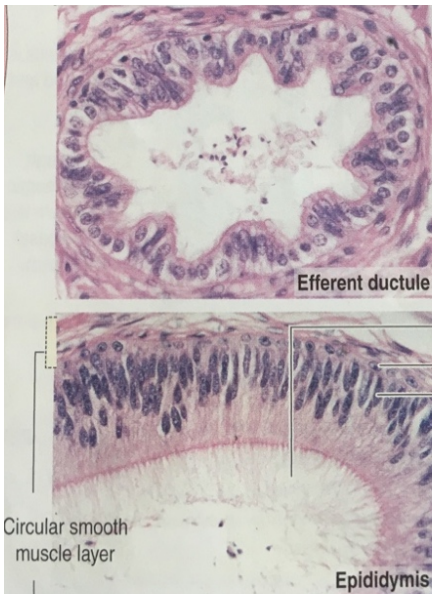

Figure 6. Structure of the efferent ducts and the epididymis in mammalian species. Figure from (Kierszenbaum, 2007).

- PHYSIOLOGY OF THE EPIDIDYMIS

Main functions of the epididymis are: 1) maturation of spermatozoa, which provides motility and the ability to recognize and bind to the zona pellucida, 2) sperm transport through peristaltic movements, 3) concentration of the sperm mass, and 4) sperm storage in a quiescent state until ejaculation (Briz *et al.*, 1995; Kierszenbaum, 2007).

The initial segment is responsible for the absortion of the majority of testicular fluid leading to a high concentration of sperm mass. Thereafter, the caput is the most active epididymal region in terms of protein synthesis and secretion but it also has an absortive function. During the transit of spermatozoa throught the caput, spermatozoa acquire the ability to move, despite they show a non-progressive movement (Kierszenbaum, 2007). Throughout the corpus, spermatozoa acquire the ability to both move progressively and to recognize oocyte coats, whereas the cytoplasmic droplet initiates the migration from the connecting piece (proximal droplet) to the annulus (distal droplet) (Zhou *et al.*, 2018).

The epididymal cauda shows a strong absortive function which is related with its storage function. Spermatozoa stored in the cauda are characterized by the presence of the cytoplasmic droplet in distal position, which keeps them in an immotile state (Briz *et al.*, 1995). Both secretory and absortive functions of the epithelial cells throughout the epididymis are responsible of the epididymal fluid, which composition is continuously changing along the duct. Such regional differences in epididymal fluid composition are responsible of sperm maturation (Dacheux *et al.*, 2012).

The combination of the secretory and absorptive activities of the epididymal epithelial cells are responsible for the formation of the perfect environment that promotes an increasing fertility in the sperm population (Zhou *et al.*, 2018).

5.2 EPIDIDYMAL MATURATION

During last stages of spermatid differentiation, spermatozoa loses transcriptional and translation ability, so maturation is not under the control of the sperm genome but it is mediated by post-translational modifications of their intrinsic protein complement (Dacheux *et al.*, 2005; Baker *et al.*, 2012). During this process, and under androgenic stimulation, spermatozoa undergo structural and biochemical modifications which are modulated by the composition of the epididymal fluid. These modifications render the spermatozoa to acquire the motility and fertilizing ability. After epididymal maturation, spermatozoa are stored in the cauda in a quiescent stage and high cell concentration until ejaculation (Dacheux & Dacheux, 2013). Alterations in epididymal functions may result in impaired sperm motility and/or low fertility ability of spermatozoa (Oyeyemi & Ubiogoro, 2005).

5.2.1 EPIDIDYMAL FLUID

One of the most important changes in the epididymal fluid is induced by water reabsortion, which principally occurs in the efferent ducts, but also in less intensity in the epididymal caput. Water absortion results in an important modification in ionic composition of the epididymal fluid (Da Silva *et al.*, 2006). As a consequence of water reabsortion, there is an increase in the luminal sperm concentration and changes in protein concentration (Fouchecourt *et al.*, 2000; Belleannee *et al.*, 2011b; Dacheux *et al.*, 2012).

Apart from water reabsortion, the epididymal epithelium secretes proteins and enzymes. Most of these proteins are very polymorphic due to glycosylation and other post-translational modifications (Dacheux & Dacheux, 2013). Secreted proteins are associated with luminal membrane vesicles named epididysomes, which are proposed to be the main way of protein transfer, as well as other molecules, from the epididymal epithelium to spermatozoa (Sullivan & Saez, 2013; Martin-DeLeon, 2015).

Some of these secreted proteins have already been identified in different mammalian species. These proteins are present in high concentrations therefore, no more than 20 proteins represent 80-90% of the total luminal proteins (e.g: lactofferin (LFT), lipocalin 5 (LCN5) and clusterin (CLU)) (Dacheux *et al.*, 2006). CLU is the most commonly secreted epididymal protein and its concentration is related to the secretory activity of the adjacent epithelium (Dacheux & Dacheux, 2013).

Many protein localization and gene expression patterns are restricted to one or more segments, so each segment acts as a distinct regulatory subunit and plays an important role in the composition of the epididymal fluid (Johnston *et al.*, 2005). Despite of this, epididymal regions also share common

patterns of gene expression which secrete the same proteins in all regions. The main function of the common secreted proteins is the protection of sperm during the epididymal transit (Dacheux *et al.*, 2006). Some of these proteins are involved in the reduction of reactive oxygen species in the epididymal fluid, such as glutathione peroxidase 5 (GPX5) (Chabory *et al.*, 2010; Taylor *et al.*, 2013), and therefore they contribute to the sperm survival during epididymal storage. Epididymal proteins may also be involved in the protection of active sites on the sperm surface (Fàbrega *et al.,* 2012), and the molecular exchange or carrying of hydrophobic components such as cholesterol, retinoic acid and androgens (Dacheux *et al.*, 2006). Eventhough, the exact function and importance of these proteins in sperm survival and maturation remains to be evaluated (Dacheux & Dacheux, 2013).

5.2.2 EPIDIDYSOMES

It is widely accepted that protein exchange from the epididymal epithelium to spermatozoa is mediated by epididysomes or soluble hydrophobic protein complexes present in the epididymal tubules, despite the exact mechanism remains to be identified (Dacheux & Dacheux, 2013). Epididysomes are proposed to be the main way of transfer of epididymal proteins (Sullivan & Saez, 2013; Martin-DeLeon, 2015).

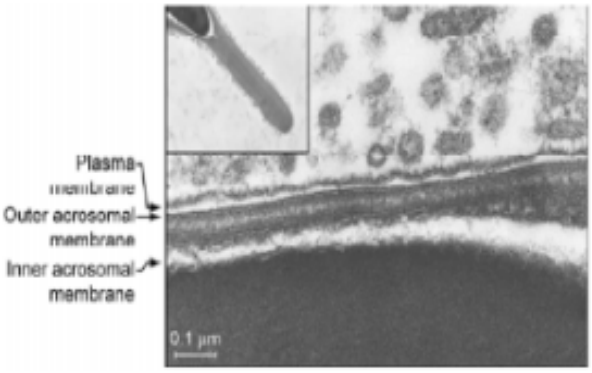

Figure 7. Electron photomicrographs showing epididysomes surrounding the plasma membrane of a spermatozoa. Figure from (Sullivan *et al.*, 2007).

Epididysomes have been described in several mammalian species such as: hamster (Legare *et al.*, 1999), mice (Griffiths *et al.*, 2008), rats (Grimalt *et al.*, 2000), bulls (Frenette *et al.*, 2001, 2002), rams (Gatti *et al.*, 2004; Ecroyd *et al.*, 2004) and humans (Thimon *et al.*, 2008). They are small spherical membranous vesicles highly heterogeneous in size and content (Figure 7), that have a very high cholesterol/phospholipid ratio. The major phospholipid constituent is sphingomyelin, which makes the association of proteins to epididymosomes very strong (Rejraji *et al.*, 2006)

Epididysomes contain different proteins, lipids and non-coding RNAs which are transfered from the epididymal ephitelium to spermatozoa. Very few proteins associated with epididymosomes have been identified (eg: sperm protein P26h/P25b, sperm adhesion molecule 1 (SPAM1) and glioma pathogenesis-related protein 1 (GPR1L1)). All these proteins can be incorporated into the plasma membrane of spermatozoa or to intracellular structures (Sullivan *et al.*, 2007). Some of them are involved in the development of sperm functions such as motility, sperm capacitation, acrosome

reaction, sperm- ZP interaction and fertilization (Sullivan *et al.*, 2005; Sullivan & Saez, 2013). Despite of this, the exact mechanisms by which these proteins exert their functions are unknown.

5.2.3 MOLECULAR CHANGES DURING EPIDIDYMAL MATURATION

Although maturation of spermatozoa is a common process in all mammalian species, significant differences among species have been reported in the composition of proteome, secretome and sperm surface proteins (Dacheux *et al.*, 2014). Epididymal maturation involves structural and functional changes in spermatozoa, among them to highlight: 1) changes in membrane phospholipid composition and in cholesterol/phospholipid ratio, 2) increase in net surface negative charge, 3) increases in the number of disulfide bonds, 4) modification, elimination or addition of surface protein and relocalization of surface antigens, and 5) migration of the cytoplasmic droplet from the connecting piece to the annulus (Briz *et al.*, 1995; Cooper & Yeung, 2006).

- CHANGES IN THE PHOSPHOLYPID COMPOSITION AND CHOLESTEROL/PHOSPHOLYPID RATIO, AND INCREASE IN THE NET SURFACE NEGATIVE CHARGE

The glycocalyx is a structure formed by glycoproteins and polysaccharides that connects the spermatozoa with its external environment. When spermatozoa enter the epididymis they lose the ability to synthesize glycans due to the abscence of Golgi apparatus; nevertheless, the existing glycans are modified and new glycans are adsorbed by the spermatozoa glycocalyx (Tecle & Gagneux, 2015). The epididymal fluid contains high concentrations of glycohydrolases and glycosyltransferases that alter the sperm surface glycocalyx (Tulsiani, 2006).

Modifications in glycocalyx are associated with an increase in the negative charge of sperm membrane during the epididymal transit, basically due to the incorporation of negatively charged sialic acid (Calvo *et al.*, 2000). The concentration of sialic acid is higher in cauda than in caput spermatozoa (Singh *et al.*, 2009). Sialic acid is closely related to sperm maturation, capacitation and sperm-oocyte recognition. It covers some antigenic deteminants to increase the survival rate of the spermatozoa in the female reprocutive tract. The loss of sialic acid is important for later sperm capacitation (Feng *et al.*, 2016).

Epididymal maturation is also associated with modifications in the lipid content of plasma membrane. A decrease in the cholesterol:phospholipid ratio occurs from caput to cauda (Jones, 2002; Saez *et al.*, 2011), which is related with an increased sperm plasma membrane fluidity (Christova *et al.*, 2002, 2004; Jones, 2002) and with an increase in sperm susceptibility to oxidative stress (Wathes *et al.*, 2007). Changes in sperm plasma membrane fluidity are essential for later events such as the acrosome reaction and the ability to fuse with the oocyte (Gervasi & Visconti, 2016).

- INCREASE IN THE NUMBER OF DISULFIDE BONDS

Epididymal maturation is associated with post-translational protein changes, which include phosphorylation and oxidation of thiol groups. The oxidation of protein thiol groups is associated with the stabilization of sperm structures, such as the nucleus and tail components, by the formation of disulfide bonds (Calvin & Bedford, 1971; Bedford & Calvin, 1974a,b). Recent analysis demonstrate an increase in disulfide bonds in the sperm tail during epididymal maturation which is important for the subsequent acquisition of sperm motility (Dias *et al.*, 2014; Ijiri *et al.*, 2014).

- REDISTRIBUTION OF SPERM SURFACE PROTEINS (PROTEIN DOMAINS) AND RELOCALIZATION OF SURFACE ANTIGENS

Surface proteins of testicular spermatozoa are modified or disappear, and new compounds can be identified in the terminal regions of the epididymis. Despite such modifications occur in all mammalian species, protein characteristics and patterns of sequential changes are different among species (Dacheux & Dacheux, 2013).

As soon as the spermatozoa enters into the epididymal caput, several surface proteins are processed and divided into peptides due to the action of proteolytic enzimes (Dacheux & Dacheux, 2013). These peptides are relocated in different plasma domains (e.g: ADAMs) or released into the surrounding medium (Gatti *et al.*, 1999). Many of the proteins resulting after modification have binding affinity to the ZP and oocyte plasma membrane; nevertheless, two proteins have been strongly associated with sperm fertility: izumo sperm egg fusion 1 (IZUMO1) and disintegrin and metallopeptidase domain 3 (ADAM3) (Dacheux & Dacheux, 2013). During the acrosome reaction, IZUMO1 changes its localization from the anterior head to the post-acrosomal region so it can bind to the oocyte receptor called folate receptor 4 (JUNO). This movement is essential for sperm-oocyte fusion (Inoue *et al.*, 2005). ADAM3 is an enzyme required for sperm transport through the utero-tubal junction (Yamaguchi *et al.*, 2009).

During the epididymal transit, specific proteins present in the epididymal fluid can bind to the sperm surface, can be adsorbed at the sperm surface by electrostatic interactions, or can be integrated into the plasma membrane (Dacheux & Dacheux, 2013).

Epididymal maturation also implies the relocation of surface antigens and the formation of new ones. These new antigens originate from the epididymal secretions and their presence or absence is correlated with the sperm capacity to ZP and oocyte membrane binding, and with the sperm movement (Dacheux *et al.*, 2005). Although this relocation and formation of antigens is important for sperm maturation and survival, their role in epididymal maturation is still not well known.

- MIGRATION OF THE CYTOPLASMIC DROPLET

The only visible morphological change during the epididymal transit is the migration of the cytoplasmic droplet from the connecting piece (proximal droplet) to the annulus (distal droplet). The cytoplasmic droplet is a residue of the germ cell cytoplasm derived from spermiogenesis (Cooper, 2011). The mechanism involved in this migration is not well-known but it occurs in the middle part of the caput, where the sperm concentration is maximum and the protein secretion is more active (Dacheux & Dacheux, 2013). When spermatozoa are stored in the cauda, the cytoplasmic droplet located on the annulus keeps the spermatozoa in an immotile state, but when they reach the female reproductive tract the droplet is released. Disturbances in the cytoplasmic droplet migration during epididymal maturation are correlated with a decrease in sperm motility (Cooper, 2005).

- LEVELS OF CAMP

During epididymal transit, levels of intracellular cAMP (Cyclic Adenosine Monophosphate) in spermatozoa increase from corpus to cauda in association with an increased metabolic capacity and ATP production. Intracellular concentrations of bicarbonate (HCO_3^-) and calcium (Ca^{2+}) are responsible for the generation of intracellular cAMP via adenylyl cyclase. Intracellular cAMP levels in spermatozoa activate subsequent protein phosphorylations, such as protein kinase A (PKA), which induce forward motility (Turner, 2006).

HCO_3^- originates in the testis, but it is partially reabsorbed by epithelial cells of the efferent ducts and epididymal caput. Reabsortion and concentration of HCO_3^- is regulated by HCO_3^- transporters (Breton, 2001; Liu *et al.,* 2012) and carbonic anhydrase activity of epididymal epithelial cells (Hermo *et al.,* 2005). High concentration of HCO_3^- is not always associated with high intracellular cAMP, and an increase in HCO_3^- concentration does not initiate the motility of spermatozoa in the caput. The transport of HCO_3^- during epididymal transtit decreases significantly from caput to cauda. Low intracellular HCO_3^- levels and, consequently, low cAMP levels help to mantain spermatozoa in a quiescent state during storage in the cauda (Dacheux & Dacheux, 2013).

Despite its exact role in sperm maturation is unclear, Ca^{2+} has an important role in the adquisition of sperm motility. Ca^{2+} levels decrease from caput to cauda. High concentrations in the caput have been correlated with mitochondrial Ca^{2+}accumulation in caput spermatozoa, the presence of the cytoplasmic droplet and the Ca^{2+} regulatory pathways (Morton *et al.*, 1978; Dacheux & Dacheux, 2013).

Despite the exact changes in Ca^{2+} and HCO_3^- concentration during the epididymal transit are still uncelar, Ca^{2+} combined with HCO_3^- speeds up the flagellar beating of mature spermatozoa in vitro (Carlson *et al.*, 2007) and changes the symmetrical flagellar wave propagation, according to the intracellular HCO_3^- levels (Dacheux & Dacheux, 2013).

- PROTEIN PHOSPHORYLATION

Testicular spermatozoa are transcriptionally inactive and unable to synthesize new proteins so post-translational modifications are used to modify their characteristics (Han *et al.*, 2007). Protein phosphorylation is a post-translational modification of proteins controlled by protein kinases and phosphatases that allows the control different cellular processes (Urner & Sakkas, 2003).

Protein phosphorylation influences almost all stages of sperm development in both male and female reproductive tract (Han *et al.*, 2007). Two signalling pathways are involved in the phosphorylation of proteins during the epididymal transit: the cAMP-PKA and the Ca^{2+} signalling pathways. Both pathways are essential for the regulation of sperm motility (Ho & Suarez, 2001). The increase of cAMP production is associated with the development of cAMP-PKA-dependent signalling pathways that leads to protein tyrosine phosphorylation and activate sperm motility (Ho & Suarez, 2001; Baker *et al.*, 2003).

Table 1. Differences in the characteristics of spermatozoa between the proximal and distal caput and cauda. Table from (Gervasi & Visconti, 2016).

Characteristics	Proximal Caput	Distal Caput	Proximal Causa	Distal Cauda
Movement	Inmotile	Non-progressive	Slow progressive	Fast progressive
Sialic acid residues	Low levels	Low levels	High levels	High levels
Disulfide bonds	Low presence	Low presence	Presence	Presence
Fertilizing ability	Unable	Unable	Able	Able
Cholesterol/phospholipid ratio	High	High	Low	Low
Cytoplasmic droplet localization	Closer to the connecting piece	Migration	Migration	At the annulus
Membrane fluidity	Low	Low	High	High

6. SPERM CAPACITATION

Sexual reproduction involves the fusion of the male gamete (spermatozoa) and the female gamete (oocyte) during fertilization in the Fallopian tube. Before the acquisition of the sperm-oocyte fusion ability, spermatozoa must complete the process of capacitation within the female reproductive tract. (Kierszenbaum, 2007)

Epididymal maturation and ejaculation are key events for further capacitation, since only ejaculated spermatozoa are capable to undergo capacitation (Fàbrega *et al.*, 2012). In humans, semen is deposited at the beginning of the cervix after mating (Kierszenbaum, 2007). In physiological conditions, a human ejaculate contains aproximately $40 \cdot 10^6$ of motile spermatozoa (De Jonge, 2005). From the deposition site, spermatozoa start a dynamic and highly complex journey to reach the fertilizing site where only a few will arrive (Barrat & Kirkman-Brown, 2006).

Capacitation is a complex process of structural and functional changes occuring in spermatozoa that 1) begins after the removal of stabilizing factors present in the seminal plasma, 2) takes place during the transit through the female reproductive tract, and 3) it is completed when spermatozoa are able to penetrate ZP by undergoing the acrosome reaction. Despite of this, capacitation and acrosome reaction are separable and distinct processes (De Jonge, 2005).

Capacitation involves modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activity, and modulation of expression of intracellular constituents. All these changes occur as a result of the activation of signaling pathway cascades (Baldi *et al.*, 2000).

Fertilization promoting peptide (FPP) is a seminal plasma component that regulates capacitation. It acts via G protein receptors positively regulating the production of cAMP in non-capacitated spermatozoa and negatively in capacitated spermatozoa (Fraser *et al.*, 2005). FPP promotes capacitation but inhibits premature acrosome reaction until the spermatozoa reaches the site of fertilization (Baldi *et al.*, 2000).

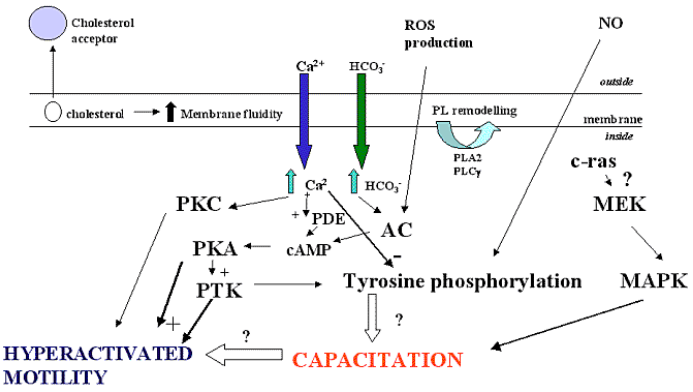

Figure 8. Schematic representation of the main events occurring during capacitation. Figure from (Baldi *et al.*, 2000).

6.1 HYPERACTIVATION AND ACROSOME REACTION

Capacitation is associated with a distinct motility pattern called hyperactivation and with the acquisition of the ability to undergo the acrosome reaction.

- HYPERACTIVATION

Hyperactivation is a motility pattern characteristic of capacitated spermatozoa, which is characterized by pronounced flagellar movements, marked lateral excursion of the sperm head and a non linear trajectory (Barbonetti *et al.*, 2008). Hyperactivated motility is associated to tyrosine phosphorilation of flagellar proteins occuring during capacitation (Baldi *et al.,* 2000). An hyperactivated movement is necessary to penetrate the cumulus and the ZP of the oocyte (Urner & Sakkas, 2003).

- ACROSOME REACTION

Sperm-oocyte interaction initiates a signal transduction cascade resulting in the exocytosis of sperm acrosomal contents, the acrosome reaction (Figure 9). Only capacited spermatozoa can undergo this process because both, capacitation and acrosome reaction, are linked processes. Acrosome reaction is a prerequisite for further penetration of the ZP and oocyte fertilization (Baldi *et al.*, 2000; Kierszenbaum, 2007).

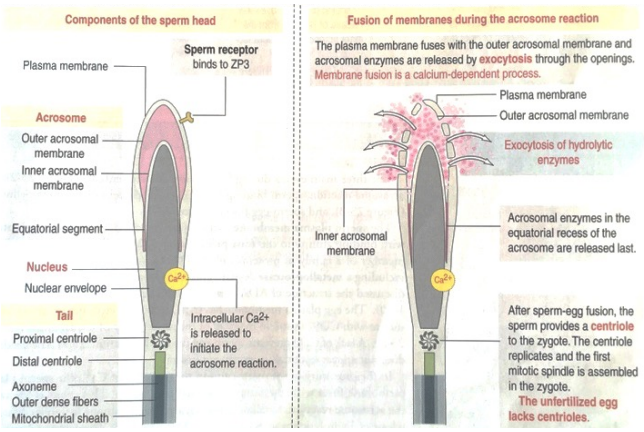

Figure 9. The acrosome reaction. Figure from (Elsevier. Kierszenbaum: Histology and Cell Biology: An Introduction to Pathology 2e).

During acrosome reaction, the plasma membrane of the sperm head fuses with the outer acrosomal membrane, thus resulting in the development of multiple fenestrations in the sperm plasma membrane by which the enzymatic content of the acrosome is released (Yanagimachi, 1994; Baldi *et al.*, 2000). Acrosome contains mainly hydrolytic enzymes which dissolve the ZP matrix locally, thus forming a channel by which the spermatozoon moves to the perivitelline space. Once in the perivitelline space, the spermatozoon can bind to the oocyte plasma membrane (oolemma). Despite few spermatozoa could bind to the oolemma, only one of them can fertilize the oocyte (Flesch & Gadella, 2000).

Different components found in the follicular fluid are related with the acrosome reaction. Progesterone is present in the follicular fluid surrounding the ovulated oocyte and it stimulates the influx of Ca^{2+} into the spermatozoa and so the acrosome reaction (Harper *et al.*, 2004). Artrial natriuretic peptide (ANP) is an other follicular fluid component and its receptor is found on the sperm plasma membrane. This peptide directly influences the sperm swimming speed and acts as a chemoattractant (Anderson *et al.*, 1994).

6.2 MOLECULAR MECHANISMS INVOLVED IN SPERM CAPACITATION

- THE CERVICAL MUCUS

Immediately after mating, ejaculated spermatozoa migrate out of the seminal plasma into the cervical mucus. This cervical mucus modifies the sperm plasma membrane and promotes their ability to penetrate ZP and acrosome reaction (De Jonge, 2005). The contact between ejaculated spermatozoa and cervical mucus leads the loss of specific sperm molecules acquired during the epididymal transit, such as vitamin E (Feki *et al.*, 2004). Vitamin E acts as a protector against oxidation by reactive oxygen species (ROS) which are produced by leukocytes and spermatozoa. Vitamin E removal makes spermatozoa more vulnerable to oxidative damage, so immature and poorly functional spermatozoa will be the first to be eliminated by ROS, while mature and functional spermatozoa will be mantained (Ford, 2004).

- INCREASE IN THE CONCENTRATION OF INTRACELLULAR Ca^{2+}

Modifications in the concentration of intracellular Ca^{2+} is one of the most important biochemical events during capacitation. In several mammalian species, including humans (Baldi *et al.,* 1991; Garcia & Meizel, 1999), an increase in Ca^{2+} levels during capacitation has been demonstrated.

Ejaculated spermatozoa are surrounded by decapacitation factors (DF) adhered to the sperm surface that keeps them in a non-capacitated state (Fraser, 1999). While adhered to the sperm surface, DF activate a Ca^{2+}-ATPase that mantains low intracellular Ca^{2+} levels in spermatozoa; however, when DF are released from the sperm surface, intracellular Ca^{2+} levels increase (Adeoya-Osiguwa & Fraser, 1996). During sperm migration throughout the female reproductive tract, DF are removed from the sperm surface, thus leading to a progressive increase of intracellular Ca^{2+} (Baldi *et al.*, 2000). Uteroglobin and transglutaminase are two seminal plasma proteins that inhibit sperm capacitation and motility, thus representing two possible DF candidates (Luconi *et al.*, 2000).

Different approaches have demonstrated that cytosolic Ca^{2+} is transported actively into the acrosome by an ATP-dependent pump and that it can be released from the acrosome through a Ca^{2+} channel (Fraser & McDermott, 1992; Fraser, 1995; Dragileva *et al.*, 1999). Other studies demonstrate the presence of Ca^{2+} channels that mediate the Ca^{2+} increase in response to ZP sperm-binding protein 3 (ZP3) (O'Toole *et al.*, 2000; Gupta, 2015).

- LOSS OF CHOLESTEROL AND INCREASE OF THE MEMBRANE FLUIDITY

Cholesterol has been identified as a seminal plasma factor that has a key role on the initiation and promotion of capacitation (Visconti *et al.*, 1999; Cross, 2003). During capacitation, it is removed from plasma membrane and this removal leads to a decrease in the cholesterol:phospholipid ratio of the sperm plasma membrane (Cross, 1998). Cholesterol removal increases the fluidity of the membrane and this is a prerequisite for the acrosome reaction (Zarantash & Cross, 1996). The uterine

environment is an important site for cholesterol removal and promotion of capacitation, due to the high presence of sterol sulphatase with great activity in the endometrium (De Jonge, 2005).

Cholesterol loss also results in an increase of the intracellular pH, entry of $Ca^{2+}$and HCO_3^- ions, activation of the monoadenylcyclase levels, increase of the cAMP levels and protein phosphorylation (Harrison, 2004).

- PROTEIN PHOSPHORYLATION

Differences in protein phosphorylation are related with the mature and capacitation state of the spermatozoa in terms of localization, intensity and proteomic expression (Fàbrega *et al.*, 2011; Naresh & Atreja, 2015). The increase in tyrosine phosphorylation of sperm proteins, mainly distributed in the flagellum, is a key step during sperm capacitation but it is still largely unknown (Naz & Rajesh, 2004). Tyrosine phosphorylation is indirectly stimulated by the phosphorylation of serine and threonine residues (PKA and MAPK) (Urner & Sakkas, 2003). Inhibition of serine-threonine kinases leads to the inhibition of protein kinase A and, therefore, of capacitation (Aitken *et al.*, 1998).

Ca^{2+} and HCO_3^- activate adenylate cyclase (AC) and this increases the generation of cAMP. Increased cAMP activates protein kinase A (PKA) and this promotes sperm tyrosine phosphorylation during capacitation (Visconti *et al.*, 1999). PKA can phosphorylate several cellular substrates so it is necessary to restric its action to specific areas to ensure specific functions. Therefore, A kinase-anchoring proteins (AKAPs) control the specific localization of PKA to the tail indicating a role for these proteins in the modulation of sperm motility (Vijayaraghavan *at al.*, 1999)

Increased tyrosine phosphorylation correlates with the acquisition of sperm-fertilizing ability, but tyrosine phosphorylation does not necessarily reflect the acquisition of sperm-fertilizing ability (Barbonetti *et al.*, 2008). Almost all ejaculated and capacitated spermatozoa show a particular phosphotyrosine expression pattern in the flagellum and sperm head that allows the binding with the ZP, thus suggesting a relationship between tyrosine phosphorylation and ZP binding (Fàbrega *et al.*, 2012)

Tyrosine phosphorylation is not an essential prerequisite for acrosome reaction (Lin *et al.,* 2006). Nevertheless, tyrosine-phosphorylated proteins present in the equatorial segment of the sperm head are suggested to be a part of the organizing center that mediates fusion leading to the acrosome reaction (Piehler *et al.*, 2006; Jones *et al.*, 2007).

Tyrosine phosphorylation of flagellar proteins is associated with the hyperactivation of sperm movement, which occurs during capacitation and it is necessary to penetrate the cumulus and the ZP of the oocyte (Urner & Sakkas, 2003). During capacitation, tyrosine phosphorylation appears first in the principal piece in all spermatozoa, and then in the midpiece in about 20% of spermatozoa.

After binding to the ZP and the oolemma, tyrosine phosphorylation is stimulated in the midpiece and almost 100% of spermatozoa have the whole flagellum phosphorylated. Soon after fusion with the oocyte, a process of dephosphorylation takes place in the entire flagellum of the fused spermatozoa (Figure 10) (Urner & Sakkas, 2003; Naz & Rajesh, 2004; Barbonetti *et al.*, 2008).

High glucose levels are necessary to support both tyrosine phosphorylation during capacitation and gamete fusion. In the absence of glucose, tyrosine phosphorylation of the principal piece is delayed, whereas tyrosine phosphorylation in the midpiece is inhibited (Urner & Sakkas, 2003).

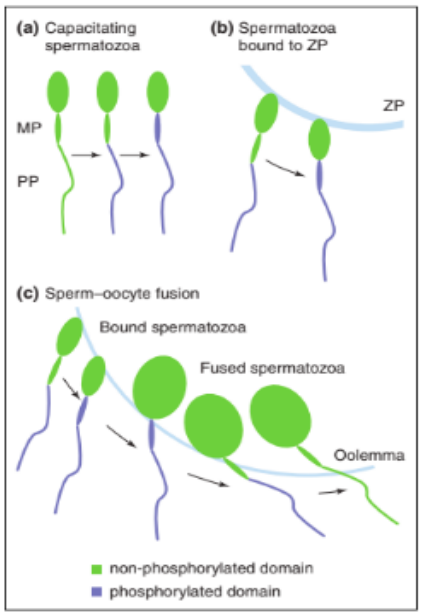

Figure 10. Pattern of protein tyrosine phosphorylation in capacitating spermatozoa in mammalian species. Figure from (Urner & Sakkas, 2003).

After capacitation, approximately 50% of spermatozoa have an incorrect phosphorylation pattern, thus indicating the presence of sperm subpopulations showing a different susceptibility to tyrosine phosphorylation. Spermatozoa that present an incorrect sequence of phosphorylation may be unable to undergo capacitation and so to fertilize an oocyte (Urner & Sakkas, 2003; Barbonetti *et al.*, 2008). Spermatozoa that are non-capacitated are all incorrectly phosphorylated, except for about 5-10% that present protein tyrosine phosphorylation in the acorsomal region. Hexokinase is the only tyrosine-phosphorylated protein found in non-capacitated spermatozoa. The phosphorylation status of hexokinase and the proportion of tyrosine phosphorylated acrosome do not vary during capacitation (Urner *et al.*, 2001).

7. CONCLUSIONS

EPIDIDYMAL MATURATION

1. During epididymal maturation, spermatozoa undergo structural and biochemical modifications modulated by the composition of the epididymal fluid to acquire the motility and fertilizing ability.

2. Each morphological region of the epididymis has a different epitelial secretory/absortive activity, thus leading to important changes in the epididymal fluid composition, which are key for sperm maturation.

3. Epididysomes are the main way of protein and enzyme transfer from the epididymal epithelium to spermatozoa.

4. Many protein localization and gene expression patterns are restricted to one or more segments, so each segment is a distinct regulatory subunit and plays an specific role in the composition of the epididymal fluid.

5. Epididymal maturation involves structural and functional changes in spermatozoa, among them to highlight:

5.1 Alterations of the sperm surface glycocalyx due to high concentrations of glycohydrolases and glycosyltransferases in the epididymal fluid, thus increasing the negative charge of the sperm plasma membrane from caput to cauda. This increase in negative charges is due to the incorporation of sialic acid, which increases the survival rate of spermatozoa in the female reproductive tract.

5.2 Decrease in the cholesterol:phospholipid ratio from caput to cauda, which results in an increase in both sperm plasma membrane fluidity and susceptibility to oxidative stress of spermatozoa. High sperm plasma membrane fluidity is essential for both acrosome reaction and sperm-oocyte fusion.

5.3 Oxidation of protein thiol groups associated with the formation of disulfide bonds and the subsequent stabilization of sperm structures, such as nucleus and tail components, which are important for the acquisition of sperm motility.

5.4 Modification, elimination or addition of surface protein and relocalization and formation of new antigens correlated with the sperm capacity to bind to ZP and oolemma.

5.5 Migration of the cytoplasmic droplet from the connecting piece to the annulus which maintains spermatozoa in a quiescent state in the epididymal cauda until ejaculation.

5.6 Increase of the cAMP intracellular levels as a consequence of the intracellular concentrations of HCO_3^- and Ca^{2+}, which activates subsequent protein tyrosine phosphorylation that induces forward motility.

SPERM CAPACITATION

6. Only ejaculated spermatozoa are capable to undergo capacitation, so epididymal maturation and ejaculation are key events. Capacitation begins after the removal of stabilizing factors acquired while resident in the seminal plasma and is it completed when spermatozoa are able to penetrate ZP.

7. Capacitation is associated with a distinct motility pattern called hyperactivation and with the acquisition of the ability to undergo acrosome reaction. Hyperactivation is a motility pattern characteristic of capacitated spermatozoa necessary to penetrate the cumulus and the ZP of the oocyte. The acrosome reaction is the process by which the acrosomal content is released by exocytosis to dissolve the ZP for subsequent penetration.

8. Sperm capacitation involves structural and functional changes in spermatozoa, among them to highlight:

8.1 Loss of decapacitation factors acquired during epididymal transit due to the contact between the spermatozoa and the cervical mucus, thus leading to an increase of the intracellular Ca^{2+}. This promotes the ability of spermatozoa to penetrate ZP and acrosome reaction.

8.2 Removal of the cholesterol, which increases the fluidity of the sperm plasma membrane, a prerequisite for the acrosome reaction.

8.3 Increase in tyrosine phosphorylation of flagellar proteins, which is associated with the hyperactivation of movement.

8. REFERENCES

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

Aitken R.J., Harkiss, D., Knox, W., Paterson, M. and Irvine, D.S. (1998). A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. *J Cell Sci,* 111, 645-656

Aitken, R.J., Nixon, B., Lin, M., Koopers, A., Lee, Y. and Baker, M. (2007). Proteomic changes in mammalian spermatozoa during epididymal maturation. *ARC Center of Excellence in Biotechnology and Development,* 554-564.

Anderson, R., Feathergill, K., Drisdel, R., Rawlins, R., Mack, S. and Zaneveld, L. (1994). Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with in vitro fertilization outcome. *J Androl*, 61–70.

Baker, M.A., Lewis, B., Hetherington, L. and Aitken, R.J. (2003). Development of the signalling pathways associated with sperm capacitation during epididymal maturation. *Molecular Reproduction and Development*, 64, 446–457.

Baker, M.A., Nixon, B., Naumovski, N. and Aitken, J. (2012). Proteomic insights into the maturation and capacitation of mammalian spermatozoa. *Systems Biology in Reproductive Medicine*, 58, 211-217.

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

Baldi, E., Luconi, M., Bonaccorsi, L., Muratori, M. and Forti, G. (2000). Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. *Frontiers in Bioscience*, 5, 110-123.

Barbonetti, A., Vassallo, M., Cinque, B., Antonangelo, C., Sciarretta, F., Santucci, R., D'Angeli, A., Francavilla, S. and Francavilla, F. (2008). Dynamics of the Global Tyrosine Phosphorylation During Capacitation and Acquisition of the Ability to Fuse with Oocytes in Human Spermatozoa. *Biology of Reproduction*, 79, 649-656.

Barrat, C. & Kirkman-Brown, J. (2006). Man-made versus female-made environment – will the real capacitation please stand up?. *Human Reproductive Update*, 12(1), 1-2.

Bedford, J.M. & Calvin, H.I. (1974a). Changes in -S-S- linked structures of the sperm tail during epididymal maturation, with comparative observations in sub-mammalian species. *J Exp Zool,* 187, 181–204.

Bedford, J.M. & Calvin, H.I. (1974b). The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. *J Exp Zool,* 188, 137–155.

Belleannee. C., Labas, V., Teixeira-Gomes, A.P., Gatti, J.L., Dacheux, J.L. and Dacheux, F. (2011b). Identification of luminal and secreted proteins in bull epididymis. *Journal of Proteomics,* 74, 59–78.

Bonet, S., Briz, M., Pinart, E., Sancho, S., García-Gil, N. and Badia, E. (2000). Morphology of Boar Spermatozoa. Barcelona.

Breton, S. (2001) The cellular physiology of carbonic anhydrases. *Journal of the Pancreas*, 2, 159–164.

Briz, M.D., Bonet, S., Pinart, E., Egozcue, J. and Camps, R. (1995). Comparative study of boar sperm coming from the caput, corpus and cauda regions of the epididymis. *Journal of Andrology*, 16, 175-188.

Calvin, H.I. & Bedford, J.M. (1971). Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J Reprod Fertil Suppl,* 13, 65–75.

Calvo, A., Pastor, L.M., Bonet, S., Pinart, E. & Ventura, M. (2000). Characterization of the glycoconjugates of boar testis and epididymis. *J Reprod Fertil*, 120, 325–335.

Carlson, A.E., Hille, B. and Babcock, D.F. (2007). External Ca^{2C} acts upstream of adenylyl cyclase SACY in the bicarbonate signaled activation of sperm motility. *Developmental Biology*, 312, 183–192.

Chabory, E., Damon, C., Lenoir, A., Henry-Berger, J., Vernet, P., Cadet, R., Saez, F. and Drevet, J.R. (2010). Mammalian glutathione peroxidases control acquisition and maintenance of spermatozoa integrity. *Journal of Animal Science*, 88, 1321–1331.

Christova, Y., James, P.S., Cooper, T.G. and Jones, R. (2002). Lipid diffusion in the plasma membrane of mouse spermatozoa: changes during epididymal maturation, effects of pH, osmotic pressure, and knockout of the c-ros gene. *J Androl,* 23, 384–392.

Christova, Y., James, P.S, Mackie, A., Cooper, T.G. and Jones, R. (2004). Molecular diffusion in sperm plasma membranes during epididymal maturation. *Mol Cell Endocrinol,* 216, 41–46.

Cooper, T.G. (2011). The epididymis, cytoplasmic droplets and male fertility. *Asian J Androl,* 13, 130–138.

Cooper, T.G. (2005). Cytoplasmic droplets: the good, the bad or just confusing?. *Human Reproduction,* 20, 9–11.

Cooper, T. & Yeung, C. (2006). Sperm maturaiton in the human epididymis. In: De Jonge C, Barratt C, editors. *The Sperm Cell. Production, Maturation, Fertilization, Regeneration. Cambridge University Press*, 72–107.

Cross, N.L. (2003) Decrease in order of human sperm lipids during capacitation. *Biol Reprod* 69, 529–534.

Cross, N.L. (1998). Role of cholesterol in sperm capacitation. *Biol Reprod,* 59, 7-11

Dacheux, J.L., Belleannee, C., Guyonnet, B., Labas, V., Teixeira-Gomes, A.P., Ecroyd, H., Druart, X., Gatti, J.L. and Dacheux, F. (2012). The contribution of proteomics to understanding epididymal maturation of mammalian spermatozoa. *Systems Biology in Reproductive Medicine*, 58, 197–210.

Dacheux, J.L, Belghazi, M., Lanson, Y. and Dacheux, F. (2006). Human epididymal secretome and proteome. *Mol Cell Endocrinol*, 250, 36–42.

Dacheux J.L., Castella, S., Gatti, J.L. and Dacheux, F. (2005). Epididymal cell secretory activities and the role of proteins in boar sperm maturation. *Theriogenology*, 63, 319-341.

Dacheux, J.L. &Dacheux, F. (2013). New insights into epididymal function in relation to sperm maturation. *Reproduction*, 147, 27-42.

Da Silva, N., Pietrement, C., Brown, D. and Breton, S. (2006). Segmental and cellular expression of aquaporins in the male excurrent duct. *Biochimica et Biophysica* Acta, 1758, 1025–1033.

De Jonge, C. (2017). Biological basis for human capacitation-revised. *Hum Reprod Update* 23(3), 289-299.

De Jonge, C. (2005). Biological basis for human capacitation. *Hum Reprod Update* 11, 205–214.

Dias, G.M., Lopez. M.L., Ferreira, A.T., Chapeaurouge, D.A., Rodrigues, A., Perales, J. and Retamal, C.A. (2014) Thiol-disulfide proteins of stallion epididymal spermatozoa. *Anim Reprod Sci,* 145, 29–39.

Dragileva, E., Rubinstein, S. and Breitbart, H. (1999). Intracellular $Ca(^{2+})$-$Mg(^{2+})$-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. *Biol Reprod,* 61, 1226-1234.

Ecroyd, H., Sarradin, P., Dacheux, J.L and Gatti, J.L. (2004). Compartmentalization of prion isoforms within the reproductive tract of the ram. *Biol Reprod*, 71(3), 993-1001. Epub 2004 May 26.

Fàbrega, A., Puigmulé, M., Bonet, S. and Pinart, E. (2012). Epididymal maturation and ejaculation are key events for further *in vitro* capacitation of boar spermatozoa. *Theriogenology*, 78, 867-877.

Fàbrega, A., Puigmulé, M., Yeste, M., Casas, I., Bonet, S. and Pinart, E. (2011). Impact of epididymal maturation, ejaculation and in vitro capacitation on tyrosine phosphorylation patterns exhibited of boar (Sus domesticus) spermatozoa. *Theriogenology*, 76, 1356-1366.

Fayomi, A.P. & Orwig, K.E. (2018). Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. *Stem Cell Research*, 29, 207-214.

Feki, N., Therond, P., Couturier, M., Limea, G., Legrand, A., Jouannet, P. and Auger, J. (2004). Human sperm lipid content is modified after migration into cervical mucus. *Mol Hum Reprod* 10, 137–142.

Feng, Y., Wang, L., Wu, Y.L, Liu, H.H and Ma, F. (2016). Roles of sialic acids in sperm maturation and capacitatio and sperm-egg recognition. *Zhanghua Nan Ke Xue*, 22(10), 944-948.

Flesch, F.M. & Gadella, B.M. (2000). Dynamics of the mammalian sperm plasma membrane in the process of fertillization. *Biochimica et Biopphysica Acta*, 1469, 197-235.

Ford, W. (2004). Regulation of sperm function by reactive oxygen species. *Hum Reprod Update* 10, 387–399.

Fouchecourt, S., Metayer, S., Locatelli, A., Dacheux, F. and Dacheux, J.L. (2000). Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. *Biology of Reproduction,* 62, 1790–1803.

Fraser, L. R, Adeoya-Osiguwa, S., Baxendale, R., Mededovic, S. and Osiguwa, O. (2005). First messenger regulation of mammalian sperm function via adenylyl cyclase/cAMP. *J Reprod Dev,* 51, 37–46.

Fraser, L.R. (1999). New insights into possible causes of male infertility. *Human. Reprod,* 14, 38-46.

Fraser, L.R. (1995). Mechanisms regulating capacitation and the acrosome reaction. *John Libbey Eurotext Ltd*, 236, 17-33.

Fraser, L. R. & McDermott, C.A. (1992). Ca2+-related changes in the mouse sperm capacitation state: a possible role for Ca2+-ATPase. *J Reprod Fertil*, 96, 363-377.

Frenette, G., Lessard, C. and Sullivan, R. (2002). Selected proteins of "prostasome-like particles" from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. *Biol Reprod*, 67(1), 308-113.

Frenette, G. & Sullivan, R. (2001). Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. *Mol Reprod Dev*, 59(1), 115-121.

Garcia, M.A. & Meizel, S. (1999). Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod,* 60, 102-109.

Gatti, J.L, Castella, S., Dacheux, F., Ecroyd, H., Metayer, S., Thimon, V. and Dacheux, J.L. (2004). Post-testicular sperm environment and fertility. *Anim Reprod Sci*, 82-83, 321-339.

Gatti, J.L., Druart, X., Guerin, Y., Dacheux, F. and Dacheux, J.L. (1999). A 105- to 94-kilodalton protein in the epididymal fluids of domestic mammals is angiotensin I-converting enzyme (ACE); evidence that sperm are the source of this ACE. *Biology of Reproduction,* 60, 937–945.

Gervasi, M. & Visconti, P. (2017). Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology*, 5(2), 204-218.

Griffiths, G.S., Galileo, D.S., Reese, K. and Martin-Deleon, P.A. (2008). Investigating the role of murine epididymosomes and uterosomes in GPI-linked protein transfer to sperm using SPAM1 as a model. *Mol Reprod Dev*, 75(11), 1627-36.

Grimalt, P., Bertini, F. and Fornes, M.W. (2000). High-affinity sites for beta-D-galactosidase on membrane-bound vesicles isolated from rat epididymal fluid. *Arch* Androl, 44(2), 85-91.

Gupta, S.K. (2015). Role of zona pellucida glycoproteins during fertilization in humans. *J Reprod Immunol*, 108, 90-97.

Han, Y., Haines, C. and Feng, H. (2007). Role(s) of the Serine/Threonine Protein Phosphatase 1 on Mammalian Sperm Motility. *Andrology*, 53(4), 169-177.

Harrison, R.A. (2004). Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol Reprod Dev*, 67, 337-352.

Harper, C., Barratt, C., and Publicover, S. (2004). Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. *J Biol Chem* 279, 46315–46325.

Hermo, L., Chong, D.L., Moffatt, P., Sly, W.S., Waheed, A. and Smith, C.E. (2005). Region- and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. *Journal of Histochemistry and Cytochemistry,* 53, 699–713.

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

Ijiri, T.W., Vadnais, M.L., Huang, A.P., Lin, A.M., Levin, L.R., Buck, J. and Gerton, G.L. (2014). Thiol changes during epididymal maturation: a link to flagellar angulation in mouse spermatozoa? *Andrology*, 2, 65–75.

Inoue, N. (2017). Novel insights into the molecular mechanism of sperm-egg fusion via IZUMO1. *Journal of Plant Research*, 130, 475-478.

Johnston, D., Jelinsky, S., Bang, H., DiCandeloro, P., Wilson, E., Kopf, G., *et al*. (2005). The mouse epididymal transcriptome: transcriptional profiling of segmental gene expression in the epididymis. *Biol Reprod*, 73, 404–413.

Jones, R. (2002). Plasma membrane composition and organisation during maturation of spermatozoa in the epididymis. *In: The Epididymis: From Molecules to Clinical Practice (eds B Robaire & BT Hinton)*, 405–416 .

Jones, R., James, P.S., Howes, L., Bruckbauer, A. and Klenerman, D. (2007). Supramolecular organization of the sperm plasma membrane during maturation and capacitation. *Asian J Androl,* 9, 438-444.

Kierszenbaum, A.L. (2007). Histology and Cell Biology, An Introduction to Pathology (2nd Edition). New York, New York: *Mosby Elsevier*.

Legare, C., Berube, B., Boue, F., Lefievre, L., Morales, C.R., El-Alfy, M. and Sullivan, R. (1999). Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. *Mol Reprod Dev*, 52(2), 225-233.

Lin, M., Lee, Y., Xu, W., Baker, M. and Aitken, R. (2006). Ontogeny of tyrosine phosphorylation-signaling pathways during spermatogenesis and epididymal maturation in the mouse. *Biol Reprod*, 75, 588–97.

Liu, Y., Wang, D.K. and Chen, L.M. (2012). The physiology of bicarbonate transporters in mammalian reproduction. *Biology of Reproduction,* 86-99.

Luconi, M., Muratori, M., Maggi, M., Pecchioli, P., Peri, A., Mancini, M., Filimberti, E., Forti, G. and Baldi, E. (2000). Uteroglobin and transglutaminase modulate human sperm functions. *J Androl* 21, 676-688.

Martin-DeLeon, P. (2015). Epididymosomes: transfer of fertility-modulating proteins to the sperm surface. *Asian J Androl* 17, 720-725.

Morton, B.E., Sagadraca, R. and Fraser, C. (1978). Sperm motility within the mammalian epididymis: species variation and correlation with free calcium levels in epididymal plasma. *Fertil Steril,* 29, 695–698.

Naresh, S. & Atreja, S.K. (2015). The protein tyrosine phosphorylation during in vitro capacitation and cryopreservation of mammalian spermatozoa. Cryobiology, 70(3), 211-216.

Naz, R.K. & Rajesh, P.B. (2004). Role of tyrosine phosphorylation in sperm capacitation/acrosome reaction. Reprod Biol Endocrinol, 9.

O'Toole, C.M.B., Arnoult, C., Darszon, A., Steinhardt, R.A. and Florman, H.M. (2000). Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol Biol Cell,* 11, 1571-1584.

Oyeyemi, M.O. and Ubiogoro, O. (2005). Spermiogram and Morphological characteristics in testicular and epididymal spermatozoa of large White Boar in Nigeria. *Int. J. Morphol.*, 23(3), 235-239.

Piehler, E., Petrunkina, A.M., Ekhlasi-Hundrieser, M. and Töpfer-Petersen, E. (2006). Dynamic quantification of the tyrosine phosphorylation of the sperm surface proteins during capacitation. *Cytometry Part A*, 69(A), 1062–1070.

Pinart, E., Bonet, S., Briz, M., Pastor L.M., Sancho, S., García, N., Badia, E. and Bassols, J. (2001). Lectin affinity of the seminiferous epithelium in healthy and cryptorchid post-pubertal boars. *Int J Androl*, 24, 153-163.

Rejraji, H., Sion, B., Prensier, G., Carreras, M., Motta, C., Frenoux, J., Vericel, E., Grizard, G., Vernet, P. and Drevet, J. (2006). Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biol Reprod* 74, 1104–1113.

Ruwanpura, S.M., McLachlan, R.I. and Meachem, S.J. (2010). Hormonal regulation of male germ cell development. *J. Endocrinol*, 205, 117-131.

Saez, F., Ouvrier, A. and Drevet, J.R. (2011). Epididymis cholesterol homeostasis and sperm fertilizing ability. *Asian J Androl,* 13, 11–17.

Schulz, R.W., Vischer, H.F., Cavaco, J.E.B., Santos, E.M., Tyler, C.R., Goos, H.J.Th. and Bogerd., J. (2001). Gonadotropins, their receptors, and the regulation of testicular functions in fish. *Comparative Biochemistry and Physiology*, 129, 407-417.

Singh, S., Malini, T., Rengarajan, S. and Balasubramanian, K. (2009). Impact of experimental diabetes and insulin replacement on epididymal secretory products and sperm maturation in albino rats. *J Cell Biochem 108*, 1094–1101.

Sullivan, R., Saez F., Girouard, J., and Frenette, G. (2005). Role of exosomes in sperm maturation during the transit along male reproductive tract. *Blood Cells, Molecules, and Diseases*, 35, 1-10.

Sullivan, R. & Saez, F. (2013). Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction*, 146(1), 21-35.

Taylor, A., Robson, A., Houghton, B.C., Jepson, C.A., Ford, W.C. and Frayne, J. (2013). Epididymal specific, selenium-independent GPX5 protects cells from oxidative stress-induced lipid peroxidation and DNA mutation. *Human Reproduction,* 28, 2332–2342.

Tecle, E. & Gagneux, P. (2015). Sugar-coated sperm: unraveling the functions of the mammalian sperm glycocalyx. *Mol Reprod Dev,* 82, 635–650.

Thimon, V., Frenette, G., Saez, S., Thabet, M. and Sullivan, R. (2008). Protein composition of human epididymosomes: a proteomic and genomic approach. *Hum. Reprod.*, Under revision.

Tulsiani, D.R. (2006). Glycan-modifying enzymes in luminal fluid of the mammalian epididymis: an overview of their potential role in sperm maturation. *Mol Cell Endocrinol,* 250, 58–65.

Turner, R.M. (2006). Moving to the beat: a review of mammalian sperm motility regulation. *Reproduction, Fertility, and Development*, 18, 25–38.

Urner, F., Leppens-Luisier, G. and Sakkas, D. (2001). Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. *Biology of Reproduction*, 64, 1350–1357.

Urner, F. & Sakkas, D. (2003). Protein phosphorylation in mammalian spermatozoa. *Reprod*, 125, 17-26.

Visconti, P.E., Galantino-Homer, H., Ning, X.P., Moore, G.D, Valenzuela, J.P., Jorgez, C.J., Alvarez, J.G. and Kopf, G.S. (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm. *J Biol Chem,* 274, 3235-3242.

Visconti, P.E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G.S. and Tezon, J.G. (1999). Roles of bicarbonate, cAMP, and tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. *Biol Reprod,* 61, 76-84.

Vijayaraghavan, S., Liberty, G.A., Mohan, J., Winfrey, V.P, Olson, G.E and Carr, D.W. (1999). Isolation and moleculatr characterization of AKAP110, a novel, sperm-specific protein kinase A-anchoring protein. *Mol Endocrinol,* 13, 705-717.

Wathes, D.C., Abayasekara, D.R. and Aitken, R.J. (2007). Polyunsaturated fatty acids in male and female reproduction. *Biol Reprod* 77, 190–201.

Yadav, R.P. & Kotaja, N. (2013). Small RNAs in spermatogenesis, *Molecular and Cellular Endocrinology Molecular and Cellular Endocrinology* [Epub ahead of print].

Yamaguchi, R., Muro, Y., Isotani, A., Tokuhiro, K., Takumi, K., Adham, I., Ikawa, M. and Okabe, M. (2009). Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. *Biol Reprod,* 81, 142–146.

Yanagimachi, R. (1994). Mammalian fertilization. *In: Physiology of Reproduction. Eds: Knobil E., Neill J. Raven Press*, New York 189-317.

Zarantash, R. & Cross, N. (1996). Unesterified cholesterol content of human sperm regulates the response of the acrosome to the agonist, proges- terone. *Biol Reprod* 55, 19–24.

Zhou, W., De Iuliis, G.N., Dun, M.D. and Nixon, B. (2018). Characteristics of the Epididymal Luminal Environment Responsible for Sperm Maturation and Storage. *Frontiers in Endocrinology*, 9, 59.