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SPERM MEMBRANE CHANNELS, RECEPTORS AND KINEMATICS

Using boar spermatozoa for drug toxicity screening

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About the cover:

The figure in the front cover displays boar spermatozoa where AQP-9 (upper-left corner), CatSper 3 (upper-right corner) and the δ -opioid receptor (lower-left corner) are immunolocalized. A representative output of the Qualisperm™ software depicting each detected spermatozoon and its motility trajectory (in yellow) is provided in the lower-right corner.

During the course of the research underlying this Thesis, Alejandro Vicente Carrillo was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University.

Forum Scientium

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ABSTRACT

Internal fertilization usually implies that a spermatozoon, with intact attributes for zygote formation, passes all hurdles during its transport through the female genitalia and reaches the oocyte. During this journey, millions to billions of other spermatozoa perish. Spermatozoa are highly differentiated motile cells without synthetic capabilities. They generate energy via glycolysis and oxidative phosphorylation to sustain motility and to maintain the stability and functionality of their plasma membrane. *In vivo*, they spend their short lifespan bathing in female genital tract fluids of different origins, or are *in vitro* exposed to defined media during diverse sperm handling i.e. extension, cryopreservation, *in vitro* fertilization, etc. Being excitable cells, spermatozoa respond *in vivo* to various stimuli during pre-fertilization (capacitation, hyperactivation, oocyte location) and fertilization (acrosome reaction, interaction with the oocyte) events, mediated via diverse membrane ion-conducting channels and ligand-gated receptors. The present Thesis has mapped the presence and reactivity (sperm intactness and kinematics) of selected receptors, water and ion channels in ejaculated boar spermatozoa. The final aim was to find a relevant alternative cell type for *in vitro* bioassays that could ease the early scrutiny of candidate drugs as well as decreasing our needs for experimental animals according to the 3R principles. Spermatozoa are often extended, cooled and thawed to warrant their availability as fertile gametes for breeding or *in vitro* testing. Such manipulations stress the cells via osmotic variations and hence spermatozoa need to maintain membrane intactness by controlling the exchange of water and the common cryoprotectant glycerol, via aquaporins (AQPs). Both AQP-7 and -9 were studied for membrane domain changes in cauda- and ejaculated spermatozoa (un-processed, extended, chilled or frozen-thawed). While AQP-9 maintained location through source and handling, thawing of ejaculated spermatozoa clearly relocated the labelling of AQP-7, thus appearing as a relevant marker for non-empirical studies of sperm cryopreservation. Alongside water, spermatozoa interact with calcium (Ca^{2+}) via the main Ca^{2+} sperm channel CatSper. Increments in intracellular Ca^{2+} initiate motility hyperactivation and the acrosome reaction. The four subunits of the CatSper channel were present in boar spermatozoa, mediating changes in sperm motility under *in vitro* capacitation-inducing conditions (increased extracellular Ca^{2+} availability and bicarbonate) or challenge by the CatSper antagonists mibefradil and NNC 55-0396. Uterine and oviduct fluids are richest in endogenous opioids as β -endorphins during mating and ovulation. Both μ - and δ - opioid receptors were present in boar spermatozoa modulating sperm motility, as *in vitro* challenge with known agonists (μ : morphine; δ : DPDPE and κ : U 50488) and antagonists (μ : naloxone; δ : naltrindole and κ : *nor*-binaltrorphimine) showed that the μ -opioid receptor maintained or increased motility while the δ -opioid receptor mediated decreased motility over time. Finally, boar spermatozoa depicted dose-response effects on sperm kinematics and mitochondrial potential following *in vitro* challenge with 130 pharmacological drugs and toxic compounds as well as with eight known mito-toxic compounds. In conclusion, boar spermatozoa expressing functional water (AQPs-7 and -9) and ion (CatSper 1-4) channels as well as μ - and δ -opioid receptors are able to adapt to stressful environmental variations, capacitation and pharmacological compounds and drug components. Ejaculated sperm suspensions are easily and painlessly obtained from breeding boars, and are suitable biosensors for *in vitro* drug-induced testing, complying with the 3R principles of reduction and replacement of experimental animals, during early toxicology screening.

Key words: plasma membrane, membrane channels, membrane receptors, kinematics, 3R-principles, spermatozoa, boar.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Spermier är mycket specialiserade celler som efter ejakulationen interagerar med många olika miljöer i den honliga reproduktionsapparaten. Under deras förflyttning till befruktningsplatsen kommer de att utsättas för en mängd endogent sekret från den honliga interna könsorgan, för att till sist nå kontakt med ägget och dess höljen. Även efter insamling för diagnostik in vitro och vidare hantering, såsom spädning, kylning, frysning-upptining och provrörs fertilisering, kommer dessa spermier att exponeras för en mångfald medier och fysikaliska processer. Denna ständiga förändring av omgivningen kräver att de har ett mycket aktivt plasmamembran, vars stabilitet och förmåga att reagera på olika stimuli är avgörande för processer både före och under befruktning. Spermerna har ett begränsat antal mitokondrier och är därför särskilt beroende av dessa för oxidativ fosforylering som, tillsammans med glykolysen, bildar den energi som behövs för rörlighet och för plasmamembranets funktionalitet. Dessutom är spermerna exciterbara celler, vars stimulering förmedlas via olika typer av jonkanaler och ligand-aktiverade receptorer. Därför är det viktigt för vår förståelse av spermernas funktionalitet och cellulära svar att kartlägga vilka receptorer och jonkanaler som de uttrycker. Det är också av stor vikt att förstå hur de svarar på olika stimulerare/hämmare av dessa receptorer. Dessutom kan spermernas rörlighet användas som en indikator på kemiska föreningars mitokondriella toxicitet, vilket även skulle kunna användas i prekliniska studier vid utveckling av läkemedel och i förlängningen kraftigt kunna minska behovet av försöksdjur. Hypotesen var att ejakulerade spermier är en relevant alternativ celltyp för bioanalyser av mitokondriefunktion och för att mäta effekter av relevanta kemikalier på olika cellulära funktioner.

För att spermier skall vara en bra cellulär modell, som kan ersätta djurförsök i studier av läkemedelsinducerad toxicitet är det av betydande vikt att man kan långtidsförvara sperma, exempelvis djupfrys i flyttande kväve. Under frysning måste cellerna kunna kontrollera vattenutbytet med extracellulärmediet för att på så sätt anpassa sig till varierande osmotiskt tryck. Flödet av vatten över cellmembranet regleras av vattenkanaler, s.k. aquaporiner (AQP). Vissa av dessa aquaporiner transporterar förutom vatten även glycerol, vilket är den mest använda frysskyddande medel vid spermafrysning. Därför studerades två sådana aquaglyceroporiner, AQP-7 och -9 för dess uttryck, lokalisation och rollen i reglering av osmotiskt tryck i spermier från gris. Uttrycket studerades med hjälp av immuncytokemi med specifika antikroppar och visualiserades med hjälp av konfokal- och elektronmikroskopi i såväl kauda epididymis som ejakulerade spermier. Spermerna exponerades ofta sekventiellt för olika medier, vilket möjliggjorde jämförelse mellan obehandlade celler och de utspädda i kylskyddande medier, kylda och frystinade efter vidare exponering för den frysskyddande glycerol. Frysning och upptining av ejakulerade spermier påverkade tydligt inmärknings av AQP-7, men inte av AQP-9. Det ter sig därför logiskt att föreslå AQP-7 som en relevant markör för vidare studier av frysningseffekter.

Kunskaper om uttryck, distribution och betydelse av olika receptorer i spermier från gris kan förhoppningsvis också ge värdefull information om dessa cellers fysiologi, vilket är relevant

för dess användning som cellmodell vid analys av läkemedelsinducerad toxicitet. Homeostasen hos celler i allmänhet och spermier i synnerhet är synnerligen beroende av kalciumjoners rörelser över plasmamembranet, eftersom de agerar som medhjälpare till flera olika enzymer och andra reaktioner i cellerna. Lika väsentliga är olika specifika receptorer, bland annat de som kan förmedla stimulering genom opioider. Dessa har klassiskt ansetts vara särskilt betydelsefulla i nerv- och endokrina celler men verkar även viktiga för spermerna. Därför studerades uttryck, distribution och funktion av både den för spermieceller viktigaste kalciumjon (Ca^{2+})-kanalen (CatSper) och opioidareceptorer i spermier från gris.

CatSper Ca^{2+} -kanalen fanns i spermernas plasmamembran. För att studera kanalens roll under spermernas kapacitering späddes spermerna i medium med varierande koncentration av Ca^{2+} , exponerades för bikarbonat, progesteron eller CatSper-hämmare, både separat eller sekventiellt i fysiologiska eller farmakologiska doser. Andelen motila spermier minskade när de var kapaciterade och en ytterligare minskning observerades när de samtidigt exponerades för hämmare av CatSper. Sammanfattningsvis verkar CatSper reglera grisarnas spermie motilitet under in vitro kapacitering.

För att ytterligare vidga förståelsen av membranreceptorers förekomst, temporala distribution och funktioner hos grisspermier studerades uttryck och lokalisering av opioidareceptorer, inklusive dess funktionella svar efter in vitro stimulering med kända agonister och antagonister. Både μ - och δ - opioidareceptorer, men inte κ -opioid receptorn, uttrycktes i cellerna. Vid tillsats av agonist till μ - och δ -opioidareceptorer påverkades spermernas motilitet. Sammanfattningsvis verkar μ -opioidreceptor agonister vara inblandade i upprätthållandet och ökningen av spermernas rörlighet, medan δ -opioidreceptor agonister minskar densamma.

För att utvärdera spermier som en ny cellmodell för att analysera läkemedelsinducerad toxicitet, utsattes spermier från gris för 130 farmakologiska läkemedel och toxiska ämnen. Dessutom mättes effekter av åtta kända mitokondriotoxiska substanser. Spermernas rörlighet kvantifierades med en halvautomatisk utrustning innehållande en specifik mjukvara för spermieanalys (ToxiSpermTM, Biophos, Schweiz) parallellt med en mätning av mitokondrie integritet. Dos-respons effekter på andelen motila spermier, deras hastighet och mitokondrieintegritet observerades för alla de testade ämnena. Spermier från gris kan därför kunna användas som lämpliga biosensorer för preklinisk toxikologisk utredning av läkemedelskandidater.

Sammanfattningsvis föreslås spermier från gris vara en lämplig cellmodell för läkemedelsinducerad toxicitet, eftersom de i sitt plasmamembran uttrycker relevanta vatten-(AQP-7 och -9) och Ca^{2+} -kanaler (CatSper) och opioidreceptorer, vilka spelar viktiga roller i spermiefysiologin.

LIST OF PAPERS

- Paper I: Vicente-Carrillo A, Ekwall H, Álvarez-Rodríguez M, Rodríguez-Martínez H. Membrane stress during thawing elicits re-distribution of Aquaporin 7 but not of Aquaporin 9 in boar spermatozoa. *Reproduction in Domestic Animals* 2016; 51:665-679.
- Paper II: Vicente-Carrillo A, Álvarez-Rodríguez M, Rodríguez-Martínez H. The CatSper channel modulates boar sperm motility during capacitation. 2016; *Submitted*.
- Paper III: Vicente-Carrillo A, Álvarez-Rodríguez M, Rodríguez-Martínez H. The mu (μ) and delta (δ) opioid receptors modulate boar sperm motility. *Molecular Reproduction and Development* 2016; 83:724-734.
- Paper IV: Vicente-Carrillo A, Edebert I, Garside H, Cotgreave I, Rigler R, Loitto V, Magnusson KE, Rodríguez-Martínez H. Boar spermatozoa successfully predict mitochondrial modes of toxicity: Implications for drug toxicity testing and the 3R principles. *Toxicology in Vitro* 2015; 29:582–591.

ABBREVIATIONS

AI	Artificial insemination
AIJ	Ampullary-isthmic junction
AKAP	A-Kinase anchor protein
AQP	Aquaporin
AR	Acrosome reaction
ATP	Adenosine triphosphate
BTS	Beltsville thawing solution
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted sperm analysis
CCD	Charge coupled device
CMOS	Complementary metal oxide silicon
CNS	Central nervous system
COC	Cumulus-oocyte complex
CPA	Cryoprotectant agent
DAG	Di-acyl glycerol
DNA	Deoxyribonucleic acid
DPDPE	[D-Pen 2,5]-enkephanile
${}_e\text{Ca}^{2+}$	Extracellular calcium
EDTA	Ethylene diamine tetraacetic acid
EU	European Union
FC	Flow cytometry
FSH	Follicle stimulating hormone
GLUT	Glucose transporter
IC50	Inhibitory concentration-50
${}_i\text{Ca}^{2+}$	Intracellular calcium

ICC	Immunocytochemistry
IP ₃	Inositol tri-phosphate
LEY	Lactose-egg yolk
LEYGO	Lactose-egg yolk-glycerol
LiU	Linköping University
NB	Northern blotting
OXPHOS	Oxidative phosphorylation
sAC	Soluble adenylyl cyclase
SEURAT	Safety evaluation ultimately replacing animal testing
SLU	Swedish University of Agricultural Sciences
SP	Seminal plasma
SR	Sperm reservoir
SRF	Sperm-rich fraction
Pre-SRF	Pre-sperm-rich fraction
Post-SRF	Post-sperm-rich fraction
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
R&D	Research and development
PKA	Protein-kinase A
PKC	Protein-kinase C
PLC	Phospholipase C
UTJ	Utero-tubal junction
WB	Western blotting
ZP	Zona pellucida

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GENERAL INTRODUCTION

Reproduction, the ability to transfer genetic material to the next generation, is the ultimate purpose of every living organism. Over evolution, two types of reproduction appear: asexual and sexual. In the first type, typical in single-cell organisms such as archae- or eubacteria, protozoa and many plants and fungi, there is no mix of the genetic material in the parental generation. In contrast, sexual reproduction implies that the parental generation will produce a very specialized haploid cell called a male gamete, whose destiny is to fuse with the female gamete to form a zygote during fertilization, leading to a new individual (Crow 1994). Sexual reproduction appears in several *Phyla* such as plants and animals, where fertilization depending on the site of occurrence is considered as either external or internal. In the case of most fishes, for example, fertilization is ex-corpore (i.e. takes part in the environment, Schulz et al 2010), while in mammals it occurs inside the female genital tract (Eddy 2006).

Regarding animal biology, the spermatozoon is the male gamete (Eddy 2006). In mammals, spermatozoa must find their way towards the oocyte inside the female reproductive tract, acquiring its final ability to fertilize during this journey (Crow 1994). Interaction with the female reproductive tract leads to sperm capacitation (Gadella et al 2008) and causes changes in sperm motility (from activated to hyperactivated, Turner 2003), to allow spermatozoa to swim through the viscous media surrounding the cumulus-oocyte complex (COC, Turner 2003). The *in vivo* interactions between spermatozoa and their surroundings occur via the sperm plasma membrane, where a series of receptors and channels are located in order to maintain homeostasis (regulating water and intracellular ion concentrations) and to respond to stimuli (hormones, β -endorphins) (Meizel 2004). *In vitro*, spermatozoa also adapt to different conditions regulating their intracellular milieu. Thus, the study of the presence, distribution and function of sperm membrane channels and receptors acquires relevance to better understand sperm physiology and mechanisms of reactivity both *in vivo* and *in vitro*.

The study of reproduction, and particularly of gamete biology, is required not only in humans to treat infertility (Agarwal et al 2015), but also in animals where two other purposes are pursued: food production (livestock) and biomedical research. Pig is the most common livestock available in Europe for meat production (Marquer et al 2015) and, together with ruminants, represent 1.2% of the animals used as research models (COM 2013, 859/final). Studies requiring the use of pigs as models focus on major areas of fundamental biomedical research, including cardiovascular research, vaccine effectiveness, gastroenterology as well as development of tissues for human xeno-transplantation (COM 2013, 859/final). In total, experimental research uses 40 to 100 million animals per year world-wide, and almost 11.5 million animals just within the European Union (EU), mostly rodents and rabbits (COM 2013, 859/final). A significant proportion (above 80%) of these research animals are used by the pharmaceutical industry to assess toxicity of drug candidates prior to clinical testing (Nuffield Council on Bioethics, May 2005). Current legislation at the EU is focused in reducing the number of animals for research (EU Directive 2010/63), in agreement with the 3R principles (Russell and Burch 1959). This calls for alternative methods for the safety

evaluation ultimately replacing animal testing (SEURAT) using ex-corpore approaches to test cell homeostasis. Spermatozoa have easily identifiable phenotypic indicators such as sperm motility as a token for viability. Commercial pigs are basically bred via artificial insemination (AI) using extended liquid semen (Wagner and Thibier 2000). Cooled pig AI-semen is a suitable source of cells for *in vitro* testing and research, an approach that would moreover aid to reduce and eventually replace conventional research animals for testing.

REVIEW OF THE LITERATURE

The pig spermatozoon

Spermatozoa are produced in the testis through a complex process called spermatogenesis. Testicular spermatozoa follow a process of maturation in the epididymis, through which they acquire capacity to move and fertilize. Mature spermatozoa are stored at the cauda epididymis in a quiescent status until ejaculation occurs; when motility is activated and they start they journey along the female internal genital tract (Eddy 2006).

Spermatogenesis

Spermatogenesis takes place in the seminiferous tubules, the exocrine parenchyma of the testis, under the modulatory action of a series of hormones produced by the somatic Leydig (testosterone) and Sertoli cells (anti-müllerian hormone, estradiol, inhibin, Setchell and Waites 1971; Maddocks and Setchell 1988). The seminiferous tubules comprise myoid cells and the seminiferous epithelium (Pinart et al 1999) where both somatic (Sertoli cells) and germ cells (from spermatogonia to elongated spermatids) reside. Spermatogenesis is divided in three specific steps. The first one is *spermatocytogenesis*, where some spermatogonia commit themselves to enter spermatogenesis generating primary spermatocytes, while others are maintained as stem cells. The second one is *meiosis*, build by two consecutive reductional meiotic divisions where the primary spermatocyte goes throughout a prolonged prophase with duplication of the genetic material, the crossing over of genomic material between chromatids, to finally divide into two recombined secondary spermatocytes which quickly, without duplication of the genomic nuclear material, yield two haploid cells; the spermatids. The final component of spermatogenesis is *spermiogenesis*, where the round spermatids transform into elongated spermatids which are ultimately shed from the epithelium as testicular spermatozoa via spermatotoleosis. After *meiosis I*, the germ cells are connected to each other via cytoplasmic bridges and are metabolically and structurally controlled by the Sertoli cells (O'Donnell et al 2011).

Spermatogenesis requires a specific ad-luminal environment for meiotic events guaranteed by the blood-testis barrier and its major component of effective tight junctions between Sertoli cells (Yazama et al 1988), which creates a physical, physiological and immunological barrier (Santon 2016) from the basal compartment, exposed to the innate and acquired immune defense. As mentioned, the Sertoli cells, continuously stimulated by follicle-stimulating hormone (FSH) and testosterone, assist germ cells during spermatogenesis providing structural support and nutrition, moving them upwards and phagocytizing degenerated germ cells as well as the bulk of cytoplasm of the maturing elongated spermatids, exception of the

residual proximal cytoplasmic droplet (Caires et al 2008). In addition, the Sertoli cells secrete an iso-osmotic fluid, higher in K^+ concentration than the blood plasma and slightly acidic (Rato et al 2010) that keeps the appropriate environment for the developing germ cells. The maintenance of this environment is guaranteed also by water-channels aquaporins (AQPs) located in the membrane of Sertoli cells, playing a crucial role in volume and osmotic regulation in the testis (Yeung et al 2010; Chen and Duan 2011).

As mentioned, differentiation from round to elongated spermatids is a complex molecular and morphological process named *spermiogenesis*, during which round spermatids undergo formation of the acrosome in relation to a dramatic condensation of the chromatin as well as development of a flagellum towards a sperm tail and a final reduction of the cytoplasm to become elongated spermatids and, when freed from the epithelium, testicular spermatozoa (Garcia-Gil et al 2002). The acrosome derives from the Golgi complex. Fusion of pro-acrosomal vesicles forms a single acrosomal vesicle that will attach to the anterior part of the nucleus, creating a flattened compartment rich in enzymes with an external and an internal membrane (Foster and Gerton 2016). Alongside, the nucleus changes its position from the center of the round spermatid to the head of the elongated spermatid. The change of position of the nucleus occurs in association with packaging and condensation of the chromatin (Kerr et al 2006). The transformation of one centriole into a flagellum builds the core of the sperm tail (Baccetti 1982). The flagellum is surrounded in the mid-piece by mitochondria (Nicander and Bane 1962) and by columns of proteins in the principal piece. Finally, the elongated spermatids are released to the lumen of the seminiferous tubules at spermatotelysis to become testicular immature spermatozoa (O'Donnell et al 2011).

Spermatogenesis in pigs lasts for 38-41 days from the moment when a spermatogonia enters the ad-luminal compartment until eight immature testicular spermatozoa are shed to the lumen to exit the seminiferous tubules towards the epididymis (Swierstra 1968; França and Cardoso 1998).

Sperm maturation

Pig spermatozoa enter the epididymis transported in testicular fluid moved by the contractions of the myoid cells, the beating of the ciliated cells of the efferent ducts and by contractions of the testicular capsule (Johnson and Howards 1975). The boar epididymis has a simple histoarchitecture, with a resorptive/secretory epithelium (principal cells, with microvilli intermingled with immune cells), surrounded by a layer of smooth muscle whose thickness increases towards the ductus deferens (Johnson and Howards 1975). The single duct is divided morphologically and functionally in three segments, the initial, mid- and terminal segments. The initial segment (caput) is where most of the testicular fluid is resorbed increasing the spermatocrite. The mid-segment (corpus) is where most maturational events (morphological and functional) occur while the terminal segment (part of the corpus and the entire cauda epididymis) is where the mature spermatozoa are stored until ejaculation

occurs. During maturation, the spermatozoa undergo several biochemical and morphological changes, regulated by specific secretions and defined ionic, solute and protein composition (Dacheux et al 1989, 2003, 2005; Rodríguez-Martínez et al 1990a). Changes comprise the final reshaping of the acrosome (Burkin and Miller 2000), the migration of the cytoplasmic droplet from a proximal to a distal location in the annulus (Kaplan et al 1984), the final compaction of the chromatin via protamines replacing histones (Rodríguez-Martínez et al 1990b), modifications in the lipid and protein composition of the sperm plasma membrane (Flesch and Gadella 2000), the acquisition of sperm motility (Bredford 2004) and, most importantly, of the fertilizing ability (Harayama et al 1993). Sperm transport and maturation in the pig epididymis are primarily regulated by androgens (Synthin et al 1999; Robaire et al 2006) although estrogens are also involved (Pearl et al 2007). Epididymal contractions are regulated by several factors such as testosterone, prostaglandins, oxytocin and vasopressin as well as temperature and adrenergic/cholinergic innervation (Robaire et al 2006). Once reaching the cauda, spermatozoa remain quiescent until ejaculation (Johnson and Howards 1975; Zimmerman et al 1979). Caudal intra-luminal sperm quiescence is ensured, besides the high cell concentration, by the low pH of the fluid, its hyperosmosis (above 390 mOsm/Kg), the very low concentration of bicarbonate (Rodríguez-Martínez 1991) and the reverse electrolyte ratios (Na:Cl and Mg:K) compared to the epididymal head (Rodríguez-Martínez et al 1990a). At ejaculation, spermatozoa are mixed with the seminal plasma, which will revert the ionic composition of the cauda epididymis fluid and activate motility, primarily by increased concentrations of bicarbonate (Zimmerman et al 1979). Sperm maturation takes from 9 to 14 days in the boar (Singh 1962; Swierstra 1968).

Structure of the ejaculated pig spermatozoon

Fully differentiated ejaculated spermatozoa have the ability to find the oocyte and fertilize it after the processes of capacitation, hyperactivation and the acrosome-reaction (AR, see below). However, a percentage of spermatozoa in the ejaculate will still be immature (presence of a proximal cytoplasmic droplet) or even depict abnormal forms (two tails, two heads, etc.) in proportion that vary depending of the species considered. In the case of pigs, due to selection of males with high sperm quality for breeding purposes, the proportions of abnormal and immature spermatozoa are low (Hirai et al 2001; Thurston et al 2001), often fewer than 15 %. In contrast, human ejaculates are still considered “normal” with a 96 % of immature and abnormal spermatozoa (WHO 2010).

At first glance, the ejaculated pig spermatozoon is 53-55 µm in length (Holt et al 2010) and has a “tennis racket”-shaped sperm head, flat in the Z-Y axis while wide in the X-axis, followed by a cylindrical tail (Nicander and Bane 1962). Head and tail are connected by the neck or connecting piece (Figure 1A). The spermatozoon is a highly compartmentalized cell with specific areas (acrosome, central part of the neck, mitochondrial segment in the mid-piece, annular area, etc.) and a plasma membrane with specific domains in the sperm head

(acrosome, equatorial segment and post-acrosome), all highly relevant for sperm interactions with the surrounding (discussed in the following section).

The sperm head is 7-9 μm long, 3.7-4.2 μm wide and 0.4 μm thick (Cummins and Woodall 1985; Thurston et al 1999, 2001), constituted by the acrosome, the nucleus, a limited amount of cytoskeletal structures and a small amount of cytoplasm (Nicander and Bane 1962). The acrosome covers around 80 % of the nucleus, forming a cap over the anterior part of the head and contains hydrolytic enzymes that, when released during the AR, will contribute to the dissolution and the forced sperm entry through the zona pellucida (ZP, Gadella 2008). The nucleus represents the major part of the sperm head and contains the main genetic material extremely packed in a haploid version, including either an X- or Y- sex chromosome (Nicander and Bane 1962).

Figure 1. Basic morphological structure (A: DIC. Scale bar: 10 μm) and membrane domains (B) of the boar spermatozoon.

The neck or connecting piece is the structure connecting the head with the tail, presenting trapezoidal shape and being 0.7 μm long and 0.5 μm thick (Nicander and Bane 1962). It is separated from the sperm head by the posterior or striated ring (Toshimori 1998) and its main components are the capitulum and the segmented columns (Nicander and Bane 1962). The capitulum is a protein structure that anchors from one side to the basal plate to the implantation fossa and from the other to the segmented columns that will fuse with the outer dense fibers, surrounding the axoneme of the flagellum (see below; Nicander and Bane 1962).

The sperm tail is divided in the mid-piece, the principal piece and the terminal piece (Figure 1A). The mid-piece is 9 μm long and 0.7 μm wide, the principal piece is 26.2 μm long and 0.4 μm wide, and the end piece or terminal piece is 2.2 μm long and 0.2 μm wide (Cummins and Woodall 1985; Thurston et al 1999, 2001). The whole tail contains in its central position

a flagellum formed by the axoneme (Baccetti 1982; Nicander and Bane 1962), surrounded by the outer dense fibers that extend from the neck to the principal piece (Nicander and Bane 1962). These structures are, as mentioned above, surrounded by the mitochondrial sheath in the mid-piece and by the fibrous sheath in the principal piece (Eddy 2006).

The mid-piece is the segment that extends from the last part of the neck and is separated from the principal piece by the annulus or Jensen's ring (Toshimori 1998). A series of 75-100 mitochondria form a spiral around the axoneme in the mid-piece just below the plasma membrane (Nicander and Bane 1962; Phillips 1977; Eddy 2006).

The principal piece is the segment of the tail that extends from the annulus to the end of the flagellum (Toshimori 1998), where the terminal or end-piece starts. The principal piece is composed by a fibrous sheath surrounding the axoneme and outer dense fibers and represents most of the length of the sperm tail (Nicander and Bane 1962). The axoneme is a complex of microtubules with the typical $9 + (9 + 2)$ structure composed by α - and β -tubulin, in association with dyneins (motor proteins with ATPase activity), which provides the sperm tail with motility (Kerr et al 2006). Around the axoneme reside the outer dense fibers, present only in the first third of the principal piece, decreasing progressively in thickness and which function is to regulate the beating of the flagellum (Phillips 1972). The fibrous sheath is transversally oriented to the microtubules of the flagellum and has a role in sperm tail directionality (Kerr et al 2006). The fibrous sheath is composed by several A-Kinase anchor proteins (AKAPs), mainly AKAP-3. These proteins have anchoring sites for cyclic Adenosine monophosphate (cAMP)-dependent protein kinase (Kerr et al 2006). In addition, the fibrous sheath contains sperm-specific components of the glycolytic pathway, which suggests that the fibrous sheath has a role in the energy production required for hyperactivation (Kerr et al 2006).

Finally, the last part of the sperm tail (terminal or end-piece) is composed exclusively by the axoneme and a small amount of cytoplasm. The axoneme progressively disappears alongside the terminal piece towards the distal part of the spermatozoon (Nicander and Bane 1962).

The sperm plasma membrane

The sperm plasma membrane is the structure that maintains homeostasis and commands interaction with the various environments the spermatozoa might be exposed to *in vivo* or *in vitro*. As any other cell membrane, the sperm plasmalemma follows the typical mosaic fluid model (Singer and Nicolson 1972). Two major components appear in the negatively charged boar sperm plasma membrane: lipids and proteins. Lipids are represented by approximately 70 % phospholipids, 25 % neutral lipids and 5 % glycolipids (Nikolopoulou et al 1985). Among the proteins, membrane receptors represent the main component. The porcine sperm membrane has relatively low amounts of cholesterol compared with other cell membranes (Nikolopoulou et al 1985; Gadella et al 2008), which might facilitate surface heterogeneity.

The particular characteristic of the sperm plasma membrane is that it can be subdivided in several domains (Figure 1B), each of which characterized by the presence of specific structures inside the cell and the presence of domain-specific membrane-receptors. Each domain plays specific roles in sperm physiology (rev. by Gadella and Luna 2014), deriving in the activation of different processes (capacitation, hyperactivation, AR) via intracellular pathways initiated by binding of extracellular ligands through membrane receptors. The plasma membrane domains of the three areas of the boar spermatozoon are: a) head: acrosome, equatorial segment and post-acrosome; b) connecting piece: neck; c) tail: mid-piece, principal piece and end-piece (Figure 1B). Some barriers develop between membrane domains to avoid diffusion of membrane proteins and lipids (e.g. the posterior ring and the annulus, Toshimori 1998).

Due to the reduced amount of sperm cytoplasm (Ekwall, 2007), the plasma membrane is in almost direct contact with some intracellular structures (e.g. the plasma membrane in the mid-piece is in contact with the mitochondria and the plasma membrane of the principal piece is in direct contact with the fibrous sheath, Gadella et al 2008).

Sperm membrane proteins

Proteins present in the plasma membrane can be either adsorbed to extracellular sites or integrated as transmembrane proteins (Singer and Nicolson 1972; Haden et al 2000). Adsorbed membrane proteins come from the testis, the epididymis and the seminal plasma and act as decapacitation factors, immune-regulators, etc. (Dacheux et al 1989; Rodríguez-Martínez et al 1998; Vadnais and Roberts 2010, Rodríguez-Martínez et al 2011). Integrated or structural membrane proteins build membrane channels and receptors relevant for sperm homeostasis and interaction (Flesch and Gadella 2000).

Adsorbed membrane proteins are usually removed from the sperm surface by fluidic interactions; *in vivo* by the uterine/oviductal secretions and *in vitro* by the extension and washing done while handling semen (Maxwell and Johnson 1999; Rodríguez-Martínez 2005). This removal exposes channels and binding sites of membrane receptors, whose action can modulate the degree of sperm interactions with the extracellular milieu and oocyte envoltures and the occurrence of capacitation, hyperactivation and AR (Rodríguez-Martínez et al 2005; Gadella et al 2008). Integrated membrane proteins play also a role in maintaining sperm membrane structure and, until the signal transduction is activated via extracellular effectors, they prevent membrane rupture and acrosome exocytosis.

A significant proportion of the energy consumed by spermatozoa is used to maintain membrane intactness (Peña et al 2009) and, particularly, the functionality of membrane channels and receptors. Among the most important events regulated by sperm membrane proteins appear volume accommodation via AQP_s (Yeung et al 2010; Chen and Duan 2011), of intracellular calcium ($[Ca^{2+}]$) levels via calcium (Ca^{2+}) channels (CatSper, Lishko et al 2011),

of pH regulation via membrane enzymes (soluble adenylyl cyclase [sAC], Litvin et al 2003), of oocyte localization and sperm motility via several membrane channels and receptors (such as CatSper [Tamburrino et al 2014] and opioid receptors [Agirregoitia et al 2006; 2012]) and the sperm-oocyte interaction and fusion via sperm membrane proteins (IZUMO1-JUNO, Aydin et al 2016; Ohto et al 2106). The main sperm membrane channels and receptors identified in pig, mouse and human spermatozoa are listed in Table 1.

Sperm metabolism

Two basic processes must function in spermatozoa to ensure homeostasis and interplay with the surrounding female genital lining epithelia: (i) interpreting signals received via the plasma membrane and (ii) maintaining motility. Both processes are energy demanding, consuming enormous amounts of adenosine triphosphate (ATP, Peña et al 2009). The ATP generated in spermatozoa is produced by cytoplasmic glycolysis and mitochondrial oxidative phosphorylation (OXPHOS, Kamp et al 2003; Silva and Gadella 2006) and, depending of the species, one of the metabolic routes has more relevance. Most of the ATP produced in boar spermatozoa comes from cytoplasmic glycolysis (Marin et al 2003). The presence of hexose transporters (glucose transporters [GLUT]) in their plasma membrane (rev by Rodríguez-Gil and Bonet 2016) guarantees the intake of glucose and other monosaccharides, which will enter glycolysis to produce ATP. At first glance, it would appear contradictory that the main energy pathway used by boar spermatozoa to produce energy is glycolysis and not OXPHOS, as the latter is more efficient than the former. *In vivo*, boar spermatozoa will be exposed to environments with different oxygen levels alongside the female genital tract, and so having two different metabolic pathways is beneficial.

Members of the GLUT family show specificity for different monosaccharides (e.g. GLUT-3 is specific for glucose while GLUT-5 is for fructose, Mueckler 1994). In the sperm plasma membrane of the pig, GLUT-3 and -5 show different, specific locations (Medrano et al 2006b; Sancho et al 2007). This would suggest that a particular hexose (glucose, for example) can only be internalized at specific plasmalemmal domains, being further metabolized at that defined location inside the spermatozoon (Rodríguez-Gil 2013). However, monosaccharides are not the only substrate that boar spermatozoa can metabolize to obtain energy, since they can also use citrate, pyruvate or glycerol (Medrano et al 2006a), although with less efficiency than sugars in terms of ATP production.

As mentioned previously, glycolysis will occur in the cytoplasm present in the sperm tail and either used for sperm motility (Marin et al 2003; Mike et al 2004) or to maintain the plasma membrane stable (Silva and Gadella 2006). Mitochondria present in the sperm mid-piece will also produce ATP via OXPHOS. Uncoupling mitochondrial activity decreases boar sperm motility (Ramíó-Lluch et al 2014), indicating how essential mitochondrial ATP production is.

Table 1. Main sperm membrane channels and receptors.

Receptor	Species	Membrane domain	Function	Method used	Reference
α_{2A} adrenergic receptors	Mouse, Human	Acrosome, post-acrosome and neck	Capacitation	WB, ICC	Adeoya-Osiguwa et al 2006
β_1 adrenergic receptors	Mouse, Human	Acrosome, equatorial segment and neck, with weaker fluorescence in the tail	Capacitation	WB, ICC	Adeoya-Osiguwa et al 2006
B ₂ adrenergic receptors	Mouse	Head and tail	Capacitation	WB, ICC	Adeoya-Osiguwa et al 2006
β_3 adrenergic receptors	Mouse, Human	Acrosome and post-acrosome and tail (midpiece)	Capacitation	WB, ICC	Adeoya-Osiguwa et al 2006
Nicotinic Acetylcholine Receptors	Human	Equatorial segment	AR and sperm-oocyte interaction	ICC	Baccetti et al 1995; Bray et al 2002
Muscarinic Acetylcholine Receptors	Human	Equatorial segment and acrosome	Sperm-oocyte interaction	ICC	Baccetti et al 1995
Dopamine D ₂ receptors	Mouse, Human	Tail	-	WB, ICC	Oth et al 2007
Dopamine D ₂ receptors	Boar	Tail, mainly in the midpiece	Sperm viability during capacitation	WB, ICC	Ramírez et al 2009
Glutamate	Human	Midpiece and tail	Fertilizing ability	ICC	Storto et al 2001
GABA _A	Human	Equatorial segment	AR	WB, ICC	Wistrom and Meizel 1993
Glycine Receptor	Mouse	3 patterns: A (tip of the acrosome) B (posterior acrosome) and C (both of A and B patterns)	AR	ICC	Sato et al 2000
Glycine Receptor _{A1, A2, A3 and B.}	Human	GlyR _{A1} and GlyR _{A2} in the equatorial region, GlyR _{A3} in the flagellum principal piece and GLR _B in the acrosome.	AR	WB, ICC	Kumar and Meizel 2008
Glycine Receptor	Boar	Acrosome	AR	ICC	Melendrez and Meizel 1996
Angiotensin II-1 Receptor	Human	Tail	Motility	ICC	Vinson et al 1995
Angiotensin II-1 and II-2 Receptors	Mouse	Acrosome and principal piece	Motility and AR	ICC	Wennemuth et al 1999
TRPV1	Mouse	Post-acrosome	Capacitation, AR, sperm-oocyte interaction	WB, ICC	Catanzaro et al 2011
TRPV1	Human	Post-acrosome	Capacitation, AR, sperm-oocyte interaction	WB, ICC	Francavilla et al 2009
TRPV1	Boar	Post acrosome and acrosome (depending on time)	Capacitation, AR, sperm-oocyte interaction	WB, ICC	Bernabò et al 2008; Maccarrone et al 2005
Cannabinoid Receptor CB1	Human	Head, midpiece and tail	Motility, AR, fertilization and mitochondrial function	RT-PCR, WB, ICC	Rossato et al 2005; Agirregoitia et al 2010

Cannabinoid Receptor CB2	Human	Post-acrosome and midpiece	Motility	RT-PCR, WB, ICC	Agirregoitia et al 2010
Cannabinoid Receptor CB1	Boar	Post-acrosome	Capacitation and AR	WB, ICC	Maccarrone et al 2005
Progesterone Receptors	Human	Acrosome, equatorial segment, midpiece and tail	AR	WB, ICC, FC	Sabeur et al 1996; Gadkar et al 2002; Thomas et al 2009
Progesterone Receptor	Boar	Acrosome, post-acrosome and midpiece	AR	WB, ICC	De Amicis et al 2012; Melendrez et al 1995
CD44	human	Generalized, mainly in the acrosome	Motility	ELISA, WB, ICC	Bains et al 2002
AQP-3	Boar	Midpiece and tail	Volume regulation	WB, ICC	Prieto-Martínez et al 2015
AQP-7	Mouse	-	-	RT-PCR, WB	Yeung et al 2009
AQP-7	Human	Midpiece, tail	Volume regulation, male fertility	RT-PCR, WB, ICC	Yeung et al 2010; Saito et al 2004; Moretti et al 2012
AQP-7	Boar	Neck	Sperm quality	WB, ICC	Prieto-Martínez et al 2016
AQP-8	Mouse	-	Volume regulation	RT-PCR, WB	Yeung et al 2009
AQP-8	Human	Tail	Volume regulation	RT-PCR, WB, ICC	Yeung et al 2010
AQP-9	Mouse	-	-	RT-PCR, WB	Yeung et al 2009
AQP-11	Boar	Head, midpiece and tail	Sperm quality	WB, ICC	Prieto-Martínez et al 2016
CatSper	Mouse	Principal piece	Motility and hyperactivated motility	NB, WB, ICC	Ren et al 2001
CatSper 1	Human	Cytoplasmic droplets, midpiece, head	Motility and AR	WB and ICC	Tamburrino et al 2014, 2015
CatSper 2	Mouse	Tail, principal piece	Motility and hyperactivated motility	WB, ICC	Quill et al 2001, 2003
CatSper 2	Human	Tail	Motility, male fertility	WB, ICC	Bhilawadikar et al 2013; Smith et al 2013
CatSper 3 and 4	Mouse, Human	Acrosome, midpiece, cytoplasmic droplet	AR, hyperactivated motility during capacitation and male fertility	Prediction software, PCR, ICC (only mouse)	Lobley et al 2003; Jin et al 2005, 2007
CatSper 4	Mouse	Principal piece	Hyperactivated motility and male fertility	ICC	Qi et al 2007
μ opioid receptor	Human	Equatorial segment and post-acrosome, tail	Motility and capacitation	RT-PCR, WB, ICC	Albrizio et al 2006; Agirregoitia et al 2006, 2012
δ opioid receptor	Human	Acrosome, equatorial segment and tail	Motility	RT-PCR, WB, ICC	Agirregoitia et al 2006, 2012
κ opioid receptor	Human	Head, midpiece and tail	-	RT-PCR, WB, ICC	Agirregoitia et al 2006, 2012

Sperm motility

Spermatozoa are basically composed by a head carrying the genetic material to be delivered to the oocyte and a tail that moves the cell to help deliver this genetic material (Silva-Villalobos et al 2014, Su et al 2012). Two types of phenotypic sperm motility can be differentiated: activated and hyperactivated (Turner 2003). Boar spermatozoa are not motile in the epididymis, albeit a vibration of the tail can be observed. Symmetric beating of the sperm tail is activated by seminal plasma, leading to the typical progressive motility depicted by ejaculated spermatozoa (Zimmerman et al 1979; Eddy 2006; Turner 2006). It is the bicarbonate present in the seminal plasma that activates the sperm sAC controlling cAMP production. Increasing levels of cAMP and Ca^{2+} activate protein-kinase A (PKA), which will phosphorylate sperm tail proteins such as AKAP-3 present in the fibrous sheath of the sperm tail (Darszon et al 2006) which, together with the phosphorylation of the microtubular dynein arms, activates the ATPase and the energy released of ATP hydrolysis is transformed in tail beating, allowing the spermatozoon to move (Turner 2006). Concentrations of bicarbonate in the female genital tract steadily increase in the oviductal fluid towards the site of fertilization (Rodríguez-Martínez et al 2005), and Ca^{2+} is finely regulated by the sequestration of Ca^{2+} in intracellular deposits (Yeste et al 2015) and by Ca^{2+} channels (Darszon et al 2006).

Hyperactivated motility is a special motility mode displayed by capacitated spermatozoa and caused by the asymmetric beating of the sperm tail. Hyperactivation allows spermatozoa to escape the eventual epithelial binding in the sperm reservoir (SR) and penetrate the viscous environment at the COC before fertilization (Suarez et al 1991; Suarez et al 1992; Ho and Suarez 2001). Hyperactivated motility will be discussed later in detail on in the section “Sperm transport in the female genital tract”.

The boar ejaculate

Caudal spermatozoa are sequentially released during ejaculation and mixed in the urethra with secretions of the various accessory sex glands to form the ejaculate (Marberger 1974; Lavon and Bournsnel 1975). The ejaculate is constituted of two parts: spermatozoa and SP, the latter building its volume (95%, Mann and Lutwak-Mann 1981).

Ejaculation, ejaculate fractions

Ejaculation is the process by which semen (spermatozoa and SP) is released by the male (Marberger 1974). The process of ejaculation is controlled by the central nervous system (CNS) via sympathetic innervation (deGroat and Booth 1980) and it is composed of two phases: *emission* (formation of semen by mixing the SP and the caudal spermatozoa in the membranous urethra) and *ejection* (Marberger 1974) via the penile urethra. In the boar, ejaculation is lengthy (5-10 min), the ejaculate being voluminous (250 mL on average), bearing a mean of 120×10^9 total spermatozoa (Mann and Lutwak-Mann 1981) and occurring in waves of ejaculation jets (fractions). The ejaculate can thus be divided in several fractions taking into account the sperm concentration and the relative contribution of each accessory gland (Lavon and Bournsnel 1975; Rodríguez-Martínez et al 2005; 2009). The three fractions that constitute the boar ejaculate are the pre-sperm-rich fraction (pre-SRF), the sperm-rich fraction (SRF) and the post-sperm-rich fraction (post-SRF, Lavon and Bournsnel 1975; Rodríguez-Martínez et al 2009).

The pre-SRF (10-15 mL) does not contain spermatozoa and it is mainly constituted by secretion of the seminal vesicles, bulbourethral glands and the prostate, but also contains some gel, urine and smegma from the prepuce and a heavy degree of contamination and of cell debris (Einarsson 1971; Lavon and Bournsnel 1975; Mann and Lutwak-Mann 1981).

The SRF (around 50 mL) is the fraction of the ejaculate that contains most spermatozoa ($0.5-2 \times 10^9$ spermatozoa / mL). The SRF contains not only spermatozoa but also the epididymal fluid where spermatozoa bathe in and secretions from the prostate and seminal vesicles (Einarsson 1971; Lavon and Bournsnel 1975; Mann and Lutwak-Mann 1981). The first 10 mL of the SRF are considered the sperm-peak portion, holding about 20-25% of all ejaculated spermatozoa (Peña et al 2006; Rodríguez-Martínez et al 2005).

The post-SRF (150-400 mL) contains a progressively decreasing number of spermatozoa (below 10^6 spermatozoa / mL) and secretions from the prostate, the seminal vesicles and bulbourethral glands (Lavon and Bournsnel 1975; Rodríguez-Martínez et al 2009). At the end of the post-SRF, a tapioca-like secretion is released by the bulbourethral glands to seal the cervix of the female (Mann and Lutwak-Mann 1981).

Seminal plasma

The pig SP is a heterogeneous mixture containing several organic and inorganic solutes (Lavon and Bournsnel 1975). Contrary to what could be thought, the SP is not just the fluid where spermatozoa bathe in, but a heterogeneous fluid which composition makes it a crucial modulator of sperm function, nutrition and immune regulation in the female reproductive tract (Maxwell et al 1996; Rodríguez-Martínez et al 2009).

The main carbohydrate present in the boar SP is glucose (Mann and Lutwak-Mann 1981) while the main organic compounds present are proteins, among which spermadhesins, antioxidant enzymes and cytokines acquire relevant importance. The first ones are involved in sperm-interaction with the female genital tract and gamete recognition (Töpfer-Petersen et al 2008), antioxidant enzymes have a role reducing the concentration of reactive oxygen species (ROS) and their oxidative damage to spermatozoa (Barranco et al 2015b; Koziorowka-Gilun et al 2011), whilst cytokines (which level varies among the fractions of the ejaculate, Barranco et al 2015a) have a signaling role towards the female reproductive tract, either pro-inflammatory for cleansing the uterus or tolerogenic (e.g. down-modulating the immune response to tolerate sperm presence in the female reproductive tract, Moldenhauer et al 2009). The most relevant inorganic compounds are Ca^{2+} and bicarbonate. The divalent cation Ca^{2+} will activate a series of intracellular pathways mainly leading to hyperactivation and, in contact with the ZP, to AR (Darszon et al 2006). The flux through the sperm plasma membrane and hence the intracellular concentration of Ca^{2+} is finely regulated (Darszon et al 2006; Yeste et al 2014). The bicarbonate concentration in SP is 10-fold higher than in the cauda (Rodríguez-Martínez et al 1990a), the molecule being primarily responsible for the activation of sperm motility (Okamura et al 1985).

Sperm transport in the female genital tract

During mating in pigs, spermatozoa are deposited in the cervix and will have to travel through the whole uterus and oviduct until they encounter the ovulated oocytes. The sow has a relatively short uterine body and two long uterine horns which, together with the oviducts sum over one meter in length, per side (Climent et al 2005). Spermatozoa are therefore primarily transported to the oviduct by contractions of the myometrium and myosalpinx (Zero bin 1966, 1968a, 1968b; Rodríguez-Martínez et al 1982; Langendijk et al 2005). During this journey, spermatozoa will undoubtedly encounter different environments, and interact with fluids and cells in order to capacitate (Viring and Einarsson 1981).

Even though billions of spermatozoa are deposited at mating, only a few thousand reach the oviductal utero-tubal junction (UTJ), the first ones within 2-3 minutes, the UTJ being replenished within an hour (First et al 1969; Hunter 1981). In the mean time, most other spermatozoa are eliminated by passive back-flow and uterine phagocytosis by polymorphonuclear leukocytes (Viring and Einarsson 1981; Steverink et al 1998). The UTJ acts as a pre-ovulatory functional sperm reservoir (SR) where viable, morphologically normal and uncapacitated spermatozoa are in close contact with the tubal epithelium (Rodríguez-Martínez et al 1990; Fazeli et al 1999; Green et al 2001; Hunter and Rodríguez-Martínez 2004; Tienthai et al 2004). During SR-storage, spermatozoa are embedded in oviductal fluid that contains variable amounts of hyaluronan (hyaluronic acid, HA, Tienthai et al 2000) and bicarbonate (Rodríguez-Martínez 2007). From the SR, spermatozoa are progressively and continuously released towards the upper parts of the oviduct, most of them in relation to ovulation (Mburu et al 1996, 1997; Hunter 2008). Alongside, bicarbonate levels increase in the tubal fluid, reaching values of the order of 33-35 mM at the site of fertilization (ampullary-isthmic junction, AIJ, Rodríguez-Martínez 2007). Such levels are the main responsible for initiating boar sperm capacitation changes, including membrane destabilization (Harrison et al 1996; Gadella and Harrison 2000; Tienthai et al 2004; Harrison and Gadella 2005).

The interactive spermatozoon (capacitation, hyperactivation, acrosome reaction, zona pellucida binding and fertilization)

Ejaculated and UTJ-stored spermatozoa are not able to fertilize the oocyte without undergoing a series of biochemical processes that prepare their membrane (sperm capacitation, Tienthai et al 2004; Rodríguez-Martínez 2007; Gadella et al 2008) and change their tail beating from activated to hyperactivated to separate from the tubal epithelium and penetrate the viscous hyaluronan-rich environment close to the oocyte and, after exocytosis of the acrosome contents (acrosome reaction, AR), penetrate the ZP (Suárez et al 1992; Gadella et al 2008 and Figure 2).

Figure 2. Overview of the processes and interactions that spermatozoa must overcome for fertilization.

Sperm capacitation, first described by Austin and Chang (Austin 1951; Chang 1951) is characterized by a series of biochemical processes that will allow spermatozoa to acquire final fertilizing capacity (Rodríguez-Martínez 2007). Sperm capacitation does not occur massively in pig spermatozoa neither *in vivo* nor *in vitro*, under short exposure to bicarbonate (Saravia et al 2007; Botto et al 2010). Sperm capacitation in pigs occurs in the oviduct while spermatozoa progress towards the oocyte (Tienthai et al 2004). When spermatozoa move towards the AIJ after being released from the SR at the UTJ (Rodríguez-Martínez et al 1982), they are washed off the decapacitating factors adsorbed from the SP and the SR. *In vivo*, this removal of adsorbed proteins and of membrane cholesterol (Flesch et al 2001), shall initiate, in combination with the increasing concentrations of bicarbonate in the tubal fluid, destabilizing changes in membrane fluidity and lipid reorganization (Flesh and Gadella 2000; Gadella and Harrison 2000) that trigger capacitation by activation of sAC. The sAC activation shall increase the levels of cAMP, activating PKA and increasing protein-tyrosine phosphorylation (Harrison and Miller 2000; Harrison 2004; Ickowicz et al 2012) and membrane Ca^{2+} permeability (Petrunkina et al 2001), leading to increased $[\text{Ca}^{2+}]$ concentration and hyperpolarization of the sperm membrane (Gadella and Boerke 2016).

The increases in $[\text{Ca}^{2+}]$ concentration and phosphorylation of proteins such as AKAP-3 during sperm capacitation leads to hyperactivation of motility (Yanagimachi 1970), where the forceful asymmetric beating of the sperm tail causes a decrease in sperm velocity and a lateral displacement of the sperm head yet displaying an increased force of propulsion but only when opposed to an obstacle, such as the cloud of hyaluronan of the expanded COC-cloud and the outer ZP (Suarez and Osman 1987; Ishijima et al 2002) (Figure 2). Hyperactivation is dependent of the increase in $[\text{Ca}^{2+}]$ concentration, facilitated by release of Ca^{2+} from intracellular deposits (Ho and Suarez 2003) and the entry of Ca^{2+} from the extracellular medium to the cytosol; via sperm Ca^{2+} channels such as CatSper (Ho et al 2009; Qi et al 2007).

Hyperactivated spermatozoa that have swum through the oviduct encounter the oocyte in the AII (Rodríguez-Martínez et al 2005) and bind to the ZP by carbohydrate interactions via membrane glycoproteins (Töpfer-Petersen et al 1985, 2008). The AR is then initiated by interaction of the spermatozoon with the protein ZP3/ZPC of the ZP, which stimulates adenylyl-cyclase leading to cAMP increases, activating PKA and causing the opening of Ca^{2+} channels in the outer acrosome membrane (Florman et al 1989; Hurtado de Llera 2014). The increases in $[\text{Ca}^{2+}]$ activates phospholipase C (PLC), producing di-acyl glycerol (DAG) and inositol tri-phosphate (IP_3), which again will stimulate the opening of Ca^{2+} channels and cause the entrance of Ca^{2+} in the acrosome (Florman et al 1992; Rice et al 2000). These intracellular changes lead to fusion of both outer and inner acrosome membranes and release of acrosome enzymes (Flesch and Gadella 2000) which role is, together with the hyperactivated motility, to facilitate the sperm advance through the ZP (Honda et al 2002).

Once the first spermatozoon has penetrated the ZP and contact the oocyte membrane, the most internal area of the ZP changes protein conformation by action of the proteases released by the cortical granules of the oocyte (cortical reaction); to avoid the entrance of more than one spermatozoon in the oocyte (polyspermy, Funahashi et al 2000; 2001). Subsequently, the equatorial and post-acrosome domains of the sperm membrane become closely associated with the oocyte membrane and membrane fusion occurs (Michelmann et al 2007) (Figure 2). Several sperm membrane proteins such as IZUMO, ADAM and CRISP and oocyte membrane proteins such as CD9 regulate sperm-oocyte membrane fusion (Ash et al 1995; Sutovsky 2009). Shortly after membrane fusion the whole spermatozoon enters the oocyte, followed by the decondensation of the sperm chromatin, and the build-up of the male pronucleus, pending its union with the female pronucleus (amphimixia), ultimately forming the zygote (Figure 2).

Sperm handling in vitro

The pig industry relies in AI for large scale breeding, which makes absolutely necessary the collection of semen and preparation of AI-doses (Knox 2016). Collection of semen is usually performed by the gloved-hand technique (Hancock and Howel 1959) or using semi-automated collection systems such as BoarMatic® (Minitube, Tiefenbach, Germany) or Collectis® (Genes Diffusion, Douai, France, Barrabes Aneas et al 2008). The obtained ejaculates are thereafter extended in commercially-prepared media to guarantee sperm survival and fertilizing capacity over time of storage (Estienne et al 2007; Knox 2016). Semen doses can be prepared from a single male (homospermic) or by pool of several males (heterospermic) and they are usually prepared at a concentration of $20\text{--}40 \times 10^6$ spermatozoa / mL in a total volume of 80-100 mL, depending of the commercial source (Knox 2016).

Boar semen extenders are classified as short-term (1-3 days) or long-term (4 and more days) (Gadea 2003). The role of semen extenders is not only to preserve sperm motility but also membrane and acrosome integrity, warranting potential fertilization after AI (Waterhouse et al 2004; Estienne et al 2007). The exact composition of commercial extenders is obviously undisclosed, but experimental extenders (from where commercial extenders usually derive) contain an energy substrate, a buffer to maintain pH, salts to maintain osmotic pressure and antibiotics to avoid bacterial growth (Gadea 2003; Althouse and Lu 2008; Bryła and Trzcińska 2015; Kuster and Althouse 2016). As example, the most common extender used in the pig AI industry, the Beltsville thawing solution (BTS), contains dextrose, sodium citrate, sodium bicarbonate (at sub-physiological levels to avoid sperm capacitation), potassium chloride and ethylene diamine tetraacetic acid (EDTA) to maintain extracellular calcium (Ca^{2+}) levels low and avoid capacitation, hyperactivation or spontaneous acrosome exocytosis (Pursel and Johnson 1975).

In vitro handling of semen implies that spermatozoa are exposed to extenders, light, oxygen, low temperatures, centrifugation, resuspension, etc., all of which contributing to reduced sperm survival by negatively affecting the plasma membrane (Leahy and Gadella 2011b); the sperm component by which spermatozoa interact with the surroundings (see above).

Factors affecting boar sperm survival in vitro

Extension of the ejaculate decreases the concentration of SP-proteins and increases the chances of structural damage and loss of sperm function during storage (Waterhouse et al 2004; Pérez-Llano et al 2009), including reduction of sperm motility (Estienne et al 2007; Gogol et al 2009). Extension also leads to alterations in membrane fluidity, this being increased with short-term extenders and decreased with long-term extenders (Dubé et al 2004; Waterhouse et al 2004; Gogol et al 2009). Increased membrane fluidity causes higher Ca^{2+} permeability and subsequent capacitation-like changes and acrosome exocytosis,

reducing the fertilizing ability of the AI-doses (Waterhouse et al 2004; Oh et al 2010; Leahy and Gadella 2011a). On the other hand, decreased membrane fluidity in short-term-extended boar semen seems caused by peroxidative damage, often in connection with the reduction in SP amounts during processing (Awda et al 2009).

Boar spermatozoa are exquisite sensitive to temperatures below 15 °C, as the plasma membrane suffers from cold-induced damage and sperm motility is reduced if they are not extended in the appropriate medium (Pursel et al 1973; Ortman and Rodríguez-Martínez 1994). Time and temperature of storage can significantly increase deoxyribonucleic acid (DNA) fragmentation in boar spermatozoa (Pérez-Llano et al 2010), but motility, membrane intactness and chromatin integrity are not affected when semen is extended in conventional semen extenders and properly stored between 16 and 20 °C for up to 5 days (De Ambrogi et al 2006). The type of extender has also an effect in DNA fragmentation, the effect being lower in extenders containing lipoproteins (Fraser and Strzezek 2004; De Ambrogi et al 2006).

The pig in research, the 3R principles

The pig is not only the livestock from where the highest amount of meat per year is produced in Europe (Marquer et al 2015), but also a large animal model for xenotransplantation (owing to the high degree of similarities between human and pig anatomy and to a certain extent physiology, despite a different body temperature and gravitational posture, Aigner et al 2010; Swindle et al 2012). Pigs are also used for research in cardiovascular diseases, vaccine effectiveness, gastroenterology, wound healing, melanoma, diabetes, cystic fibrosis, traumatic brain injury, neurodegenerative diseases and development of tissues for human transplantation (Aigner et al 2010; COM 2013, 859/final; Swindle et al 2012). Considering anatomical and physiological similarities, using the pig as research model acquires particular relevance as it has recently been shown that animal models (non-human primates, dogs, rodents and rabbits) are poor predictors of human drug-induced toxicity (Bailey et al 2013; 2014; 2015). For the previous reasons, pigs and mini-pigs are being used in toxicological research to assess toxicity of human drug candidates (Svendsen 2006; Swindle et al 2012).

Toxicological testing for bioscience and research and development (R&D) in medicine, veterinary or dentistry requires about 8 % of total animal use for research within the EU and Switzerland (Daneshian et al 2015). Increasing evidence of the lack of predictability of the most commonly used animal models for human toxicology (Hartung and Leist 2008; Bailey et al 2013; 2014; 2015) has led to the development of alternative methods for toxicity testing.

Alternative methods to animal experimentation have been suggested several decades ago together with the 3R principles to *Reduce*, *Replace* and/or *Refine* animal experimentation (Russel and Burch 1959). Although postulated more than half a century ago, these principles are still valid and increasingly applied today (EU Directive 2010/63; Parker et al 2014; Törnqvist et al 2014; Burden et al 2015; Fleetwood et al 2015), as the use of animals for research has been reduced over the past years (COM, 2010, 511/final 2 vs. COM 2013, 859/final). Moreover, European legislation is, in agreement with the 3R principles, focusing on alternative methods to animal research for assessing toxicity of chemicals, as shown by several research initiatives within the EU such as SEURAT (Gocht et al 2015) or EU-ToxRisk (EU-ToxRisk 2015; Daneshian et al 2016) among others. Within this framework, using boar spermatozoa from commercial AI-doses to assess drug-induced toxicity of chemicals and drug candidates, would fit both current legislation and research initiatives within the EU towards the improvement of alternative methods to animal research.

This Thesis aimed to study the presence and reactivity (intactness and kinematics) of selected receptors, water and ion channels in ejaculated boar spermatozoa, with the final goal of finding a relevant alternative cell type for bioassays to ease the early scrutiny of candidate drugs as well as decreasing our needs for experimental animals (3R principles).

Specifically, four sub-studies were performed to:

- study the presence and distribution of AQP_s-7 and -9 alongside *in vitro* sperm handling and cryopreservation (**Paper I**),
- identify the presence and distribution of CatSper 1, 2, 3 and 4 subunits and evaluate the functionality of the CatSper channel (**Paper II**),
- study the presence, distribution and function of the μ -, δ -, and κ -opioid receptors (**Paper III**) and finally, to
- evaluate spermatozoa as a suitable cell type for toxicity assessment in contrast to conventional cell types (hepatocytes) (**Paper IV**).

METHODOLOGICAL CONSIDERATIONS

Ethical permissions

All experiments performed during this Thesis were in accordance with current regulations of the EU (European Community Directive 2010/63/EU) and in compliance with current Swedish legislation (SJVFS 2012:26). Experiments were performed at the Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden, the Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden and the Drug Safety and Metabolism Laboratory, AstraZeneca R&D, Alderley Park, United Kingdom. All experimental protocols had previously been reviewed and approved by the Local Ethical Committee for Experimentation with Animals at Linköping (nr 74-12 for pigs and nr 24-15 for mice) and Uppsala (nr C-119-4), and the Local Ethical Committee for Human Studies at Linköping (Ethical Permission Number 2015/387-31), Sweden.

Monitoring sperm motility

Sperm motility was assessed using the QualispermTM software (Biophos SA, Lausanne, Switzerland; Rigler et al 2008; 2011) connected via a Complementary Metal Oxide Silicon (CMOS) camera (UEye, IDS Imaging Development Systems GmbH, Ubersulm, Germany) to an upright Zeiss Axio Scope A1 light microscope using a 10x/1.25 phase contrast objective (Carl Zeiss, Stockholm, Sweden).

The QualispermTM technology is based on fluorescence correlation spectroscopic analysis of single particles (spermatozoa) in confocal volume elements (Rigler 2010), yielding a regression fluctuation algorithm of sperm numbers and translation classes. The fluctuation of the number of particles with respect to the detection areas is analyzed by the correlation function of the fluctuation signal, which is generated by light emission of the particles. Individual spermatozoa are projected on a pixel grid of the CMOS camera and the algorithm calculates the number of fluctuations in each pixel by correlation function of sperm numbers and translation classes, which is used to calculate the distribution of spermatozoa per velocity. This system benefits from a high throughput (usually 4 fields per minute), being able to analyze >2,000 spermatozoa/field but restricts the output of information to numerical sperm classes, based on their velocity of translation in a large field (Figure 3A-B).

Figure 3. A representative output of the Qualisperm™ software after analysis of a pig sperm sample. In (A) each detected spermatozoon, and its trajectory, in a typical microscope field appears marked in yellow, note the large numbers analysed per field. In (B) the results are depicted as various variables including velocity classes.

The capability of the Qualisperm™ technology of analyzing a large number of spermatozoa per field heavily contrasts with available Computer Assisted Sperm Analysis (CASA) systems, where many of the variables delivered are recalculated from absolute values of, for instance, curvilinear velocity (VCL). Although providing a large series of variables, the trajectory analysis of a CASA system fails to analyze high sperm concentrations, due to crossing trajectories. The Qualisperm™ technology, being independent of trajectories makes it possible to measure high sperm numbers at one time (Figure 3A). The method has been validated for boar spermatozoa against conventional CASA systems (Tejerina et al 2008; Rodríguez-Martínez et al 2008) and further tested for cryopreservation trials (Siqueira et al 2011). It has also been validated for use in the analysis of spermatozoa from stallion (Tejerina et al 2009; Johannisson et al 2009).

Assessing sperm integrity

Sperm integrity was assessed by flow cytometry (FC), enabling the analysis of multiple sperm characteristics in a short time frame thanks to the combination of several probes in one single experiment (Martínez-Pastor et al 2010; Hossain et al 2011). Membrane integrity was assessed with propidium iodide (PI, Garner et al 1986; Pintado et al 2000; Martínez-Pastor et al 2010), Annexin V (Peña et al 2003; Petrunkina et al 2005), or YO-PRO-1 (Peña et al 2005, 2007). Acrosome integrity was assessed with *Arachis hypogaea* (peanut) agglutinin (PNA) conjugated with Alexa Fluor 488 (Cross and Meizel 1989; Hossain et al 2011), and mitochondrial integrity with MitoTracker Deep Red (Hallap et al 2005). In addition, all FC-assessments included Hoechst 33342, a probe that binds DNA-containing particles and

fluoresces in the blue spectrum, allowing discrimination of spermatozoa from eventual debris present in the semen sample (Martínez-Pastor et al 2010).

Detecting membrane proteins

Membrane proteins were detected with Western blotting (WB, to determine presence and molecular weight) and indirect immunocytochemistry (ICC) at light and scanning electron microscopy levels (for specific cellular localization). Both methods (ICC and WB) followed the same principle: a primary antibody binds specifically to the protein of interest and a secondary antibody conjugated with a detection system (enzymatic, fluorescent or electron-dense) adequate to the visualization method used, binds specifically to the primary antibody (Fritschy and Härtig 2001; Mathews et al 2009; Burry 2010; Mahmood and Yang 2012; Shakes et al 2012).

Stressing spermatozoa (cryopreservation, osmotic and drug challenges)

Cryopreservation of living cells makes necessary to extend them in media containing a cryoprotectant agent (CPA) such as the commonly used glycerol (Meryman 1971) to avoid freezing-induced cellular damage. Cooling and freezing are done progressively to facilitate water flux out of the spermatozoa in exchange for glycerol and avoid intracellular ice crystal formation (Rodríguez-Martínez and Wallgren 2010). This situation causes formation of extracellular ice crystals and thus the sperm cytoplasm is filled with high concentrations of toxic solutes, surrounded by a frozen, extremely hyper-osmotic medium (Storey and Storey 2013). At thawing, the extracellular ice crystals change phase and the liquid water rapidly enters the cytoplasm causing a dramatic, often only transient, expansion of the plasma membrane (Mazur 1984; Jeyendran 1992). The capacity of boar spermatozoa to react to media with different osmotic pressures as those encountered during cryopreservation was mimicked *in vitro* by exposing boar spermatozoa to hyper- and hypo-osmotic media.

In order to evaluate boar spermatozoa as alternative cell model for drug-induced toxicity screening, boar spermatozoa were exposed to eight different concentrations of a battery of 130 toxic compounds and drugs and eight known mito-toxins. After exposure, both boar sperm kinematics and mitochondrial integrity (assessed by JC-1 and MitoTracker Red CMXRos) were assessed. The sperm-kinematic analysis was performed in an IX71 Olympus light inverted microscope equipped with a thermal plate, positive phase contrast optics (10x/0.30 objective, Olympus AB, Stockholm, Sweden), and a charge coupled device (CCD) camera (UI-1540LE-M-HQ, IDS Imaging Development Systems, Germany), connected via a personal computer to the ToxiSperm™ software (Biophos SA, Lausanne, Switzerland). The ToxiSperm™ software is a further developed version of the QualiSperm™ (see above)

sharing the same principles for use in drug screening due to its capability of performing semi-automatic assessment of sperm kinematics, increasing the speed of analysis.

Mitochondrial integrity after drug-exposure was assessed using MitoTracker Red CMXRos and JC-1 probes to confirm that changes in sperm kinematics were due to inactivation of sperm mitochondria by the toxic compounds. For this purpose, the IX71 Olympus microscope was equipped with fluorescence optics and each sperm sample examined for kinematics could thus be also examined for mitochondrial integrity. MitoTracker Red CMXRos accumulates in active mitochondria and emits red fluorescence while JC-1 accumulates in active mitochondria forming monomers and emitting green fluorescence. In the presence of high mitochondrial potential, the monomers form aggregates and photoconvert to emit orange fluorescence (Martínez-Pastor et al 2010).

Cytotoxicity in human hepatocytes

The human hepatoma cell line HepG2, purchased from the American Type Culture Collection (Rockville MD, USA), was challenged at the Drug Safety and Metabolism Laboratory, AstraZeneca Research and Development, United Kingdom with the same 130 toxic compounds and drugs and the eight known mito-toxins with the objective of comparing the results obtained with the boar sperm bioassay and the standard *in vitro* cell assay used by the pharmaceutical industry (Gómez-Lechón et al 2003). Ten different concentrations of each compound were used and ATP-concentration was determined post incubation using a commercial kit (Cell Titer Glo Luminescent Cell viability assay, Promega Biotech AB, Sweden).

Experimental design, Paper I

Membrane water channels AQP-7 and -9, that also transport glycerol and small non-polar solutes (Verkman et al 1996; Borgnia et al 1999), were studied for presence (WB) and distribution (ICC, at confocal and electron microscopy levels) in boar spermatozoa from different origin: a) the cauda epididymis, b) the portions P1 and P2 of the ejaculate and, c) the SRF, extended in BTS. Moreover, the distribution of AQP-7 and -9 was studied in SRF/BTS-extended spermatozoa alongside freezing/thawing and challenged with hyper- or hypo-osmotic media. Due to the previous reported lack of specificity of commercially available antibodies for AQPs (Yeung 2010), three commercially available antibodies were used against AQP-7 (bs-2506 [Bioss Inc., MA, USA], NBP1-30862 [Novus Biologicals, Abingdon, UK] and ab15213 [Abcam, Cambridge, UK]) and two against AQP-9 (bs-2060 [Bioss Inc., MA, USA] and NBP1-30865 [Novus Biologicals, Abingdon, UK]) as well as two different protocols for ICC (with and without extra permeabilization) were evaluated.

Experimental design, Paper II

The presence (WB), distribution (ICC) and function (sperm motility [QualispermTM], membrane, acrosome and mitochondrial integrity and Ca^{2+} concentration [Flow Cytometry]) of the four subunits of the main sperm Ca^{2+} channel, the CatSper (Lishko et al 2011; Tamburrino et al 2014) were studied in ejaculated, SRF, extended boar spermatozoa. The spermatozoa were extended in media containing different Ca^{2+} availability, exposed to the capacitation-trigger bicarbonate, to progesterone or challenged by CatSper inhibitors (mibefradil and NNC 55-0396) at physiological and toxicological doses separately or sequentially. The challenges were performed up to 60 min incubation and both dose-response and time effects were studied. In addition, the membrane enzyme ABHD2 was investigated for its presence in boar spermatozoa, as it has been reported to be involved in CatSper activation via progesterone in human spermatozoa (Miller et al 2016).

Experimental design, Paper III

The G-protein coupled μ -, δ -, and κ -opioid receptors (Ong and Cahill, 2014) were investigated for presence (WB) and distribution (ICC) in boar spermatozoa. Moreover, the relation to *in vitro* sperm kinematics (QualispermTM), sperm viability, mitochondrial integrity, and early membrane destabilization (Flow Cytometry) was studied using specific agonists (μ : morphine; δ : DPDPE; κ : U 50488) and antagonists (naloxone [μ]; δ : naltrindole; κ : *nor*-binaltorphimine).

Experimental design, Paper IV

Boar spermatozoa were evaluated as a suitable cell type for toxicity assessment by challenging them with eight different concentrations of 130 pharmacological drugs and toxic compounds as well as with eight known mito-toxic compounds, using a semi-automated motility analyser (ToxiSpermTM) together with real-time fluorescent probing of mitochondrial potential (MitoTracker & JC-1). Results obtained with this sperm assay (obtained after 30 min) were compared to results obtained with a hepatome-cell line (HepG2) for cell viability by determination of ATP-content (data obtained after 24-48 h).

GENERAL RESULTS

Membrane stress during thawing elicited re-distribution of Aquaporin 7 but not of Aquaporin 9 in boar spermatozoa (Paper I)

Extra membrane permeabilization was not needed for epitope presentation

Analysis of immunocytochemistry (ICC) protocols with/without extra permeabilization revealed that simple fixation already caused a desirable degree of permeabilization and that extra permeabilization (0.25 % Triton X-100 for 10 min) did not further increase the availability of epitopes located in the intracytoplasmic site of the plasma membrane (antibody NBP1-30862).

AQPs-7 and -9 were present in boar spermatozoa from various sources, although their distribution differed among the commercial primary antibodies tested

Western Blotting (WB) confirmed the presence of both AQPs in boar spermatozoa, depicting a different band pattern for each of the antibodies used. The ICC also revealed that each antibody gave a different location for each AQP. As such, AQP-7 was detected in the membrane over the principal piece with the bs-2506 antibody while AQP-9 was detected at the mid-piece plasma membrane with the NBP1-30862 and the ab15123 antibodies. Interestingly, all antibodies showed that AQP-7 was relocated post-thaw towards the membrane over the acrosome. AQP-9 was distributed over the membrane of the acrosome with the antibody bs-2060 and over the post-acrosome domain with the antibody NBP1-30865. AQP-9 distribution did not change after thawing.

In caudal spermatozoa, the AQP-7 was distributed over the entire tail membrane and the cytoplasmic droplets with the antibody bs-2506; over the membrane of the post-acrosome and cytoplasmic droplets with the antibody NBP1-30862 or over the plasmalemma over the cytoplasmic droplet and the principal piece of the tail with antibody ab15123. There were no differences observed among ejaculated spermatozoa from different fractions, but there were differences among antibodies. AQP-7 was detected over the principal piece with the antibody bs-2506, over the membrane the post-acrosome with the antibody NBP1-30862 and over the mid-piece membrane with the antibody ab15123.

In caudal spermatozoa, the AQP-9 was detected in the cytoplasmic droplet and also over the acrosomal membrane with the antibody bs-2060, or the post-acrosome membrane with the antibody NBP1-30865. Among ejaculated spermatozoa, AQP-9 was distributed over the membrane over the acrosome in either ejaculate portion with the antibody bs-2060, while for the antibody NBP1-30865 it was detected over the post-acrosome plasma membrane.

Distribution of AQPs-7 and -9 during cryopreservation and thawing

Cooling/freezing and thawing of ejaculated spermatozoa modified the distribution of AQP-7 as confirmed by all tested antibodies. After BTS-extension and at +15 °C in lactose egg-yolk (LEY)-extender, the AQP-7 was mainly distributed over the membrane of the principal piece for the antibody bs-2506, or on the membrane over the mid-piece for the antibodies NBP1-30862 and ab15123. However, when extended in lactose egg-yolk glycerol (LEYGO), cooled at +5°C and post-thawed, the distribution of AQP-7 was observed in the plasma membrane over the mid-piece for all antibodies, but also over the acrosome in thawed spermatozoa.

AQP-9 was not redistributed alongside cryopreservation and thawing. However, the specific location of AQP-9 varied with the antibody, as it was distributed in the membrane over the acrosome with the antibody bs-2060 or over the post-acrosome membrane domain with the antibody NBP1-30865.

In vitro challenge with hyper/hypo osmotic media

Exposure of SRF collected, BTS-extended spermatozoa to a hyper-osmotic solution (LEYGO extender) at room temperature (RT) did not modify the distribution of AQP-7 or -9 compared to the control (SRF-collected, BTS-extended spermatozoa, isosmotic, 320 mOsm/Kg). When LEYGO-extended spermatozoa were exposed to hypo-osmotic media (75 or 150 mOsm/Kg), both AQPs were distributed on the plasma membrane over the principal piece and the acrosome. Sperm exposure to the 150 mOsm/Kg hypo-osmotic solution for 30 or 60 min at 38 °C changed the location of AQP-7 to the plasma membrane over the acrosome while when exposed to 75 mOsm/Kg solution but not to 150 mOsm/Kg solution or the BTS-control AQP-9 was also identified in the plasma membrane over the tail.

Conclusions

Epididymal and ejaculated boar spermatozoa express both AQPs-7 and -9. Different commercial antibodies show varying molecular weights and unequal distribution of these AQPs over the sperm plasma membrane. However, all tested commercial antibodies confirmed that conventional cryopreservation, thawing in particular, modified domain localization of AQP-7 but not of AQP-9, as further demonstrated by *in vitro* hyper/hypo osmotic challenge.

Both AQPs-7 and -9 seemed involved in osmotic regulation during freezing and thawing and AQP-7 appeared as a relevant marker for non-empirical studies of sperm cryopreservation.

The CatSper channel modulated boar sperm motility during capacitation (Paper II)

The CatSper channel and the membrane enzyme ABHD2 were present in ejaculated spermatozoa

All four CatSper subunits were present in BTS-extended boar spermatozoa as confirmed by WB. The ICC analysis revealed that the four main CatSper subunits were localized over the sperm neck, tail and cytoplasmic droplets; albeit CatSper -1 presented additional distribution on the membrane over the acrosome and CatSper -2 and -4 on the post-acrosome membrane domain. The sperm membrane enzyme ABHD2 was detected via WB, confirming its presence in boar spermatozoa.

Challenge of non-capacitated spermatozoa with bicarbonate, progesterone or CatSper inhibitors (mibefradil or NNC 55-0396)

Challenge of spermatozoa extended in BTS (non-capacitating conditions) with bicarbonate (35 mM), progesterone (0.01 to 10 μ M) or CatSper inhibitors (mibefradil, 10 to 320 μ M; NNC 55-0396, 2.5 to 80 μ M) did not affect sperm motility unless sperm viability was affected over time.

Incubation of spermatozoa in capacitating-inducing conditions

Extension of spermatozoa in a medium with increased $_{e}Ca^{2+}$ availability (BTS without EDTA) and 35 mM of bicarbonate (capacitating-inducing conditions) caused a decrease ($p < 0.05$) in sperm motility, sperm viability, intact mitochondria and increased ($p < 0.05$) the amounts of spermatozoa displaying acrosome exocytosis. In addition, incubation in capacitating-inducing conditions caused an increase in $_{i}Ca^{2+}$ concentration as soon as after 15 min of incubation.

Exposure of capacitated spermatozoa to progesterone

Incubation with progesterone at physiological (0.01 μ M) or pharmacological (10 μ M) concentrations did not affect any of the sperm motility variables compared to when spermatozoa were solely exposed to capacitating-inducing conditions. Furthermore, addition of progesterone to capacitated spermatozoa did not modify $_{i}Ca^{2+}$ concentration compared to capacitated boar spermatozoa without exposure to progesterone.

Exposure of capacitated spermatozoa to CatSper inhibitors and sequentially to progesterone

Exposure of capacitated spermatozoa to 40 μM of mibefradil or 20 μM of NNC 55-0396 caused a decrease ($p < 0.05$) in sperm motility, not reverted by the addition of progesterone at physiological (0.01 μM) or pharmacological (10 μM) doses.

Conclusions

The four CatSper subunits were present and functional in ejaculated, extended, boar spermatozoa. Ca^{2+} availability and 35 mM bicarbonate appear to initiate sperm capacitation, hyperactivation and acrosome exocytosis. However, neither bicarbonate nor progesterone acted as specific CatSper agonists. On the other hand, mibefradil and NNC 55-0396 acted as specific CatSper antagonists effectively blocking the CatSper channel in capacitated spermatozoa.

The mu (μ) and delta (δ) opioid receptors modulated boar sperm motility (Paper III)

Presence and distribution of the opioid receptors in ejaculated spermatozoa

The WB and ICC analyses indicated that only the μ - and δ -opioid receptors were present in ejaculated spermatozoa. The ICC revealed that both μ - and δ -opioid receptors were located over the plasma membrane covering the acrosome, neck, and principal piece.

Modulation of sperm motility by the opioid receptors

Challenge with the μ -agonist morphine maintained and increased sperm motility over time while the δ -agonist [D-Pen 2,5]-enkephanile (DPDPE) reduced sperm motility over time. No effect was observed after challenge with the μ -antagonist naloxone and only a decrease in sperm viability was observed when boar spermatozoa were incubated for 60 min with the highest concentration (100 μ M) of the δ -antagonist naltrindole. Following the absence of the κ -opioid receptor as revealed by WB and ICC, no effects were observed after challenge with κ -agonist/antagonist in sperm kinematics without a decrease in sperm viability over time.

Conclusions

Both μ and δ -opioid receptors, but not the κ -opioid receptor, were present in ejaculated, extended boar spermatozoa and appeared to play a role modulating sperm motility as the μ -opioid receptor agonist morphine caused maintenance or increases in sperm motility while the δ -opioid receptor agonist DPDPE decreased sperm motility over time.

Boar spermatozoa successfully detected mitochondrial modes of toxicity: Implications for drug toxicity testing and the 3R principles (Paper IV)

Boar spermatozoa as detectors of drug-induced toxicity

Using sperm motility, progressive motility and sperm velocity as endpoints, dose-response curves were obtained for all the tested compounds, showing that the studied sperm variables decreased in response to increasing concentrations of toxic compounds ($p < 0.05$ in all curves), proving the disruption of the sperm energy production machinery and, thus, justifying the kinematic-based model assayed. Inhibitory Concentrations-50 (IC₅₀) values for each compound were calculated and the statistical analysis showed that the first point being different ($p < 0.05$) within the curve was close to the IC₅₀ for each dose-response curve.

Mitochondrial potential was assessed in order to confirm that decreases in sperm kinematics were caused by decreases in mitochondrial activity and thus a progressive decrease in mitochondrial potential was observed with increasing concentrations of the tested compounds.

Comparison between boar spermatozoa and HepG2 cells

The IC₅₀s obtained with spermatozoa and the HepG2 cells were compared between assay systems to determine the validity of the sperm model. The analysis showed that 62.5 % of the compounds with high toxicity (low IC₅₀) in HepG2 cells (IC₅₀ < 250 μ M) were also detectable as high toxicity compounds with boar spermatozoa (IC₅₀ < 300 μ M). Moreover, 87 % of the compounds with low toxicity (high IC₅₀) in HepG2 cells (IC₅₀ > 250 μ M) were also detected as low toxicity compounds with boar spermatozoa (IC₅₀ > 300 μ M), albeit diverging in 20% of the tested compounds. In addition, significant correlations ($p < 0.01$) between methods (boar spermatozoa and HepG2 cells) and sperm kinematic variables were found.

Conclusions

Boar spermatozoa were able to detect drug-induced mitochondrial toxicity using the endpoint of sperm kinematics. Both the sperm motility bioassay (30 min) and the conventional HepG2 testing (24-48 h) showed a high degree of agreement in detection of toxic compounds. The boar sperm bio-assay appears, thus, as a faster and relevant alternative method for detection of mitochondrial modes of toxicity based on sperm kinematics, including fulfilment of the 3R principles for reduction and ultimately replacement of experimental animals.

GENERAL DISCUSSION

This Thesis demonstrated that the pig spermatozoon expresses a series of functional water (**Paper I**) and ion (**Paper II**) channels as well as membrane receptors (**Paper III**), whose stimulation or blockade causes measurable responses. This information could be used to detect mitochondrial modes of toxicity by ejaculated spermatozoa, based on a rather simple assay quantifying sperm kinematics (**Paper IV**).

Spermatozoa have been the main cells used in this Thesis, albeit from different origins and species (pig, mouse, human). Human spermatozoa were obtained from consent ejaculates (**Paper III**), those of mice were obtained from the cauda epididymis of sacrificed animals (**Paper II**) and those of boar were both obtained from ejaculates and from the ductus epididymides (**Papers I-IV**). Boar spermatozoa, specifically used for experimentation, were obtained from breeding sires, either as commercial AI-doses (Svenska Köttföretagen, Hållsta, Sweden), or ejaculates/epididymides from breeding boars kept at Linköping University (LiU, Linköping, Sweden) or at the Swedish University of Agricultural Sciences (SLU, Uppsala, Sweden). Besides the different sources (including different pig breeds), results with boar spermatozoa were similar between commercial AI-doses or semen collected from animals kept at LiU or SLU as well as among breeds, which reflect the high degree of selection for semen quality among breeding boars reached over the years (Knox 2016). Boar semen was collected with the gloved-hand technique as already described (Hancock and Howel 1959), which allowed for the joint or separate collection of the different fractions and portions within fractions that characterize the boar ejaculate, for each of the different experiments (see General Results). Murine and human spermatozoa were used as positive controls for WB and ICC experiments, as the majority of the work already performed regarding membrane channels and receptor mapping and functionality has been done using either murine or human spermatozoa (see Table 1). Extraction of spermatozoa from cauda epididymis was performed by carefully dissecting the structures, keeping an adequate temperature and buffer conditions as well as avoiding blood contamination of the extracted semen sample. Mice used for sperm extraction were bred for educational purposes and belonged to strict stocks of wild type individuals, what guarantees a certain degree of homogeneity among animals and thus among extracted sperm samples. In contrast to the homogeneity of porcine and murine samples, human spermatozoa are not only heterogeneous among individuals but also among different ejaculates of the same individual (WHO 2010). The ejaculates used were provided, after consent ruled by ethical permission, from fertile voluntary donors (**Paper III**). Besides the named heterogeneity of human ejaculates, results obtained in **Paper III**, where human spermatozoa were used as a positive control, are in agreement with those already published in the literature (Agirregoitia et al 2006; Albrizio et al 2006), which could indicate a high conservation of the primary protein structure for the opioid receptors among species (see below) and/or the relevance of those receptors for the physiology of spermatozoa.

The mapping of membrane proteins via WB and ICC requires the use of antibodies (Mahmood and Yang 2012; Shakes et al 2012). The antibodies used in this Thesis were

purchased from commercial sources, which present a series of advantages (wide offer, no need for the cumbersome own production of the antibodies) but also some disadvantages (varying availability and uncertain specificity of a particular antibody, as already reported, for example, in the case of AQPs [Yeung 2010, **Paper I**]). The quality of antibodies available from commercial sources is highly dependent of the production process and thus, the affinity, quality and specificity (in terms of efficacy of binding to the antigen) are not consistent among batches and, more dramatically, among different commercial sources (see **Paper I** for specific examples). Most of the antibodies used in this Thesis were polyclonal, generated in rabbit and designed against a particular epitope of the protein (human) of interest (see **Papers I, II, III**). Even though commercial antibodies guarantee some cross-reactivity among some species, the binding specificity is not as accurate as expected in most cases when used for another species than the one designed for (excess of background, unspecific binding, Mahmood and Yang 2012; Shakes et al 2012). In consequence, a thoughtful literature study of homology between the immunogen protein and the protein to be detected must be performed (when available), to select the adequate antibody and thus reduce the negative impact of the lack of specificity. Nevertheless, both positive and negative controls should be included in order to detect unspecific immune binding (see **Papers I, II and III**). Moreover, a potential lack of specificity can also be reduced using not only substantial blocking solutions during the experimental procedures (Mahmood and Yang 2012; Shakes et al 2012) but by blocking the antibodies with a specific blocking peptide. However, it is not common that commercial sources make available the specific blocking peptide for their antibodies, as it was the case with the commercial antibodies used during this Thesis.

Spermatozoa are very specialized cells which, to fulfil their final role of fertilizing the oocyte/s, must interact via their plasma membrane with a variety of environments either *in vivo* or *in vitro* (Eddy 2006; Gadella et al 2008). The sperm plasma membrane contains a series of channels and receptors responsible of regulating sperm interactions with the surroundings (Meizel 2004). For instance, before ejaculation, pig spermatozoa in the epididymal cauda bathe in a hyper-osmotic intraluminal fluid containing a very low amount of bicarbonate (10 times lower than in blood plasma) (Rodríguez-Martínez 1991). At ejaculation, there is a sudden reduction of osmotic pressure and a 10-fold increase of bicarbonate concentration (Zimmerman et al 1979) which results in sudden changes in the volume of the spermatozoon but, particularly, the activation of their motility. Following exposure to upraising levels of bicarbonate in the oviduct close to ovulation (when progesterone dominates the media where spermatozoa bathe in) and the contact with the coverings of the newly ovulated oocyte/s, biochemical changes under the name of sperm capacitation occur. This exemplifies how membrane interactions with the environment (*in vitro* media or *in vivo* fluids, secretions or cells) can result in particular, monitorable, phenotypic responses: sperm motility changes (Gadella et al 2008). Such phenotypic responses can be tracked *in vitro* and be related to a defined extracellular medium composition (e.g., an exact concentration of an agonist/antagonist of a particular sperm channel/receptor or a compound whose effect wants to be studied), paving the way to the development of *in vitro* assays for detection of compounds with defined actions on spermatozoa.

Cryopreservation of boar spermatozoa, particularly thawing and extension in hypo-osmotic media, causes a relocation of AQP-7 from the membrane over the sperm tail to the membrane over the acrosome domain (**Paper I**). Spermatozoa from breeding boars are often subjected to various handling procedures, including cooling, freezing and thawing, thus compromising the intactness and stability of the plasma membrane (Mazur 1984; Jeyendran 1992). More importantly, freezing implies use of specific cool-protecting substances (as egg yolk) and CPA such as glycerol (Lovelock and Polge 1954), which increases the osmolarity of the media (see **Paper I**). Spermatozoa adapt to variations in osmotic pressure exchanging water via membrane AQPs (Yeung et al 2010; Chen and Duan 2011), provided these are functional within a stable, intact plasma membrane. In addition, an intact cytoskeleton is also required (Petrunkina et al 2004), which marks how relevant sperm maturation is to complete the development of all sperm structures, particularly of the plasma membrane, in relation to osmo-adaptation (Druart et al 2009). Several AQPs have been identified up to now in boar spermatozoa (AQP-3: Prieto-Martínez et al 2015; AQP-7: **Paper I**; AQP-9: **Paper I**; AQP-11: Prieto-Martínez et al 2016) although only a relationship between volume regulation and AQP-7 might appear due to the characteristic relocation of AQP-7 in the sperm plasma membrane in response to hypo-osmotic challenge (**Paper I**).

Due to the previously reported lack of specificity of antibodies against AQPs (Yeung 2010), three different antibodies were used for AQP-7 and two for AQP-9 and each of them showed a different membrane domain location for each of the studied AQPs (ICC, **Paper I**) and a different band pattern (WB, **Paper I**). Interestingly, however, a relocation of AQP-7 from the tail to the acrosome plasma membrane domains was observed with all tested antibodies, at either light or scanning electron microscopy levels (**Paper I**), thus confirming the relocation of this particular AQP-7 after thawing. It could be argued that cryopreservation alters the membrane structure and thus the relocation observed is nothing but unspecific binding. To test this possibility, an *in vitro* hyper/hypo-osmotic challenge was performed, mimicking what occurs during freezing and thawing, obtaining the same relocation of AQP-7 towards the plasma membrane on the acrosome domain (**Paper I**). Spermatozoa from different fractions of the ejaculate and from cauda epididymis were used in **Paper I**. The distribution of both AQPs did not differ among them, although a higher background was observed in the second portion of the ejaculate (seminal vesicle-dominated), probably due to a higher amount of protein-rich SP (Rodríguez-Martínez et al 2005). Interestingly, epididymal caudal spermatozoa depicted a strong labelling for both AQPs, what could explain why epididymal spermatozoa survive handling and cryopreservation procedures better than ejaculated spermatozoa do (Alkmin et al 2014).

The relocation of AQP-7 in the plasma membrane after thawing would make of this AQP a relevant marker for studies of sperm osmo-adaptation and sperm handling, contributing to the improvement of cryopreservation protocols of boar semen, an area still dominated by empirical studies. Furthermore, if used as alternative cell for drug toxicity screening, cryopreserved spermatozoa would further reduce the need of animals and increase sample availability for testing.

Boar spermatozoa express the four subunits of the main sperm Ca^{2+} channel (CatSper; Lishko et al 2011; Tamburrino et al 2014) in their plasma membrane. The CatSper becomes functional when boar spermatozoa are exposed to capacitation-inducing conditions; modulating sperm motility (**Paper II**). As mentioned above, the exposure of spermatozoa at ejaculation with a bicarbonate-rich SP, results in a sudden activation of sperm motility, becoming progressive and rectilinear (Zimmerman et al 1979). The presence of bicarbonate *in vitro* at levels encountered in the female oviduct (35 mM, Rodríguez-Martínez 2007) combined with Ca^{2+} availability (extender without EDTA, see **Paper II**), initiates capacitation-like changes in boar spermatozoa as depicted by an increase in membrane fluidity, in $[\text{Ca}^{2+}]$ concentration and stronger local motility (**Paper II**; Gadella et al 2008). The initiation of boar sperm capacitation is not dependent of CatSper activation; it is dependent on time and bicarbonate levels *in vitro* or *in vivo* (Tienthai et al 2004; Harrison and Gadella 2005), and it does not occur massively in boar spermatozoa (Tienthai et al 2004; Saravia et al 2007). However, sperm capacitation seems permissive for CatSper channel activation, as demonstrated in **Paper II** with clear increases in $[\text{Ca}^{2+}]$ after capacitation and an effective reduction of progressive sperm motility caused by CatSper antagonists (mibefradil and NNC 55-0396) among spermatozoa previously exposed to capacitating-inducing conditions.

A battery of sperm membrane ion channels, including the CatSper channel (Ren et al 2001; Qi et al 2007), has been suggested to control male fertility in human and murine spermatozoa (Navarro et al 2008; Lishko et al 2012), possibly due to their role regulating sperm motility and hyperactivation. Whether the CatSper channel is implicated in regulating pig male fertility remains to be proven. Contrary to human spermatozoa where the CatSper channel seems to be activated by progesterone via the ABDH2 enzyme (Miller et al 2016), and despite this enzyme is present in boar spermatozoa, progesterone did not appear to be a CatSper agonist (**Paper II**). Noteworthy, progesterone is not a CatSper agonist for mouse spermatozoa (Lisko et al 2011; Miller et al 2016). Progesterone has been reported to increase pig sperm motility as determined by absorbance of a particular cell suspension (Campbell et al 2013). In **Paper II**, progesterone did not affect boar sperm motility (using a proven motility analyzer) neither at physiological nor pharmacological concentrations irrespective of the cells being previously subjected, or not, to capacitation-inducing conditions.

In **Paper III** both the μ - and δ -opioid receptors were identified and proven functional, modulating sperm motility. Agonist-stimulation of the μ -opioid receptor with morphine maintains and increases sperm motility over time, while agonist-stimulation of the δ -opioid receptor causes decreases over time. Presence and activity of the κ -opioid receptor were also explored, but, although the positive control confirmed the specificity of the antibody (see Agirregoitia et al 2006 and **Paper III**), neither its presence nor its function could be determined in the pig spermatozoon. When challenged with the μ -agonist morphine, a biphasic change in sperm motility was seen, with an immediate decrease followed by a time-dependent increase (**Paper III**). These results fully agree with the typical biphasic effect of opioids (Feigenbaum and Howard 1997) and with previous observations in equine spermatozoa (Albrizio et al 2005), albeit they significantly contrast with observations reported in human (Agirregoitia et al 2012). Moreover, boar sperm motility was reduced

when challenged with the δ -opioid receptor agonist DPDPE, but not by the δ -opioid receptor antagonist naltrindole (**Paper III**). In equine, naltrindole increased sperm motility at low concentrations but decreased it at high concentrations (again a typical biphasic effect; Albrizio et al 2010). In human, naltrindole decreased sperm motility (Agirregoitia et al 2012). Interestingly, although the μ - and δ -opioid receptors depict a similar distribution pattern among species and probably share a conserved primary protein structure (inferred by the consistency of the results obtained in this Thesis and by other authors, see above) their regulatory role on sperm motility is species-dependent.

The main endogenous opioid is β -endorphin, which presents differential binding affinity for the opioid receptors, highest for μ - and lowest for κ -opioid receptors (Subirán et al 2011). The presence of β -endorphin in the porcine follicular fluid, with a peak pre-ovulation (Kaminski et al 2000), logically justifies the presence of opioid receptors in spermatozoa, suggesting that endogenous opioids present in the porcine follicular and oviductal fluid may modulate sperm motility *in vivo*, regulating sperm progression towards the oocyte, a matter supported by the *in vitro* observations of modulation of sperm motility in **Paper III**. It appears contradictory, however, that β -endorphin might stimulate both the μ - and δ -opioid receptors but with opposite output. Such scenario claims for further investigation to elucidate the specific contribution of each receptor to the regulation of boar sperm motility *in vivo*.

Figure 4 intends to summarize the possible intracellular pathways in boar spermatozoa that would ultimately lead to sperm capacitation and hyperactivation. Once boar spermatozoa have been exposed to a capacitating agent as bicarbonate (at 35 mM level), the increase in intracellular pH caused by the entry of bicarbonate might activate the CatSper channel, causing a Ca^{2+} influx from the extracellular medium ultimately leading to hyperactivation. As well, the dual entry of bicarbonate and increases in $[\text{Ca}^{2+}]_i$ possibly stimulates the sAC, which would initiate the PKA pathway and phosphorylation of proteins, leading also to changes in motility (hyperactivation). Moreover, when spermatozoa are progressing towards the oocyte they may encounter increasing levels of endorphins and enkephalins, found at highest concentrations in the pre-ovulatory follicular fluid (Kaminski et al 2000). Endogenous opioids bind to opioid receptors (with different affinities depending of the opioid receptor subtype, Subirán et al 2011), which are G-protein coupled receptors (GPCR) whose signal transduction seem to block Ca^{2+} channels (Minoia and Sciorci 2001) as CatSper and open K^+ channels, which in turn would cause depolarization of sperm membrane and thus reduce sperm motility. Interestingly, stimulation of the opioid receptors with low doses or short exposure times would block the sAC (Law et al 2000; Lee and Ho 2013), inhibiting sperm motility as the PKA pathway is blocked by the lack of production of cAMP. At sustained stimulation (e.g. higher doses/longer exposure times) the effect might be the opposite: the opioid receptors would stimulate the sAC (Law et al 2000; Lee and Ho 2013), increasing thus the production of cAMP and stimulating the PKA pathway, increasing sperm motility and in the worst case finally causing acrosome exocytosis and thus cell “infertility” (ultimately death) unless the spermatozoon has reached the ZP and can acrosome-react (Figure 4). The differences between acute/chronic stimulation of opioid receptors are in agreement with the

known biphasic effect of opioids (Feigenbaum and Howard 1997), a response also observed in boar spermatozoa when challenged *in vitro* with the μ -agonist morphine (**Paper III**).

Figure 4. Possible intracellular pathways and sperm response after: entrance of bicarbonate in spermatozoa (blue), activation of the CatSper channel (red) and the Opioid Receptors (green). Yellow: Potassium channel. Green lines indicate stimulation or activation and red lines blockade or inhibition.

In conclusion, boar spermatozoa express a series of functional channels (**Papers I and II**) and receptors (**Paper III**) whose stimulation or blockade derives in traceable phenotypic responses *in vitro*, linked to a possible function *in vivo* (see above). Pursuing the aim of using boar spermatozoa as an alternative cell model to detect drug-induced toxicity, traceable sperm reactivity after compound-challenge as expressed by changes in sperm kinematics and/or protein membrane distribution, settled the basis for deeper analysis and further studies of the model. As such, boar spermatozoa can detect toxic compounds in an *in vitro* assay based on sperm kinematics (**Paper IV**). This proposed boar sperm model was able to detect drug-induced toxicity with a rather similar specificity and sensitivity as that shown by the currently model used by the pharmaceutical industry (HepG2 cells, see Gómez-Lechón et al 2003 and **Paper IV**). Moreover, the sperm model provided results after barely 30 min (compared to the 24-48h consumed by the assays currently used in the pharmaceutical industry), significantly increasing the speed of analysis (**Paper IV**). Disturbance of mitochondrial function is the leading cause of drug-induced toxicity, causing cardio- and

hepatotoxicity and thus attrition of drug candidates (Dyken and Will 2007). Despite motility in boar spermatozoa consumes ATP mostly produced by cytoplasmic glycolysis than by OXPHOS in mitochondria (Marin et al 2003), uncoupling mitochondrial activity clearly decreased sperm motility (Ramió-Lluch et al 2014; **Paper IV**), reassuring the validity of sperm kinematics as detectors of mitochondrial modes of drug-induced toxicity (**Paper IV**). Moreover, boar spermatozoa can be obtained from AI-doses, available from a variety of breeding companies. Such origin of cells for *in vitro* testing remarkably ameliorates the use of animals, since these breeding boars are not subjected to painful procedures or sacrificed for the purpose.

Both the current legal framework within the EU (EU Directive 2010/63), as well as society, claim for a reduction of the number of research animals (directly as animals *per se* or indirectly as a source of tissues) in agreement with the 3R principles postulated over 50 years ago (Russel and Burch 1959). The number of animals used in research has consequently been reduced during the last years (COM, 2010, 511/final 2 vs. COM 2013, 859/final), reflecting the efficacy of legislation and dedication of researchers to a responsible use of animals in research (Parker et al 2014; Törnqvist et al 2014; Burden et al 2015; Fleetwood et al 2015). Furthermore, development of alternative methods acquires relevant importance within current toxicological research (EU-ToxRisk 2015; Gocht et al 2015; Daneshian et al 2016), contributing not only to reduce animals and animal-explant tissues in research but also increasing the speed of analysis. Both situations will lead to a reduced and selected number of drug candidates to be tested in a further reduced number of clinical trials, optimizing drug screening with the concomitant benefits not only for animal welfare but also for the development of new and safe drugs.

Boar spermatozoa detect drug-induced mitochondrial toxicity (**Paper IV**), and have previously been validated as detectors of toxins causing disruption of cell ion homeostasis, energy generation and mitochondrial function (Andersson et al 2006; Apetroaie-Constantin et al 2009; Hoornstra et al 2003), proving they are equally sensitive as somatic cell lines used to indicate cytotoxicity by other mechanisms (Severin et al 2005; Jääskeläinen et al 2003). Spermatozoa from other animal species than porcine could be used for the bioassays, such as human, laboratory animals or other livestock. Human semen presents, as previously mentioned, a significantly lower sperm quality and higher heterogeneity than the semen of other animal species (WHO 2010). Mouse spermatozoa, recently demonstrated to detect bisphenol A toxicity by a CatSper dependent mechanism (Wang et al 2016), might be used as detectors of toxicity, but requires castration or killing of animals to retrieve the spermatozoa from the epididymis (Dusells and Vrana 2007). Bull spermatozoa have also been reported as detectors of toxic compounds (Eskov et al 2008, 2009), but with a proven lower sensitivity to detect toxic compounds compared to boar spermatozoa (Jääskeläinen et al 2003). Both pig and cattle breeding rely on AI, but while AI in pigs is performed with liquid semen (Wagner and Thibier 2000), frozen/thawed semen is mostly used in cattle (Rodríguez-Martínez and Barth 2007). The interference of freezing is not to be neglected, and must be further tested. Ram spermatozoa present, as bull (Katz & Dott 1975), a higher speed than boar spermatozoa, apparently depending of higher dependence of OXPHOS for sperm motility. This acquires

particular relevance as ram spermatozoa express both CatSper channel and opioid receptors (Vicente-Carrillo et al 2015). In view of all the differences, availability and homogeneity of the previously mentioned spermatozoa from different species, boar semen is so far the best source for this sperm *in vitro* assay due to the higher sperm output per ejaculate (a mean of 120×10^9 total spermatozoa, Mann and Lutwak-Mann 1981), and the high sperm homogeneity in terms of sperm morphology, viability and motility. The proposed *in vitro* boar sperm model for detection of drug-induced toxicity relies in the proper and fast quantification of sperm kinematics (**Paper IV**). For that purpose, sperm motility analysis should be performed in a semi-automated basis and using a software such as the ToxiSpermTM that allows for a fast measurement of a high number of spermatozoa per sample. Further development of the software, combining other relevant end-points of cell reactivity (i.e. sperm capacitation markers) is hoped for.

In sum, boar spermatozoa, obtained from commercial AI-doses, appear to be an outstanding alternative *in vitro* cell model to detect mitochondrial drug-induced toxicity based on variations in sperm kinematics. These cells express channels and receptors in their plasma membrane, with measurable phenotypic responses upon challenge: sperm motility, mitochondrial activity, membrane integrity or acrosome exocytosis. Among all channels and receptors that potentially could be present in the membrane of boar spermatozoa (see Table 1), only the water channels AQP-7 and -9 (**Paper I**), the Ca^{2+} -channel CatSper (**Paper II**) and the G-protein coupled opioid receptors (**Paper III**) have been studied under this Thesis. Other receptors are present in boar spermatozoa, including melatonin (González-Arto et al 2016) and hyaluronan (Vicente-Carrillo and Rodríguez-Martínez 2016) both of utmost relevance for reproductive events, and whose malfunction would lead to infertility. In addition, studies covering the functionality of sperm membrane channels and receptors and their implications in drug metabolism should be carried out to obtain a deeper understanding of the physiology of these gametes and the full establishment of their use in the pharmaceutical industry as an alternative cell model for drug toxicity screening.

GENERAL CONCLUSIONS

- Boar spermatozoa regulate changes in osmotic pressure during freezing/thawing via membrane AQPs-7 and -9. The AQP-7 appears as a relevant marker for non-empirical studies of sperm freezing and osmo-adaptation to handling.
- The CatSper channel is present and functional in boar spermatozoa, regulating sperm motility during *in vitro* capacitation. Progesterone does not seem to act as specific CatSper agonist.
- The μ and δ -opioid receptors are present in boar spermatozoa, modulating sperm motility *in vitro*.
- The use of semen from commercially available breeding boars to detect *in vitro* drug-induced mitochondrial toxicity, through disruption of sperm kinematics as endpoint, reduces the number of animals required for toxicity testing and ultimately foresees their replacement, in agreement with the 3R principles.

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Papers

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