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UNRAVELING THE MECHANISMS OF SPERMICIDAL ACTIVITY BY SURFACTANTS: THEIR POTENTIAL USE AS SAFE MULTI-FUNCTIONAL COMPOUNDS

Tese de Doutoramento na área científica de Biologia, especialidade Biologia Celular, orientada pelo Professor Doutor João Ramalho-Santos do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnolologia da Universidade de Coimbra, e apresentada à Faculdade de Ciências e Tecnolologia da Universidade de Coimbra.

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Unraveling the mechanisms of spermicidal activity by surfactants:

their potential use as safe multi-functional compounds

Tese de Doutoramento apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia (Especialidade Biologia Celular), realizada sob a orientação científica do Professor Doutor João Ramalho-Santos (Universidade de Coimbra e Centro de Neurociências e Biologia Celular da Universidade de Coimbra).

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny'..."

(Isaac Asimov)

“Our doubts are traitors,
And make us lose the good we oft might win
By fearing to attempt.”

(William Shakespeare)

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Houve um dia em que uma criança teve um Avô Pai. É essa a história de amor que pretendo perpetuar.

Para o meu Avô.

Palavras houvesse que pudessem descrever a minha gratidão por ti. Ensinas-me todos os dias muito mais do que alguma vez eu te poderei ensinar a vida toda.

Sabes-me bem.

Para a Maria Beatriz.

“Olha lá,
Já se passaram alguns anos
Nem sequer vinhas nos meus planos
Saíste-me a sorte grande

E eu cá vou
Gozando os louros deste achado
Contigo de braço dado para todo o lado

Eu vou até morrer ser teu se me quiseres
Agarrado a ti vou sem hesitar
E se o chão desabar que nos leve aos dois
Vou agarrado a ti

Meu amor na roda da lotaria
Que é coisa escorregadia
Saíste-me a sorte grande.”

Sorte Grande, de João Só e Abandonados

Por seres a força que me move, o equilíbrio que me sustém.

We can burn brighter than the Sun.

Para o Filipe.

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List of abbreviations

$[Ca^{2+}]_i$ – Intracellular calcium concentration

AIDS – Acquired Immunodeficiency syndrome

ART - Assisted Reproductive Techniques

ARV – Antiretroviral

ASAs – Antisemen antibodies

BSA – Bovine serum albumine

C_{10}TAB - decyltrimethylammonium bromide

C_{12}BZK - dodecyl-N-benzyl-N,N-dimethylammonium (Benzalkonium) bromide

C_{12}PYR – N-dodecylpyridinium bromide

C_{12}TAB - dodecyltrimethylammonium bromide

C_{14}TAB - tetradecyltrimethylammonium bromide

C_{16}TAB – hexadecyltrimethylammonium bromide

Ca2+ - Calcium ion

cAMP – Cyclic adenosine monophosphate

CASA - Computer-Aided Sperm Analysis

CMC – Critical micelle concentration

C_nTAB – n-alkyl-N,N,N-trimethylammonium bromides

CP – Central pair

DA – Dynein arms

DAG – Diacylglycerol

DAPI - 4',6-diamidino-2-phenylindole

DCFDA – 2’,7’-dichlorofluorescein diacetate

DDPS - N-dodecyl-N,N-dimethylammonium-propanesulfonate

DNA – Deoxyribonucleic acid

DSE - Disulfide esthers

ETC – Electron transport chain

FITC-PSA - Pisum sativum agglutinin linked to fluorescein isothiocyanate

FS – Fibrous sheath

HIV – Human immunodeficiency virus

HOST – Hypo-osmotic swelling test

ICAM – Intracellular adhesion molecules

ICC – Immunocytochemistry

ICSI – Intracytoplasmic sperm injection

IMM – Inner mitochondria membrane

IP3 – Inositol-3 phosphate

IRB - Internal Review Board

ISX – Isoxazolecarbaldehydes

IVF – *in vitro* fertilisation

JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

KO – Knock out

LC – Longitudinal columns

LD 90 – Lethal dose 90

LD10 – Lethal dose 10

LD50 – Lethal dose 50

MMP – Mitochondrial membrane potential

MS – Mitochondrial sheath

N-9 – Nonoxynol-9

NNRTI – Non-nucleoside reverse transcriptase inhibitor

NRTI – Nucleotide reverse transcriptase inhibitor

ODFs – Outer dense fibers

OGB – Oregon green BAPTA 1-AM

OMDA – Outer microtubule doublets of the axoneme

OMM – Outer mitochondria membrane

PBS – Phosphate-buffered saline

PDL - poly-D-lysine

PI – Propidium iodide

PKA – Protein kinase A

PKC – Protein kinase C

PLC – Phospholipase C

PM – Plasma membrane

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RS – Radial spokes

RT – Reverse transcriptase

RVI - Rabbit vaginal irritation test

SDS - Sodium dodecyl sulphate

sEBSS – Supplemented Earle's balanced salt solution

SEM – Standard error of the mean

SHIV – Simian/Human immunodeficiency virus (HIV and SIV combined virus)

STDs – Sexually transmitted diseases

TK – Tyrosine kinase

TR – Transverse ribs

TX-100 - Triton X-100

WHO – World Health Organization

ZP – Zona pellucida

Abstract

The human population is steadily increasing. The lack of women-controlled contraceptives is one of the major causes that lead to the exponential increase in population growth, especially in undeveloped regions, since women do not have the social and economic power to persuade their partners. And accompanying this increase is poverty, hunger, lack of health care and the dissemination of infectious diseases such as sexually transmitted infections (STDs). So, the urgent need for new cheap, safe, easy-to-use and easy-to-store women-controlled spermicides makes surfactants an attractive choice for avoiding unwanted pregnancies.

However, the efficacy and safety of this kind of product is not always as obvious as would be expected. Past formulations that have been proposed as efficient spermicides (e.g., Nonoxynol-9) turned out to be unsafe for female use. For this reason, and in order to avoid the same mistakes, a more fundamental knowledge concerning surfactant toxicology and structure-activity correlation is required.

In this work, we tested the *in vitro* toxic effects of different families of commercially available surfactants – non-ionic (Triton X-100), zwitterionic (DDPS), anionic (SDS) and cationic (C_nTAB, n=10 to 16; $C_{12}PYR$ and $C_{12}BZK$) – through tests that evaluate basic parameters for sperm cell survival, such as viability, motility and membrane integrity. Other finer aspects of sperm cell function were also assessed, such as mitochondrial membrane potential, sperm capacitation, sperm acrosome status and calcium signaling.

The results showed that the toxicity of the surfactants tested was dose- and time-dependent. Data also suggested that their toxicity is dependent on the chemical structure. Indeed, Triton X-100, DDPS and SDS were toxic to sperm at concentrations around their critical micelle concentration (CMC), suggesting a non-selective mechanism of action involving cell membrane disruption, a typical mode of action for surfactants. On the other hand, all the cationic surfactants (with the exception of $C_{16}TAB$) were toxic at concentrations far below their CMC, suggesting that these surfactants are not acting here as typical detergents, killing solely via membrane disruption, but targeting more

specific pathways, subsequently leading to sperm cell death. Cationic amphiphiles of the C_nTAB family, differing only in the carbon number of their hydrophobic chain, revealed a linear dependence of their toxicity on the alkyl chain length; it was also demonstrated that surfactant toxicity is dependent on the chemical structure of the polar head group, given that surfactants with larger polar head groups ($C_{12}PYR$ and $C_{12}BZK$) and more delocalized positive charge ($C_{12}PYR$) were the most toxic to sperm. The distinct action of cationic surfactants is also observed regarding the effects on the physiological aspect of the cell membrane: they were shown to impair predominantly the physiological, rather than the structural, sperm membrane integrity, which suggests that before killing the sperm cell they may be affecting important physiological events occurring in the sperm plasma membrane.

As cationic surfactants were the most efficient and interesting compounds since they were acting below the CMC, other aspects of sperm cell function were evaluated in order to find different targets for surfactants toxicity. It was then observed that all the compounds tested ($C_{12}TAB$, $C_{12}PYR$ and $C_{12}BZK$) impaired mitochondrial membrane potential, indicating that they may interfere with sperm energy metabolism. An inhibition of sperm capacitation (a required step for sperm to become competent and fertilize the oocyte) was found after incubation with $C_{12}PYR$ and $C_{12}BZK$. A reduction in the percentage of intact acrosomes (sperm must have an intact acrosome in order to suffer acrosome reaction in the proper time – near the oocyte – and thus, be able to enter inside the female gamete and fuse) was achieved in the presence of $C_{12}PYR$ and $C_{12}TAB$. Experiments with sperm calcium movements revealed that all the compounds were able to increase sperm intracellular calcium, with $C_{12}PYR$ and $C_{12}TAB$ the most efficient, possibly explaining downstream effects on capacitation and acrosomal status. This data on calcium signaling, besides being the first time that spermicides are evaluated on this parameter, seemed to be particularly sensitive compared with other assays in detecting sperm function changes.

Finally, the data presented in this work contributes to the understanding of the possible mechanisms mediating surfactant effects on sperm cells, which may be helpful for the design of more efficient surfactants as topical spermicides. The results also highlight the importance of using more efficient *in*

vitro methodologies in order to better evaluate possible spermicidal candidates, since they seem to offer vital clues on the applicability and safety of a variety of compounds.

Resumo

A população humana está em constante crescimento. A ausência de contraceptivos controlados pela mulher é uma das principais causas que levam ao aumento exponencial da população, particularmente nas regiões subdesenvolvidas, uma vez que as mulheres não possuem poder social e económico suficiente para persuadir os seus parceiros. E o crescimento populacional vem geralmente acompanhado de pobreza, fome, cuidados de saúde precários e disseminação de doenças infecciosas como as doenças sexualmente transmissíveis (DSTs). Portanto, a necessidade urgente de encontrar novos espermicidas que sejam pouco dispendiosos, seguros, fáceis de usar e de armazenar, e que possam ser controlados pelas mulheres, tornam os surfactantes numa escolha atractiva para tentar evitar gravidezes indesejadas.

Contudo, a eficácia e a segurança deste género de produto nem sempre são tão evidentes como seria de esperar. Formulações anteriores, que foram propostas como espermicidas eficazes (e.g. Nonoxynol-9), acabaram por se mostrar prejudiciais para o uso feminino. Por este motivo, tentando evitar o mesmo tipo de erros, é fundamental obter um conhecimento elementar mais preciso acerca da toxicologia associada aos surfactantes e à sua relação estrutura-actividade.

Neste trabalho, testámos os efeitos tóxicos *in vitro* de diferentes famílias de surfactantes comercialmente disponíveis – não-iónicos (Triton X-100), zwiteriónicos (DDPS), aniónicos (SDS) e catiónicos (C_nTAB, n= 10 a 16; $C_{12}PYR$ e $C_{12}BZK$) – através de testes que avaliam parâmetros básicos relacionados com a sobrevivência dos espermatozóides, como sejam a viabilidade celular, mobilidade e integridade membranar. Outros aspectos mais específicos da função celular do espermatozóide também foram avaliados, como o potencial de membrana mitocondrial, capacitação, estado do acrossoma e sinalização por cálcio.

Os resultados mostraram que a toxicidade dos surfactantes testados é dependente da dose e do tempo de exposição utilizados. Os dados sugerem também que a sua toxicidade é dependente da estrutura química.

Efectivamente, o Triton X-100, DDPS e SDS são tóxicos para os espermatozóides a concentrações aproximadas à sua concentração micelar crítica (CMC), sugerindo assim um mecanismo de acção não-selectivo envolvendo disrupção membranar, uma forma de actuar típica dos surfactantes. Por outro lado, todos os surfactantes catiónicos (à excepção do C_{16}TAB) mostraram ser tóxicos a concentrações bastante abaixo da CMC, sugerindo que estes surfactantes não estão aqui a actuar como detergentes típicos, induzindo um efeito de morte celular somente via disrupção membranar, mas tendo como alvo vias mais específicas que subsequentemente levam à morte dos espermatozóides. Os compostos catiónicos anfifílicos da família C_nTAB, diferindo apenas no número de carbonos na sua cadeia hidrofóbica, apresentaram uma dependência linear da sua toxicidade em relação ao comprimento da cadeia alquil; tendo sido igualmente demonstrado que a toxicidade dos surfactantes é dependente da estrutura química do grupo polar, uma vez que surfactantes com grupos polares maiores (C_{12}PYR e C_{12}BZK) e carga positiva mais deslocalizada (C_{12}PYR) mostraram ser os mais tóxicos para os espermatozóides. A acção distinta dos surfactantes catiónicos é também observada relativamente aos efeitos na fisiologia da membrana celular: foi demonstrado que estes deterioram predominantemente a integridade fisiológica, mais que a estrutural, da membrana dos espermatozóides, o que sugere que estes compostos podem, antes mesmo de induzir morte celular, afectar eventos fisiologicamente importantes que ocorrem na membrana celular dos espermatozóides.

Uma vez que os surfactantes catiónicos foram os mais eficientes e promissores, actuando abaixo da CMC, outros aspectos da função celular nos espermatozóides foram analisados, de maneira a desvendar outros alvos para a toxicidade dos surfactantes. Foi então observado que os compostos testados (C_{12}TAB, C_{12}PYR e C_{12}BZK) danificam o potencial de membrana mitocondrial, indicando que podem interferir com o metabolismo energético nos espermatozóides. Foi ainda encontrada uma inibição do fenómeno de capacitação (um passo vital para o espermatozóide se tornar competente e fertilizar o ovócito) aquando da incubação com C_{12}PYR e C_{12}BZK, bem como uma diminuição da percentagem de acrossomas intactos nos espermatozóides na presença de C_{12}PYR e C_{12}TAB (o espermatozóide deve apresentar o

acrossoma intacto, por forma a sofrer reacção acrossómica no momento adequado – próximo do ovócito – e assim ser capaz de penetrar e fundir-se com o gâmeta feminino). As experiências envolvendo movimentos de cálcio no espermatozóide revelaram ainda que todos os compostos provocam um aumento do cálcio intracelular, sendo C_{12}PYR e C_{12}TAB os mais eficientes, explicando assim potencialmente os efeitos, mais tardios, na capacitação e estado do acrossoma. Estes dados relativos à sinalização por cálcio, além de constituírem uma novidade na avaliação de espermicidas, parecem ser particularmente sensíveis quando comparados com outros testes utilizados na avaliação de alterações funcionais nos espermatozóides.

Finalmente, os resultados apresentados neste trabalho contribuem para uma melhor compreensão dos possíveis mecanismos mediados por surfactantes nos espermatozóides, o que pode revelar-se útil no desenvolvimento de surfactantes mais eficientes como espermicidas de aplicação tópica. Os dados evidenciam ainda a importância de metodologias *in vitro* mais eficazes para uma melhor avaliação de potenciais espermicidas, uma vez que esta metodologia oferece pistas fundamentais relativamente à aplicabilidade e segurança na utilização de uma variedade de compostos eventualmente espermicidas.

Chapter I. General Introduction

Part of this chapter was published in the following paper:

Baptista M and Ramalho-Santos J (2009) Spermicides, microbicides and antiviral agents: recent advances in the development of novel multi-functional compounds. Mini Rev Med Chem 9 (13): 1556-67.

Synopsis

1.1. The human spermatozoon – A unique cell

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1.4. Aims of the work

The possibility of female-controlled pregnancy has had a major societal impact in most communities. However, this is not a common worldwide phenomenon. Indeed, in less developed countries women do not have social and financial control to allow for contraceptive choices and, thus, to protect their health, subjecting themselves to the will of sexual partners (Zaneveld et al., 2002). This leads to several other related problems, such as the spreading of sexually transmitted diseases (STDs) and unwanted pregnancies.

Lately, scientists have tried to direct their attention towards the development of new cheap, topical multi-function spermicides, capable of protecting subjects from STD transmissions while, at the same time, providing anticonceptional action. The recent introduction of molecules that act on a specific cellular target may give important clues for the design of more effective compounds (Kelly and Shattock, 2011). Lastly, and crucially, the assessment of spermicide safety should never come in second place, including the proper evaluation of mucosal and epithelial integrity, inflammation and infection of the tissues in contact with the prospective formulation (Su et al., 2011; Zalenskaya et al., 2011).

Therefore, the development of novel cost-effective, safe, female-controlled contraceptives that could offer a STD-preventive action is in order, and research on this topic has been ongoing.

1.1. The human spermatozoon – A unique cell

The unique physiology of sperm exists for one sole reason: the transmission and union of the paternal and maternal genomes. Membrane dynamics and membrane fusion are crucial for sperm function, being involved in events such as sperm capacitation, acrosome reaction, and sperm-oocyte fusion itself (Ramalho-Santos et al., 2002).

The sperm cell is the final product of an extensive process called spermatogenesis that occurs in the seminiferous tubules, within the testis, hormonally controlled by the hypothalamic-pituitary axis (de Kretser et al., 1998). During spermatogenesis, diploid spermatogonial stem cells suffer a series of mitotic cell divisions followed by meiosis, which gives rise to the production of haploid spermatids (Fox, 2006) (Figure 1.1.).

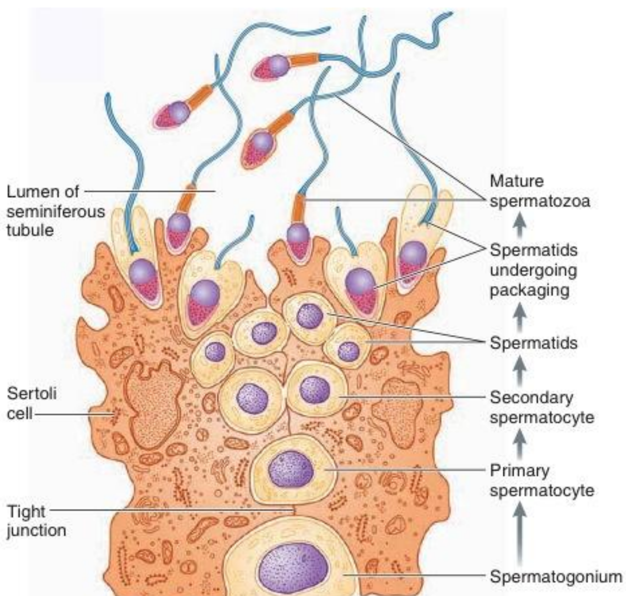


Figure 1.1. Spermatogenesis. Spermatogonia undergo mitotic division in which they replace themselves and produce a daughter cell that will undergo meiotic division (primary spermatocyte). Upon completion of first meiotic division, the daughter cells are called secondary spermatocytes. Each of these completes a second meiotic division to form spermatids (interconnected cells). Each spermatid will give rise to a mature spermatozoon.
From Sherwood, 2011.

Then, intricated post-meiotic morphologic alterations (spermiogenesis) will finally produce sperm, the differentiated, polarised and hydrodynamic-shaped male gamete (Figure 1.2.).

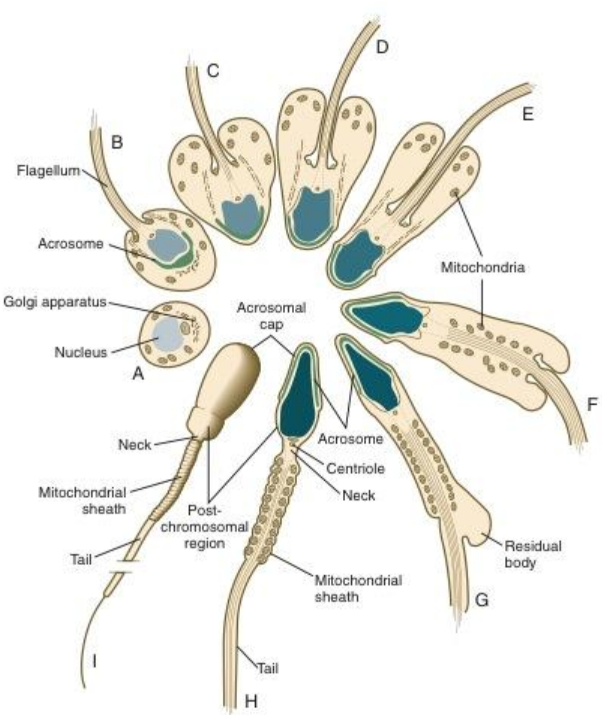


Figure 1.2. The processing of spermatids into spermatozoa (Spermiogenesis). As the spermatids develop into spermatozoa, intricated post-meiotic alterations will occur to form the differentiated, polarised and hydrodynamic-shaped male gamete.
From Carlson, 2009.

The main morphologic changes contributing for the final “design” of normal and functional spermatozoa are:

1) The highly compacted nucleus, achieved by the removal of histones, which includes their sequential change by transitional proteins and substitution by protamines (Dadoune, 2003);

2) The loss of the cytoplasm and all the other common cell organelles, in a phagocytic process carried out by Sertoli cells (Bustos-Obregon et al., 1975);

3) The formation of the acrosome out of the Golgi complex, located in the anterior zone of the sperm head, operating as an enzymatic vesicle (Sinowatz and Wrobel, 1981);

4) The migration and rearrangement of the remaining mitochondria in elongated tubular structures, packed helically around the midpiece (Otani et al., 1988);

5) The development of a flagellum, allowing sperm to move up to its target, the oocyte.

The highly specialized sperm structural characteristics mirror their distinctive functional activities. So, after spermiogenesis, the newly formed sperm cell acquires three principal components: the head, the midpiece and the tail or flagellum (Figure 1.3.).

The head encloses a haploid nucleus with a much reduced volume than the nucleus of a somatic cell, as common nuclear histones are partially replaced during spermiogenesis by protamines, as described above. Protamines are, in relation to histones, relatively small DNA binding proteins, rich in arginine and cysteine, and stabilized by disulfide bonds when integrating the DNA-protamine complex (Grimes, 1986; Eddy and O'Brien, 1994). Thereby, and taking into account the hypercondensation of the sperm nucleus, it is not surprising that these cells are considered transcriptionally silent (Ramalho-Santos et al., 2007), despite some reported evidences for translation of mRNAs in sperm (Gur and Breitbart, 2006). Surrounding the anterior part of the head (like a cap), is the acrosome, a Golgi-derived secretory vesicle that harbours proteases and

receptors required for sperm interaction with the glycoproteins from the zona pellucida of the oocyte (Moreno et al., 2000).

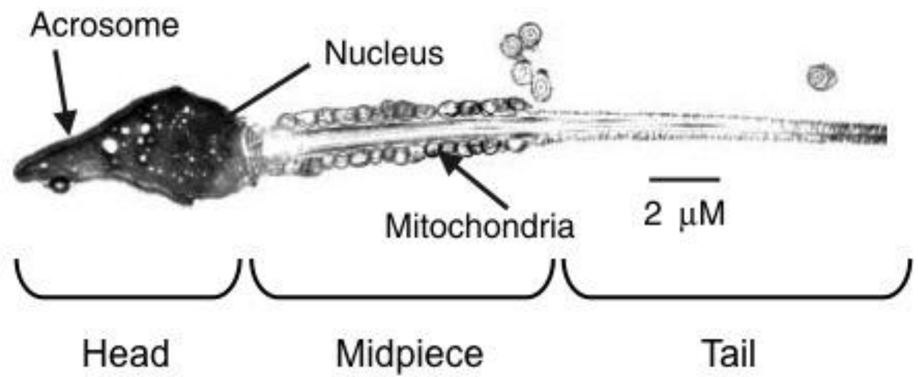


Figure 1.3. A human spermatozoon. The human sperm is composed of three main regions: the head (which comprises the nucleus and the acrosome), the midpiece (where mitochondria are localised) and the tail (or flagellum).
From Costello et al., 2009.

The tail or flagellum is the responsible for sperm movement. The driving force is based on a 9+2 arrangement of microtubules dubbed the central axoneme (Kierszenbaum, 2002). This complex structure is surrounded by fibrous sheath, an exclusive cytoskeletal formation that wraps the outer dense fibers, defining the scope of the sperm flagellum (Eddy et al., 2003). Associated to microtubules are the dynein-ATPases, extremely important motor proteins for the production and synchronization of the tail movement (Lindemann et al., 1992). These features all together will provide flexible yet firm support during flagellar movement (Figure 1.4.).

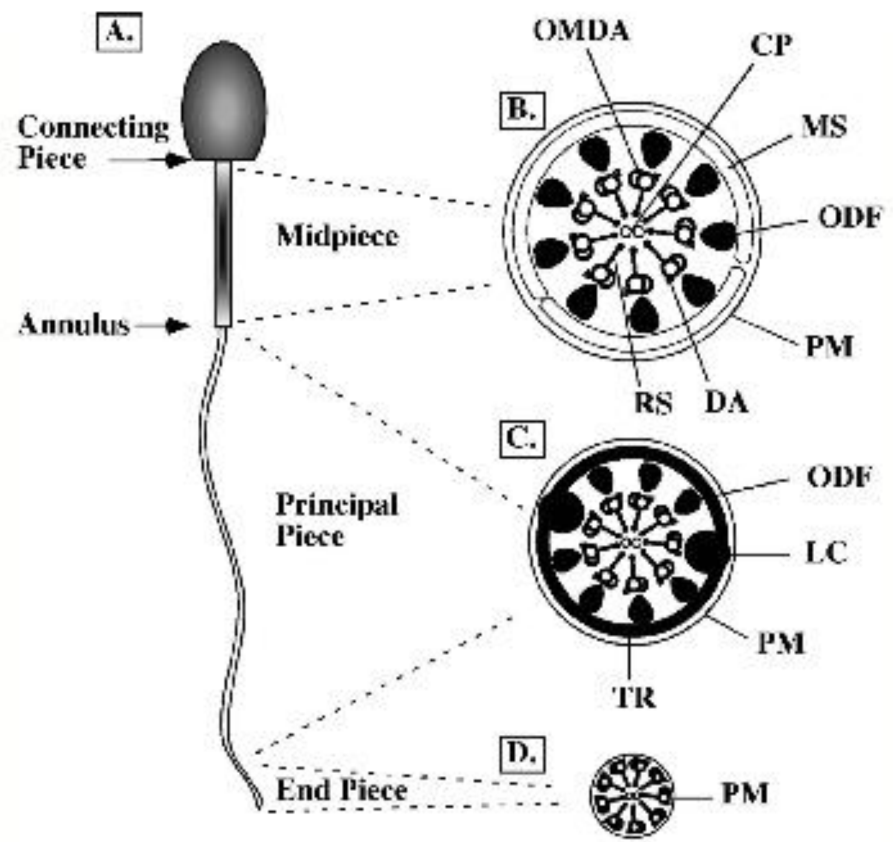


Figure 1.4. Ultrastructure of the human sperm tail. (A) Sperm flagella are structurally divided into four areas: the connecting piece, midpiece, principal piece, and end piece. (B) Schematic cross-section of the midpiece showing the plasma membrane (PM) and mitochondrial sheath (MS) surrounding the nine outer dense fibers (ODFs). Within the ODFs are the axoneme components: the nine outer microtubule doublets of the axoneme (OMDA) with associated dynein arms (DA) and radial spokes (RS) and the central pair (CP) of singlet microtubule. (C) Schematic cross-section of the principal piece showing the PM surrounding seven ODFs. ODFs 3 and 8 have been replaced by the two longitudinal columns (LC) of the fibrous sheath. The two columns are connected by transverse ribs (TR). The axonemal components are unchanged. (D) Schematic cross-section of the end piece. The ODFs and fibrous sheath (FS) tapered at the termination of the principal piece and are no longer present in the end piece, thus leaving only the PM to surround the axoneme.
From Turner, 2003.

The midpiece, the connecting part between the head and the tail, is constituted, apart from the axoneme, by a variable number of elongated

mitochondria disposed in a double helix form (Otani et al., 1988). Mitochondria present in sperm, spermatids and late spermatocytes present a more condensed (which means more metabolically efficient) form than spermatogonia and early spermatocytes, harbouring orthodox mitochondria. Indeed, mitochondria undergo morphological changes during spermatogenesis. The exact role of mitochondria and the origin of the ATP that fuel motility (and other important processes in spermatozoa) is still debatable, as some authors support that ATP is derived by OXPHOS and others by glycolysis (Ruiz-Pesini et al., 2007; Ramalho-Santos et al., 2009; Pena et al., 2009; Rajender et al., 2010). Moreover, each mitochondria carries multiple copies of the paternal mitochondrial genome, the paternal mitochondrial DNA (mtDNA), which is posteriorly and for dubious reasons eliminated by targeted proteolysis inside the egg (Sutovsky et al., 2000). The mtDNA has, therefore, an exclusively maternally inherited pattern.

1.1.1. Specific human sperm functionalities

In the end of sperm formation, these specialized cells are released into the lumen of the seminiferous tubules in the testis and travel to the epididymis, in a process termed spermiation. In the epididymis, where sperm is stored until ejaculation, the highly differentiated sperm undergo post-testicular maturation, acquiring the ability to move and to fertilise an oocyte (Yanagimachi, 1994). However, the full functionality of ejaculated sperm is only achieved in the female reproductive tract. The poorly understood process of final maturation, dubbed “capacitation”, involves several steps including membrane re-arrangements, the

activation of intracellular signalling cascades and the phosphorylation of certain proteins (Visconti and Kopf, 1998; Salicioni et al., 2007). This fertilizing competence acquisition can also be carried out *in vitro* by appropriate capacitating media mimicking the female reproductive tract environment. In most cases, these media are constituted by energy substrates (pyruvate, lactate and glucose), a cholesterol acceptor (usually serum albumin) and electrolytes (sodium bicarbonate and Ca^{2+}); (Davis et al., 1979; Dow and Bavister, 1989; Visconti and Kopf, 1998; Abou-haila and Tulsiani, 2009).

Capacitation

At the molecular level, and as stated before, capacitation is characterized by a complex process involving activation of signalling cascades. This involves intracellular increases of cAMP, Ca^{2+} and pH, plasma membrane fluidity changes (cholesterol efflux), ROS production and protein phosphorylation (on serine, treonine and, mostly, tyrosine residues) (de Lamirande et al., 1997; de Lamirande and O'Flaherty, 2008; Gadella et al., 2008; Abou-haila e Tulsiani, 2009; de Lamirande e Lamothe, 2010). The second messenger cAMP has an important role in the mediation of the phosphorylative state of proteins vital for several events within the sperm cell. ATP conversion to cAMP is done by the Ca^{2+}-dependent cyclase adenilate (partially controlled by calmodulin), allowing then the phosphorylation of specific protein residues by cAMP-dependent protein kinases. (Eddy and O'Brien, 1994; Visconti et al., 1995; Pukazhenthi et al., 1998; Si and Okuno, 1999; Yeung et al., 1999; Visconti et al., 2002). Thus, the monitorization of capacitation can be done through the detection of

phosphorylated protein tyrosines, by immunocytochemistry (Ramalho-Santos et al, 2007). It should be noted that capacitation is reversible, as the addition of some factors (called decapacitating factors) can functionally reverse the process, causing cells to regress to a non-fertilising state (Bedu-Addo et al., 2005; Fraser, 2010).

Sperm hyperactivation

The sperm hyperactivation event is one of the first changes that can be observed during capacitation. The extremely vigorous, extravagant and coordinated motility pattern is obtained as a result of Ca^{2+} influx causing an increased flagellar curvature, which aids the penetration of the layers that surround the egg (Suarez and Pacey, 2006). The sperm motility achievement seems to be related with sperm plasma membrane maturity. Indeed, scientists observed that demembranated immature testicular sperm that were later exposed to ATP, cAMP and Mg^{2+} become as active as the mature ejaculated sperm (Mohri and Yanagimachi, 1980; White and Volglmayr, 1986; Ishijima and Witman, 1987). This information undoubtedly associates the sperm motility acquisition with plasma membrane changes during the epididymal route (Yanagimachi, 1994).

Finally, the sperm population of a human ejaculate is very heterogeneous, including sperm with no motility, in situ motility or progressive motility. The evaluation of this parameter is made immediately (using optical microscopy linked to CASA - Computer-Aided Sperm Analysis), so artefacts as temperature or dehydration will be avoided (WHO, 2010).

Acrosome Reaction

Following capacitation, sperm undergo a regulated Ca^{2+}-dependent secretory membrane fusion event, called the acrosome reaction, during which hydrolytic enzymes that digest the oocyte's zona pellucida are released, leading to sperm penetration (Ramalho-Santos et al., 2000, 2002, 2007). Concomitantly, the sperm plasma membrane is again reorganized, acquiring specific fusogenicity towards the oocyte plasma membrane. Therefore, lack of acrosomal reaction renders sperm non-functional on these levels: sperm-oocyte penetration and plasmalemma rearrangements.

The acrosome reaction and sperm-oocyte fusion are the major membrane fusion-related events during sperm-oocyte interaction, and it is known that these actions are facilitated by the increase in membrane fluidity in capacitated sperm (the efflux of cholesterol renders the plasma membrane less rigid); (Primakoff and Myles, 2002).

The mechanism responsible for the acrosome reaction starts with the recognition and binding of capacitated sperm to the zona pellucida (ZP) of the oocyte (Yanagimachi, 1994). The loss of coating proteins and membrane changes would expose sperm specific surface receptors through which the ZP glycoproteins induces the acrosome reaction, triggering a signaling cascade. The stimulus activates the production of cAMP and IP3 (inositol-3 phosphate) as well as the activation of Ca^{2+} channels, which will cause an intracellular Ca^{2+} increase. The increase in cAMP triggers protein kinases that lead to protein phosphorylation, and the rise in intracellular Ca^{2+} and other second messengers activates a signalling cascade that elevates internal sperm pH and triggers the

fusion of the plasma membrane and the outer acrosomal membrane (Abou-haila and Tulsiani, 2009) (Figure 1.5.).

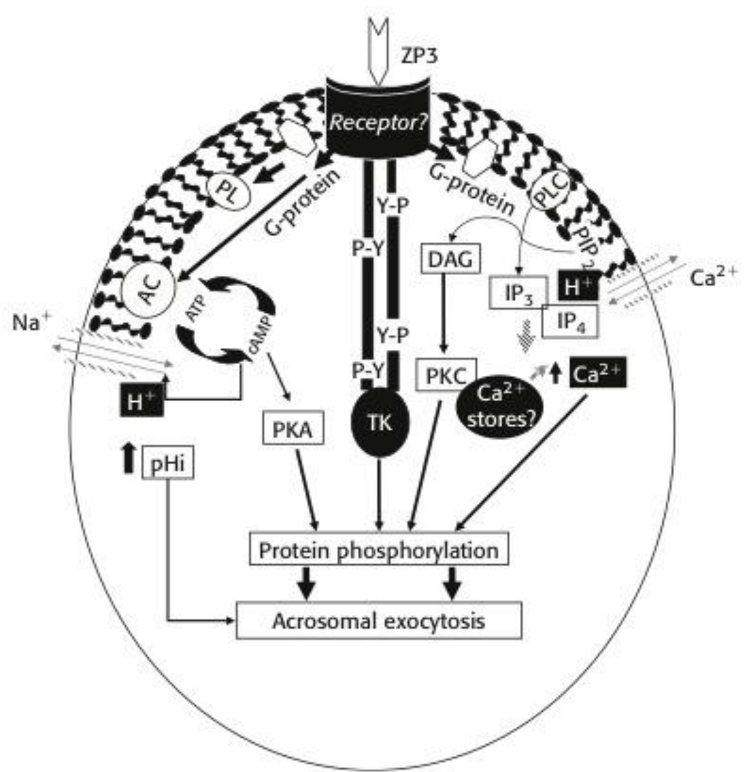


Figure 1.5. Putative intracellular cascades leading to the acrosome reaction. Upon binding of ZP3 with its putative receptor, one (or all) of three main phosphorylation systems may be activated resulting in acrosomal exocytosis. These second messenger systems are: activation of a tyrosine kinase (TK), G-protein–cAMP–protein kinase A (PKA), and phospholipase C (PLC)-diacylglycerol (DAG)-protein kinase C (PKC). In addition, increased intracellular calcium levels may result after mobilization from internal stores and following influx from activated membrane channels. An efflux of H^+, which in turn determines a rise of intracellular pH, may also accompany this event.
From Oehninger and Franken, 2006.

It cannot be forgotten the essential role the steroid hormone progesterone has in this event. Progesterone, present in high concentrations in the cumulus cells, is a known stimulator of the acrosome reaction, enabling the Ca^{2+} influx. It has been shown that progesterone exerts a priming effect on the

ZP-stimulated acrosome reaction in the mouse (Roldan et al., 1994) and in the human (Schuffner et al., 2002).

Interestingly, sperm can also undergo acrosome reaction "spontaneously", before and without the interaction of ZP (Bedford, 1968; Chen and Sathananthan, 1986). However, the spontaneously acrosome-reacted sperm will have no fertilizing ability, since it will not be able to penetrate the oocyte. In fact, reaching the female gamete with a partially destroyed acrosome will not allow sperm-ZP binding and attachment, essential for posterior penetration and fusion.

Capacitated human sperm respond not only to the acrosome reaction inducer ZP but also to a number of physiological (e.g. progesterone) and non-physiological substances (e.g. calcium ionophores, neoglycoproteins, lectins). The application of these stimuli enables the *in vitro* study of the acrosome reaction phenomenon (Gadella and van Gestel, 2004).

Calcium signalling

All the events addressed before have something in common: regulation by intracellular Ca^{2+}. In fact, and taking into account that there is little or almost no regulation of sperm function by translation/transcription, post-translational mechanisms gain greater importance in the control of all activities in the cell. Sperm intracellular [Ca^{2+}] regulates the acrosome reaction (Evans and Florman, 2002), hyperactivation (Suarez and Ho, 2003; Harper and Barratt, 2004), chemotaxis (Kaupp and Hildebrand, 2006), and has a pivotal role in capacitation (Breitbar, 2002) - all the activities that a sperm must undergo to

fertilise an oocyte (Figure 1.6.). Moreover, a sperm cell must “guarantee” that each event is initiated at the appropriate time. These days it is well known that sperm with impaired Ca^{2+} regulation and signalling is associated with male subfertility (Krausz et al., 1995; Baldi et al., 1999; Espino et al., 2009).

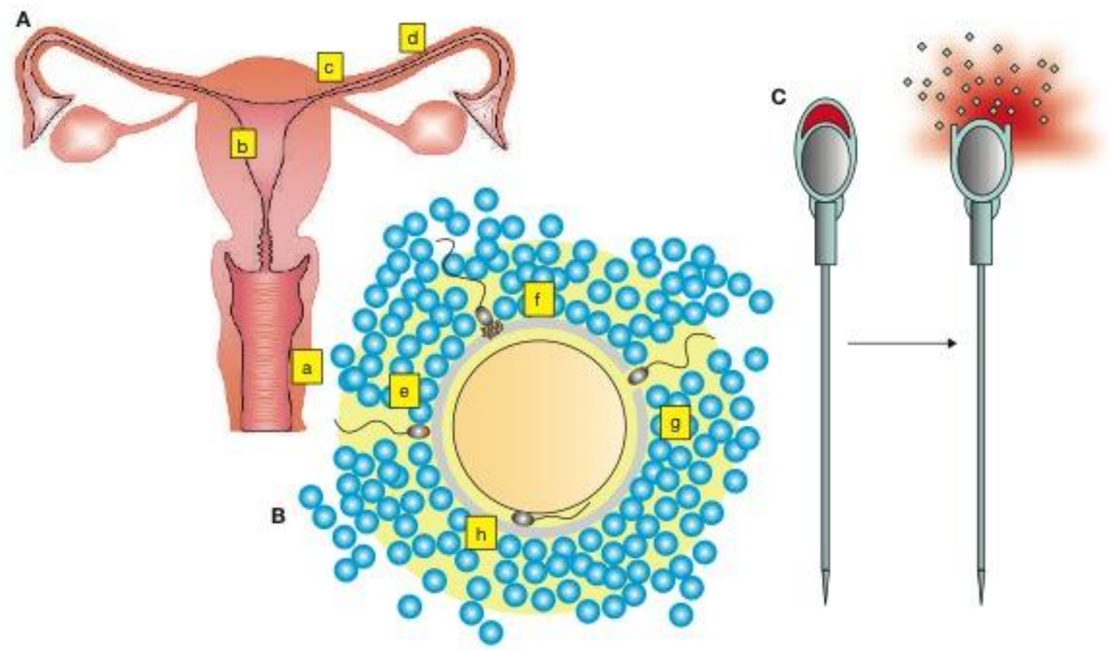


Figure 1.6. The journey of a human sperm. (A) After intercourse, sperm enter the cervical mucus (a). At this time, the cells begin capacitation. During passage to the uterus (b) the sperm induce a host reaction. Leucocytes, outnumbering the sperm 100:1, engulf normal and abnormal spermatozoa. In humans, only one in 14,000,000 ejaculated human sperm reach the oviduct (c), functioning as a sperm store. Before ovulation, sperm detach and detached cells have higher $[Ca^{2+}]_i$ and more vigorous motility. Fertilization occurs in the oviduct (d). (B) When the sperm reach the egg, hyperactivated beating of the flagellum enables penetration of the surrounding layer of cumulus cells (blue) embedded in matrix (e) and the sperm attaches to the zona pellucida (grey). Modulation of flagellar beat by $[Ca^{2+}]_i$-signals induced by progesterone is potentially important here. Rapid acrosome reaction now occurs in response to binding of the zona pellucida (f). This, together with hyperactivated motility, permits penetration of the zona (g) and fusion with the oolemma (h). (C) The acrosome reaction releases the contents of the acrosomal vesicle (red). The outer membrane of the acrosome fuses with the overlying plasmalemma and exposes the inner membrane of the acrosome, which is required for fusion with the oolemma.
From Publicover et al., 2007.

Mature sperm are very different from somatic cells, lacking endoplasmic reticulum and several other organelles that usually serve as Ca^{2+} stores. However, has been found evidence for the existence of functional Ca^{2+} stores in sperm, at least one in the acrosomal region and another in the region of the sperm neck and midpiece (Costello et al., 2009). Also, the rise in $[Ca^{2+}]_i$ can come from the inside (from the stores previously described) or from the extracellular milieu, mediated by plasma membrane Ca^{2+} channels (Jimenez-Gonzalez et al., 2006), and these Ca^{2+}-signals vary in size and “shape”. The two Ca^{2+} stores may indicate different roles in the regulation of sperm function as they are located in different places of the cell (Publicover et al., 2007). Indeed, the acrosomal store was shown to be strongly implicated in regulation of exocytosis of the acrosomal vesicle itself (Publicover and Barratt, 1999); and the store located in the neck/midpiece region functions as a regulator of sperm motility and hyperactivation (Marquez et al., 2007).

Still, although there is no scientific doubt that the storage organelle in the acrosomal region of the sperm head is the acrosome itself, the identity of the Ca^{2+} storage organelle in the midpiece region is less obvious (Costello et al., 2009). It is probable that this store consists of more than one structure. Furthermore, the interaction of the neck/midpiece region store with sperm specific plasma membrane ion channels (CatSpers) is still not very clear. CatSper channels are required for normal hyperactivation and are present only in the main part of the flagellum (sperm-specific). Knockout (KO) mice for these channels present sperm with impaired Ca^{2+} signalling not able to hyperactivate, resulting in male infertility (Ren et al., 2001; Quill et al., 2003; Carlson et al., 2003). Recently, it has also been found that progesterone stimulates this

plasma membrane channel (non-genomic response, with no transcription involved), in order to induce immediate influx of Ca^{2+} for sperm functional regulation (Strünker et al. 2011; Lishko et al., 2011). This way, CatSper represents an excellent drug target for non-hormonal male contraception.

Mitochondria

Sperm mitochondria are, like in other eukaryotic cells, double membrane organelles, with outer (OMM) and inner membranes (IMM), divided by the intermembrane space (Figure 1.7.). The inner membrane encloses the mitochondrial matrix and forms the mitochondrial cristae (invaginations), where electron transport chain complexes (ETC) are localised.

The ETC is formed essentially by enzymes involved in oxidative phosphorylation (OXPHOS), which are organized into five known complexes: complex I (NADH-dehydrogenase); complex II (succinate dehydrogenase); complex III (cytochrome c dehydrogenase); complex IV (cytochrome c oxidase); and complex V (ATP synthase) (Rajender et al., 2010).

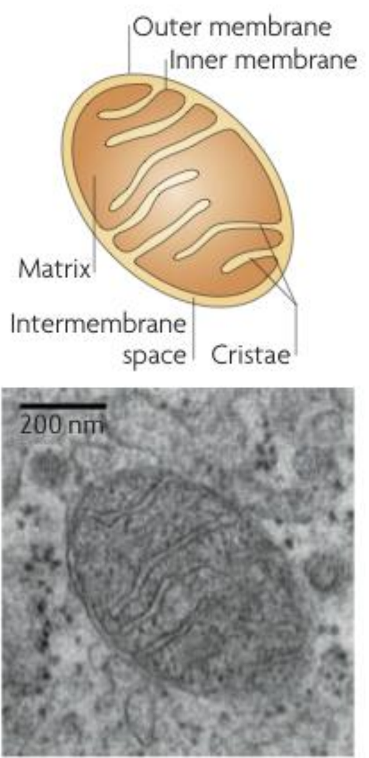


Figure 1.7. Mitochondria. This organelle is composed of an outer and inner membrane. The inner membrane encloses the matrix and forms the cristae. Between the two membranes is the intermembrane space.
From Westermann, 2010.

The relationship between mitochondria functionality and sperm quality is well studied, supporting that mitochondria may have a relevant role in several processes such as sperm survival, capacitation and motility, ROS production, Ca^{2+} storage, among others (Ruiz-Pesini et al., 2007; Amaral et al., 2013). In fact, sperm with an abnormal midpiece, such as a shorter midpiece, can cause low motility (Bartoov et al., 1980; McClure et al., 1983; Rawe et al., 2001); and such patients show a decrease in oxidative phosphorylation performance (Ruiz-Pesini et al., 2000). There are also studies indicating a correlation between sperm motility and mitochondrial membrane potential (MMP) (Troiano et al.,

1998; Donnelly et al., 2000; Barroso et al., 2006; Paoli et al., 2011; Wang et al., 2012). Furthermore, sperm MMP also correlates with acrosome function and fertilisation ability (Kasai et al., 2002; Gallon et al., 2006; Marchetti et al., 2002, 2004, 2012), expressing in a clear way the crucial role of mitochondria on sperm function.

Finally, it is to be noted that, despite the above evidence on the importance to sperm mitochondria, the exact role of this organelle is still a matter of serious discussion.

1.1.2. Defining a good sperm sample

The human ejaculate is very heterogeneous, containing sperm cells very different from each other. When a patient needs to do a sperm analysis, several parameters are taken into account and measured in accordance with the guidelines and references postulated by the World Health Organization (WHO), the directing and coordinating authority for health within the United Nations system. This specialized agency is responsible for defining the procedures for semen handling and processing, the parameters to be evaluated (that together compose the standardised laboratory sperm analysis called spermiogram) and the thresholds given for these parameters (WHO, 1999, 2010). In fact, infertility (inability to conceive after one year of regular intercourse in the absence of contraception) already affects 15% of the couples in reproductive age, and the spermiogram is essential for the diagnosis of male infertility, which is found to be the cause of infertility in half of the cases. In order to help these couples, scientists have developed the assisted reproductive techniques (ART). Of

these, *in vitro* fertilisation (IVF), in which sperm is placed in contact with unfertilized oocytes to ease fertilisation (Lopata et al., 1978); and intracytoplasmic sperm injection (ICSI), where a single sperm is inserted into an oocyte with the use of micromanipulation (Palermo et al, 1992), are the most used and widespread techniques (Andersen et al., 2008).

The semen analysis parameters routinely evaluated in the lab are concentration/count, motility and morphology. And for each one the cut off values were found and established and are presented on Table 1.1..

Table 1.1. Reference values for the evaluation of human sperm quality (WHO, 2010). Samples below these values are considered abnormal. The three principal parameters are in bold.

Parameter	Reference value (WHO, 2010)
Ejaculate volume	≥1.5 ml
Ejaculate pH	≥ 7.2
Total sperm number	≥ 39 million sperm/ejaculate
Sperm concentration	≥ 15 million sperm/ml ejaculate
Sperm motility	≥ 40% total motile sperm
Sperm morphology	≥ 4% normal forms
Sperm vitality	≥ 58% live sperm

* According to the strict criteria (Kruger et al., 1986).

With that in mind, a semen sample presenting all three parameters above the cut off values is considered a normozoospermic sample. However, low values of concentration, motility and morphology are called oligozoospermia, asthenozoospermia and teratozoospermia, respectively. In more severe cases, the patient can be diagnosed with oligoasthenoteratozoospermia, when the sample is abnormal for the three

parameters (values below the threshold). In any case, semen analysis should be considered cautiously in terms of an absolute diagnosis, since the result of a spermiogram cannot accurately predict if the patient will become a biological progenitor (Bonde et al., 1998; Agarwal and Allamaneni, 2005), meaning that some individuals with high semen quality cannot conceive and others with male factor infertility prognosis can. For this reason a number of tests have been carried out recently to monitor different aspects of sperm function and to understand which sperm characteristics are essential for fertility (such as motion parameters, capacitation, acrosome reaction and sperm-oocyte interaction); (Aitken, 2006; Lewis, 2007), contributing to the establishment of the true fertility potential of an individual.

1.2. Particularities on contraception

1.2.1. Main approaches

Recent data suggest an alarming world population growth rate (Sitruk-Ware, 2006). At the same time, even though there is an increment in the use of contraceptives worldwide, a high percentage of couples still have limited access to this technology, contributing to an increase in unwanted pregnancies (Sitruk-Ware, 2006). More than 400.000 maternal deaths related with unwanted pregnancies were directly linked to risky abortions, especially in developing countries between 1995 and 2000 (Global Health Council, 2008). Thus, it seems necessary to develop new contraceptive formulations that can be easily distributed worldwide (Aitken et al., 2008). As observed in previous studies,

reductions in maternal death rates (related to unsafe abortions), child mortality rates, and demographic growth rates are positively correlated with the use of contraception (Kumar, 1994). These results are very appealing, mainly if we consider the population growth problem in poorer regions.

However, it is important to note that there is a great disparity in relation to the access and use of contraceptive methods between different regions in the world (Anderson and Baird, 2002). Thus, the prevalence of contraception is, as expected, much higher and constant for industrialized countries, while in less developed countries, especially those located in sub-Saharan Africa, the opposite is true.

The acceptance of different contraceptive choices also varies from region to region, noting that the developed countries prefer contraception that relies on immediate and reversible action, such as the condom or the oral contraceptive pill. In turn, in less developed countries, long-term (and sometimes irreversible) methods are the majority of contraception used, such as the female and male sterilization or intrauterine device (Figure 1.8.).

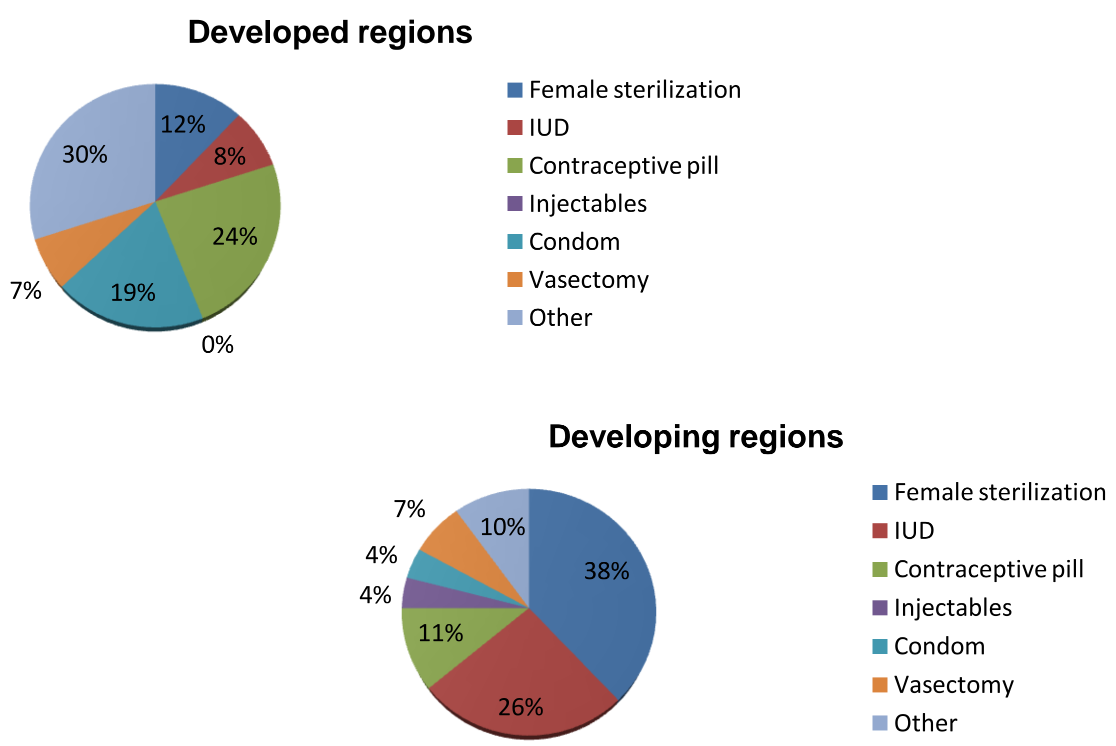


Figure 1.8. Distribution of the different methods of contraception in developed and developing regions of the world.
Adapted from Anderson and Baird, 2002.

Currently, available contraceptive methods can be divided into definitive (tubectomy and vasectomy), natural (safe periods, coitus interruptus), barriers (condoms, spermicides, intra-uterine devices), reproductive-endocrine interventions (steroidal contraceptives) and post-coital methods. The implementation of efficient contraceptive methods, such as the condom (the only method offering a dual anti-microbial and anti-viral protection), has made spermicides seem less attractive. However, their potential action against both HIV and other STD pathogens, and, especially, the possibility of being female-controlled, brought these compounds again to the forefront of the debate (Lech, 2002).

Yet, it must be noted that all spermicidal compounds must possess specific requirements to be considered effective and safe. Namely, they need to rapidly kill or immobilize sperm on contact, or otherwise render them incapable of fertilizing an oocyte. They should also have no adverse effects on a putative embryo/fetus. Finally, non-irritating properties against vaginal and penile mucosa and the long-term absence of toxicity are other important requirements (Lee, 1996).

Spermicidal agents have to undergo a screening process involving several types of tests. One should consider the Sander-Cramer test (to examine effects on sperm motility) and the hypo-osmotic swelling test – HOST (to determine changes in sperm membrane integrity), among other tests (Sander and Cramer, 1941; Yanagimachi et al., 1976; Jeyendran et al., 1984). The viability tests basically rely on two techniques: the optical microscopy-based eosin-nigrosin assay; and the fluorescence-based live-dead assay (using propidium iodide (PI) and SYBR-14 dyes) (Garner and Johnson, 1995; Björndahl et al., 2003). Both methods correlate very well (Chalah and Brillard, 1998; Ramalho-Santos et al., 2007). It should also be noted that some compounds can affect several aspects of sperm activity and act as *de facto* spermicides, even though sperm may retain some functional properties.

1.2.2. Historical perspective on novel contraceptive development strategies

One of the first times the word “contraception” was mentioned in human history was on an Egyptian medical papyrus called Ebers Papyrus, dated 1550

B.C. (Lech, 2002). In fact, condoms, the very well-known contraceptive devices, were invented many centuries ago, but its spreading occurred much more recently. And one of the reasons for their success was the emergence of a lethal sexually transmitted virus: the Human Immunodeficiency Virus (HIV), the causal agent of Acquired Immunodeficiency Syndrome (AIDS) (Stone, 1990).

In the early 1980s HIV had spread exponentially. Almost 35 years later, one can state that HIV/AIDS is the leading cause of death between reproductive age women, having already killed about 30 million people since its discovery (WHO, 2009). Today, around 34 million people live with HIV, and in 2011 alone 2.5 million were newly infected while 1.7 million people died (UNAIDS, 2012). To note that, the virus has now shifted to a predominantly heterosexual transmission for the majority of new infections (Gaym, 2006).

The condom is still the only available method that can protect against both contraception and Sexually Transmitted Diseases (STDs), as mentioned before. But, regardless of the regular use of the condom among couples in more developed countries, data indicate that this method is still insufficient to protect women from less developed countries and, curiously, especially those that are in a stable long-term heterosexual relationship (Herold et al., 2011). Unfortunately, and mainly in countries from sub-Saharan Africa, the condom is not a very accepted method for men, and women still do not have the social and economical power to make their own choices. In fact, in a study made by Bisika in Malawi (Bisika, 2009), with couples living in a stable marital relationship, it was concluded that “It is clear that men do not want a female-controlled method of HIV prevention, fearing that this may encourage women to engage in extramarital sex knowing that they will be protected from STDs and pregnancy.

What these men do not realize is that the women are in fact trying to protect themselves from unfaithful husbands”.

Therefore, many attempts have been made to provide a strategy that could fight HIV spreading, and on the top of the list one can find efficient vaccines and topical microbicides (Cutler and Justman, 2008). It is also very important to note that topical prophylactic microbicides, besides providing female-controlled protection, can also have an additional contraceptive effect (Shattock and Rosenberg, 2012). Taking this into account, many scientists shifted their interests to the discovery of a compound that could fulfill all those requirements, plus being easy to use, cheap and easily distributed. One can also never forget that the development of efficient multi-function molecules should focus on possible common cellular targets, such as the structure and function of the biomembranes involved, or in cell interaction and fusion (Doncel, 2006). In terms of putative multipurpose uses, there were few compounds purported to exert both spermicidal and antiviral activities, such as polyanions (or anionic polymers) and surface-active agents (Doncel and Mauck, 2004). In fact, some of these molecules were even included in preclinical trials, such as Nonoxynol-9 (N-9) (Iyer and Poddar, 2008). However, all have failed and, in some cases, worsened the health of trial subjects by causing inflammation and other pathological conditions often leading to an actual increase in STD transmission (Fichorova et al., 2001; Feldblum et al., 2008), in exact opposition to the initial goal. This might be unsurprising, since that most of the studies referred did not choose a systematic approach to the problem, or even performed an *in vitro* analysis of compound toxicology before moving on to *in vivo* models or to more expensive and risky clinical human studies.

Thus, and knowing that there are no alternatives on the market, there is an urgent need of identifying a new generation of spermicides and/or microbicides that would fill the existing gap. The development of new compounds that would simultaneously act as antiviral and anticonceptional agents will require a detailed and systematic knowledge of how these molecules interact with membranes and how they would, for example, inhibit membrane fusion in relevant systems, including both gametes and viruses.

The variety of multi-functional compounds developed until now may act on several targets. Namely, these molecules may exert their function at the vaginal/cervical/rectal mucosa (as a physical barrier or enhancing protective vaginal flora, for example), at the cell or virus surface (HIV cell receptor "blockers", for example) and even inside the cell, interfering with the viral replication cycle (Balzarini and Van Damme, 2007) (Figure 1.9.).

1.3. Multi-functional compounds

1.3.1. Common cellular mechanisms as ideal targets for multi-functional substances

To develop compounds that would both inactivate HIV or other STD agents and, at the same time, disable sperm, there are two logical strategies: either to design safe and effective spermicides that will also interfere with pathogenic microorganisms (Hughes et al., 2007); or use well-known anti-HIV/STD drugs and test their effects on sperm function (D'Cruz et al., 1999; D'Cruz et al., 2004).

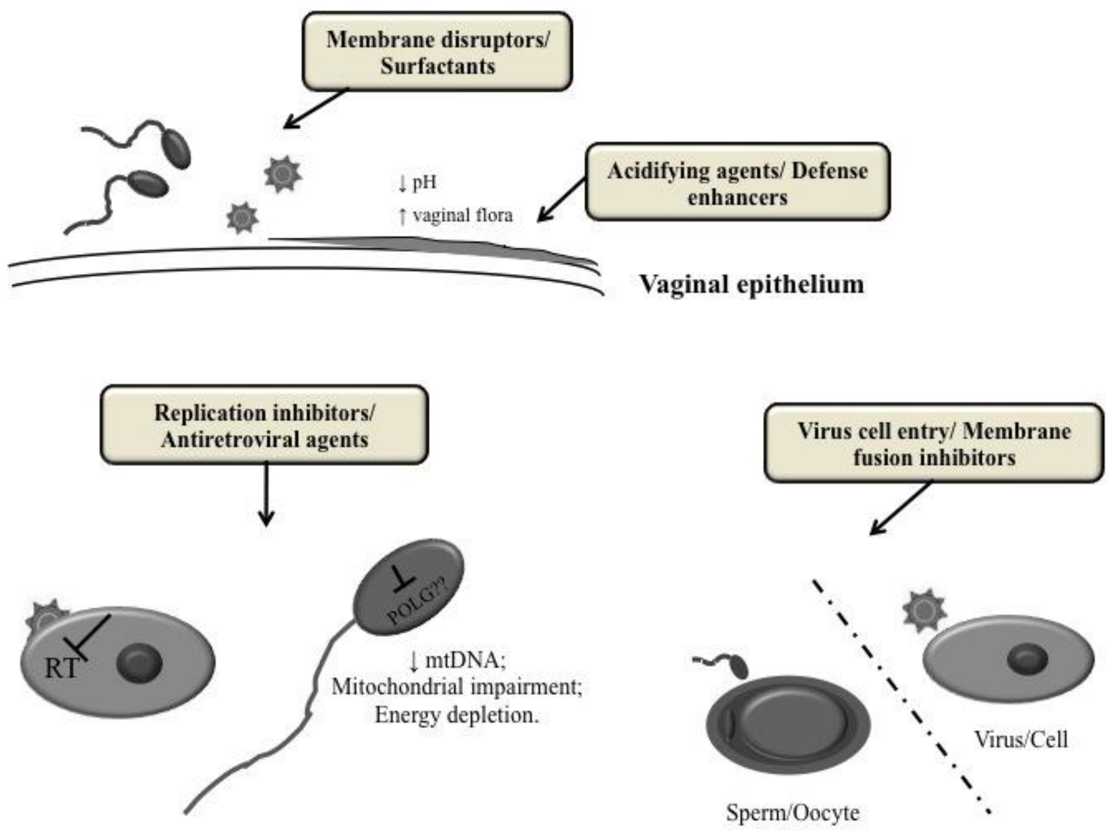


Figure 1.9. Several mechanisms through which multi-function compounds may act. Surfactants disrupts the sperm and pathogen biomembranes, causing damage and inactivation. Acidifying agents enhance vaginal defenses, through the maintenance of a protective mucosal pH. Viral cell entry/Membrane fusion inhibitors block attachment and fusion of pathogens/sperm into target cells, through the interaction with important entry/fusion receptors and co-receptors. Antiretroviral agents inhibit viral DNA replication, leading to the inactivation of virus. Although it has been proven that replication inhibitors block viral RT, they can also inhibit cellular DNA polymerases, particularly POLG (DNA polymerase Gamma), which can potentially lead, in sperm, to the depletion of mtDNA, with spermicidal effects such as loss of motility (probably due to energy depletion).

The strategy focused on the development of multi-active compounds has in its favor the fact that, although different receptors are involved, oocyte fertilisation and genital infection by STDs share the same functional and anatomical context; namely the same route of transmission and anatomical environment in terms of membrane fusion/cell penetration, thus offering the possibility of simultaneous inhibitory action (Doncel, 2006). Furthermore, possible common targets have been actively researched. Among them, the structure and function of both sperm and HIV membranes are extensively mentioned, since both have lipid bilayers that can be physically disrupted, for example by surfactant agents through formation of mixed micelles (Wong et al., 2002; Hillier et al., 2005). Additionally, localized membrane changes, such as those achieved by the disruption of lipid rafts, can also lead to remarkable modifications in sperm or pathogen function (Cross, 1999; Liao et al., 2001). Lipid rafts are microdomains in biological membrane, enriched in cholesterol, sphingolipids and glycosylphosphatidylinositol-anchored proteins (Simons and Ikonen, 1997), and are present in both sperm and HIV. In sperm, they seem to have a role in capacitation, the acrosome reaction and sperm motility (Ramalho-Santos et al., 2000) and the disruption of these domains (for example, by cholesterol depletion) can lead to dramatic changes in sperm function (Parinaud et al., 2000). These domains are also very important for HIV infection and replication, since virions leave the host cells through lipid rafts, and their disruption causes a drop in viral infectivity (Campbell et al., 2001; Graham et al., 2003).

Membrane oxidative damage may also play a role in this process. Even though low and controlled concentrations of reactive oxygen species (ROS),

such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-), are crucial for proper sperm function, higher amounts of ROS can cause sperm pathologies, including loss of motility and viability (Chaki and Misro, 2002). ROS have similar effects on HIV, acting as potential anti-viral agents (Klebanoff and Coombs, 1991).

Besides membranes, other strategic events should be considered. One of them involves molecules that contribute for cell interaction and fusion, as both sperm and STD pathogens need to recognize and fuse with a target cell to achieve their goal (fertilize/infect) (Cohen and Melikyan, 2004; Primakoff and Myles, 2007). Thus, compounds that would interact with binding receptors, such as gp120/41 and CD4 (in HIV infection), or zona pellucida and CD9 receptors (in fertilisation), may inhibit attachment (Oehninger et al., 1991; Kensinger et al., 2004). Although the molecular mechanisms involved in virus-cell and cell-cell fusion are still not well elucidated, there have been some agents developed for this purpose, such as anionic polymers (Anderson et al., 2002).

1.3.2. Initial approaches to spermicides/microbicides: mechanisms of action

Surfactants – The particular case of Nonoxynol-9

As mentioned above, the efforts to find a good contraceptive/microbicide led to the development of numerous studies in preclinical investigations and clinical trials (Hughes et al., 2007). From all the topically applied female-controlled compounds, one of the first generation strategies explored was a

non-ionic surfactant-based substance called Nonoxynol-9, a compound that was previously observed to inactivate HIV and other STD pathogens *in vitro*, with an associated spermicidal effect (Malkovsky et al., 1988; Batár, 2010). These “surface active agents” are known to act via membrane disruption by the formation of mixed micelles, which can damage the lipid bilayer of target cells and also change the membrane electrostatic surface potential (le Maire et al., 2000; Vieira and Carmona-Ribeiro, 2006), although some studies suggest another possible mechanism of action, this time in the cell cytoplasm, leading to a toxic effect exerted inside the cell (Strupp et al., 2000). Indeed, once the detergent gets into the cell it may interact with cytoplasmic components and change their activity, causing for example changes in protein synthesis or inducing cellular death through apoptotic mechanisms by activating caspases (Strupp et al., 2000).

Possible targets for surfactants inside the cell may also include nucleic acids (evidently targeted by cationic detergents, as DNA and RNA are negatively charged polymers) (Dias et al., 2005). Indeed, a few studies have noted surfactant-induced DNA fragmentation (Dietrich et al., 2007), while others have attempted to discriminate genotoxic actions (direct action on the DNA) from the extragenomic cytotoxic action (indirect effect, also eventually leading to DNA fragmentation and subsequent cell death); (Vock et al., 1998).

What characterizes most this class of compounds is that, regardless of the recognized mechanisms of action, they will act non-specifically on sperm cells and STD pathogens as well as on other cells that were not supposed to be harmed, such as vaginal and penile epithelia (Table 1.2.); (Hillier et al., 2005). The use of N-9 showed to induce inflammatory responses in the female

reproductive tract, which can lead to increased risk of HIV transmission (Fichorova et al., 2001).

Table 1.2. Classes of multi-function compounds.

	Specificity	Mechanism of Action	Examples
Surfactants	Not target-specific	Membrane disruption of cells and bacteria/virus	N-9 SDS (Invisible Condom) C31G (Savvy)
pH buffers	Not target-specific	Acidification of vaginal pH (semen buffers)	Carbopol 974P (BufferGel) AcidForm (Amphora)
Entry inhibitors	Not target-specific (Anionic polymers)	Interference of HIV binding to host cells	Cellulose Sulfate (Ushercell) Carrageenan (Carraguard) Naphthalene Sulphonate (PRO2000)
	Target-specific (CCR5 blockers)	Interference of HIV attachment to host cells, blocking CCR5 co-receptor (white blood cells)	PSC-RANTES
ARV drugs	Target-specific	Interference with HIV RT enzyme, blocking virus replication	Tenofovir (NRTI) Dapivirine/TMC-120 (NNRTI)

Regardless of the extended debate between non-surfactant and surfactant supporters, the discontinuation of this kind of surfactant-based approach did not last for a long time. Indeed, evidence of that are the preclinical and clinical trials carried out with that class of substances over time. Compounds such as Benzalkonium Chloride (Tévi-Bénissan et al., 2000; Xu et al., 2006), Sodium Lauryl Sulfate (also called Sodium Dodecyl Sulphate (SDS) or Invisible Condom (Université Laval, Quebec, Canada)) (Piret et al., 2002; Haineault et al., 2003), saponins (Ojha et al., 2003) and C31G (or Savvy; Cellegy Pharmaceuticals, Quakertown, PA, USA) (Wyrick et al., 1997; Krebs et al., 1999; Mauck et al., 2004) were exhaustedly evaluated, showing some

positive results despite the disappointment related to the rejection of a phase III clinical trial in Nigeria with C31G. In fact, after four phase I trials, where most of the tested concentrations of C31G resulted in doubtful findings such as symptoms of irritation, epithelial disruption and vaginal colonization, the phase III study, using lower concentrations, concluded that no significant difference between the product and the placebo was found, despite being less toxic than N-9 (Feldblum et al., 2008).

pH Buffers

The protective effect of acidic vaginal pH (lower than 4.5) is well known, preserving a vigorous microflora. Consequently, a healthy microflora is the first and natural barrier to infection, producing substances (such as hydrogen peroxide) that contribute for the inactivation of STD pathogens (Hillier, 1998). Taking that into account, the use of compounds that can control vaginal pH, in particular by neutralizing the alkaline pH of semen after sexual intercourse (one of the principal causes of vaginal colonization and infection by opportunistic pathogens), was another strategy proposed (Table 1.2.); (Karim and Baxter, 2013). In fact, some of these non-specific compounds were tested in clinical trials, such as BufferGel (ReProtect, Baltimore, MD, USA) and AcidForm (or Amphora; Instead Inc., San Diego, CA, USA), both acting by regulating the pH of vaginal mucosa and having a multi-function role (inactivation of HIV and other STD pathogens as well as sperm immobilization and damage) (Zeitlin et al., 2001; Mayer et al., 2001; Tuyama et al., 2006). Actually, even though BufferGel did not go further because of its ineffectiveness in a large-scale Phase III trial

(Abdool Karim et al., 2011), the bioadhesive-containing lactic acid vaginal gel AcidForm is still in the microbicide pipeline, integrated in a Phase I trial (Keller et al., 2012). More specifically, in what concerns BufferGel, the product was found to be safe but it did not change the risk of getting HIV, when compared with the placebo “gel” and “no-gel” arms (Ramjee et al., 2010). The differences between the results on phase I and phase III trial can possibly be due to the lower exposure frequency on the latter (Abdool Karim et al., 2011). Regarding AcidForm, it should be mentioned that all the findings related to this compound until now should be considered premature and requiring additional safety studies. However, for now one can say that, despite mild vaginal irritation and a decrease on anti-inflammatory proteins, AcidForm also augmentated mucosal defences evidenced by the decrease of vaginal pH, an increase in bactericidal activity of genital secretions and no increase in pro-inflammatory cytokines/chemokines (Keller et al., 2012).

It should be noted that, although these compounds are developed by chemical technicians and synthesized by pharmaceutical companies with a very clear goal in mind, the idea of using compounds that lower the vaginal pH to help improving women sexual health is not a completely new approach in some societies. Indeed, the importance of the use of “homemade” compounds such as lemon juice and vinegar (Fletcher et al., 2008), or applying more “probiotic” procedures such as the treatment with “live” microflora to promote vaginal colonization (Ojha et al., 2003; Rao et al., 2005), should not be excluded in this context.

Entry Inhibitors: Anionic polymers

Lastly, there is another group of compounds that also merited some attention. These substances, polyanions or anionic polymers, can non-specifically block pathogen attachment, membrane fusion or entry of virus into target cells, in addition of having spermicidal effects, through the inhibition of sperm-oocyte attachment and fusion (Table 1.2.); (Cutler and Justman, 2008). In the particular case of HIV, the mechanism of action of anionic polymers relies on the interaction of the negative charges of the compound with viral envelope proteins, interfering with the attachment of the virus to $CD4^+$ cells and, ultimately, avoiding host cell co-receptor activation (Schols et al., 1990). Examples of compounds included in this class are cellulose sulfate, carrageenan or carraguard and PRO 2000 (naphthalene sulphonate) (Dhawan and Mayer, 2006). The first two compounds went to phase III trials, but were immediately shown to have no significant differences to the placebo regarding HIV prevention (Skoler-Karpoff et al., 2008; Van Damme et al., 2008). Adding to that, and unlike carrageenan, cellulose sulfate suggested some safety concerns (Van Damme et al., 2008). After that, scientists raised high expectations on Pro 2000, a compound that had promising results on a phase II trial, showing a 30% level of effectiveness in preventing HIV infection (Abdool Karim et al., 2011). However, the following study, a large-scale phase III study, revealed disappointing results, despite its proven safety towards the vaginal mucosa (Nunn et al., 2009).

1.3.3. Recent approaches for spermicides/microbicides: mechanisms of action

Until now we have addressed non-specific approaches, meaning that the mechanism of action does not rely on a particular molecular target. For this reason, and in addition to the fact that, so far, all the compounds above exhibited unsatisfactory outcomes, a shift in multi-function microbicides research was observed, leading the way towards development of different, functional and more daring approaches.

Entry Inhibitors: CCR5 blockers

Inside the entry inhibitors group, CCR5 inhibitors are possible target-specific strategies to fight STD pathogens. CCR5 is a protein on the surface of white blood cells that is involved in the immune system as it acts as a receptor for chemokines. This is the process by which T cells are attracted to specific tissue and organ targets. Many forms of HIV initially use CCR5 to enter and infect host cells. The most famous drug in this group is PSC-RANTES, a synthetic CCR5 antagonist (Table 1.2.). Previous studies noticed that RANTES, a natural CCR5 ligand, was efficient in blocking HIV infection (Alkhatib et al., 1996) and also that individuals with a polymorphism in the CCR5 HIV co-receptor have a slower progression of AIDS (McDermott et al., 1998). PSC-RANTES binds to CCR5 and blocks viral infection, interfering with the attachment of the virus to host cells, as shown primarily in animal models. In these studies a high and non-toxic protection against SHIV (simian/human

immunodeficiency virus), a chimeric simian/human immunodeficiency virus, was observed in rhesus macaques (Lederman et al., 2004). Also, this molecule was shown to be effective against all HIV clades and to block HIV infection of Langerhans cells (very important cells for vaginal epithelium HIV transmission) (Torre et al., 2000; Kawamura et al., 2004).

Antiretroviral mechanisms (Replication inhibitors)

As mentioned before, the shift in microbicide research was caused by the emergence of novel tools, namely the development of highly specific inhibitors of viral replication. Some therapeutic agents for chronic HIV infection have been recently exploited as potential multi-function microbicides (for HIV and unwanted pregnancy prevention). The main purpose of these compounds is to act when other drugs can no longer block pathogens, i.e., once they are inside the host cell (Table 1.2.). These antiretroviral (ARV) substances, besides being potent and efficient inhibitors of viral reverse transcriptase (RT) activity, were also shown to negatively affect sperm and other STD pathogens, if used topically (D'Cruz et al., 2004). Nevertheless, the main drawback pointed out to ARV drugs is the potential emergence of antiretroviral resistance (McGowan, 2006).

The proof that the current focus of microbicide research is represented by ARV compounds relies on the percentage of ongoing clinical trials, involving either nucleotide reverse transcriptase inhibitors (NRTI), such as Tenofovir; or non-nucleoside reverse transcriptase inhibitors (NNRTI), such as Dapivirine (Tenofovir and Dapivirine studies fulfill 100% of Phase II/III ongoing clinical

trials) (AVAC, 2013). The difference between NRTI and NNRTI compounds is that the latter do not need to be metabolized for conversion into an active form (via phosphorylation by intracellular nucleoside kinases), which is an important advantage. In summary, these substances act by inhibiting viral RT, a crucial enzyme needed for the replication of retroviruses (eg: HIV), converting viral RNA into complementary DNA for posterior insertion into the host genome (Pierson et al., 2004). These promising candidates have shown great *in vitro* and *in vivo* results, and are presently being assessed in large-scale human clinical trials (McGowan, 2010). Currently, and despite several ongoing experiments, there is a great optimism about these compounds, also associated with some improvements related to the way the drugs can be delivered into the organism (including vaginal rings, tablets, suppositories, creams, foams, gels), which is a very important issue (Nuttall et al., 2007; Verma et al., 2011).

Novel mechanisms under investigation

Multi-function compounds are experiencing a phase of fast evolution. Research is always moving forward to identify new pipeline candidates and to achieve improved formulations. Therefore, regardless of the ongoing trials testing all the compounds already mentioned, new original and hopefully better ways of reaching the proposed goals are being exploited.

Example of this is the work of a research group that has synthesized and evaluated a number of novel and promising structures, leading to the discovery of a number of non-detergent spermicidal prototypes, such as the disulfide esthers (DSE) of carbothioic acid and the isoxazolecarbaldehydes (ISX) (Jain et

al., 2010). Regarding the spermicidal, antifungal and anti-*Trichomonas* activity, the recent outcomes were very positive, showing that compounds selectively kill sperm or render them unable to fertilize the oocyte and inhibit *Candida* and *Trichomonas* vaginal activity, sparing the microflora and the vaginal epithelium to ensure a safer contraception (Gupta et al., 2005; Jain et al., 2007; Kumar et al., 2008; Jain et al., 2009). The same lab tried another very clever approach by modifying an already known effective drug against *Trichomonas* (Metronidazole) in order to insert pharmacophores that promote spermicidal activity. The results with this modified compound evidenced spermicidal, anti-*Trichomonas* and antifungal activities, as well as non-toxic effects against an epithelial model (HeLa cells); (Kumar et al., 2010; Jain et al., 2011).

Screening active compounds derived from plants also led to the discovery of new promising drugs with efficient spermicidal action. Studies with plant extracts used in traditional western and Chinese medicine were tested on *in vitro* human sperm for spermicidal activity and revealed to cause strong sperm immobilization with low adverse effects in some cases (Khillare and Shrivastav, 2003; Paul et al., 2006; Souad et al., 2007; Alvarez-Gomez et al., 2010; Qiu et al., 2011).

One of the major drawbacks of the compounds that have failed in these experiments was that they were not specifically targeted to sperm/pathogens (in order to avoid cytotoxicity in other cells). With that in mind, a number of researchers found in immunocontraception and, in particular, the targeting of antibodies to sperm-specific antigens implicated in sperm-egg binding and fertilisation an attractive approach to control fertility (Diekman and Herr, 1997; Frayne and Hall, 1999; Suri, 2005). A study done by Chen and collaborators

tested antisemen antibodies (ASAs) in order to evaluate their spermicidal action. This hypothesis relies on the fact that if sperm can generate an immune response capable of inducing a contraceptive state (immune infertility), then ASAs can probably be used as spermicides (Chen et al., 2009). This specific antibody immobilized and agglutinated sperm, along with the decrease in sperm MMP. It also blocked sperm-zona pellucida binding, as well as *in vitro* and *in vivo* fertilisation (all at low doses in terms of cytotoxicity). But to assert that ASAs are a suitable and safe strategy, more data should be provided.

It is known that mutations and deletions in sperm-specific ion channels can negatively influence male fertility, as a result of sperm motility and oocyte fertilisation impairment, without affecting other physiological functions, and thus representing an excellent target to non-hormonal contraceptives (Ren et al., 2001). In fact, sperm ion channels, mainly CatSper, as previously mentioned, have been confirmed to regulate male fertility, as these are the channels through which progesterone triggers a rapid influx of Ca^{2+}, ultimately signalling all the important intracellular responses for efficient fertilisation (Publicover and Barratt, 2011; Barratt, 2011; Lishko et al., 2012). Therefore, the uniqueness of CatSper, makes it an ideal target for contraception. Li and collaborators thought of the immunocontraception technique as a tool to inhibit the cationic channel of sperm (similar to what Chen et al. used on their work). Namely, they discovered that antibodies against CatSper blocked the hyperactivation motility, besides leading to a reduction in the *in vitro* fertilisation rate (Li et al., 2009).

Other important aspect associated to sperm motility and fertilisation is related to the so-called cell energy machine: the mitochondrion. This tiny but vital organelle is believed to play a crucial role, given that sperm needs ATP for

the cellular events involved in sperm capacitation (Ramalho-Santos et al., 2009; Shivaji et al., 2009). Indeed, a recent study revealed that a candidate compound was able to target mitochondria and specifically blocked sperm motility, induced the loss of sperm MMP and decreased ATP generation (Wang et al., 2010), leading to mitochondrial dysfunction.

One should note that there are multiple targets for STDs infection, besides the fact that a multi-function compound should also meet the requisite of disable sperm, meaning that the synergistic combination of microbicides/spermicides will probably confer greater protection. Combining agents with dissimilar mechanisms of action may be the best strategy for the success of multi-function microbicides. Definitely, the forthcoming reality is that no single method will be able to stop the spread of STDs on its own, given that pathogen transmission has redundant mechanisms. Consequently it is important to provide several lines of defence to guarantee effective protection (Cutler and Justman, 2008).

1.3.4. Current clinical/preclinical compounds under evaluation

Several agents have been evaluated in a variety of trials for the potential development of safer topical spermicides and/or microbicides (Gaym, 2006). Preclinical and clinical trials are absolutely essential given that it is the only way to assess the compound safety and effectiveness, as well as its level of acceptance. So, before the product is widely available it should pass several steps/trials.

Briefly, studies can be divided into preclinical and clinical trials, the latter including Phase I, II and III evaluations (defined by the U.S: Food and Drug Administration - FDA). Preclinical trials provide the basis for clinical trial testing, which takes place in humans. Phase I clinical trials test a new drug using a small group of people (dozens) to evaluate its safety, determine a safe dosage range, and identify possible side effects. In Phase II clinical trials, the effectiveness and safety of the compound is assessed in a larger group of people (few dozen to about 300 people). In Phase III clinical trials, the drug is given to even larger groups of people (several hundred to about 3000 people) in order to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the product to be used safely. Obviously, each of these phases is tremendously expensive, with costs increasing in value with progressing stages.

A summary of the compounds undergoing clinical trials outlined in this work are listed in Table 1.3..

1.3.5. Importance of evaluating candidate compound safety and acceptability

Cytotoxicity is a big issue in vaginal spermicide research. After the disappointing results of N-9 and the subsequent awareness messages by the scientific community, several cautions have been taken in what concerns toxicity monitoring. Basically, when N-9 was launched as a microbicide, few if any *in vitro*/animal testing preceded its exhaustive use by women (Fichorova et al., 2001; Van Damme et al., 2002).

Table 1.3. Status of some microbicide candidates in terms of STD-related clinical trials. Adapted from Cutler and Justman, 2008.

Class	Product	Status
Surfactants	Nonoxynol-9	Several subsequent clinical trials reached the consensus that, for safety reasons, it should not be used anymore.
	SDS	Completed Phase I/II trial. No current studies planned.
	Savvy (C31G)	Phase III trials halted due to low HIV incidence rate in the study population (Peterson et al., 2007; Feldblum et al., 2008).
pH buffers	Carbopol 974P (BufferGel)	Completed Phase II trial. Failed for not showing efficacy in HIV prevention, despite being a good contraceptive. No safety concerns (Abdool Karim et al., 2011).
	AcidForm (Amphora)	Ongoing phase III trial in Madagascar testing diaphragm with AcidForm for prevention of *N gonorrhoeae* and *C trachomatis*.
Entry inhibitors	Cellulose Sulfate (Ushercell)	Completed Phase III trial. Failed to show efficacy in HIV-infection prevention (Van Damme et al., 2008; Halpern et al., 2008).
	Carrageenan (Carraguard)	Completed Phase III trial. Failed to show efficacy in prevention of HIV infection. No safety concerns (Skoler-Karpoff, 2008).
	Naphthalene sulfonate (Pro 2000)	Completed Phase III trial. Despite the good expectations from Phase II/IIb trials, Phase III failed, not showing statistical effect on HIV infection. No harm was observed (Nunn et al., 2009; Abdool Karim et al., 2011).
	PSC-RANTES	Protected macaques from SHIV with no evidence of systemic absorption or toxicity (Lederman et al., 2004).
ARV	Tenofovir	Completed Phase II trial, with very optimistic results and no adverse effects. Ongoing Phase III trials (Abdool Karim et al., 2010).
	Dapivirine (TMC-120)	Completed Phase I/II studies. Planned Phase III trial, currently recruiting.

It is therefore important to reinforce that a healthy cervicovaginal milieu is a pre-requisite to supply a robust first line of defence against STDs. But what renders women and, hence, the female genital tract so susceptible is what we will discuss below.

First of all, besides the already discussed reduced social and economic power of women in many cultures, the incidence of HIV transmission is gender-related; indeed, sexual transmission from male-to-female is more efficient than the opposite, contributing to a higher vulnerability of the female sex (Saracco et al., 1993). Also, the diagnosis of STDs in women is much harder to obtain, which again puts them in a weaker position, since there is a strong positive correlation between the presence of STDs and the enabling of HIV dispersion (Wilkinson et al., 1999). Once in the genital tract, one should take into consideration some histological issues: the epithelium along the female reproductive tract varies in cellular organization, being the vagina/cervix composed by a stratified squamous epithelium (lacking HIV surface receptors and thus rendering it somewhat more protected from possible infections), while the endocervix/uterus is composed of a single-layer columnar epithelium (much thinner and with primary receptors for HIV on the surface, turning it in a much more vulnerable region for HIV infection) (Pudney et al., 2005).

After some cases of clear failure (such as N-9), and taking into account the specific characteristics of female genital tract, researchers have thought of implementing stricter rules, such as that new candidates should go through various *in vitro* and animal tests prior to their acceptance for human clinical trials. A number of papers have thus proposed *in vitro* experiments involving a variety of parameters that could be used as markers of microbicide and

contraceptive safety. Among them, the production of pro-inflammatory molecules, such as cytokines and interleukins, and mitochondrial dysfunction assessment are promising cytotoxicity biomarkers (Fichorova, 2004; Su et al., 2011; Zalenskaya et al., 2011; Inácio et al., 2013).

In addition, several *in vitro* models mimicking the vaginal epithelium have been developed to evaluate tissue irritation and inflammation. Alternative models to the commonly used rabbit vaginal irritation test (RVI), such as the EpiVaginal test system or the dual-chamber model, were created in order to be able to select the most active but least toxic candidate microbicide before undergoing clinical trials (Gali et al., 2010; Ayehunie et al., 2011). With those models it is possible to study the interaction between representative female epithelial cells and the candidate compounds, and how much the vaginal tissue can be susceptible to infection by STD pathogens, making these tests important easy-to-use and reproducible tools for the screening of effective microbicides (that do not damage or interfere with epithelial integrity and function). However, more research should be done in this area, since the models are quite recent and the actual impact of biological fluids on microbicidal activity is still undetermined, implying that more refined tests closer to real physiological conditions should be proposed (Herold et al., 2011).

No less important is the determination of the optimal dosage of the microbicide or/and the design of the formulation in which the drug will be delivered (low release devices – e.g. the vaginal ring - are preferred in order to avoid possible systemic toxicity). These parameters, combined with a discrete use and the non-interference with sexual intercourse, will ultimately determine the user acceptability (Nuttall et al., 2007).

1.4. Aims of the work

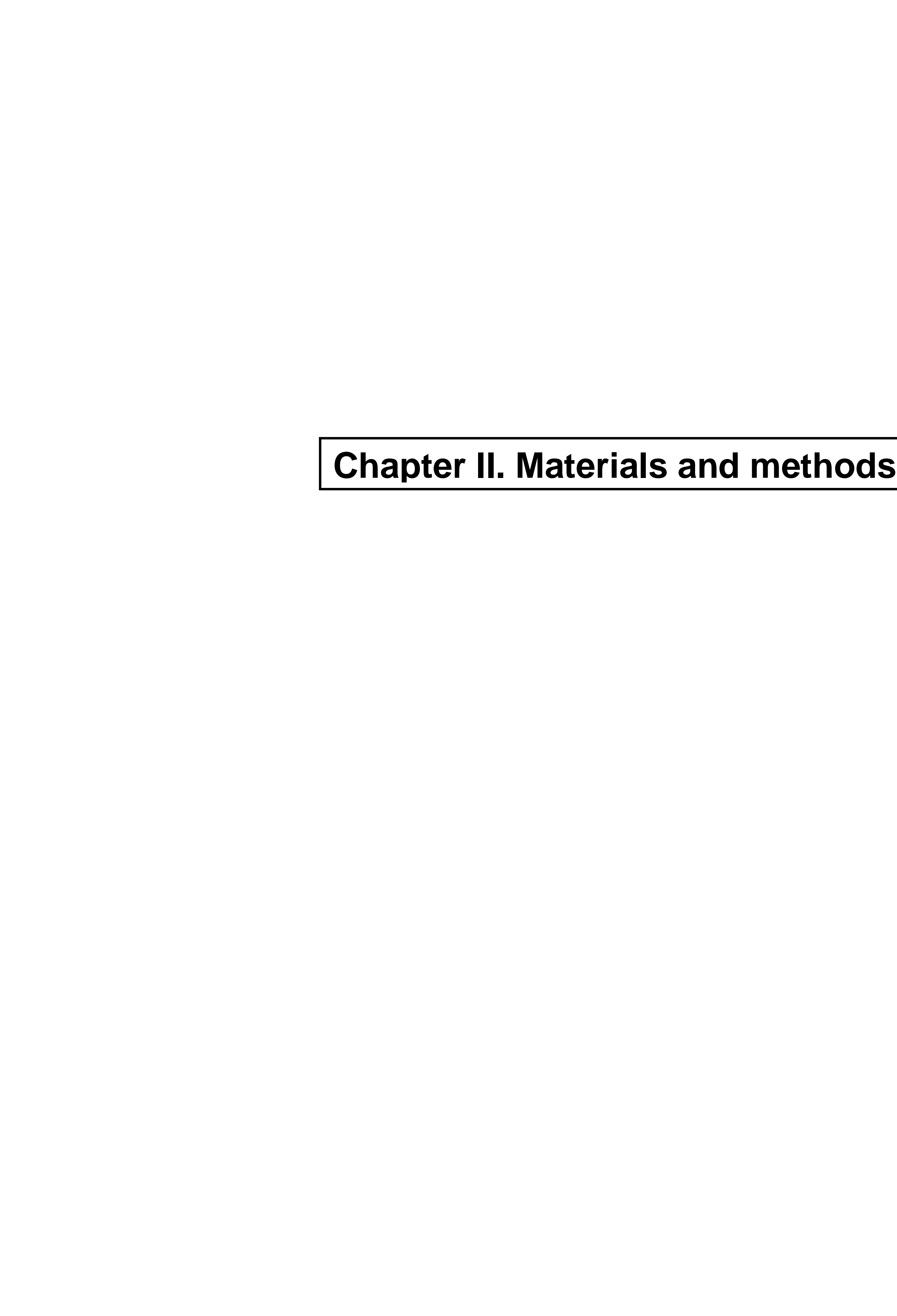
In the present work, the most important aims to retain are:

1) To explore the toxic effects of different classes of surfactants on human sperm cells, monitoring viability and other parameters important for sperm cell function and fertilisation, namely motility, mitochondrial status, capacitation, acrosome reaction and intracellular Ca^{2+};

2) To analyze the spermicidal effects taking into account the surfactant critical micelle concentration (CMC), as it is known to condition their effects on lipid bilayers (Aránzazu Partearroyo et al., 1990);

3) To establish a relationship between the surfactants toxicity and their chemical structure, in order to provide insights for the design of new more effective compounds;

4) To determine an efficient *in vitro* pre-screening methodology for the simple and quick evaluation of spermicidal candidates.



Chapter II. Materials and methods

Synopsis

2.1. Reagents

2.2. Biological material

2.3. Sperm motility

2.4. Sperm plasma membrane status/integrity (HOST)

2.5. Sperm viability

2.6. Sperm mitochondrial membrane potential

2.7. Sperm capacitation status

2.8. Sperm acrosomal status

2.9. Sperm calcium signaling (single-cell imaging)

2.10. Statistical analysis

2.1. Reagents

The LIVE/DEAD® Sperm Viability Kit, the probes 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and Oregon Green BAPTA 1-AM were purchased from Molecular Probes®, Eugene, Oregon, USA). Decyltrimethylammonium bromide (C_{10}TAB) was supplied from Fluka (St. Louis, MO, USA). All the other chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), except when noted. All reagents used were of the highest grade of purity commercially available.

In Table 2.1. we present the representatives of all the families of commercially available surfactants tested in this work.

2.2. Biological material

Patients undergoing routine semen analysis or fertility treatment involving both in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI) were recruited from the Fertility Clinic (University Hospitals of Coimbra, Portugal). All patients signed informed consent forms, and all human material was used in accordance with the appropriate ethical and Internal Review Board (IRB) guidelines provided by the University Hospitals of Coimbra.

Fresh semen samples were obtained by masturbation after 3 to 5 days of sexual abstinence and were allowed to liquefy for 10-20 min at room temperature or at 37ºC in cases of high viscosity. In order to isolate sperm from both seminal plasma and round cells, semen samples were prepared by density gradient centrifugation using Isolate® Sperm Separation Medium (Irvine Scientific, Santa Ana, CA, USA), according to the manufacturer's protocol, and

sperm were then washed and suspended in Sperm Preparation medium (SPM; Medicult, Jyllinge, Denmark). Seminal analysis was carried out in conformity to the World Health Organization Guidelines (WHO, 2010). Samples were categorized based on sperm concentration, motility and morphology. All samples used were normozoospermic for concentration (i.e., ≥ 15 million sperm/ml semen) for motility (i.e., ≥ 40 % total motile sperm) and for morphology (≥ 4% normal forms).

2.3. Sperm motility

Motility was assessed at time 0 minutes and after 20, 60, 180 and 540 minutes of incubation with distinct compounds in PBS-Glucose-BSA medium (phosphate-buffered saline (PBS; pH=7.2-7.4) supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.3% (w/v) bovine serum albumin (BSA), 5 mM glucose and 1% (v/v) GIBCO penicillin/streptomycin (Invitrogen, Paisley, UK)), by phase contrast microscopy, according to the WHO guidelines. Results were expressed as % of progressive motile sperm.

Table 2.1. Surfactants chemical structure.

Family	Surfactant	Abbreviation	Molecular formula	Chemical structure
Non-ionic	Triton X-100	TX-100	$C_{16}H_{26}O_2$	$(H_3C)_3C-CH_2-C(CH_3)_2-C_6H_4-O-[CH_2CH_2O]_n-H$
Zwitterionic	N-dodecyl-N,N-dimethylammonium-propanesulfonate	DDPS	$C_{17}H_{37}NO_3S$	$CH_3(CH_2)_{10}CH_2-N^+(CH_3)_2-(CH_2)_3-SO_3^-$
Anionic	Sodium dodecyl sulfate	SDS	$C_{12}H_{25}NaO_4S$	$CH_3(CH_2)_{10}CH_2O-S(=O)_2-ONa$
Cationic	Decyltrimethylammonium bromide	$C_{10}TAB$	$C_{13}H_{10}BrN$	$H_3C(H_2C)_8H_2C-N^+(CH_3)_3\ Br^-$
	Dodecyltrimethylammonium bromide	$C_{12}TAB$	$C_{15}H_{34}BrN$	$CH_3(CH_2)_{10}CH_2-N^+(CH_3)_3\ Br^-$
	Tetradecyltrimethylammonium bromide	$C_{14}TAB$	$C_{17}H_{38}BrN$	$CH_3(CH_2)_{12}CH_2-N^+(CH_3)_3\ Br^-$
	Hexadecyltrimethylammonium bromide	$C_{16}TAB$	$C_{19}H_{42}BrN$	$H_3C(H_2C)_{15}-N^+(CH_3)_3\ Br^-$
	N-dodecylpyridinium bromide	$C_{12}PYR$	$C_{17}H_{30}BrN$	$C_5H_5N^+-(CH_2)_{11}CH_3\ Br^-$
	Dodecyl-N-benzyl-N,N-dimethylammonium bromide	$C_{12}BZK$	$C_{21}H_{38}BrN$	$CH_3(CH_2)_{10}CH_2-N^+(CH_3)_2-CH_2C_6H_5\ Br^-$

2.4. Sperm plasma membrane status/integrity (HOS test)

The hypo-osmotic swelling test (HOST) (Jeyendran et al., 1984) was used to determine the effect on the physiological integrity of the sperm membrane. As in some other species, human spermatozoa “swell” under hypo-osmotic conditions due to the influx of water and the subsequent expansion of the membranes. The sperm tail appears to be particularly susceptible to such hypo-osmotic stress, and based on the vigor of the sperm, different patterns of tail swelling can be observed (Figure 2.1.).

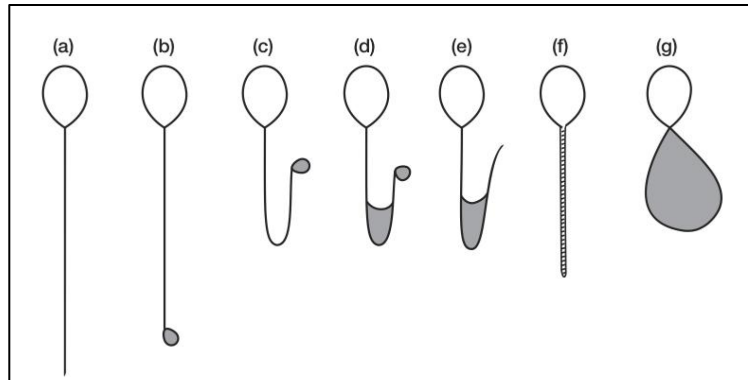


Figure 2.1. Typical morphological changes in human spermatozoa subjected to hypo-osmotic stress. (a) No change; (b)–(g) Various types of tail changes. Swelling in tail is indicated by the grey area.
Adapted from Jeyendran et al., 1984.

Human sperm treated with spermicide solution were pelleted, treated with hypo-osmotic solution (a mixture of equal parts of fructose and sodium citrate, 150mOsm) and mixed gently. The suspension was incubated for 30 min at 37°C. Samples were mounted and observed under a phase-contrast microscope and 200 spermatozoa in at least four different fields were classified

concerning the morphological changes in the sperm tail - “swollen” spermatozoa=coiled tails, means that the functional integrity of the membrane is not affected; “unswollen” spermatozoa=uncoiled tails, means that sperm is already affected in terms of membrane physiology and the transport of fluids across the membrane is compromised.

2.5. Sperm viability

The LIVE/DEAD® Sperm Viability kit consists of two DNA-binding fluorescent dyes: SYBR-14, which is membrane-permeant and thus all sperm stain with bright green fluorescence in the nucleus; and propidium iodide (PI), that only penetrates sperm nuclei with compromised membrane integrity, fluorescing in the red channel, and usually overwhelming the SYBR-14 signal.

Primary stock solutions of 1 mM SYBR and 2.4 mM PI were prepared in water (PI) or in DMSO (SYBR), and aliquots stored at -20 ˚C protected from light. Secondary stock solutions were prepared in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.01 M PO_4 buffer, 0.003 M KCl, pH 7.2-7.4 (GIBCO – Invitrogen, Paisley,UK); pH 7.2), immediately prior to use. Live sperm suspensions ($10x10^6$ sperm/mL) were incubated with 100 nM SYBR-14 and 240 nM PI, for 20 min, at 37°C, in the dark. Sperm suspensions were mounted on a microscope slide and observed using a Zeiss Axiophot II microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter and categorized according to the patterns of staining in the sperm head. Results were expressed as % of viable sperm (with integral membrane; *i.e.*, green heads). For each slide 200 sperm were analyzed, in at least four different fields.

2.6. Sperm mitochondrial membrane potential

5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a mitochondrial potential-dependent lipophilic cationic dye. Thus, a high active mitochondrial membrane potential (MMP) leads to an increase in intra-mitochondrial JC-1 accumulation, indicated by orange/red fluorescence. This happens due to the formation of JC-1 aggregates, leading to a shift in fluorescence emission (from green to red). Conversely, low MMP leads to the accumulation of JC-1 in its monomeric form, and thus exhibit green fluorescence.

Primary stock solutions of 3.8 mM JC-1 was prepared in DMSO, and aliquots stored at -20 ˚C protected from light. Secondary stock solutions were prepared in PBS, immediately prior using. For labeling, live sperm suspensions ($10x10^6$ sperm/mL) were incubated with 2 µM JC-1 for 10 min, at 37°C, in the dark. Sperm suspensions were mounted on a microscope slide and observed using a Zeiss Axioplan II Imaging fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter and categorized according to the patterns of staining in the midpiece. Results were expressed as % of sperm with “positive” MMP: orange (almost always accompanied with green – high MMP) and green (low MMP) stained midpieces. For each slide 200 sperm were analyzed, in at least four different fields.

2.7. Sperm capacitation status

Sperm capacitation status was monitored by the detection of phosphotyrosines by immunocytochemistry (ICC) as described (Ramalho-

Santos et al., 2007). Briefly, after incubation with the surfactants, sperm samples were fixed with 2% (v/v) formaldehyde in phosphate-buffered saline (PBS; pH= 7.2) for 60 min, permeabilised in PBS with 1% Triton X-100 (v/v) for 30 min, and finally incubated in blocking solution (PBS with 2 mg/mL Bovine Serum Albumin (BSA) and 100 mM glycine). Samples were then incubated overnight at 37ºC with the primary antibody rabbit anti-human phosphotyrosine polyclonal (Zymed, South San Francisco, CA, USA) diluted 1:10 in blocking solution. After washing, samples were incubated with the secondary antibody (anti-rabbit) diluted 1:200, for 30 min at 37ºC. After washing, cells were counterstained with 4,6-diamino-2-phenylindole (DAPI; Molecular Probes®), and coverslips were mounted in VectaShield mounting medium (Vector Labs, Burlingame, CA) and sealed with nail polish. Slides were analysed by fluorescence microscopy using a Zeiss Axioplan II Imaging fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter. Sperm showing red staining in the whole tail region were considered to be capacitated; while unstained sperm, or sperm presenting an irregular staining in the tail region, were considered uncapacitated. 200 sperm per slide were counted in at least four different fields, in order to determine the percentage of stained sperm. Secondary antibody-only experiments were carried out as negative controls.

2.8. Sperm acrosomal status

Acrosomal status was determined using the acrosome content marker *Pisum sativum* agglutinin, linked to fluorescein isothiocyanate (FITC-PSA). The

procedure was performed as described before (Ramalho-Santos et al., 2007). Sperm samples were fixed, permeabilised and blocked, as previously described. Samples were then incubated 1h at 37ºC with FITC-PSA diluted 1:200 in blocking solution. After rinsing, samples were mounted as formely described and observed using a Zeiss Axioplan II Imaging fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter. Sperm showing green staining in the whole acrosomal region were considered to present intact acrosome; while unstained sperm, or sperm presenting green staining in the equatorial region or an irregular staining in the acrosome region, were considered acrosome-reacted. 200 sperm were counted in at least four fields and the percentage of sperm with an intact acrosome was determined.

2.9. Sperm calcium signaling (single-cell imaging)

Healthy donors were recruited at the University of Birmingham, in accordance with the Human and Embryology Authority Code of Practice (University of Birmingham Life and Health Sciences ERC 07-009 and ERN-12-0570; Tayside Committee of Medical Research Ethics B 08/S1402/6). After liquefaction, sperm were prepared by direct swim-up into supplemented Earle's balanced salt solution (sEBSS) containing 1.01 mM NaH_2PO_4, 5.4 mM KCl, 0.81 mM $MgSO_4.7H_2O$, 5.5 mM D-glucose, 2.5 mM Na-pyruvate, 19 mM Na-lactate, 1.8 mM $CaCl_2.2H_2O$, 52.4 mM $NaHCO_3$, 118.4 mM NaCl, and 15 mM HEPES (pH 7.3-7.4; 285–295 mOsm), supplemented with 0.3% (w/v) fatty acid-

free BSA, and adjusted to 6 million cells/ml. Sperm were allowed to capacitate at 37°C and 5% CO_2 for at least 3 hours.

For cell Ca^{2+} imaging cell density was reduced to 4 million sperm/ml and samples were loaded with 12 µM Oregon Green BAPTA 1-AM (OGB; Molecular Probes®, Eugene, Oregon, USA; constituted with 0.6% dimethyl sulfoxide (DMSO) and 0.12% Pluronic F-127) for 30 minutes and transferred to a purpose-built, perfusable, imaging chamber incorporating a coverslip previously coated with 1% poly-D-lysine (PDL) for another 30 minutes (all at 37°C and 5% CO_2). The imaging chamber was then connected to the perfusion apparatus and perfused with fresh medium (25°C) before starting recording, in order to remove unattached cells and excess dye. All experiments were performed at 25 ± 0.5°C in a dark room with a continuous medium flow (perfusion rate of ≈0.4 ml/minute); (as described in Baptista et al., 2013).

Cells were imaged with a Nikon TE200 inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA). Real time recordings were performed every 2.5 seconds using an x40 oil objective and an IQ aqquisition software platform (Andor Technology, Belfast, UK).

To evaluate the effects of cationic surfactants, sperm cells were treated following a proposed timeline (Figure 2.2.). In the first minutes sEBSS medium was added to the perfusion chamber ("control" period), and later on the tested compound was added, previously diluted in sEBSS. After the surfactant reaches a plateau in the Ca^{2+} increase, sperm were washed with sEBSS and 3.2 µM progesterone was added to determine if they were responding properly to the physiological stimuli (internal "positive control"), given that with progesterone ≥98% of cells should react by increasing Ca^{2+} influx.

Analysis of images, background correction and normalization of data was performed as described in previous studies (Kirkman-Brown et al. 2000). The region of interest was drawn around the posterior head and neck region of each cell and raw intensity values were imported into Microsoft Excel and normalized using the equation $\Delta F = [(F - F_{basal}) / F_{basal}] \times 100\%$, where ΔF is percentage change in intensity, F is fluorescence intensity at time t and F_{basal} is the mean basal F established in the beginning of each experiment before application of any stimulus. Each cell was considered to respond when the mean of 10 determinations of normalized F during the exposure period significantly differed from the mean of 10 determinations of normalized F during control treatment ($p < 0.05$). Mean amplitudes and percent responsive cells were calculated for each concentration in each sperm sample analyzed.

Figure 2.2. Design of the single-cell imaging experiments.

2.10. Statistical analysis

Results were expressed as the means ± standard error of the mean (SEM), unless otherwise stated. The cell viability dose-response curves were fitted with a four-parameter sigmoid equation (Seefeldt et al., 1995; Knezevic et al., 2007) through computer-assisted curve fitting (SigmaPlot® 11.0, SPSS Inc., USA). The fitted equation was:

$$y = y_0 + \frac{y_{max} - y_0}{1 + e^{-\left(\frac{\log(x) - \log(LD_{50})}{b}\right)}}$$

Where x is the surfactant concentration, y_{max} is the maximal percentage of cell viability, y_0 is the basal cell viability, b is the curve slope between its maximum and minimum thresholds and LD_{50} is the lethal dose 50 (LD_{50}). From these data, it was possible to calculate the lethal dose (LD) 90, 50 and 10 concentrations for each individual data set.

Statistical analysis was performed using SPSS for Windows (version 19.0, IBM SPSS Inc., USA). All variables were tested for normal distribution using the Shapiro-Wilk test (designed for small samples). One-way ANOVA parametric test (or the Kruskal-Walli's non-parametric test for not normal distributions) was performed to compare the results between the different concentrations. If the previous test rejected the hypothesis of equality of the means, the Post-Hoc test Dunnett was used for multiple comparisons. Statistical significance was considered for $p < 0.05$.

Chapter III. Studying the spermicidal action of surfactants: *in vitro* pre-screening methodologies

Part of this data was published in the following papers:

Vieira OV, Hartmann DO, Cardoso CM, Oberdoerfer D, **Baptista M**, Santos MA, Almeida L, Ramalho-Santos J and Vaz WL (2008) Surfactants as microbicides and contraceptive agents: a systematic *in vitro* study. PLoS One 3 (8): e2913. doi: 10.1371/journal.pone.0002913.

Inácio ÂS, Mesquita KA, **Baptista M**, Ramalho-Santos J, Vaz WL and Vieira OV (2011) *In vitro* surfactant structure-toxicity relationships: implications for surfactant use in sexually transmitted infection prophylaxis and contraception. PLoS One 6 (5): e19850. doi: 10.1371/journal.pone.0019850.

Synopsis

3.1. Introduction

3.2. Results

3.2.1. Effect of surfactant type, concentration and exposure time on the viability and motility of sperm cells

3.2.2. Effect of surfactant exposure on the sperm plasma membrane (HOST)

3.2.3. Cationic surfactant structure-toxicity relationship

3.3. Discussion

3.1. Introduction

According to the 2010 Revision of World Population Prospects (Zaneveld et al., 2002) it is believed that the world population will increase from 6.9 billion in mid-2011 to 9.3 billion in 2050, reaching 10.1 billion by 2100. If fertility remains constant in each country at the level registered in 2005-2010 the world population could reach nearly 27 billion by 2100. Related to this issue is the failure in proper contraception, mainly in less developed countries, which is usually accompanied by poverty, hunger and lack of health care, leading to the transmission of STDs (Kelly and Shattock, 2011; Su et al., 2011; Zalenskaya et al., 2011). The condom is, until now, the only method capable of successfully preventing both unwanted pregnancies and STDs. However, it is not woman controlled, which is decisive in some developing countries due to social constraints (Stone, 1990; Lech, 2002). This has led to the search for alternatives, such as topical (e.g. vaginal gels) female-controlled spermicides. Until now it has been identified and studied several promising spermicidal compounds, but none of them have shown real efficacy (see Chapter I).

There is an urgent need for more refined *in vitro* assays, more closely mimicking physiological conditions and monitoring more relevant functional parameters (Bisika, 2009), before resorting to expensive and time consuming preclinical and clinical trials (Zalenskaya et al., 2011). So, despite the negative results obtained from clinical trials (that included surfactants, among several other types of compounds), it seemed necessary, in this work, to do a step-by-step, systematic investigation of the toxicity of different sorts of compounds, in this case on surfactants, in order to answer the following questions: are

surfactants really useful as vaginal contraceptives? Are there good laboratory models to test their utility in this regard?

Surfactants are amphiphilic molecules consisting of at least two parts: an apolar hydrophobic tail and a polar hydrophilic head. They are often classified taking into account the charge of the polar head group. According to this, they are divided into non-ionic (without charge), anionic (negative charge), cationic (positive charge) and zwitterionic (both positive and negative charge) (Holmberg et al., 2003). Because of their amphiphilic nature, surfactants are generally believed to act at the level of the cell membrane. This interaction is related to their partition coefficient between the aqueous and membrane phases which, in its turn, is related to the critical micelle concentration (CMC). The CMC is defined as the surfactant concentration at which the surfactant monomers form the thermodynamic stable aggregates called micelles (Tanford, 1991). At concentrations below the CMC an equilibrium is established between the surfactant monomers in the aqueous phase and the surfactant in the membrane. At concentrations above the CMC, surfactant micelles co-exist with the surfactant-containing membranes and phospholipids are transferred from the membrane to the micelles. At a high enough concentration of micelles the equilibrium state is a solution of mixed surfactant/phospholipids micelles and the membrane is dissolved. However, long before membrane dissolution occurs, non-ideal miscibility of surfactant and phospholipids in membranes may lead to perturbations of membrane properties, affecting physiologically important processes (Balgavý and Devínsky, 1996; Heerklotz, 2008). Therefore, in this study we find important to compare the toxic effects of the tested surfactants to their respective CMC (Table 3.1.).

Table 3.1. Critical micelle concentrations (CMC) of the surfactants used.

Surfactant		CMC (M)	
Family	**Name**	**Literature**	**Reference**
Non-ionic	TX-100	2.0×10^{-4}	(Brito and Vaz, 1986)
Zwitterionic	DDPS	2.0×10^{-3}	(Brito and Vaz, 1986)
Anionic	SDS	2.6×10^{-3}	(Brito and Vaz, 1986)
Cationic	C_{10}TAB	4.0×10^{-2}	(Brito and Vaz, 1986)
	C_{12}TAB	3.5×10^{-3}	(Brito and Vaz, 1986)
	C_{14}TAB	2.8×10^{-4}	(Brito and Vaz, 1986)
	C_{16}TAB	2.6×10^{-5}	(Brito and Vaz, 1986)
	C_{12}PYR	5.0×10^{-3}	(Simoncic and Span, 1998)
	C_{12}BZK	5.0×10^{-3}	(Kopecky, 1996)

With this in mind, the effects of concentration, exposure time and surfactant structure on the *in vitro* viability and motility of human sperm cells were assessed. Also, a more detailed assay was performed to assess surfactant functional alterations in the sperm plasma membrane (using the HOS test). Representatives of all families of commercially available surfactants were evaluated: non-ionic – Triton X-100 (TX-100); anionic – sodium dodecyl sulfate (SDS); cationic – a homologous series of n-alkyl-N,N,N-trimethylammonium bromides (C_nTAB with n from 10 through 16), N-dodecylpyridinium bromide (C_{12}PYR), and dodecyl-N-benzyl-N,N-dimethylammonium (also known as Benzalkonium) bromide (C_{12}BZK); and zwitterionic – N-dodecyl-N,N-dimethylammonium-propanesulfonate (DDPS). The class of compounds chosen have a combination of characteristics essential in a good spermicide: they are stable and have relative long shelf lives, their synthesis is not expensive and

were previously suggested to have bactericidal and anti-viral properties (Gilbert and Moore, 2005).

3.2. Results

3.2.1. Effect of surfactant type, concentration and exposure time on the viability and motility of sperm cells

In order to better understand the mechanisms of toxicity of the surfactants, we tested the effect of four classes of commercially available surfactants towards the viability of normozoospermic sperm cells. The surfactants studied were: the non-ionic TX-100, the zwitterionic DDPS, the anionic SDS and the cationics C_nTAB. $C_{12}BKZ$ and $C_{12}PYR$. We tested surfactant-dependent sperm cell viability by percentage of sperm heads with only SYBR-14 (green fluorescence – live sperm) or also with propidium iodide (red fluorescence – dead sperm) (Figure 3.1.), immediately after surfactant exposure.

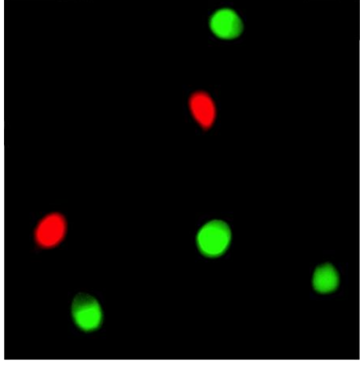


Figure 3.1. Representative image of the LIVE/DEAD sperm viability assay. DNA of live sperm stained green and DNA from dead sperm stained red. Magnification is x1000.

They were exposed to different concentrations of surfactants during 20, 60, 180 and 540 minutes with the different surfactants. We also evaluated sperm cell motility in the presence of the compounds. The data of each independent experiment was fitted to a four parameter logistic equation (Seefeldt et al., 1995; Knezevic et al., 2007) and the Lethal Dose 90 (LD_{90}), Lethal Dose 50 (LD_{50}) and Lethal Dose 10 (LD_{10}), surfactant concentrations at which cell viability was, respectively, 10%, 50% and 90% of the control, were determined for each exposure time (Table 3.2.).

Table 3.2. Lethal doses of surfactant towards human sperm cells.

Surfactant			Lethal Dose (M) after Exposure Times of			
Family	**Name**		**20 min**	**60 min**	**180 min**	**540 min**
Non-ionic	Triton X-100	LD_{90}	$1.37 \times 10^{-4} \pm 4.35 \times 10^{-6}$	$1.37 \times 10^{-4} \pm 2.21 \times 10^{-6}$	$1.35 \times 10^{-4} \pm 4.20 \times 10^{-6}$	$1.28 \times 10^{-4} \pm 3.22 \times 10^{-6}$
		LD_{50}	$1.18 \times 10^{-4} \pm 4.75 \times 10^{-6}$	$1.19 \times 10^{-4} \pm 7.79 \times 10^{-7}$	$1.08 \times 10^{-4} \pm 4.10 \times 10^{-6}$	$9.76 \times 10^{-5} \pm 5.24 \times 10^{-6}$
		LD_{10}	$9.75 \times 10^{-5} \pm 1.57 \times 10^{-6}$	$9.54 \times 10^{-5} \pm 5.05 \times 10^{-6}$	$6.92 \times 10^{-5} \pm 1.69 \times 10^{-6}$	$4.81 \times 10^{-5} \pm 7.47 \times 10^{-6}$
Zwitterionic	DDPS	LD_{90}	$1.32 \times 10^{-3} \pm 4.36 \times 10^{-5}$	$1.30 \times 10^{-3} \pm 5{,}50 \times 10^{-5}$	$1.26 \times 10^{-3} \pm 4.30 \times 10^{-5}$	$1.13 \times 10^{-3} \pm 2.71 \times 10^{-5}$
		LD_{50}	$1.10 \times 10^{-3} \pm 3.51 \times 10^{-5}$	$1.08 \times 10^{-3} \pm 4{,}03 \times 10^{-5}$	$9.87 \times 10^{-4} \pm 7{,}04 \times 10^{-5}$	$9.18 \times 10^{-4} \pm 6.72 \times 10^{-5}$
		LD_{10}	$8.46 \times 10^{-4} \pm 4.18 \times 10^{-5}$	$6.45 \times 10^{-4} \pm 2.04 \times 10^{-5}$	$6.53 \times 10^{-4} \pm 1.35 \times 10^{-5}$	$6.81 \times 10^{-4} \pm 1.08 \times 10^{-4}$
Anionic	SDS	LD_{90}	$1.19 \times 10^{-3} \pm 1.73 \times 10^{-5}$	$1.16 \times 10^{-3} \pm 1.49 \times 10^{-4}$	$1.17 \times 10^{-3} \pm 1.42 \times 10^{-4}$	$1.16 \times 10^{-3} \pm 1.61 \times 10^{-4}$
		LD_{50}	$1.09 \times 10^{-3} \pm 1.71 \times 10^{-5}$	$1.04 \times 10^{-3} \pm 9.01 \times 10^{-6}$	$1.03 \times 10^{-3} \pm 3.45 \times 10^{-5}$	$9.20 \times 10^{-4} \pm 2.74 \times 10^{-4}$
		LD_{10}	$9.33 \times 10^{-4} \pm 7.66 \times 10^{-5}$	$8.64 \times 10^{-4} \pm 1.78 \times 10^{-5}$	$7.13 \times 10^{-4} \pm 1.20 \times 10^{-5}$	$6.25 \times 10^{-4} \pm 2.89 \times 10^{-4}$
Cationic	$C_{10}TAB$	LD_{90}	$6.95 \times 10^{-3} \pm 2.44 \times 10^{-4}$	$2.51 \times 10^{-3} \pm 5.62 \times 10^{-4}$	$1.88 \times 10^{-3} \pm 1.74 \times 10^{-4}$	$1.44 \times 10^{-3} \pm 2.79 \times 10^{-4}$
		LD_{50}	$4.57 \times 10^{-3} \pm 1.89 \times 10^{-4}$	$1.11 \times 10^{-3} \pm 2.38 \times 10^{-4}$	$9.23 \times 10^{-4} \pm 8.10 \times 10^{-5}$	$1.10 \times 10^{-3} \pm 5.93 \times 10^{-4}$
		LD_{10}	$2.73 \times 10^{-3} \pm 1.03 \times 10^{-4}$	$5.54 \times 10^{-4} \pm 1.06 \times 10^{-5}$	$5.08 \times 10^{-4} \pm 5.90 \times 10^{-5}$	$5.73 \times 10^{-4} \pm 2.55 \times 10^{-4}$
	$C_{12}TAB$	LD_{90}	$5.69 \times 10^{-4} \pm 2{,}05 \times 10^{-5}$	$5.28 \times 10^{-4} \pm 2.42 \times 10^{-5}$	$4.34 \times 10^{-4} \pm 5.42 \times 10^{-5}$	$3.13 \times 10^{-4} \pm 3.40 \times 10^{-5}$
		LD_{50}	$4.01 \times 10^{-4} \pm 6.27 \times 10^{-6}$	$2.87 \times 10^{-4} \pm 1.80 \times 10^{-5}$	$1.49 \times 10^{-4} \pm 4.39 \times 10^{-5}$	$1.32 \times 10^{-4} \pm 2.20 \times 10^{-5}$
		LD_{10}	$2.50 \times 10^{-4} \pm 3.32 \times 10^{-6}$	$1.35 \times 10^{-4} \pm 2.44 \times 10^{-5}$	$5.52 \times 10^{-5} \pm 2.30 \times 10^{-6}$	$6.51 \times 10^{-5} \pm 6.04 \times 10^{-6}$
	$C_{14}TAB$	LD_{90}	$1.07 \times 10^{-4} \pm 9.49 \times 10^{-7}$	$1.10 \times 10^{-4} \pm 9.14 \times 10^{-6}$	$3.56 \times 10^{-5} \pm 1.39 \times 10^{-6}$	$3.09 \times 10^{-5} \pm 7.95 \times 10^{-6}$
		LD_{50}	$1.00 \times 10^{-4} \pm 9.49 \times 10^{-7}$	$3.92 \times 10^{-5} \pm 1.66 \times 10^{-6}$	$1.65 \times 10^{-5} \pm 7.25 \times 10^{-6}$	$1.58 \times 10^{-5} \pm 1.96 \times 10^{-6}$
		LD_{10}	$8.89 \times 10^{-5} \pm 5.19 \times 10^{-6}$	$1.03 \times 10^{-5} \pm 5.95 \times 10^{-6}$	$5.00 \times 10^{-6} \pm 2.65 \times 10^{-7}$	$1.83 \times 10^{-6} \pm 3.15 \times 10^{-7}$
	$C_{16}TAB$	LD_{90}	$5.72 \times 10^{-5} \pm 3.32 \times 10^{-7}$	$5.92 \times 10^{-5} \pm 6.80 \times 10^{-7}$	$5.55 \times 10^{-5} \pm 2.01 \times 10^{-6}$	$4.86 \times 10^{-5} \pm 1.17 \times 10^{-6}$
		LD_{50}	$4.51 \times 10^{-5} \pm 1.97 \times 10^{-6}$	$4.75 \times 10^{-5} \pm 8.60 \times 10^{-7}$	$4.81 \times 10^{-5} \pm 7.98 \times 10^{-7}$	$4.01 \times 10^{-5} \pm 5.37 \times 10^{-7}$
		LD_{10}	$3.40 \times 10^{-5} \pm 1.41 \times 10^{-6}$	$3.17 \times 10^{-5} \pm 1.20 \times 10^{-6}$	$3.14 \times 10^{-5} \pm 4.24 \times 10^{-6}$	$2.76 \times 10^{-5} \pm 2.20 \times 10^{-6}$
	$C_{12}PYR$	LD_{90}	$1.40 \times 10^{-4} \pm 1.74 \times 10^{-5}$	$1.11 \times 10^{-4} \pm 4.91 \times 10^{-6}$	$8.28 \times 10^{-5} \pm 1.22 \times 10^{-6}$	$5.99 \times 10^{-5} \pm 1.28 \times 10^{-5}$
		LD_{50}	$1.07 \times 10^{-4} \pm 5.44 \times 10^{-6}$	$8.45 \times 10^{-5} \pm 7.05 \times 10^{-6}$	$6.12 \times 10^{-5} \pm 8.01 \times 10^{-6}$	$3.99 \times 10^{-5} \pm 1.69 \times 10^{-6}$
		LD_{10}	$8.01 \times 10^{-5} \pm 5.83 \times 10^{-6}$	$6.03 \times 10^{-5} \pm 8.91 \times 10^{-6}$	$4.22 \times 10^{-5} \pm 5.99 \times 10^{-6}$	$2.08 \times 10^{-5} \pm 1.47 \times 10^{-6}$
	$C_{12}BZK$	LD_{90}	$5.72 \times 10^{-5} \pm 1.60 \times 10^{-6}$	$2.96 \times 10^{-5} \pm 2.63 \times 10^{-6}$	$1.63 \times 10^{-5} \pm 1.49 \times 10^{-6}$	$1.55 \times 10^{-5} \pm 3.93 \times 10^{-6}$
		LD_{50}	$3.08 \times 10^{-5} \pm 9.31 \times 10^{-6}$	$2.07 \times 10^{-5} \pm 3.71 \times 10^{-6}$	$1.19 \times 10^{-5} \pm 1.13 \times 10^{-6}$	$9.53 \times 10^{-6} \pm 2.12 \times 10^{-6}$
		LD_{10}	$1.69 \times 10^{-5} \pm 5.45 \times 10^{-6}$	$1.45 \times 10^{-5} \pm 3.84 \times 10^{-6}$	$8.47 \times 10^{-6} \pm 8.36 \times 10^{-7}$	$5.95 \times 10^{-6} \pm 1.45 \times 10^{-7}$

Data are shown as Mean ± SD of 5 independent experiments. LD_{90} – lethal dose 90; LD_{50} – lethal dose 50; LD_{10} – lethal dose 10.

The results show that all surfactants used in this study revealed concentration- and time-dependent toxic effects. It is also shown that the compounds exhibited different degrees of toxicity depending on the charge of the polar head. For TX-100, DDPS and SDS, cytotoxicity was not observed up to concentrations close to the CMC (Figure 3.2.), whereas the toxicity of the cationic surfactants, C_{10-14}TAB, C_{12}BZK and C_{12}PYR, was at concentrations that were much lower than its CMC (with the only exception of C_{16}TAB that was not toxic at concentrations below its CMC) (Figure 3.3.). Indeed, the LD_{50} for sperm cells exposed to TX-100, DDPS or SDS after 180 minutes is much higher than the LD_{50} for sperm exposed to C_nTAB, C_{12}BZK and C_{12}PYR after the same time exposure (approximately 0.5 x CMC for the first three compounds, and 0.002-0.06 x CMC for the rest).

The effects on sperm cell motility were also assessed because this parameter is essential for sperm to reach the egg, and once motility is impaired then it is obvious that fertilisation will not take place. Therefore, the tested surfactants led to a dose- and time-dependent toxicity, very similar to the effect observed with viability, as was expected (Figure 3.2. and 3.3.). Indeed, the same pattern as for viability was found, which means that TX-100, DDPS and SDS induced sperm motility loss at concentrations close to CMC, in contrary to the cationic compounds (with the exception of C_{16}TAB), which had effects at concentrations below the CMC. However, it should be stressed out that, although effects on viability and motility were parallel, percentages of motile sperm were always lower than the values of viable sperm, as was expected, given that when challenged with toxic substances, sperm loses motility before dying.

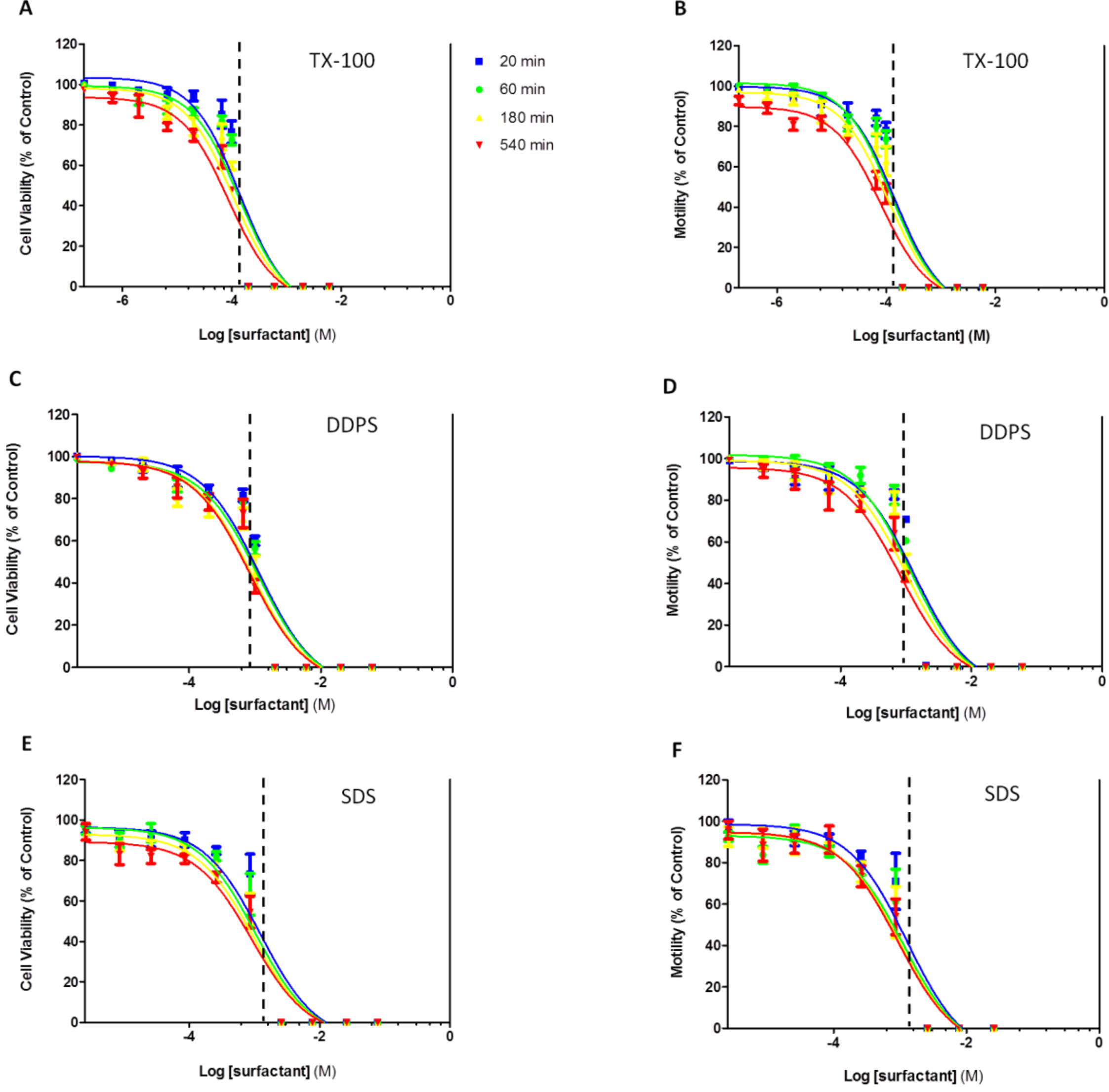
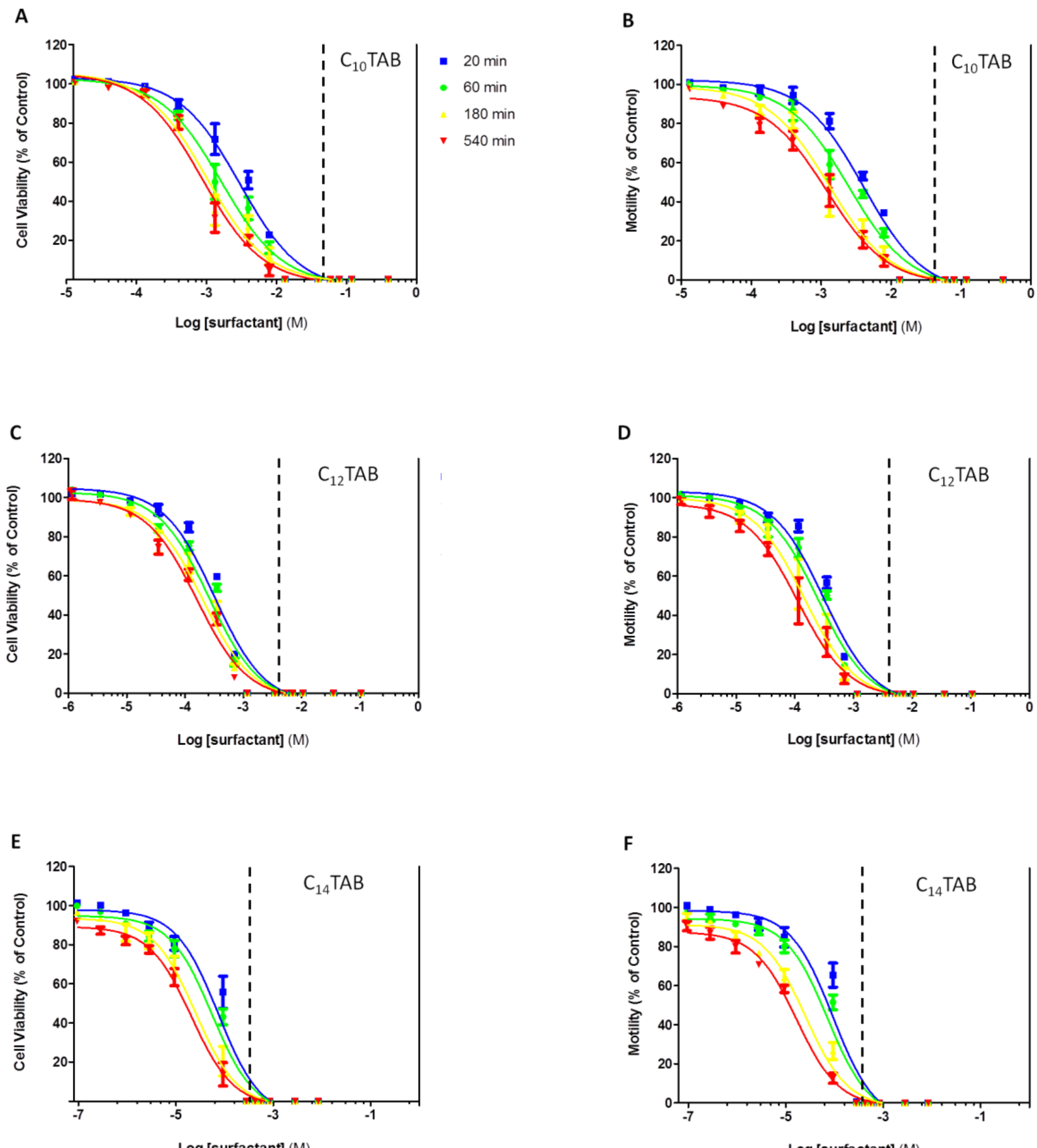


Figure 3.2. Effect of different compound classes on sperm cell viability and motility. The compounds evaluated were: non-ionic TX-100 (**A**, **B**), zwitterionic DDPS (**C**, **D**) and anionic SDS (**E**, **F**) Cell viability and motility were assessed 20, 60, 180 and 540 minutes after the cells had been exposed to different concentrations of surfactant. Cell viability and motility is expressed as percentage in relation to the control. The data of each independent experiment was fitted to a four parameter logistic equation and the LD_{10}, LD_{50} and LD_{90} concentrations determined for each time point. The CMC of each surfactant is represented by the black dashed line. Data are presented as Mean ± SD of 5 independent experiments for each compound.


A
B
C
D
E
F
C_{10}TAB
C_{12}TAB
C_{14}TAB
20 min
60 min
180 min
540 min
Cell Viability (% of Control)
Motility (% of Control)
Log [surfactant] (M)

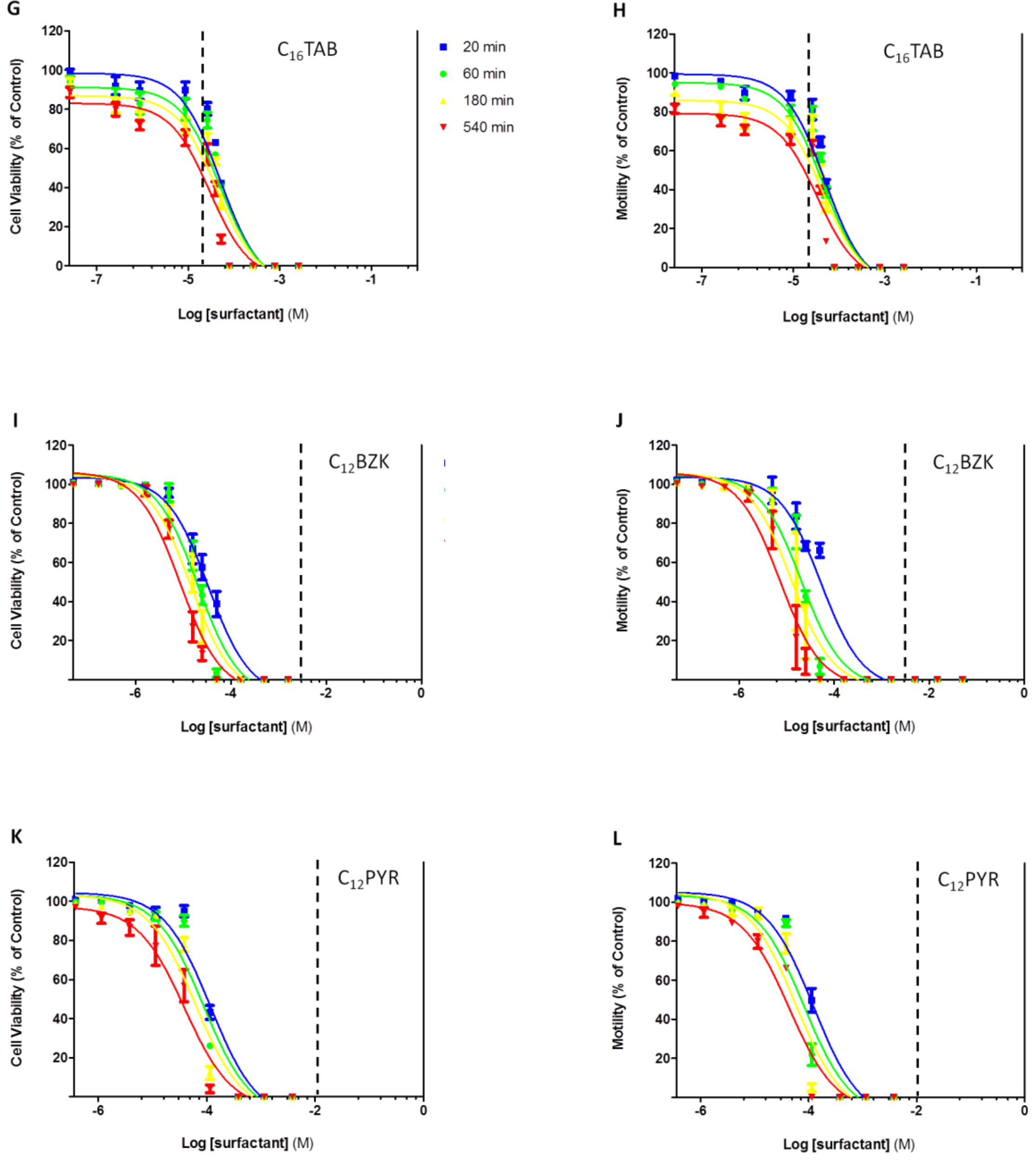


Figure 3.3. Effect of cationic compounds on sperm cell viability and motility. The compounds evaluated were: C_{10}TAB (**A**, **B**), C_{12}TAB (**C**, **D**), C_{14}TAB (**E**, **F**), C_{16}TAB (**G**, **H**), C_{12}BZK (**I**, **J**) and C_{12}PYR (**K**, **L**). All data is as detailed in Figure 3.2.

3.2.2. Effect of surfactant exposure on the sperm plasma membrane (HOST)

In the diagnosis of male infertility, routine andrology laboratories assess, among many other characteristics, the functional integrity of the sperm membrane. This is an important parameter to be in consideration because non-functional sperm plasma membrane will undoubtedly affect the metabolism and the obligatory changes in the dynamics of the membrane, affecting sperm capacitation and the acrosome reaction (Ramu and Jeyendran, 2013). Therefore, the assessment of the membrane function may be a helpful indicator of the fertilizing potential of sperm.

To measure the functional/physiological integrity of the sperm plasma membrane in contact with the tested surfactants, we have used the hypo-osmotic swelling test (HOST), which evaluates the physiological integrity of the membrane by determining the ability of the sperm membrane to reach equilibrium between the sperm cell and its environment. After incubation with a hypo-osmotic solution sperm with impaired plasma membrane physiological integrity will not be able to "swell", not causing the tail to coil. On the contrary, sperm with intact plasma membrane physiological integrity will curl their tails in response to the hypo-osmotic stress caused by the hypo-osmotic solution (Jeyendran et al., 1984) (see Chapter II for more details).

The results show that, for the LD_{50} of all the compounds tested (TX-100, DDPS, SDS, C_{10-16}TAB. C_{12}BKZ and C_{12}PYR), the cationic compounds (with the exception of C_{16}TAB) promote a higher impairment of sperm plasma membrane physiology than TX-100, DDPS and SDS (Figure 3.4.). This means

that for a 50% loss of structural membrane integrity (LD_{50}: 50% of sperm PI-stained), the cationic compounds were the only ones having more than 50% of sperm-membrane physiological integrity loss (between 70%-80%), which suggests that they first affect the physiological integrity of sperm membrane rather than the structural integrity.

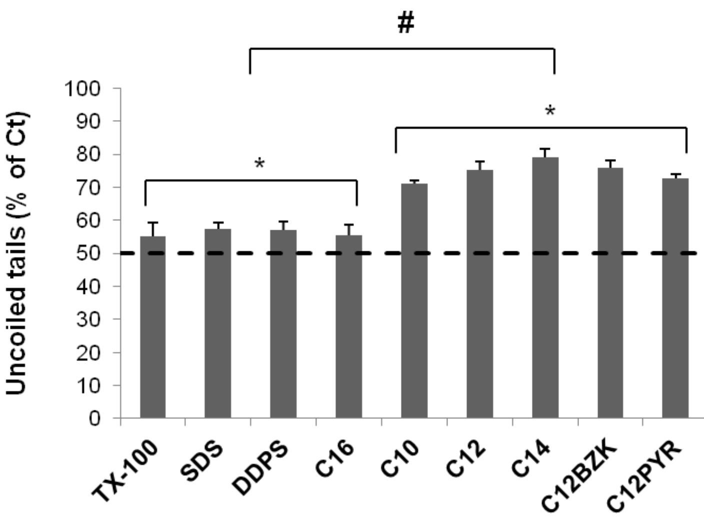


Figure 3.4. Effect of different compound classes on sperm physiological sperm membrane integrity.

All compound classes were evaluated: non-ionic TX-100, zwitterionic DDPS, anionic SDS and cationics $C_{10\text{-}16}$TAB, C_{12}BZK and C_{12}PYR. Sperm membrane physiology was assessed after the cells had been exposed to the concentration LD_{50}. Uncoiled sperm tails were expressed as percentage in relation to the control. The LD_{50} is represented by the black dashed line. Data are presented as Mean ± SEM of 5 independent experiments for each compound. Asterisks denote statistically significant differences from control ($P<0.05$); and the cardinal signs denote difference between experimental groups ($P<0.05$).

3.2.3. Cationic surfactant structure-toxicity relationship

Despite the fact that clinical trials using surfactant-based gels have failed, it has been shown that several quaternary ammonium compounds, with various alkyl chain lengths and polar head groups, exert spermicidal activity, antibacterial activity, as well as against some pathogenic species of fungi and protozoa (Vieira and Carmona-Ribeiro, 2006; Ayotte and Colin, 2002). The study of the relation between cationic surfactant structure and its toxic effects is crucial to understand the mechanisms involved in surfactant toxicity and make predictions of the impact that new surfactants will have in cell viability. For this purpose we tested the effects of the hydrocarbon chain length and polar head group structure of the cationic surfactants upon cell viability. To do so, we treated the cells with surfactants of a homologous series of cationic Alkyl-N,N,N-trimethylammonium bromides ($C_{10–16}$TAB), C_{12}PYR and C_{12}BZK.

The results indicate that for the homologous series of cationic surfactants examined the toxicity to sperm cells was linearly dependent upon the surfactant hydrophobic chain length (Figure 3.5.). This means that the concentration needed to kill 50% of sperm population is much lower for C_{10}TAB than the one needed for C_{12}TAB, and so on. The toxicity ranking of the surfactants studied towards sperm cells, normalized with respect to their respective CMCs, was C_{10}TAB≥C_{12}TAB>C_{14}TAB>C_{16}TAB, for all the exposure times.

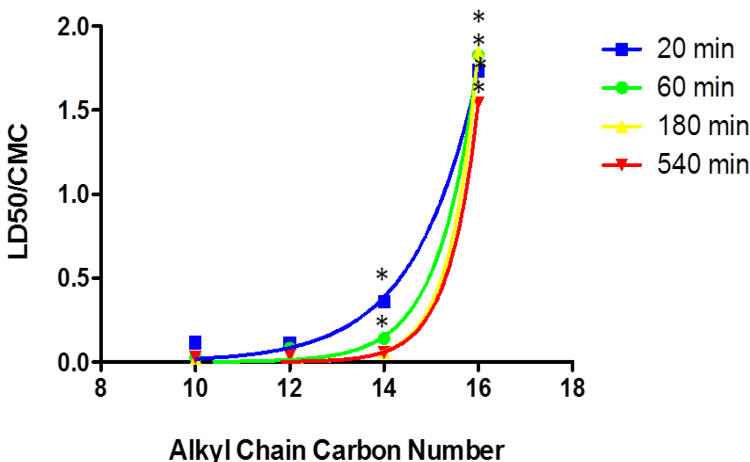


Figure 3.5. Effect of C_nTAB compounds hydrophobic chain length on sperm cell viability. The graph show the LD_{50} concentration of the surfactants $C_{10\text{-}16}TAB$ after exposure times of 20, 60, 180 and 540 minutes. LD50 concentrations of each surfactant are normalized with respect to the CMC. Data are presented as Mean ± SD of 5 independent experiments. Asterisks denote statistically significant differences between experimental groups ($P<0.05$).

The effect of the polar head group of the cationic surfactants was also evaluated by comparing the effects of three surfactants with a 12 carbon n-alkyl chain: $C_{12}TAB$, $C_{12}BZK$ and $C_{12}PYR$. Data revealed that $C_{12}BZK$ and $C_{12}PYR$ are between 10 to 15 times more toxic (using the CMC-normalized concentration scale) than $C_{12}TAB$ (Figure 3.6.). The reason for this difference is unclear but could be related to the larger polar head groups ($C_{12}BZK$ and $C_{12}PYR$ have benzene and pyridine rings, respectively) or to the more delocalized positive charge ($C_{12}PYR$) and may merit further investigation.

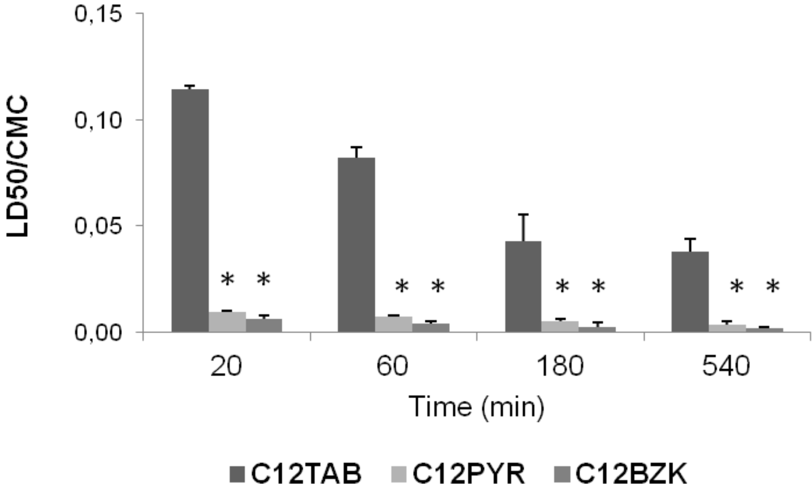


Figure 3.6. Effect of the polar head structure of cationic compounds on sperm cell viability. The LD50 concentration of the three cationic surfactants with similar hydrophobic chain length, but different polar head groups, was evaluated after exposure times of 20, 60, 180 and 540 minutes. LD50 concentrations of each surfactant are normalized with respect to the CMC. Data are presented as Mean ± SD of 5 independent experiments. Asterisks denote statistically significant differences between experimental groups ($P<0.05$).

3.3. Discussion

Given the imperative demand of a new spermicidal and/or microbicidal compound, its evaluation should begin with *in vitro* screening before using animal models and human studies. Surfactants, if safe and effective, would be ideal candidates since they fulfill most of the stipulated criteria to be used on all the regions of the world (e.g. affordable, stable, easiness of storage).

In this work we have screened the *in vitro* effects of concentration, exposure-time and surfactant structure on the viability and motility of normozoospermic sperm samples.

The results reported here indicate that all surfactants used in this study revealed concentration and time-dependent toxic effects at both parameters tested (viability and motility) but exhibited different degrees of toxicity depending on the chemical nature of their polar head, which is in agreement with previous *in vitro* (Krebs et al., 2000) and *in vivo* studies (Cone et al., 2006), done in other cell types as well. For Triton X-100, DDPS and SDS (non-ionic, zwitterionic and anionic surfactants, respectively), cytotoxicity was not observed up to concentrations close to the Critical Micelle Concentration (CMC), whereas the toxicity of cationic surfactants occurred at concentrations much lower than the surfactant CMC, with the exception for C_{16}TAB. However, the reasons for the different C_{16}TAB response in relation to the other cationic surfactants would be speculative without precise information concerning, for example, different affinities of the surfactant for the different, possibly multiple, sites of their action. The interpretation of this data can be that TX-100, DDPS and SDS act mainly at the level of the plasma membrane of the cells probably by causing structural changes, or even its solubilization, as expected at concentrations close to the surfactant CMC (Aránzazu Partearroyo et al., 1990). On the other hand, the cationic surfactants are probably being toxic at a more subtle level, because their toxicity acts at concentrations that are not sufficient to cause significant damage to the membrane integrity. These effects could even be at the intracellular level, conditioned by membrane partitioning (Abreu et al., 2004; Sampaio et al., 2005) and/or translocation (Moreno et al., 2006) across the membranes.

The distinct action of cationic surfactants C_nTAB, C_{12}BZK and C_{12}PYR relative to the rest of the compounds is also observed with the HOST

experiments. Indeed, the first group of compounds showed to impair predominantly the physiological rather than the structural sperm membrane integrity, which suggests that before killing the sperm cell they may be affecting important physiological events occurring in the sperm plasma membrane. Results shown with TX-100, DDPS and SDS indicate that a 50% disruption of structural (LD_{50}; 50% PI-stained) membrane integrity is accompanied by a similar % of physiological integrity membrane disruption, probably meaning that they act exclusively by the classic "detergent" mechanism of action, disrupting the sperm plasma membrane structure. This kind of observation was also found in other works, where surfactant and non surfactant-based compounds were tested and similar results to ours were also achieved (Jain et al., 2007).

For the homologous series of cationic surfactants examined, the results show that the toxicity to sperm cells was linearly dependent upon surfactant hydrophobic chain length ($C_{10}TAB \geq C_{12}TAB > C_{14}TAB > C_{16}TAB$). The evaluation of the effect of the polar head group of the cationic surfactants showed that $C_{12}BZK$ and $C_{12}PYR$, which have the larger polar head groups and more delocalized charge, were the more toxic surfactants, being 10 to 15 times more toxic than $C_{12}TAB$. Delocalized charge on the surfactant head group makes its ionic radius considerably larger and reduces the work required for translocation of the polar group from one side of the membrane to the other (Honig et al., 1986; Gennis, 1989).

A good spermicide should not harm the vaginal epithelia. Despite the innumerous reports about the possible use of surfactants as spermicides, their mechanism of action still has to be further investigated. Indeed, efforts to use surfactants as both contraceptives and microbicides relied upon their capacity

to destroy viral, bacterial and sperm membranes but did not seem to take into account that if they destroyed those membranes they would also destroy the membranes of cells of the vaginal epithelium. However, our results indicate that destruction of cell membranes is not the only mechanism of surfactant toxicity, as is evidenced in the case of cationic surfactants. As argued above, their toxic effects probably do not involve merely the disassembly of the cell membrane but maybe some more delicate effects (such as toxic effects on the cell cytoplasm). Candidate mechanisms that have been proposed in the literature include modulation of membrane curvature elastic stress and consequent reduction of membrane bound protein activity (Dymond and Attard, 2008), alteration of the electrostatic surface potential of membranes (Vieira and Carmona-Ribeiro, 2006), or interaction with anionic polymers (DNA and RNA) in the cytoplasm or cell nucleus following translocation across the cell plasma membrane (Patrzykat et al., 2002). Cationic surfactants are known to bind strongly to DNA and RNA (Spink and Chaires, 1997; Zhu and Evans, 2006) and induce drastic conformational changes in the structure of these polymers (Dias et al., 2005; Dietrich et al., 2007).

As mentioned previously, the efficacy of the spermicidal activity depends on the balance between the concentration required for spermicidal function and the concentration that causes damage to the vaginal epithelium, and this can be measured by a therapeutic index (defined as the ratio of the LD_{50} value for vaginal cells to the LD_{50} value for sperm cells, both treated with the same surfactant for the same exposure time) (Burns, 1999; Finkel et al., 2009). Taking this into account, data obtained from collaboration with a laboratory that measured the LD_{50} in mammalian epithelial cells after surfactants exposure

(Caco-2, mimicking the vaginal epithelia), showed very interesting results (Inácio et al., 2011). Indeed, it was observed that, of all the surfactants tested, only the cationic surfactants (namely, C_{12}BZK and C_{12}PYR) presented more toxicity to sperm than they do to other cells typically found in the vagina (all the other surfactants were as toxic to sperm as to the polarized epithelial cells). But even in these two cases their narrow therapeutic indices imply that their use as contraceptive agents should be carefully controlled. This result was somehow unexpected for C_{12}BZK which is commonly used, and at surprisingly high concentrations, as a contraceptive in contraceptive sponges (Ayotte and Colin, 2002) and condoms (Hitchcock, 2000). Our results show that the *in vitro* LD_{50} for the surfactants examined here are some orders of magnitude lower than the ‘‘contraceptive’’ doses used in these devices, calling into question if many if not most of the contraceptive sponges and spermicide gels on the market today should be licensed for use.

In conclusion, the systematic study of structure-toxicity relationship in *in vitro* models is of extreme importance in the understanding of the mechanisms underlying surfactant use as contraceptive agents. The detailed approach we have used in this work should be a mandatory first-line screening of possible spermicide candidates. These results attempt to contribute to the understanding of the mechanisms involved in surfactant toxicity and can be used to make predictions about the safety of these molecules, which would be helpful in the design of new, more effective and less harmful surfactants for use as vaginal gels. Our results clearly show that among the surfactants tested, cationic surfactants may be the only ones acting in a different way from the “classic”

surfactants, and this should be considered in future surfactant design, for this purpose.

Chapter IV. *In vitro* effects of cationic compounds on functional human sperm parameters

This data was included in the following paper:

Baptista M, Publicover SJ and Ramalho-Santos J (2013) *In vitro* effects of cationic compounds on functional human sperm parameters. Fertil Steril 99 (3): 705-12. doi: 10.1016/j.fertnstert.2012.11.008.

Synopsis

4.1. Introduction

4.2. Results

- 4.2.1. Effect of cationic surfactants on sperm mitochondrial membrane potential
- 4.2.2. Effect of cationic surfactants on sperm capacitation
- 4.2.3. Effect of cationic surfactants on sperm acrosomal status
- 4.2.4. Effect of cationic surfactants on sperm intracellular calcium movements

4.3. Discussion

4.1. Introduction

In order for mammalian sperm to fertilize an oocyte they must undergo a series of complex molecular signal transduction pathways, generally called capacitation. This process involves numerous aspects, starting with sperm cholesterol efflux, changes in plasma membrane permeability/fluidity, calcium influx, activation of protein kinases with subsequent phosphorylation of tyrosine residues, and resulting in exocytosis of acrosomal contents (acrosome reaction) near the oocyte, leading to sperm zona pellucida (ZP) penetration, and fusion with the oocyte plasma membrane (Abou-haila and Tulsiani, 2009; Zaneveld et al., 1991). Therefore, when one thinks of impairing sperm function to prevent undesired pregnancies, all the steps noted before can be thought of as useful targets for a potential contraceptive. Even if the promising compound does not kill sperm, it can render the male gamete enable to fertilize the oocyte, which is also valid for an efficient spermicide.

In this part of the study we have attempted to use an *in vitro* approach in order to dissect the way different spermicidal candidates affect important human sperm functional parameters, with the ultimate goal of determining more efficient methodologies for compound evaluation. The chosen compounds were representatives of the cationic surfactants only, once they were the more efficient surfactants regarding not only the spermicidal activity, but also their safety towards other cells (Inácio et al., 2011). Also, for comparison purposes we chose molecules with the same hydrophobic chain but different polar heads.

Thus, we attempted to determine exactly how benzalkonium bromide (C_{12}BZK), dodecylpyridinium bromide (C_{12}PYR) and dodecyltrimethylammonium

bromide ($C_{12}TAB$), influence sperm function, by monitoring the mitochondrial status, capacitation, acrosomal status, and, for the first time in this type of study, intracellular Ca^{2+} levels. Indeed, we suggest that calcium movements may constitute a previously unrecognized sensitive parameter to determine the putative effect of novel molecules thought to have spermicidal properties.

4.2. Results

4.2.1. Effect of cationic surfactants on sperm mitochondrial membrane potential

Mitochondrial transmembrane potential maintains the integrity of mitochondrial polarization for normal energy generation and dissipation (Bains et al., 2006), and a significant drop in this potential may indicate initiation of apoptotic process (Chaoui et al., 2006). Therefore, in order to better understand the effects of cationic surfactants on mitochondria and its function, we assessed the mitochondrial membrane potential which is essential for sperm function.

Having previously determined the LD_{10} and LD_{50} of the three compounds, we asked under what conditions might mitochondrial membrane potential (MMP) be affected, given that this parameter is also a sensitive indication of sperm function (Sousa et al., 2011). Using the fluorescent mitochondrial-specific and MMP-dependent probe JC-1, several different sperm labeling patterns were considered: orange (almost always accompanied with green – high MMP); green only; and unstained (no MMP). The first two populations contain all sperm with “positive” MMP (Figure 4.1.).

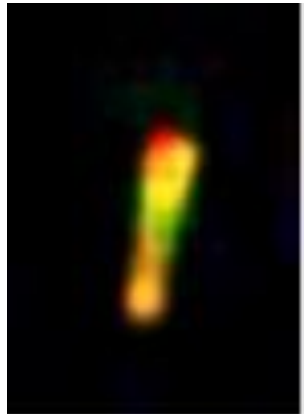
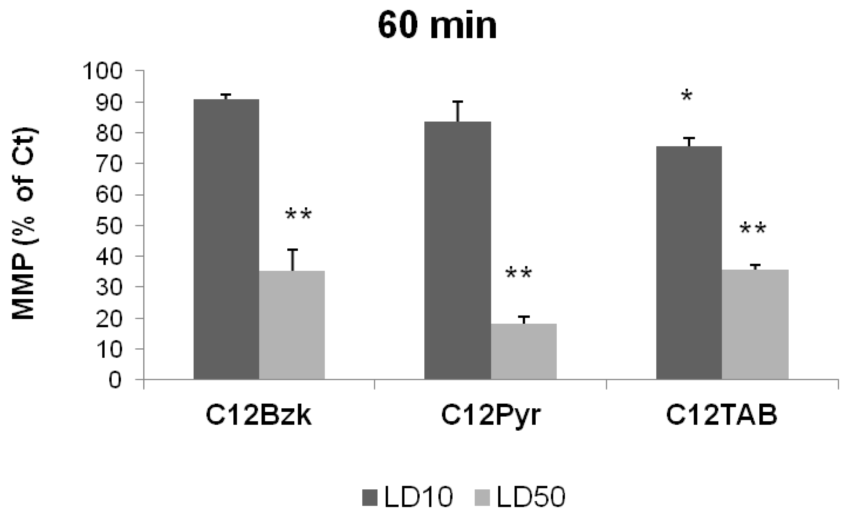


Figure 4.1. Representative image of the sperm MMP assay. Mitochondrial membrane potential monitored with the JC-1 probe, showing a midpiece stained orange (accompanied with green), indicating high MMP. Magnification is x1000.

For all compounds results were magnified with incubation times (60 and 180 minutes). There were significant effects for both LD_{10} and LD_{50} at 180 minutes, although at 60 minutes LD_{50} was universally effective, whereas LD_{10} showed a slight effect only in the case of C_{12}TAB (Figure 4.2.). A significant loss of MMP with all compounds, even at the lower concentration, suggests important mitochondrial impairment.



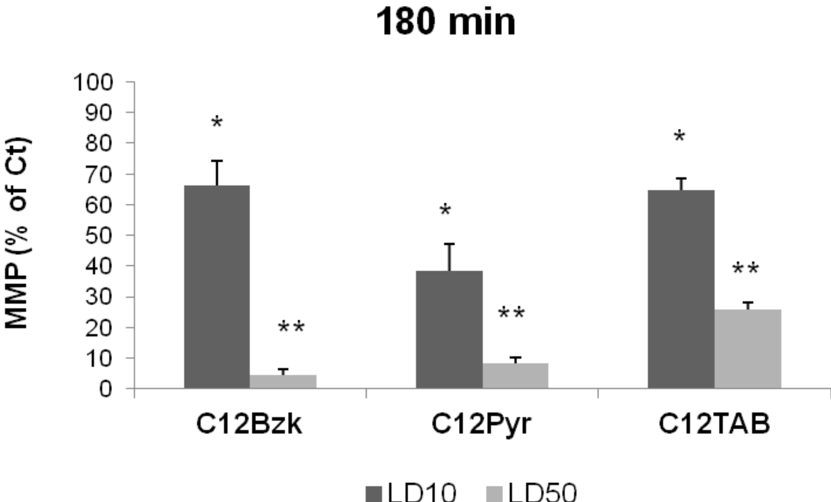


Figure 4.2. Effect of different cationic compounds on sperm mitochondrial membrane potential. Three compounds were evaluated (C_{12}BZK, C_{12}PYR and C_{12}TAB). *In vitro* sperm MMP was assessed by the JC-1 assay 60 and 180 minutes after the cells had been exposed to different concentrations of surfactants (LD_{10} and LD_{50}). Sperm MMP is expressed as percentage in relation to the control. Data are presented as Mean ± SEM of 5 independent experiments for each compound. Asterisks denote statistically significant differences from control (*P<0.05 and **P<0.01).

4.2.2. Effect of cationic surfactants on sperm capacitation

To determine if the cationic compounds could lead to any functional impairment potentially preventing sperm from becoming competent, we tested their effect on capacitation. The immunocytochemistry (ICC) performed for the detection of phosphorylated protein tyrosine residues was used as an indicator of sperm capacitation. The sperm labeling pattern was mostly seen in the tail (Figure 4.3.).

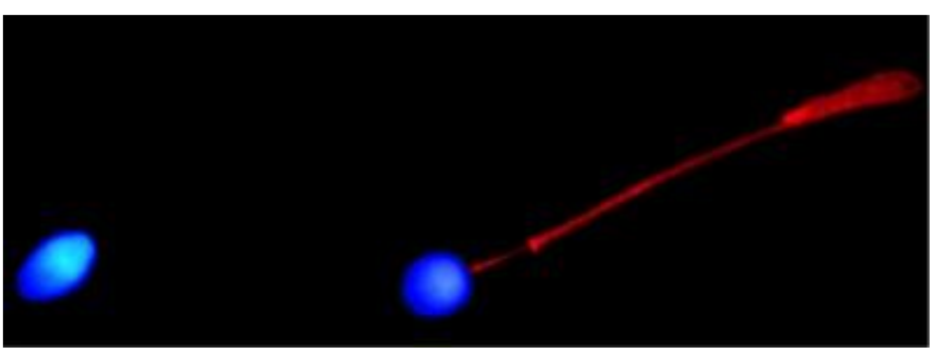
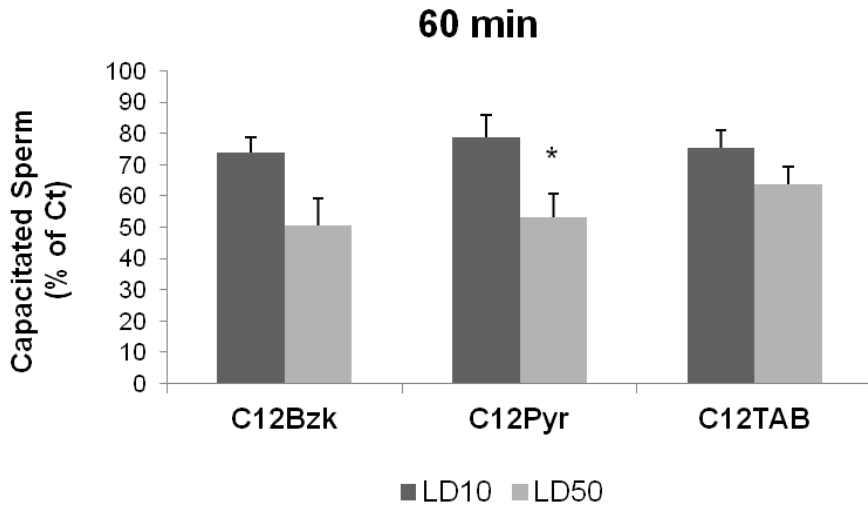


Figure 4.3. Representative image of the capacitaton assay. Capacitation monitored by the detection of phosphotyrosines (red) in capacitated (right) but not uncapacitated (left) sperm. DAPI (blue) was used as a nuclear stain. Magnification is x1000.

The results obtained showed a strong inhibition of capacitation with C_{12}BZK after exposure for 180 minutes at the higher dose, LD_{50} (46.04 ± 8.32, $p<0.05$; Figure 4.4.). At the same dose, C_{12}PYR also diminished tyrosine phosphorylation after 60 minutes of incubation, which was further enhanced after 180 minutes (53.34 ± 7.30 and 36.32 ± 9.33, respectively; $p<0.05$; Figure 4.4.). No statistically significant differences were found at the lower dose, LD_{10}, for both compounds. The compound C_{12}TAB was the only one that did not have any significantly effect on sperm capacitation at the concentrations tested ($p>0.05$; Figure 4.4.).



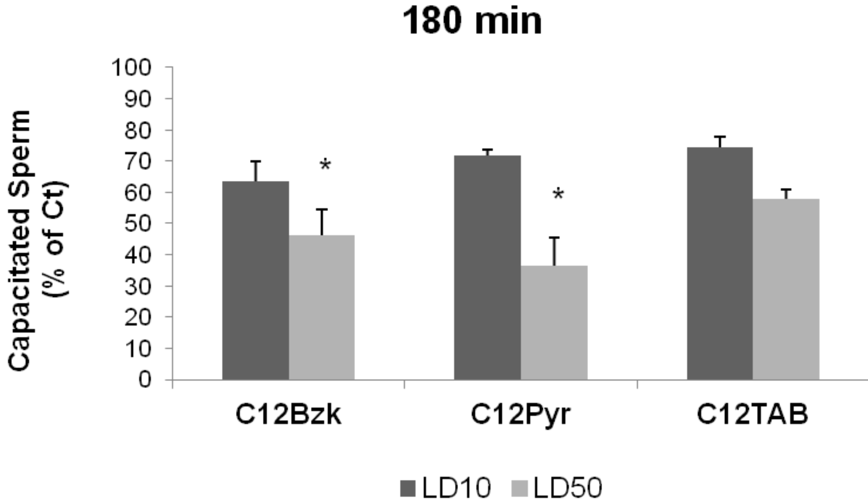


Figure 4.4. Effect of different cationic compounds on sperm capacitation (protein tyrosine phosphorylation). Three compounds were evaluated (C_{12}BZK, C_{12}PYR and C_{12}TAB). *In vitro* sperm capacitation was assessed 60 and 180 minutes after the cells had been exposed to different concentrations of surfactants (LD_{10} and LD_{50}). Capacitated sperm is expressed as percentage in relation to the control. Data are presented as Mean ± SEM of 5 independent experiments for each compound. Asterisks denote statistically significant differences from control ($P<0.05$).

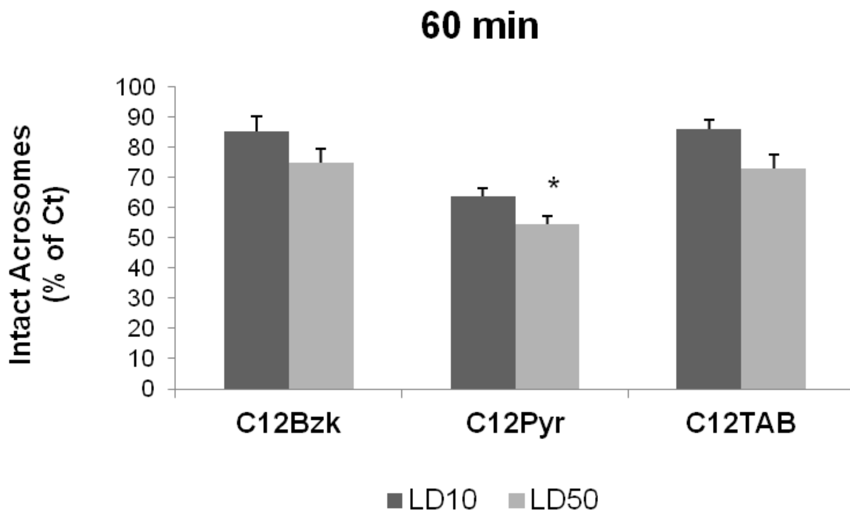
4.2.3. Effect of cationic surfactants on sperm acrosomal status

The acrosome is a membranar structure essential for fertilisation. Indeed, when the spermatozoon with a damaged acrosome reaches the oocyte, it will not be able to penetrate through the zona pellucida, since the enzymes inside the vesicle were lost long ago. Therefore, since this parameter reflects the ability of sperm to fertilize an oocyte, the presence or absence of the acrosomal matrix was assessed with the fluorescent acrosome content marker FITC-PSA, after sperm plasma membrane permeabilization (Figure 4.5.).

Figure 4.5. Representative image of the acrosome integrity assay. Acrosomal status monitored with FITC-PSA (green). Intact acrosomes are shown as a green cap, which is lacking in sperm that have lost their acrosome (middle cell). DAPI (blue) was used as a nuclear stain. Magnification is x1000.

We could see that C_{12}BZK did not have any effect on sperm acrosomal status ($p>0.05$; Figure 4.6.). However, a robust reduction in the percentage of intact acrosomes occurred with C_{12}PYR for both times (60' and 180') with LD_{50} (54.31 ± 2.97 and 39.87 ± 4.70, respectively; $p<0.05$; Figure 4.6.). Moreover, at LD_{50}, C_{12}TAB also led to a significant spontaneous acrosomal loss after 180 minutes of exposure (61.23 ± 1.66, $p<0.05$; Figure 4.6.), suggesting that both

C_{12}TAB and C_{12}PYR can cause a substantial impairment in sperm acrosome homeostasis.



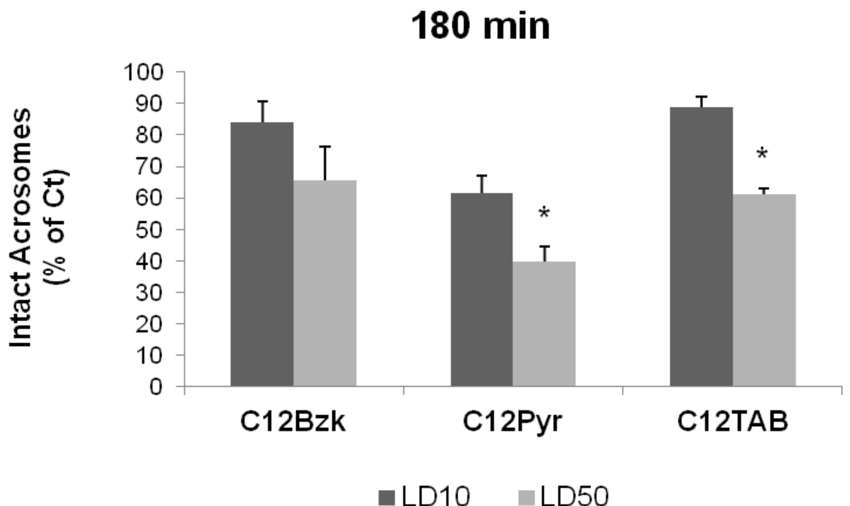


Figure 4.6. Effect of different cationic compounds on the percentage of intact acrosomes. Three compounds were evaluated (C_{12}BZK, C_{12}PYR and C_{12}TAB). *In vitro* sperm acrosome status was assessed 60 and 180 minutes after the cells had been exposed to different concentrations of surfactants (LD_{10} and LD_{50}). Acrosome-intact sperm is expressed as percentage in relation to the control. Data are presented as Mean ± SEM of 5 independent experiments for each compound. Asterisks denote statistically significant differences from control ($P<0.05$).

4.2.4. Effect of cationic surfactants on sperm intracellular calcium movements

Because sperm homeostasis is altered under certain conditions in terms of both capacitation and acrosome reaction, and given that both these events involve calcium influx and calcium-dependent signaling, we attempted to determine if the compounds tested could affect this parameter, especially at the more effective concentrations of LD_{50}. To assess calcium intracellular fluxes a dynamic analysis of live single cell imaging was performed, using the fluorescent probe Oregon Green Bapta (OGB) (Figure 4.7.).

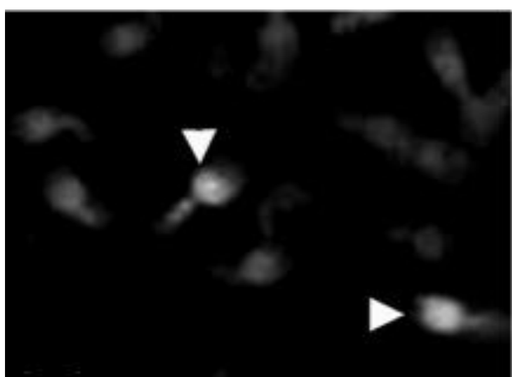


Figure 4.7. Representative image of the single sperm cell imaging assay. Intracellular calcium monitored with the use of OGB. Sperm with increased calcium concentration stain brighter than the background fluorescence signal (arrowheads). Magnification is x400.

Indeed, the results have shown that C_{12}BZK significantly increased sperm intracellular calcium concentration $[Ca^{2+}]_i$ in 20.2% of cells (Figure 4.8.). However, C_{12}PYR and C_{12}TAB induced a higher increase of $[Ca^{2+}]_i$ in a substantial proportion of sperm cells analysed, (81.7% for C_{12}PYR and 67.9% for C_{12}TAB; Figure 4.8.). These results suggest that all of the compounds may

modulate sperm calcium signaling, especially C_{12}PYR and C_{12}TAB. It should be noted that the exposure to compounds in this experiment was much shorter than what was noted in earlier assays, and that the effect was sustained as long as compounds were present.

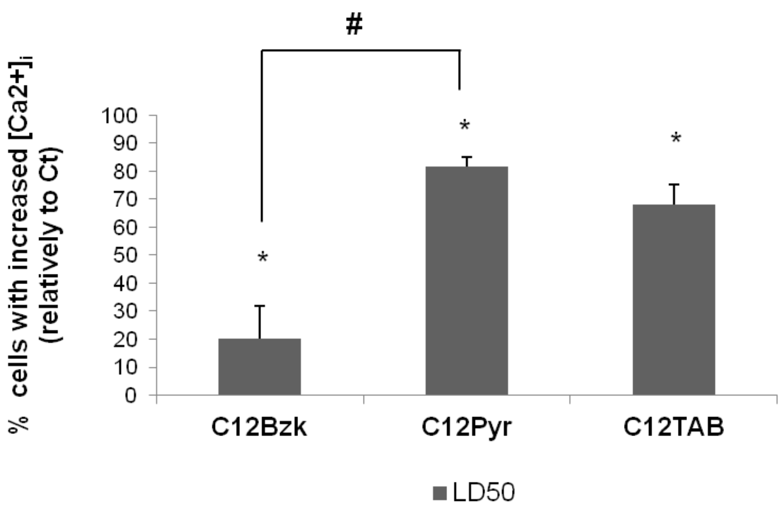


Figure 4.8. Effect of different cationic compounds on intracellular sperm calcium concentration. Three compounds were evaluated (C_{12}BZK, C_{12}PYR and C_{12}TAB). Sperm $[Ca^{2+}]_i$ was assessed after the cells had been exposed to different concentrations of surfactants (LD_{10} and LD_{50}). Data are presented as Mean ± SEM of the percentages of increased $[Ca^{2+}]_i$ sperm. At least 5 independent experiments for each compound was performed. Asterisks denote statistically significant differences from control (P<0.05); and the cardinal sign denotes difference between experimental groups (P<0.05).

4.3. Discussion

In evaluating a putative contraceptive agent acting as a spermicide, the first requirement is that the compound act rapidly and efficiently to kill or immobilize sperm on contact or to render the male gamete incapable of fertilisation by affecting crucial sperm functional parameters (Lee, 1996).

Therefore, many other sperm parameters, besides viability, can cause a complete blockage of fertilisation and, ultimately, prevent unwanted pregnancies. In an attempt to provide more contraceptive choices to the most STD-vulnerable sex (Saracco et al., 1993; Wilkinson et al., 1999), i.e., women from undeveloped regions that do not have the economic power to negotiate their sexual health (Moschicki, 2008), we evaluated three compounds of interest. The choice of cationic surfactants as promising topical women-controlled spermicides was based on collaboration studies that proposed cationic compounds as vaginal disinfectants and suggested their possible use without harming the vaginal epithelia. We used the three compounds with the same hydrophobic side chain in order to control that aspect of membrane interaction and then we attempted to determine the toxic effects on crucial sperm parameters.

As described previously, if these compounds suggest that they are not merely acting as typical detergents, what could their targets be? One important aspect in sperm function is mitochondrial status. All compounds were able to inflict a functional change in this parameter, especially C_{12}TAB, which caused mitochondrial impairments at both LD_{10} and LD_{50} at shorter (60 minutes) and longer (180 minutes) times of exposure. These results are relevant, because the correlation between poor sperm mitochondrial function and diminished fertilisation ability is well known (Sousa et al., 2011; Nishimura et al., 2006). The compounds studied were able to depolarize sperm mitochondria, which indicates that they may interfere with sperm energy metabolism and/or other pathways that involve this organelle, such as apoptosis-related processes

(Chaoui et al., 2006). Other studies that evaluate candidates for contraceptive agents have also shown changes in MMP (Jain et al., 2007; Chen et al., 2009).

Before becoming fully functional, sperm must undergo several maturation steps involving membrane rearrangements and cell signalling, collectively termed "capacitation" (Salicioni et al., 2007). Capacitation is later followed by the acrosome reaction, an exocytotic event involving the release of enzymatic contents that digest the zona pellucida (Ramalho-Santos et al., 2002). Therefore, inhibiting either capacitation or the acrosome reaction will render the sperm incapable of fertilising the oocyte, even if alive and motile. Indeed other authors have previously proposed capacitation and the acrosome reaction as appropriate targets for novel contraceptives (Zaneveld et al., 2002; Gupta et al., 2005). In the present assays, C_{12}PYR was the stronger compound: it significantly inhibited capacitation and resulted in more sperm with compromised acrosome integrity at LD_{50} (at 60 and 180 minutes). On the other hand, the other compounds affected only one of these two parameters, causing the sperm population to be either less capacitated or with reduced intact acrosomes (C_{12}BZK and C_{12}TAB, respectively; both at LD_{50} after 180 minutes).

Intracellular Ca^{2+} modulates both capacitation and the acrosome reaction, and the first step in the acrosome reaction is a huge influx of calcium ions (Publicover et al., 2007). All the compounds triggered significant $[Ca^{2+}]_i$ increases in a large number of sperm cells, suggesting that they act by modulating sperm Ca^{2+} signaling. This $[Ca^{2+}]_i$ increase was detected for all compounds at much lower exposure times, and sperm ability to control $[Ca^{2+}]$ seems therefore impaired by the compounds, possibly explaining downstream effects on capacitation and acrosomal status. Importantly, the experiments

seem to reveal that sperm with a lower percentage of intact acrosomes also had a higher number of cells with an increase in $[Ca^{2+}]_i$, thus suggesting a direct link between these parameters, and indicating that an induced calcium increase may be causing the premature loss of sperm acrosomes, rendering them non-functional. Furthermore, calcium imaging seemed to be particularly sensitive compared with other assays in detecting changes to sperm function.

Finally, as we previously stressed out, the compounds tested here are more toxic to sperm cells that they are to Caco-2 cells (fully polarized columnar epithelial human cells), which are used to mimic the vaginal epithelia (Inácio et al., 2011). This feature should be considered in future surfactant design, because it can selectively kill sperm cells without harming the vaginal/penile epithelial cells, and, as we show here, without necessarily acting as a classic surfactant/detergent.

These results suggest that the commercially available cationic surfactants tested are able to impair sperm at functional and physiologic levels, although C_{12}PYR was shown to be more effective as a putative spermicide than C_{12}BZK or C_{12}TAB, at least when considering nontypical spermicidal events (inhibition of capacitation and modulation of acrosomal status and calcium signaling). To our knowledge, this is the first study reporting the result of possible spermicides on sperm calcium signaling, which seems a promising new tool in these studies, given its sensitivity.

Although further studies using sperm samples from donors with proven fertility will be important to confirm these findings, the validation of this kind of comprehensive *in vitro* study for the evaluation of new promising compounds is very important, because these techniques are cheaper, quicker and safer than

those usually used to test the toxicity of a substance *in vivo*. This information may be helpful in the design of more effective and less harmful surfactants that could be used in vaginal gels.

Chapter V. General Discussion

The need for women-controlled devices to prevent unwanted pregnancies has been an issue of great concern among the scientific community (Lech, 2002). The lack of contraception affects mainly the less developed regions, where, besides the difficult accessibility to get the contraceptive, women (the most affected gender) also do not have the social power to have their contraceptive choices and, consequently, to protect their health, subjecting themselves to the will of sexual partners.

Taking this into account, the research of a compound that could ally the contraceptive effect to the possibility of being women-controlled (topically applied), easy to use and to store (high stability) and cheap, drew attention to a class of compounds, surfactants, where all those attractive characteristics are present. Candidate surfactants, such as Nonoxynol-9 and C31G (surfactants that combine a microbicide effect along with the anticonceptional effect) were tested in clinical trials. However, they failed to pass since they were found to disrupt the membrane of sperm cells/bacteria/virus in the same level they destroyed the membrane of the vaginal epithelia, rendering them unsuitable for human use due to their cytotoxicity (Fichorova et al., 2001; Feldblum et al., 2008). Still, some surfactants such as Benzalkonium, are currently available in the market as contraceptives, even though they can have dubious safety issues towards the vaginal/penile epithelia.

After these drawbacks, why should surfactants still trigger interest concerning their potential use as topical contraceptives? First of all, it should be highlighted that the pre-clinical and even clinical human trials involved in the evaluation of candidate surfactants did not take into consideration the chemical properties of the compounds, such as that the surfactants form micelles

(establishing an equilibrium with the membranes) when they reach the surfactant concentration CMC. In Phase III trials, Nonoxynol-9 (CMC = 0.05 mg/mL) was tested at concentrations between 5 and 100 mg/mL (Wilkinson et al., 2002) and the surfactant mixture C31G (CMC = 0.005 mg/mL) was used at concentrations between 0.005 and 0.02 mg/mL (Peterson et al., 2007; Feldblum et al., 2008), which are higher concentrations than the CMC, where membrane disruption is achieved (unspecific mechanism of action). The results obtained in those pre-clinical and clinical trials clearly showed that the causes of the failure were related to their lack of selectivity, leading to the impairment of the vaginal epithelia at the same time they damaged other membranes (sperm/bacteria/virus); (Fichorova et al., 2001; Catalone et al., 2005; Cone et al., 2006).

However, despite all the pessimistic results found in relation to surfactant toxicity, a recent collaboration work evidenced that cationic surfactants could inflict their effects through a mechanism of action different from the one exerted by the other surfactant families (Vieira et al., 2008). The study revealed that cationic surfactants had bactericidal activity against a range of bacterial models at sub-lethal concentrations to epithelial cell lines mimicking the vaginal epithelia (MDCK). Therefore the negative results obtained in advanced clinical trials should not serve to immediately exclude the potential use of these compounds but to develop better *in vitro* models for the assessment of new spermicidal candidates.

In the present work we attempted to create an *in vitro* model useful for the evaluation of the toxicity of candidate compounds that, besides being efficient, more economic and simple, can also be used as the first line of action

before resorting to the more complex and expensive *in vivo* models. In fact, although *in vivo* models and clinical trials offer more complete experimental conditions, the *in vitro* methodologies proposed in this work should be seen as required pre-conditions since they seem to provide crucial clues on the applicability and safety of a variety of compounds, enabling their immediate sorting/screening.

One of the decisive steps in correctly evaluating and comparing the efficacy of different surfactants is to analyse the results taking into account the respective critical micelle concentration (CMC). These are amphiphilic molecules, which mean that the preferential site of interaction with cells will be the phospholipidic cell membranes. This interaction is related to their partition coefficient between the aqueous and membrane phases which, in its turn, is related to the surfactant concentration CMC. Taking this into account, it was possible to identify a group of cationic surfactants with potential to be used as safe spermicides, and perhaps as dual-function compounds, having effects at a microbicidal level.

C_nTAB surfactants with shorter hydrophobic chains (10 and 12 carbons) seemed to be the most efficient ones, with higher spermicidal activity always dose- and time-dependent. $C_{16}TAB$ was the only exception of this group, exerting spermicidal effects only above its CMC concentration. Because of that, this compound should not be taken into account in advanced studies. The efficiency of C_nTABs is highly improved by modifying their polar head structure to larger polar heads, as it was observed in the particular case of $C_{12}BZK$ and $C_{12}PYR$, in relation to $C_{12}TAB$.

These results should be seen as a guideline since they provide insights on the most efficient structures that can be used for the design of new molecules, which in this case seem to be compounds with short alkyl-chains and a large polar head group with delocalized positive charge.

It also should be noted that this work was done in collaboration with another laboratory, so the same compounds have been tested in other cell types. And this is important, since the efficacy of the spermicidal activity will always depend on the balance between the concentration required for harming sperm and the concentration that causes damage to the vaginal epithelium. Indeed, positive results were also found with cationic surfactants, since they had efficient and selective action against different cell types (Inácio et al., 2011). It was observed that surfactants were slightly more toxic to sperm cells than to MDCK and Caco-2 cells (polarized epithelial cells). This difference may be due to the different aspects of the cell membranes: epithelial cells seem to have a more ordered and thicker plasma membrane than sperm cells, also related to the extensive microvillation that they present (Butor and Davoust, 1992). Surfactant toxicity is dependent on the ability to partition between the aqueous phase and the cell membrane and may also depend upon their ability to subsequently cross the membrane and enter the cytoplasm. The fact that amphiphile partition coefficients as well as the rate constants for their insertion into membranes and translocation across them are lower for more ordered membranes (Abreu et al., 2004; Sampaio et al., 2005; Moreno et al., 2006; Estronca et al., 2007) explain why the thinner and more accessible sperm plasma membrane may be more susceptible to surfactant toxicity than fully polarized and confluent epithelial cells such as MDCK and Caco-2 cell lines.

It should also be highlighted that the same collaboration found that, with the exception of $C_{16}TAB$, all cationic surfactants were able to have an antimicrobial activity (*E. coli*) at sub-lethal concentrations to mammalian epithelial polarized cells. This is of extreme importance, given that the development of a dual-function compound (spermicide and microbicide, safe to the reproductive tract epithelium) would answer some of the issues related to unwanted pregnancies and STDs.

The finer aspects of spermicidal action were studied on cationic compounds $C_{12}TAB$, $C_{12}BZK$ and $C_{12}PYR$, regarding their spermicidal action at concentrations far below the CMC. Therefore, as they were not acting as typical detergents at that concentration it was necessary to understand which pathways they could be targeting. In fact, other studies propose that surfactant concentrations close to CMC can cause acute toxicity and necrosis due to the general disruption of the cell plasma membrane, while lower concentrations induce a more controlled apoptotic cell death (Perani et al., 2001; Enomoto et al., 2007). Our insight to explain the results in this work are: 1) the LIVE/DEAD viability test, which evaluates the sperm plasma membrane integrity, is an indicator of irreversible cell death either due to cell membrane damage directly caused by the surfactants or due to loss of plasma membrane integrity posterior to cell death due to reasons that have nothing to do with direct membrane damage by the surfactants; 2) at concentrations above the CMC, the compounds act as classic surfactants, forming “holes” in the plasma membrane, detected by the viability test; 3) for concentrations below the CMC, the observed sperm cell death (in this case, only caused by the cationic surfactants) suggests an independent effect from membrane permeabilization. As our results indicate,

a specific targeting of sperm membrane physiology without affecting the structure, a depolarization of the MMP (that may interfere with apoptosis-related processes), a strong inhibition of capacitation, and a rapid and huge increase in intracellular [Ca^{2+}], which triggers acrosome reaction (loss of the acrosome), and consequently, the disruption of sperm plasma membrane, immediately detected by the viability assay, was observed (Figure 5.1.).

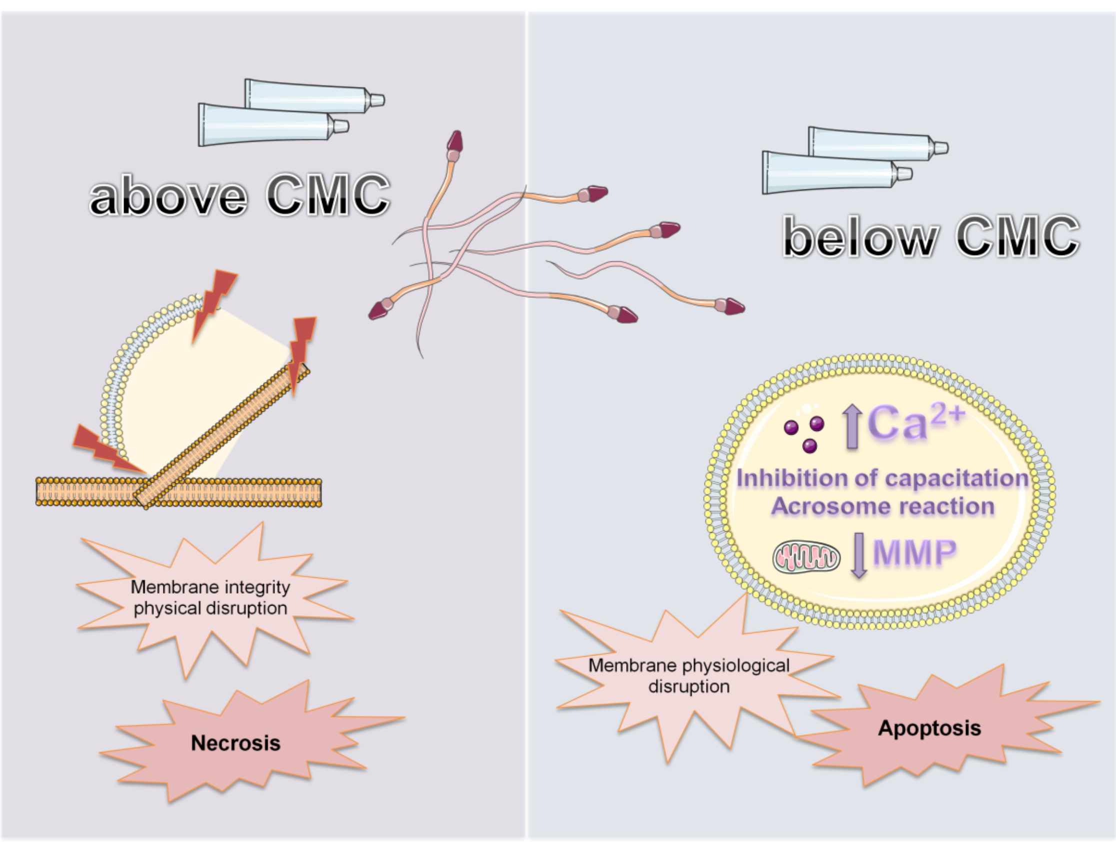


Figure 5.1. Scheme illustrating the different mechanisms of cell dead performed by surfactants on sperm cells. At concentrations above the CMC, the surfactants act as classic surfactants, disrupting the plasma membrane, causing necrosis. For concentrations below the CMC, the observed sperm cell death (in this case, performed by the cationic surfactants) suggests an independent effect from the exclusive membrane permeabilization.

To note that other aspects of spermicidal activity should be addressed in the future for further validation of these results, such as the evaluation of the sperm plasma and acrosomal membrane surface by electron microscopy; the performance of flow citometry with annexin-V to clarify the exact mechanism by which surfactants are leading to sperm cell death; the evaluation of oxidative stress by measuring ROS production (with the DCFDA fluorescent probe) and SOD activity (SOD assay kit); the detection of phosphotyrosines by western blotting, to reinforce the results obtained by fluorescence microscopy.

In summary, this work aimed to provide some insights about the mechanisms of action that mediate surfactant toxic effects in sperm cells. A lot has still to be done, however this work will help to answer some questions related to the *in vitro* surfactant structure-toxicity relationships. It was also our intention to find more efficient *in vitro* methodologies in order to evaluate promising effective and targeted-based spermicides, and alert to the importance of these methodologies. Easily systematized *in vitro* methodologies should be seen as a mandatory first-line procedure before more advanced tests, since they seem to offer vital clues on the applicability and safety of a variety of compounds. Thus, it would be possible to perform sorting/screening of a large variety of molecules to decide which ones should continue to more complex, lengthy, stringent, ethically challenging and expensive experimental models.

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