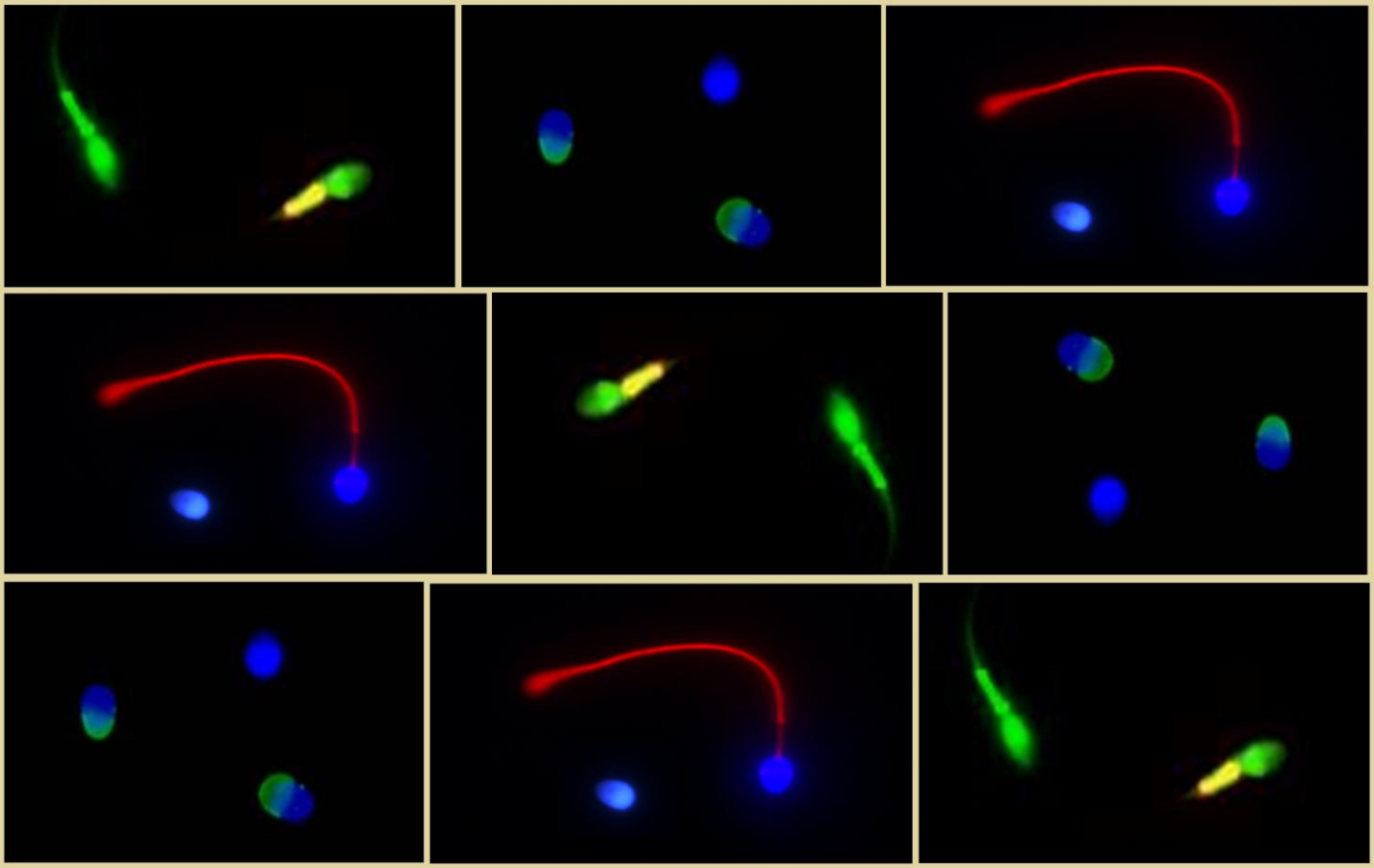

Renata Santos Tavares

A TWO-SIDED TALE: THE INFLUENCES OF ENVIRONMENTAL ENDOCRINE DISRUPTORS AND SPERM CHROMATIN STATUS ON MALE FERTILITY

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 Tecnologia da Universidade de Coimbra, e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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A TWO-SIDED TALE: THE INFLUENCES OF ENVIRONMENTAL ENDOCRINE DISRUPTORS AND SPERM CHROMATIN STATUS ON MALE FERTILITY

Renata Santos Tavares

Dissertation presented to the Faculty of Sciences and Technology of the University of Coimbra as a requirement for the degree of Doctor of Philosophy in Biology (specialty Cell Biology), under the scientific supervision of Professor João Ramalho-Santos, Associate Professor in the Department of Life Sciences of the University of Coimbra and Principal Investigator in the Center for Neurosciences and Cell Biology.

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra com vista à obtenção do grau de Doutor em Biologia (especialidade Biologia Celular) sob orientação científica do Professor Doutor João Ramalho-Santos, Professor Associado no Departamento de Ciências da Vida da Universidade de Coimbra e investigador principal no Centro de Neurociências e Biologia Celular.

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“A scientist in his laboratory is not a mere technician; he is also a child confronting natural phenomena that impress him as though they were fairy tales”

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

Marie Curie

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Table of Contents

List of Abbreviations

AB - Aniline blue

ADP - Adenosine diphosphate

AhR - Aryl hydrocarbon receptor

ANOVA - One-way analysis of variance

AO - Acridine orange

AOT - Acridine orange test

AR - Acrosome reaction

ARec - Androgen receptor

ART - Assisted Reproductive Technology

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

BPA - Bisphenol A

Ca^{2+} - Calcium

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

Catsper - Cationic sperm channel

CMA3 - Chromomycin A3

COCs - Cumulus-oocyte-complexes

COMET - Single cell gel electrophoresis assay

Cs^{+} - Caesium

DAPI - 4,6-diamino-2-phenyl-indole

DDT - Dichlorodiphenyl trichloroethane

DFI - DNA fragmentation index

DGC - Density gradient centrifugation

DMSO - Dimethyl sulphoxide

DNA - Deoxyribonucleic acid

dUTP - Deoxyuridine triphosphate nucleotide

DVF - Divalent-free caesium-based medium

EDs - Endocrine disruptors

EDTA - Ethylenediaminetetraacetic acid

EDTA-Na - Ethylenediaminetetraacetic acid disodium salt

EGTA - Ethylene glycol tetraacetic acid

ER - Estrogen receptor

ETC - Electron transfer chain

FSH - Follicle stimulating hormone

GnRH - Gonadotropin-releasing hormone

hCG - Human chorionic gonadotropin

HCO_3^- - Bicarbonate

HEPES - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

hMG - Human menopausal gonadotropin

HPLC - High-performance liquid chromatography

HS - HEPES-buffered solution

ICSI - Intracytoplasmic sperm injection

IP3 - Inositol 1,4,5-triphosphate

ISNT - In situ nick translation

IUI - Intrauterine insemination

IVF - In Vitro Fertilization

JC-1 - 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide

LH - Luteinizing hormone

Mibefradil - (1S,2S)-2-[2[[3-(2-Benzimidazolyl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride

MMP - Mitochondrial membrane potential

NNC 55-0396 - (1S,2S)-2-(2-(N-[(3-Benzimidazol-2-yl)propyl]-N-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride

OXPHOS - Oxidative phosphorylation

P1 - Type 1 protamine

P2 - Type 2 protamine

P_4 - Progesterone

PBS - Phosphate buffered saline

PCBs - Polychlorinated biphenyls

PCB 47 - 2,2',4,4'-Tetrachlorobiphenyl

PCB 77 - 3,3',4,4'-Tetrachlorobiphenyl

PCB 118 - 2,3',4,4',5-Pentachlorobiphenyl

PCB 126 - 3,3',4,4',5-Pentachlorobiphenyl

PCB 153 - 2,2',4,4',5,5'-Hexachlorobiphenyl

PCDDs - Polychlorinated dibenzo-p-dioxins

PCDFs - Polychlorinated dibenzofurans

PI - Propidium iodide

PN - Pronuclei

p,p' - DDE - p,p'-dichlorodiphenyldichloroethylene

PSA-FITC - *Pisum sativum* agglutinin coupled to fluorescein isothiocyanate

PUFA - Polyunsaturated fatty acids

ROC - Receiver-operating characteristic

ROS - Reactive oxygen species

SCD - Sperm chromatin dispersion test

SCSA - Sperm chromatin structure assay

sEBSS - Supplemented Earle's balanced salt solution

SEM - Standard error of mean

SPM - Sperm preparation medium

TB - Toluidine blue

TCDD - 2,3,7,8-Tetrachlorodibenzo-p-dioxin

TdT - Terminal deoxynucleotidyl transferase

TEF - Toxicity equivalency factor

TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

VOCCs, Ca_V -Voltage-operated Ca^{2+} channels

WHO - World Health Organization

ZP - Zona pellucida

Abstract

Exposure to environmental toxicants, mainly endocrine disruptors, has been associated with a decline in sperm quality paralleled by a trend for increased testicular cancer and anomalies in male reproductive organs, suggesting that these substances play a role in male reproductive dysfunction and infertility. Of the more prevalent pollutants, organochlorine compounds such as the classical dioxin TCDD, PCBs and the major DDT metabolite, p,p'-DDE, are potentially serious hazards to the general human population. Although these substances preferentially accumulate in body fat, important amounts are present at the site of fertilization *in vivo*, representing an important direct route of exposure for spermatozoa that should not be neglected. Moreover, while often relying on the evaluation of standard semen parameters to infer about the potential damaging effects of such compounds in human male fertility, studies may have underestimate their true toxicity, mainly at low physiological concentrations. Thus, the evaluation of more precise sperm functional parameters is mandatory. One of these parameters is sperm chromatin/DNA integrity, crucial for a correct delivery of the paternal genome to the embryo. Despite the multitude of tests available, the lack of valid cut-offs and the involvement of extensive protocols and/or expensive reagents and equipment have hampered the introduction of sperm DNA damage as an additional parameter in routine semen analysis and limited its evaluation in large-scale studies.

In the first part of our work an *in vitro* approach allowed to mimic the putative *in vivo* continuous exposure of human sperm to TCDD, PCB 77 and p,p'-DDE in the female reproductive tract, for several days. Although exposure to TCDD did not affect sperm function, both the dioxin-like PCB 77 and p.p'-DDE were able to directly target human sperm, promoting several alterations that culminated in cell death. p,p'-DDE exposure was found to decrease both mitochondrial function and cellular ATP levels which, collectively, resulted in a notable reduction of sperm motility. Similarly, PCB 77 affected both mitochondria and motility, but by firstly causing a decline in sperm motility followed by mitochondrial dysfunction, a different mechanism of action was suggested when compared to p,p'-DDE. Consistent with this idea, sperm capacitation was inhibited only by p,p'-DDE treatment. Furthermore, Ca^{2+} homeostasis, which is vital for proper sperm function, was not significantly affected by PCB 77, but was strikingly altered upon p,p'-DDE exposure, even at doses as low as 1 pM and 1 nM. The remarkable increase and sustained elevation of intracellular Ca^{2+} levels promoted by p,p'-DDE exposure resulted from an influx from the external environment triggered by the activation of the Ca^{2+}-

permeable cation channel Catsper localized at the plasma membrane. The acrosome reaction, which is strongly Ca^{2+}-dependent, was prematurely triggered by p,p'-DDE.

In the second part of our work we sought to determine the value of our simple, fast and inexpensive Diff-Quik staining assay in evaluating sperm chromatin status and predicting ART fertility outcomes in post-prepared samples. Similarly to what was reported using other tests, this recently described assay was able to detect an improvement of sperm chromatin integrity after sperm selection. Moreover, lower percentages of chromatin damage were associated with better quality embryos in both IVF and ICSI cycles and with clinical pregnancy success, but only in IVF treatments, where some degree of "natural" sperm selection may occur. In this case, a threshold value beyond which clinical pregnancy was significantly compromised was established at 34.25% of damaged sperm monitored by the Diff-Quik staining assay.

Overall, we provide evidence that p,p'-DDE and PCB 77 promote direct (non-genomic) effects in human sperm function via different mechanisms of action, ultimately compromising male fertility. Given the involvement of Ca^{2+} in many vital processes relevant for sperm function, p,p'-DDE-induced non-regulated Ca^{2+} entry via Catsper and overload may be, at least in part, accountable for the general sperm dysfunction observed. In contrast, TCDD was ineffective *in vitro* suggesting that its effects on human sperm parameters may be indirect, i.e. rather resulting from alterations at other levels. To our knowledge this is the first time that Catsper has been associated with the action of endocrine disruptors.

Furthermore, the Diff-Quik staining assay was confirmed to be a clinically valuable tool that provides useful information concerning the success ART, particularly in IVF cycles, and is thus suggested as an alternative method to detect sperm chromatin status in minimal clinical settings, when no other well-established assays are available.

Resumo

A exposição a contaminantes ambientais, em particular a disruptores endócrinos, tem sido associada a uma diminuição da qualidade espermática e a uma tendência acrescida de cancro testicular e anomalias nos órgãos reprodutores masculinos, dados que sugerem estes compostos como potenciais causadores de disfunção reprodutora masculina e infertilidade. De entre os mais conhecidos contaminantes com este tipo de propriedades incluem-se substâncias organocloradas como a clássica dioxina TCDD, os PCBs e o p,p'-DDE (principal metabolito do pesticida DDT), as quais representam sérios riscos para a população humana, dada a ampla disseminação e persistência das mesmas. Muito embora estas substâncias acumulem tendencialmente no tecido adiposo, têm também sido encontradas no tracto reprodutor feminino, estando assim em contacto directo com os espermatozóides. No entanto, a análise apenas de parâmetros seminais clássicos para avaliar os potenciais efeitos nefastos destes compostos na fertilidade masculina pode subestimar a sua toxicidade, sobretudo a baixas concentrações. Torna-se assim imperativa a avaliação de parâmetros funcionais mais precisos. Um destes parâmetros é a integridade da cromatina/ADN, crucial para uma correcta transmissão da informação genética paterna ao embrião. Apesar do vasto número de técnicas disponíveis para a sua análise, a ausência de valores-limite e a utilização de protocolos extensos e/ou de reagentes e equipamento dispendiosos têm dificultado a introdução da integridade da cromatina/ADN como parâmetro adicional aquando da realização de um espermograma, bem como limitado a sua avaliação em estudos de larga escala.

Na primeira parte do trabalho uma abordagem *in vitro* permitiu mimetizar a exposição contínua aos compostos TCDD, PCB 77 e p,p'-DDE a que os espermatozóides estão sujeitos *in vivo* durante vários dias no tracto reprodutor feminino. Embora a exposição a TCDD não tenha comprometido os parâmetros funcionais estudados, tanto o PCB 77 como o p,p'-DDE afectaram directamente a função espermática, culminando em morte celular. A exposição a p,p'-DDE levou a uma diminuição da função mitocondrial que, em simultâneo com o decréscimo dos níveis de ATP celular, resultou numa acentuada redução da mobilidade espermática. A exposição a PCB 77 afectou igualmente a mitocôndria e a mobilidade espermática, no entanto o declínio na mobilidade precedeu a disfunção mitocondrial, sugerindo distintos mecanismos de acção para os diferentes compostos. Reforçando esta ideia, uma inibição da capacitação foi observada apenas durante a exposição a p,p'-DDE. Além disso, a homeostase do Ca^{2+} não foi significativamente afectada pelo PCB 77 mas foi, pelo

contrário, substancialmente alterada durante a exposição a p,p'-DDE, inclusive a concentrações tão diminutas e fisiologicamente relevantes como 1 pM e 1 nM. De facto, o notável e persistente aumento dos níveis de Ca^{2+} intracelular proveniente do meio externo foi despoletado pela activação do canal de Ca^{2+} Catsper localizado na membrana plasmática. Por seu lado, a reacção acrossómica, evento fortemente dependente de Ca^{2+}, foi também prematuramente induzida pela exposição a p,p'-DDE.

Na última parte do trabalho determinámos o valor do método de coloração Diff-Quik na avaliação do estado da cromatina espermática após selecção dos espermatozóides para utilização nas técnicas de Reprodução Assistida, e a sua relação com os resultados de fertilidade obtidos. Recentemente desenvolvido pelo nosso grupo de trabalho, este método simples e barato detectou um aumento da percentagem de espermatozóides com cromatina íntegra após o processo de selecção. Adicionalmente, amostras com baixas percentagens de espermatozóides com danos na cromatina foram associados a uma melhor qualidade embrionária após ICSI e FIV e à obtenção de uma gravidez clínica após FIV, onde, apesar de toda a manipulação inerente ao procedimento, é possível ocorrer uma certa selecção "natural". Neste último caso, 34,25% de espermatozóides com danos na cromatina foi estabelecido como valor de referência acima do qual a obtenção de uma gravidez poderá estar comprometida.

Em conclusão, os compostos organoclorados p,p'-DDE e PCB 77 afectam directamente a função espermática humana via diferentes mecanismos de acção, comprometendo, em última análise, a fertilidade masculina. Tendo em conta o envolvimento do Ca^{2+} em diversas vias de sinalização, o seu influxo não regulado e excessivo via Catsper poderá ser responsável, pelo menos em parte, pelo declínio da função espermática observada durante a exposição ao p,p'-DDE. Pelo contrário, a exposição ao composto TCDD mostrou-se ineficaz sugerindo que os efeitos previamente descritos em espermatozóides humanos resultam de um outro mecanismo, possivelmente indirecto. Este é o primeiro estudo que descreve o envolvimento do canal de Ca^{2+} Catsper na acção de disruptores endócrinos.

Ademais, o método de coloração Diff-Quik revelou-se uma importante ferramenta clínica com capacidade de predizer o sucesso das técnicas de Reprodução Assistida, em particular após FIV, sendo portanto sugerido como método alternativo para detecção do

estado da cromatina em espermatozóides humanos em qualquer laboratório de Andrologia, quando não estejam disponíveis técnicas mais elaboradas.

Chapter 1 General introduction

1.1 Male infertility

Infertility, generally defined as the inability to conceive following at least 1 year of regular and unprotected intercourse, affects approximately 15% of couples (Dada *et al.* 2011) and in many cases the male factor is the sole reason behind this problem (Schulte *et al.* 2010). Currently, male infertility diagnosis relies on the microscopic assessment of standard semen parameters - sperm concentration, motility and morphology - in the native sample based on the World Health Organization (WHO) guidelines and reference values (WHO 2010). To this extent, a sperm sample is considered normal, and thus normozoospermic, if it contains ≥15 millions of spermatozoa *per* ml of semen, ≥32% of sperm with progressive motility or ≥40% of spermatozoa with total motility (progressive motility and *in situ* motility) and ≥4% of morphologically normal forms (WHO 2010). If one of these parameters is below the reference value, the sample is considered abnormal and defined as oligozoospermic, asthenozoospermic or teratozoospermic according to the low values of concentration, motility or morphology displayed, respectively (WHO 2010).

The evaluation of sperm concentration, motility and morphology, although relevant, is not sufficient for a complete male infertility diagnosis. It has become evident that these parameters may not accurately predict fertility outcomes (Agarwal & Allamaneni 2005) as normozoospermic individuals may be infertile (Agarwal & Allamaneni 2004) and men presenting abnormal values in one, two or even all sperm parameters may father a child. As a result, these parameters seem to only address few aspects of sperm quality and function of the male reproductive system and not the ability of spermatozoa to fertilize an oocyte (Sousa *et al.* 2009). Inevitably, this further stimulated the search for better markers of male fertility and consequently the development of assays that may give clear indications of a subject fertilizing potential, such as the ones detecting capacitation status, acrosome reaction (AR), sperm-oocyte interaction and chromatin/deoxyribonucleic acid (DNA) integrity, among others (Aitken 2006). The detection of different aspects of sperm function by these tests certainly provides valuable information for the diagnosis of male infertility as well as for understanding how environmental contamintants may influence human fertility.

1.2 Spermatogenesis - the production of spermatozoa

The seminiferous tubules are considered the functional units of the testis (Cheng & Mruk 2002) and it is within these unique structures that spermatogenesis occurs. Inside the tubules two different types of cells can be found: the somatic Sertoli cells and the germ cells. Sertoli cells play a paramount role in all the spermatogenic process nourishing and providing physical support to the germ cells located within their invaginations and creating an privileged microenvironment that controls substances entrance and antigen/antibody exchanges, through the formation of the blood-testis barrier (Holstein *et al.* 2003). Not less important are the intertubular space located Leydig cells, which are vital for the regulation of spermatogenesis and the differentiation of male sexual organs and secondary sex characteristics by secreting the steroid hormone testosterone (Holstein *et al.* 2003).

The highly dynamic and metabolically active process of spermatogenesis, by which spermatogonia give ultimately rise to spermatozoa, involves different steps of proliferation and differentiation and culminates with the release of newly formed cells to the lumen of seminiferous tubules (De Kretser *et al.* 1998; Holstein *et al.* 2003). Spermatogenesis starts at puberty and occurs continuously throughout life because of the self-renewing spermatogonia stem cell pool present in the germinal epithelium (Holstein *et al.* 2003). The duration of each spermatogenic cycle, i.e. the time required to obtain spermatozoa from the progenitor spermatogonia, is species-specific (França *et al.* 1998) and in men it takes approximately 64 days on average (Misell *et al.* 2006).

Spermatogenesis can be divided in three distinct and sequential phases: spermatogoniogenesis, meiosis and spermiogenesis (Figure 1.1). In this first phase, spermatogonial stem cells present at the basement membrane undergo numerous mitotic divisions to continuously reload the germinal epithelium (De Kretser *et al.* 1998; Holstein *et al.* 2003). At this point, the daughter cells can either remain dormant and renew the stem cell reservoir (A-type spermatogonia) or initiate a sequential and gradual transformation that will finally give rise to spermatozoa (committed B-type spermatogonia; Holstein *et al.* 2003).

After the last spermatogonial division, B-type spermatogonia differentiate into primary spermatocytes that enter the first meiotic division. From this reducing division secondary spermatocytes are generated. These cells do not undergo DNA replication

dividing quickly to form round spermatids in the second and last meiotic division (Holstein *et al.* 2003). Round spermatids soon undergo a series of complex alterations that allow their differentiation into spermatozoa in a process termed spermiogenesis (De Kretser & Kerr 1994). During this profound remodeling step, chromatin becomes highly condensed and the haploid nucleus elongated as a consequence of the replacement of histones by smaller basic nuclear proteins, the protamines. The Golgi apparatus undergoes conformational changes contributing to the formation of the acrosome, which connects to the nucleus surface. Conversely, the flagellum is formed in each cell in the opposite direction. Further reorganization of organelles and loss of most cytoplasm and other structures complete the series of morphological changes (Holstein *et al.* 2003).

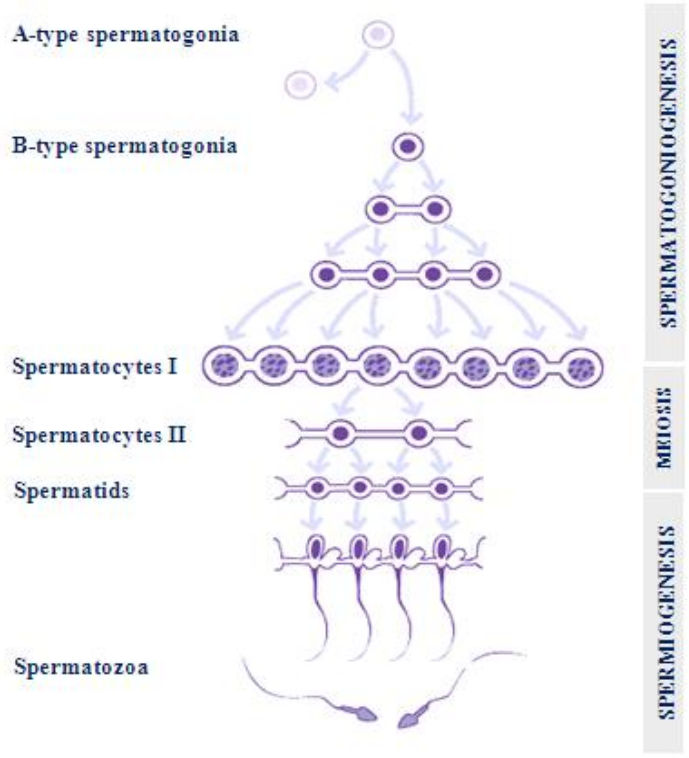

Figure 1.1. Spermatogenesis.

Spermatogenesis is a highly dynamic and metabolically active process which allows the formation of spermatozoa from the progenitor spermatogonia. It involves different steps of proliferation and differentiation and can be divided into three distinct phases. Spermatogoniogenesis is defined by the proliferation of spermatogonia and development of primary spermatocytes that quickly enter meiosis. After two meiotic divisions, the initial primary spermatocyte leads to the formation of four haploid round spermatids that during spermiogenesis experience terminal differentiation. Spermatocytes I - primary spermatocytes; spermatocytes II – secondary spermatocytes (adapted from http://www.embryology.ch/anglais/cgametogen/spermato03.html).

At the end of spermiogenesis cells are released to the lumen of seminiferous tubules in a process known as spermiation (De Kretser *et al.* 1998; Holstein *et al.* 2003). Spermatozoa can then leave the testis towards the rete testis by peristaltic contraction of the seminiferous tubules (Romano *et al.* 2005) aided by the testicular fluid secreted by Sertoli cells (Holstein *et al.* 2003). It is throughout the epididymal ducts that spermatozoa suffer posttesticular maturation, acquiring significant physiological attributes such as the ability to swim progressively (Yanagimachi 1994; Cooper 2007).

1.2.1 Hormonal regulation of spermatogenesis

Spermatogenesis is a highly regulated process mainly dependent upon testosterone and the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH; Holdcraft & Braun 2004). Upon LH stimulation in response to the hypothalamic gonadotropin-releasing hormone (GnRH; Holstein *et al.* 2003; Figure 1.2), Leydig cells expressing LH receptors produce testosterone which is absolutely crucial for adult spermatogenesis, especially in the progression and completion of both meiosis and spermiogenesis (McLachlan *et al.* 2002a; Haywood *et al.* 2003; Ruwanpura *et al.* 2010). Testosterone acts via androgen receptors (ARec) localized in Sertoli and Leydig cells and it is generally accepted that its actions on spermatogenesis are transduced by the Sertoli cells (McLachlan *et al*. 2002b). However, these receptors have also been identified in germ cells (Kimura *et al.* 1993; Zhou *et al.* 1996) and spermatozoa (Aquila *et al.* 2007; Rago *et al.* 2007; Kotula-Balak *et al.* 2012), suggesting that testosterone may possibly have another mechanism of action by direct interaction with these cells. Furthermore, by a negative feedback mechanism testosterone also inhibits LH secretion (Holstein *et al.* 2003).

FSH, on the other hand, acts on Sertoli cells through specific G-protein coupled surface receptors (Simoni *et al.* 1997; McLachlan *et al*. 2002b) stimulating their proliferation at specific time windows of mammalian development (Sharpe *et al.* 2003). This is particularly relevant given that each Sertoli cell only supports a limited number of germ cells (Rodriguez *et al.* 1997; Sinha Hikim & Swerdloff 1999), determining the quantity of germ cells present in the seminiferous tubule and consequently, sperm output (Ruwanpura *et al.* 2010). Additionally, FSH may regulate spermatogonial

proliferation, meiosis and spermiogenesis. FSH may stimulate spermatogonial division (Krishnamurthy *et al.* 2000; Haywood *et al.* 2003; O'Shaughnessy *et al.* 2010) and also act as a survival factor by controlling apoptosis in spermatogonia (Meachem *et al.* 1999; Ruwanpura *et al.* 2008a,b), spermatocytes (Meachem *et al.* 1999; Ruwanpura *et*

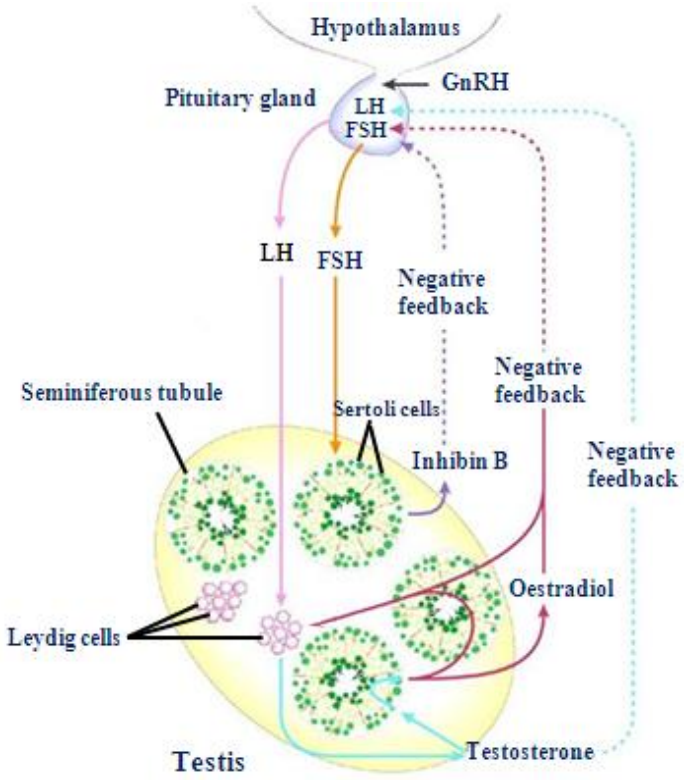

Figure 1.2. Hormonal regulation of spermatogenesis.

The hypothalamus regulates the production of FSH and LH by the anterior pituitary gland through GnRH secretion. LH stimulates Leydig cells to secrete testosterone which is crucial for the development of spermatogenesis, particularly of meiosis and spermiogenesis. Besides having a local action, testosterone enters the blood stream and inhibits LH secretion (negative feedback). FSH, on the other hand, exerts its effects through Sertoli cells. Both circulating inhibin B secreted by Sertoli cells and oestradiol, converted from testosterone by the enzyme aromatase, decrease FSH secretion by the hypophysis (negative feedback). Estrogens play an important role in the development of spermatogenesis. GnRH - gonadotropin-releasing hormone; FSH - follicle stimulating hormone; LH - luteinizing hormone (adapted from Cooke & Saunders 2002).

al. 2008a) and spermatids (Chandolia *et al.* 1991; Meachem *et al.* 1999). Similar to the role of testosterone on later spermatogenic cells (spermatocytes and spermatids; Erkkilä

et al. 1997), FSH also appears to be important in supporting germ cell development to spermatids through the regulation of apoptosis in men (Tesarik *et al.* 2000; Matthiesson *et al.* 2006). FSH may also interfere with the adhesions between spermatids and Sertoli cells. Saito and colleagues have found a higher percentage of cells failing to spermiate following one week of passive FSH immunization in adult rats, stressing its importance in the spermiation process (Saito *et al.* 2000). These authors also found that combined FSH and testosterone suppression led to a 50% failure in spermiation, a far higher percentage than the approximately 15% observed for each hormone individually, clearly highlighting the need of both substances for the development of a normal spermiation process (Saito *et al.* 2000). In fact, many reports show that both FSH and testosterone can act co-operatively and thus optimize the spermatogenic process. The synergy among FSH and testosterone is also observed at the spermatocyte level in which combined FSH and testosterone treatment was found to be much more effective in the maintenance or re-establishment of meiosis than individual hormone treatment (McLachlan *et al.* 2002a). Through a negative feedback mechanism FSH secretion is inhibited by inhibin B secreted by Sertoli cells (Holstein *et al.* 2003) and, paradoxically, by the "female hormone" oestradiol (Hayes *et al.* 2001).

In reality, increasing evidence identifies estrogens as potential regulators of spermatogenesis in many mammalian species including humans (Carreau *et al.* 2010, 2012). Both the biologically active enzyme aromatase, which irreversibly converts testosterone to oestradiol (the major physiological estrogen), and the estrogen receptors (ERs), the main mediators of estradiol actions, are localized in several testicular cell types such as Leydig, Sertoli and germ cells (Cavaco *et al.* 2009; Lardone *et al.* 2010; Carreau *et al.* 2010, 2012). Additionally, ERs have also been detected in mammalian spermatozoa (Aquila *et al.* 2004; Rago *et al.* 2007; Carreau *et al.* 2010; Kotula-Balak et al. 2012). Estrogens were found necessary for the progression of adult spermatogenesis, especially during meiosis and differentiation of spermatozoa (Carreau *et al.* 2010, 2012). Furthermore, oestradiol was also found to act as a potent germ cell survival factor in human testis (Pentikainen *et al.* 2000). On the other hand, increased concentrations of estrogens in the male testis may impair spermatogenesis (Carreau *et al.* 2012).

1.3 Spermatozoa – highly specialized and functional cells

Spermatozoa are terminally differentiated and hydrodynamic-shaped cells that have only one but rather gigantic "goal", to achieve fertilization and transmit their genetic information to the progeny. In order for this to happen, each one of these notable cells is composed of three main regions: head, midpiece and tail (Figure 1.3).

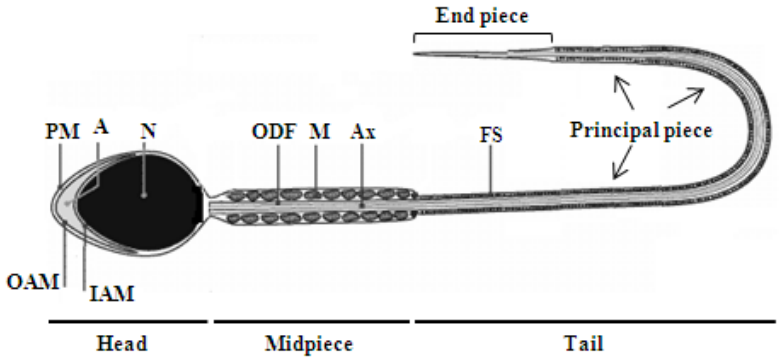

Figure 1.3. Longitudinal schematic representation of a mammalian spermatozoon.
Spermatozoa consist of three main regions: the head (which comprises the acrosome and the nucleus), the midpiece (where mitochondria are restricted) and the flagellum (composed of a proximal piece and an end piece). PM - plasma membrane; OAM - outer acrosomal membrane; A - acrosomal matrix; IAM - inner acrosomal membrane; OAM + A + IAM - acrosome; N - nucleus; ODF - outer dense fibers; M - mitochondrion; Ax - axoneme; FS - fibrous sheath (adapted from http://www.embryology.ch/anglais/cgametogen/spermato05.html).

1.3.1 Sperm head

Within the sperm head two organelles are easily distinguished: a large secretory vesicle dubbed acrosome and the nucleus, which contains a haploid set of chromosomes (Holstein *et al.* 2003).

1.3.1.1 Acrosome

The acrosome is a double membrane organelle (comprises an inner and an outer membrane) located in the anterior portion of the sperm head, overlaying the nucleus

(Florman *et al.* 2008; Abou-haila & Tulsiani 2009). In mammals, both the shape and the size of acrosome are species-specific and normally follow sperm head morphology, which in humans assume a paddle shape form (Abou-haila & Tulsiani 2009). The acrosome may comprise 40-70% of the sperm head (WHO 2010) and contains hydrolytic enzymes, such as acrosin, thought to facilitate sperm penetration through the oocyte zona pellucida (ZP; Ramalho-Santos *et al.* 2007). Because certain proteins with hyaluronidase activity (Lin *et al.* 1994; Hunnicutt *et al.* 1996) are present in the sperm surface, it is believed that acrosome-intact spermatozoa can safely penetrate the hyaluronic acid rich-extracellular matrix between the cumulus cells that surround and support the oocyte (Lin *et al.* 1994; Abou-haila & Tulsiani 2009), allowing sperm to move forward. However when spermatozoa interact with the translucent glycoprotein-based ZP, AR takes place. AR is a calcium (Ca^{2+})-dependent exocytotic event in which the outer acrosomal membrane and the plasmalemma fuse at multiple sites, conducing to the release of hydrolytic enzymes (important for digesting and penetrating the ZP) and the exposure of new membrane domains, both of which essential for fertilization to occur (Breitbart 2002; Florman *et al.* 2008; Abou-haila & Tulsiani 2009). Binding to the ZP seems to trigger a signaling cascade that involve second messengers, Ca^{2+} influx, internal pH rise and activation of protein kinases that promote protein phosphorylation (Abou-haila & Tulsiani 2009). This will ultimately lead to exocytosis of the acrosomal contents by stimulating the fusion between the outer acrosomal membrane and plasma membrane (Abou-haila & Tulsiani 2009). Besides ZP, a number of physiological inducers, i.e. substances that spermatozoa will encounter during *in vivo* fertilization, are also known to induce AR. Progesterone, which is released by cumulus cells, was recently found to trigger acrosomal exocytosis by directly activating a cation channel which promotes Ca^{2+} influx apparently without involving metabotropic receptors (Strunker *et al.* 2011).

AR is indispensable for fertilization to occur both *in vivo* and *in vitro* (except in the case of Intracytoplasmic Sperm Injection [ICSI] where spermatozoa are directly injected into the oocyte). Sperm cells that experience AR prematurely or do not possess an acrosome due to defective spermiogenesis have no fertilizing ability *in vivo*. Furthermore, it has been reported that human samples with low percentages of acrosome-intact spermatozoa also present low *in vitro* fertilizing potential (Liu & Baker 1988; Albert *et al.* 1992; Chan *et al.* 1999).

Human spermatozoa are not capable of experiencing AR and thus fertilization oocyte immediately after ejaculation. It is only after being in the female reproductive tract (Abou-haila & Tulsiani 2009) for a suitable amount of time (Yanagimachi 1994) that spermatozoa undergo a series of poorly understood maturation steps collectively dubbed capacitation (Visconti & Kopt 1998; Ramalho-Santos *et al.* 2007). This fertilizing competence acquisition includes increased plasma membrane permeability and fluidity due to cholesterol efflux, influx of ions specially Ca^{2+} and bicarbonate (HCO_3^-), internal pH rise and protein phosphorylation, typically on tyrosine residues (Visconti & Kopf 1998; Ramalho-Santos *et al.* 2007; Abou-haila & Tulsiani 2009). The increased membrane fluidity in capacitated spermatozoa was found to facilitate fusion-related events during spermatozoa-oocyte interactions such as AR and sperm-oocyte fusion (Primakoff & Miles 2002). Capacitation is also related to striking changes in sperm swimming patterns, designated as sperm hyperactivation (Suarez 2008; Abou-haila & Tulsiani 2009). The hyperactive beat pattern of the flagellum is believed to be required for sperm to penetrate both the cumulus complex and the ZP, being therefore another important factor that regulates fertilization (Suarez 2008)

The process of capacitation may also be achieved *in vitro* using chemically-defined media complemented with energy substrates (e.g. lactate, pyruvate and glucose), ions (e.g. Ca^{2+} and HCO_3^-) and serum albumin, a cholesterol acceptor (Visconti & Kopf 1998; Abou-haila & Tulsiani 2009). Spermatozoa may also revert their capacitated state when treated with decapacitating factors, causing cells to fall back to a non-fertilizing condition (Fraser 2010). With time, spermatozoa can recapacitate and recover their fertilizing ability, showing that the process of capacitation itself is reversible (Fraser 2010). This *in vitro* manipulation of capacitation has allowed researchers to determine its importance in mammalian fertility.

1.3.1.2 Nucleus – chromatin/DNA structure

Spermatozoa are responsible for the delivery of the paternal genetic information, distinctively packaged in the haploid nucleus, to the future embryo. Unlike the relatively loose chromatin structure present in somatic cells, spermatozoa possess an extremely organized and highly compacted chromatin due to the unique associations between the DNA and sperm nuclear proteins, primarily protamines (Ward & Coffey

1991; Brewer *et al.* 1999). In fact, while the somatic cell nuclear DNA is wrapped around histone octamers forming nucleosomes that will further coiled into solenoids (McGhee *et al.* 1980) therefore increasing chromatin volume, spermatozoa experience a different type of chromatin packaging that results in a reduced nuclear size (Ward & Coffey 1991; Figure 1.4).

During spermiogenesis sperm chromatin undergoes several alterations in which the histones are replaced by transition proteins and later on by protamines (Dadoune 1995; Lee *et al.* 1999; Steger *et al.* 2000; Kierszenbaum 2001). The DNA strands are tightly wrapped around these latter proteins, forming supercoiled doughnut-like structures named toroids, the basic packaging units of sperm chromatin (Tanphaichitr *et al.* 1978; Ward & Coffey 1991; Brewer *et al.* 1999). These structures, in addition to the solenoids

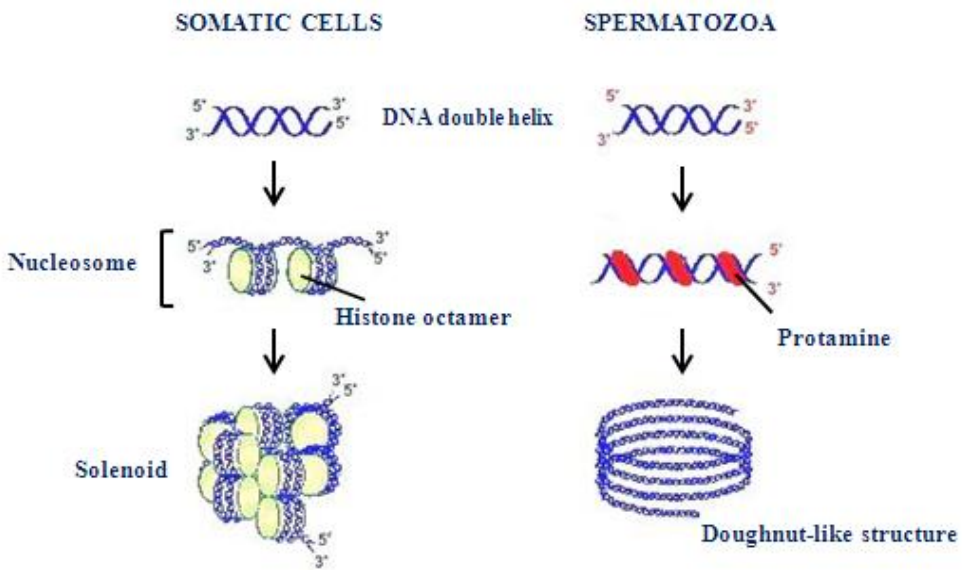

Figure 1.4. DNA packaging in somatic cells and spermatozoa.

Somatic and sperm cells do not share the same type of DNA packaging. While in somatic cells the DNA double helix is wrapped around histone octamers forming nucleosomes further coiled into solenoids, the DNA from spermatozoa is wrapped around protamines forming supercoiled doughnut-like structures (toroids; adapted from Sakkas *et al.* 1999a and http//compbio.med.wayne.edu/protamines.html).

in somatic cells, are further organized into loop domains attached to a nuclear matrix (Ward & Coffey 1991). Progressive oxidation of the thiol (-SH) groups among cysteine-rich protamines will lead to inter- and intramolecular disulfide cross-links further

allowing compaction and stabilization of the sperm nuclei (Kosower *et al.* 1992), thus reducing sperm chromatin to one-sixth the volume taken up in somatic cells nuclei (Fuentes-Mascorro *et al.* 2000). This chromatin rearrangement is thought to be important, not only for protecting the paternal genome during the transport through the male and female reproductive tracts, but also in guaranteeing its proper delivery to the oocyte, hence allowing accurate embryo development (Sakkas *et al.* 1999a). Interestingly, altered protamine expression may be detrimental. Human spermatozoa contain both type-1 and type-2 protamines (P1 and P2, respectively), but a higher prevalence of P2 may cause DNA to become more susceptible to damage since P2 contains fewer cysteine residues and consequently less disulfide cross-links (Corzett *et al.* 2002). Torregrosa and coworkers have suggested a relationship between deficient P2 processing and decreased DNA integrity in infertile patients (Torregrosa *et al.* 2006) and others have shown a common altered P2 expression in these men (Carrell *et al.* 2001).

It is important to note that not all histones are replaced by protamines and up to 15% are actually retained in human sperm (Gatewood *et al.* 1987; Bench *et al.* 1996). It is believed; however, that these histones mark the genes that will be preferentially activated in early stages of embryonic development (Gatewood *et al.* 1987; Gineitis *et al.* 2000) by remaining less tightly compacted, thus providing an easy access to DNA. In contrast, an excess of nuclear histones may be associated with reduced chromatin compaction and enhanced susceptibility to DNA damage in both human and mice spermatozoa (Cho *et al.* 2001, 2003; Aoki *et al.* 2005, 2006). Furthermore, a higher histone to protamine ratio among infertile men has been described when compared to fertile controls (Steger *et al.* 2000; Oliva 2006; Zhang *et al.* 2006; Zini *et al.* 2007).

1.3.2 Sperm tail and midpiece

The ultrastructure of the mammalian sperm tail is highly conserved and is comprised of a number of cytoskeletal structures whose proper assembly is decisive for sperm motility (Turner 2003). The sperm tail is composed by an axoneme displaying a central pair of single tubulin microtubules and nine peripheral doublets in a classic 9+2 microtubular arrangement, further surrounded by the outer dense fibers and a fibrous

sheath (Kierszenbaum 2002; Ramalho-Santos *et al.* 2007). These cytoskeletal elements provide strength and resistance to the tail (Turner 2003; Ramalho-Santos *et al.* 2007) and also differentiate the sperm flagella in its principal piece and end piece (Turner 2003). Both the outer dense fibers and the fibrous sheath narrow in the end of the principal piece and are no longer present in the end piece, letting the plasma membrane as the sole structure encircling the axoneme (Turner 2003).

During spermiogenesis mitochondria suffer elongation and become arranged end to end in the midpiece, where they are wrapped helically around both the axoneme and outer dense fibers in the anterior portion of the tail (Otani *et al.* 1988; Olson & Winfrey 1992; Ho & Wey 2007). It is believed that since mitochondria are retained in a specialized region of spermatozoa and do not contribute to the formation and development of the embryo, they might have an essential role on sperm function (Ramalho-Santos *et al.* 2009). As in other cells, sperm mitochondria may be important for numerous events including the regulation of apoptosis, Ca^{2+} storage, reactive oxygen species (ROS) generation and energy production in the form of adenosine triphosphate (ATP), among others (Ramalho-Santos *et al.* 2009; Amaral *et al.* 2013a). Sperm mitochondria are composed of an outer and inner membrane separated by the intermembrane space (Figure 1.5). The inner membrane, which encloses the mitochondrial matrix, is folded, forming cristae that contain enzymes of the electron transfer chain (ETC) involved in the oxidative phosphorylation (OXPHOS) process. OXPHOS is the major provider of cellular ATP and relies on the activity of five ETC complexes (Ramalho-Santos *et al.* 2009; Amaral *et al.* 2013a). The oxidation processes occurring at complexes I (nicotinamide dinucleotide-dehydrogenase) and II (succinate dehydrogenase) generate electrons that are transferred to complexes III (cytochrome c dehydrogenase) and IV (cytochrome c oxidase) and accepted by oxygen at complex IV, synthesizing water. The electron transport throughout complexes I, III and IV is accompanied by the pumping of protons from the mitochondrial matrix to the intermembrane space, producing a transmembrane electrochemical gradient commonly termed mitochondrial membrane potential (MMP). This electrochemical gradient is further used by complex V (ATP synthase) to catalyze the production of ATP from adenosine diphosphate (ADP) and inorganic phosphate.

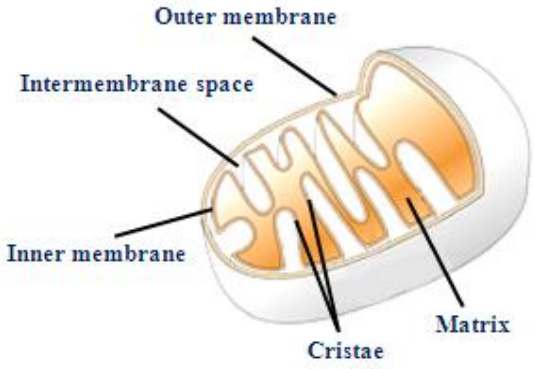

Figure 1.5. Schematic representation of a mitochondrion.

Mitochondria are composed of an outer and an inner membrane. The inner mitochondrial membrane enfolds the matrix and forms numerous invaginations, the cristae, where the ETC complexes are located. The area between the two membranes is termed intermembrane space (adapted from Rajender *et al.* 2010).

As mitochondria generate ATP through OXPHOS they might provide sperm with energy for key events such as flagellar propulsion. In fact, multiple studies support the involvement of mitochondria in sperm motility. Patients presenting asthenozoospermic samples have shown decreased activities of the ETC complexes I, II and IV when compared to men with no sperm motility issues (Ruiz-Pesini *et al.* 1998). Furthermore, *in vitro* inhibition of the ETC enzymes (for instance, with antimicin A, rotenone or potassium cyanide) have resulted in ATP depletion and/or reduced sperm motility in different mammalian species including humans (Ford & Harrison 1981; Fisher-Fischbein *et al.* 1985; De Lamirande & Gagnon 1992; Pascual *et al.* 1996; Krzyzosiak *et al.* 1999). Additionally, strong associations between decreased mitochondrial function, determined by a lower MMP, and reduced sperm motility has been observed in both humans (Donnelly *et al.* 2000; Marchetti *et al.* 2004; Sousa *et al.* 2011; Sharbatoghli *et al.* 2012) and other mammals (Garner *et al.* 1997; Martinez-Pastor *et al.* 2004). More importantly, fertilization capability, measured as fertilization rates after In Vitro Fertilization (IVF), have been robustly correlated with MMP, and therefore with mitochondrial function in humans (Kasai *et al.* 2002; Marchetti *et al.* 2002; Gallon *et al.* 2006).

Nevertheless, the complete dependence of sperm motility in OXPHOS-derived ATP has been challenged, as compartmentalization of mitochondria in the midpiece

may limit the availability of OXPHOS-derived ATP for the ATPases placed in the principal piece, as it is uncertain that enough ATP can diffuse to distant parts of the tail, particularly in species with long sperm tails (e.g. rodents; Ramalho-Santos *et al.* 2009). Accordingly, several studies have suggested that the ATP needed for sperm motility may be produced by the glycolytic pathway, given that mammalian sperm possess various glycolytic enzymes compartmentalized in the fibrous sheath of the principal piece (Mukai & Okuno 2004; Ford 2006; Krisfalusi *et al.* 2006; Kim *et al.* 2007; Storey 2008). Furthermore, male homozygous knockout mice for the sperm glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase-S (a mouse sperm-specific enzyme) has shown slow sperm movement without forward progression and no alterations in mitochondrial oxygen consumption, stressing the importance of glycolysis as opposed to OXPHOS in sperm motility (Miki *et al.* 2004).

However, latest findings suggest that pathways other than glycolysis and OXPHOS may also provide human sperm with the ATP necessary to fuel motility. Specifically, Amaral and colleagues have reported the presence of various components involved in the mitochondrial fatty acid β-oxidation pathway by proteomic analysis and described a decrease in motility when sperm cells were incubated with the fatty acid oxidation inhibitor etomoxir (Amaral *et al.* 2013b). Overall, the origin of the ATP generated for mammalian sperm motility is a largely debated topic and evidence suggest that OXPHOS, glycolysis and fatty acid β-oxidation may supply ATP required for sperm motility. The prevalence of one of these metabolic pathways may vary among species and according to the substrates available for spermatozoa to use.

Besides the large ATP expenditure for motility, ATP is also required for many events and ultimately for maintaining sperm viability. It is also well established that ROS, which in spermatozoa appear to be mainly produced by the mitochondrial ETC, is normally balanced by various cellular antioxidant defences but at high concentrations it may cause cellular oxidative stress (De Lamirande & Gagnon 1992; De Lamirande *et al.* 1997; Turrens 2003). Small amounts of ROS stimulate several key events including sperm motility, capacitation, hyperactivation and fusion-related events such as AR and sperm-oocyte fusion (De Lamirande & Gagnon 1993; Aitken *et al*. 1995, 1997; Griveau & Le Lannou 1997), but excessive ROS has been linked to DNA fragmentation, peroxidation of plasma membrane lipids, decreased motility and apoptosis in mammalian sperm (De Lamirande *et al.* 1997; Agarwal *et al.* 2008). Thus, the fine

balance between ROS generation and ROS scavenging is of paramount importance for sperm to successfully fertilize the oocyte.

1.3.3 Ca^{2+} stores and channels

Despite their small size and low cytoplasm content spermatozoa are equipped with extraordinary mechanisms for the tight regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and production of complex Ca^{2+} signals, exhibiting a specialized 'toolkit' of channels, pumps and stores (Jimenez-Gonzalez *et al.* 2006). In fact, Ca^{2+} signaling is of particular significance in spermatozoa, given its crucial regulatory role in many key processes including AR (Kirkman-Brown *et al.* 2002), motility, hyperactivation (Ren *et al.* 2001; Carlson *et al.* 2003; Qill *et al.* 2003) and capacitation (Jimenez-Gonzalez *et al.* 2006). Impairment of Ca^{2+} signaling is therefore associated with decreased male fertility (Krausz *et al.* 1995; Nikpoor *et al.* 2004; Espino *et al.* 2009; Alasmari *et al.* 2013).

Signaling through $[Ca^{2+}]_i$ is achieved by allowing Ca^{2+} from the extracellular environment and/or from intracellular organelles, where Ca^{2+} concentration is extremely high, to enter the sperm cytoplasm where Ca^{2+} is maintained at very low (resting) levels (Costello *et al.* 2008). Evidence supports the existence of two storage organelles in mammalian spermatozoa, one in the acrosomal region, i.e the acrosome itself, and another in the sperm neck/midpiece (Naaby-Hansen *et al.* 2001; De Blas *et al.* 2002; Herrick *et al.* 2005; Costello *et al.* 2008). The acrosomal store is strongly implicated in the regulation of AR as stored Ca^{2+} mobilization through inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} channels, which are located mostly in the outer acrosomal membrane, is essential for AR induction (De Blas *et al.* 2002; Herrick *et al.* 2005). On the other hand, the store present in the neck/midpiece region seems to function as a regulator of sperm motility (Costello *et al.* 2008). It is well established that mammalian sperm mitochondria can accumulate Ca^{2+} into the matrix (Storey & Keyhani 1974; Babcock et al. 1976; Vijayaraghavan & Hoskins 1990). However, in pathological conditions, Ca^{2+} uptake by these organelles may conduce to mitochondrial Ca^{2+} overload and consequent disruption (Bianchi *et al.* 2004).

Before 2001, sperm plasma membrane voltage-operated Ca^{2+} channels (Ca_V; VOCC) were believed to be responsible for Ca^{2+} entry into the cell, being also understood as the main sperm Ca^{2+} conductance (Florman *et al.* 1998; Darszon *et al.* 1999). This idea was borne by the discovery of Ca_V channels in spermatocytes using the patch-clamp technique (Arnoult *et al.* 1996; Santi *et al.* 1996) and also by a putative voltage-gated Ca^{2+} influx observed in mature spermatozoa upon application of a high pH extracellular medium (Wennemuth *et al.* 2000). However, male mice deficient in high- (Ca_V 2.2, Ca_V 2.3) and low-voltage activated channels (Ca_V3 subfamily), were found to be fertile, showing that these VOCCs were not fundamental for sperm physiology or worked redundantly (Kim *et al.* 2001; Beuckmann *et al.* 2003). Knockouts of the high-voltage channel subfamily Ca_v1, particularly Ca_V 1.2, and Ca_V 2.1 were lethal, therefore preventing the evaluation of Ca^{2+} rise in spermatozoa (Jun *et al.* 1999; Seisenberger *et al.* 2000).

In 2001 a novel low voltage-dependent Ca^{2+}-permeable cation channel (Catsper), with a transmembrane pore sequence that resembles that of VOCCs, was described exclusively in spermatozoa (Ren *et al.* 2001; Jimenez-Gonzalez *et al.* 2006). So far, seven subunits composing this heteromeric channel have been identified (Figure 1.6), with the four α subunits that form the Ca^{2+}-selective pore - CatSper1-4 - and the auxiliary subunit CatSper δ - being indispensible for appropriate channel formation and function (Ren *et al.* 2001; Lobley *et al.* 2003; Qill *et al.* 2003; Carlson *et al.* 2005; Qi *et al.* 2007). Though interaction of the two other auxiliary subunits (CatSper β and CatSper γ) with the CatSper complex has been revealed (Liu *et al.* 2007; Wang *et al.* 2009), whether they are necessary for functional CatSper channel assembly is still unclear (Lishko *et al.* 2012).

Due to Catsper channel localization in the principal piece of the sperm flagellum (Ren *et al.* 2001), its involvement in the regulation of motility has been suggested. Consistent with this notion, studies reported that Catsper gene and protein expressions were reduced in human sperm samples presenting low motility (Nikpoor *et al.* 2004; Bhilawadikar *et al.* 2013). Furthermore, Ren et al. also found that CatSper-null mouse presented a severe decrease in sperm motility and consequently CatSper-null spermatozoa were unable to fertilize intact oocytes (Ren *et al.* 2001). Additionally, others have also shown the preponderant role of Catsper in sperm hyperactivation, a hallmark of capacitation. It was found that in the absence of at least one of the Catsper α

subunits a remarkable decrease or a complete lack of hyperactivated motility under capacitating conditions was observed (Carlson *et al.* 2003; Qill *et al.* 2003; Jin *et al.* 2007; Qi *et al.* 2007), further supporting the idea that all Catsper α proteins are required for hyperactivation and thus male fertility (Qill *et al.* 2003; Jin *et al.* 2007). Furthermore, as the CatSper channel is gated by internal pH alterations, intracellular alkalinization caused by extracellular application of the Catsper agonist ammonium chloride or by increased patch-clamp pipette pH solution potentiated $[Ca^{2+}]_i$ elevation (Kirichov *et al.* 2006; Marquez & Suarez 2007) and elicited hyperactivation in mammalian spermatozoa (Marquez & Suarez 2007).

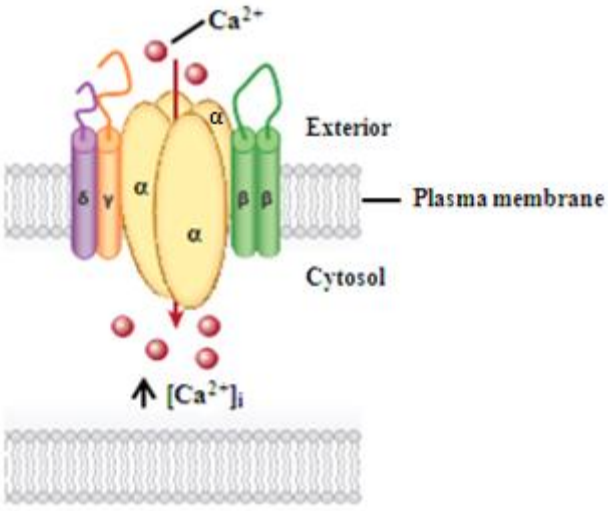

Figure 1.6. Schematic representation of the Catsper channel.

CatSper is a plasma membrane channel formed by four α subunits that enclose the Ca^{2+}-selective pore and three auxiliary proteins - CatSperβ, CatSperγ and CatSperδ - of unknown stoichiometry (Liu *et al.* 2007; Wang *et al.* 2009; Ren & Xia 2010). Upon stimulation, Catsper allows Ca^{2+} influx from the external environment promoting an increase in $[Ca^{2+}]_i$. All CatSper subunits are located in the principal piece of the sperm flagellum (adapted from Lishko *et al.* 2012).

Interestingly, Catsper has also being newly implicated in the robust Ca^{2+} influx and concomitant AR induced by progesterone in human sperm (Lishko *et al.* 2011; Strünker *et al.* 2011). Progesterone was found to stimulate human CatSper at low doses by changing the voltage dependency of the channel towards the physiological range. Progesterone action was fast and independent of metabotropic receptors and intracellular ATP, guanosine diphosphate, cyclic nucleotides, Ca^{2+}, or additional soluble

intracellular messengers (Lishko *et al.* 2011; Strünker *et al.* 2011). The authors suggested that the progesterone-binding site may be accessible from the extracellular environment and may be either located on one of the CatSper subunits or on a currently unknown protein associated with the CatSper complex (Lishko *et al.* 2011). Besides membrane potential, intracellular pH and progesterone, Catsper is also activated by the cumulus cells-secreted prostaglandins, known to stimulate Ca^{2+} influx, odorants and other organic molecules (Lishko *et al.* 2011; Strünker *et al.* 2011; Brenker *et al.* 2012), apparently acting as a polymodal sensor for numerous chemical stimuli that support spermatozoa through their journey in the female reproductive tract.

Finally, it is worth mentioning that in spermatozoa as well as in all other eukaryotic cells, Ca^{2+} clearance is essential for the control of Ca^{2+} signaling events (Berridge *et al.* 2000). As such, the maintenance or reduction of $[Ca^{2+}]_i$ back to resting levels after stimulation is undertaken by ATP-requiring Ca^{2+} pumps (Ca^{2+}-ATPases) or Na^{+}-Ca^{2+} exchangers, which expel Ca^{2+} either out of the cell, or into intracellular stores (Michelangeli *et al.* 2005).

1.4 Relevance of sperm chromatin/DNA damage on male fertility

For the past years emerging reports have shown that sperm DNA integrity may be a better predictor of male fertilizing potential than standard sperm parameters (Agarwal & Allamaneni 2004; Bungum *et al.* 2011). Indeed, infertile patients often exhibit substantially higher levels of DNA damage than fertile donors (Zini *et al.* 2001, 2002; Saleh *et al.* 2003a; Sergerie *et al.* 2005) which may negatively influence both *in vivo* and *in vitro* fertility outcomes. Although sperm DNA damage was found to correlate with poor sperm parameters in several studies (Muratori *et al.* 2000; Zini *et al.* 2001, 2002; Sousa *et al.* 2009), the truth is that standard parameters do not give any information about the status of sperm chromatin/DNA. This idea is strengthened by studies showing that infertile individuals with normal semen parameters display increased DNA damage compared to fertile men (Saleh *et al.* 2002a, 2003a; Venkatesh *et al.* 2011) and that morphologically normal spermatozoa may also possess DNA fragmentation (Avendaño *et al.* 2009). Thus, an important aspect of sperm status is not being routinely considered in most Andrology laboratories.

1.4.1 Mechanisms of chromatin/DNA damage

Sperm DNA damage may occur within the seminiferous tubules, during sperm transit along the male reproductive tract and/or following ejaculation (Lewis & Agbage 2008), but so far the exact mechanisms behind this impairment in human spermatozoa are not completely understood (Schulte *et al.* 2010). Nevertheless, three major theories including defective sperm chromatin packaging, abortive apoptosis and oxidative stress have been suggested (Schulte *et al.* 2010). One should note that these mechanisms are not mutually exclusive and damage may arise from combinations of all three (Aitken & De Iullis 2010). To date, several factors have been described to induce these mechanisms, including exposure to organochlorines (Rignell-Hydbom *et al.* 2005a; De Jager *et al.* 2006) and reproductive disorders such as varicocele, a pathological dilatation of testicular veins caused by venous reflux (Saleh *et al.* 2003b; Enciso *et al.* 2006; Sadek *et al.* 2011), among others.

1.4.1.1 Defective sperm chromatin packaging

Studies performed in both animal models and humans have proposed that in order for a proper chromatin packaging to occur temporary DNA nicks may be required to facilitate the replacement of histones by protamines (McPherson & Longo 1993; Marcon & Boissonneault 2004). These nicks, induced by the endogenous nuclease DNA topoisomerase II, are believed to relieve the torsional stress generated as DNA is condensed and packaged into the differentiating sperm head (McPherson & Longo 1992; Sakkas *et al.* 1999a; Marcon & Boissonneault 2004). The transitory nicks are then usually repaired by this same enzyme before the end of spermiogenesis (McPherson & Longo 1992, 1993). However, if they remain unrepaired, spermatozoa with fragmented DNA will be present in the ejaculate (Manicardi *et al.* 1995; Sakkas *et al.* 1999a; Muratori *et al.* 2006), thus indicating anomalies during the later stage of spermatogenesis (Manicardi *et al.* 1995).

Furthermore, damage may also arise as a result of defective chromatin compaction. For example, protamine deficiency (absolute or relative) can potentially result in suboptimal compaction (Aravindan *et al.* 1997) turning chromatin more prone to

external assault. This deficiency may ultimately induce sperm DNA damage (Cho *et al.* 2001, 2003; Aoki *et al.* 2005; Torregrosa *et al.* 2006).

1.4.1.2 Abortive apoptosis

It has been argued that sperm DNA damage may also be a consequence of abortive apoptosis. Throughout mammalian spermatogenesis, apoptosis usually occurs in order to 1) avoid an overproduction of germ cells and 2) selectively eliminate the damaged ones (Sinha Hikim & Swerdloff 1999). When the formation of these cells is excessive a mechanism such as apoptosis is required to match the size of the germ cell population with the supportive capacity of Sertoli cells (Rodriguez *et al.* 1997; Sinha Hikim & Swerdloff 1999). It has been reported that this apoptotic pathway is activated by the interaction between the Fas ligand (FasL) produced by the Sertoli cell and the Fas receptor in the germ cell surface (Lee *et al.* 1997; Pentikainen *et al.* 1999; Xu *et al.* 1999; Francavilla *et al.* 2000). Nonetheless, the Fas system may not be the only one promoting apoptosis in the testis since Hikim and colleagues have observed that FasL defective mice presented germ cell apoptosis (Hikim *et al.* 2003). Regardless, this proposed mechanism may not be as efficient in clearing Fas-bearing cells and thus defective germ cells may undergo chromatin remodeling and differentiate into spermatozoa, particularly in men with poor sperm parameters (Figure 1.7; Sakkas *et al.* 1999b). As a result, sperm cells displaying apoptotic markers such as Fas positivity and DNA damage will appear in the ejaculate (Sakkas *et al.* 1999b). Indeed, although a very small percentage of Fas-positive spermatozoa has been observed in normozoospermic men, individuals with abnormal sperm parameters present greater proportion of sperm expressing Fas in the ejaculate (Sakkas *et al.* 1999b). These results have further led to the hypothesis that in men with abnormal sperm parameters, cells may possibly undergo a process termed “abortive apoptosis”, i.e. they initiated the apoptotic pathway but subsequently managed to escape (Sakkas *et al.* 1999b).

Furthermore, a study carried out by McVigar and coworkers reported that no spermatozoa from men considered fertile were labeled with the anti-Fas antibody while 70% of the infertile patients exhibited samples with Fas positivity up to 55%. From these latter samples it was also found that 96% of the couples were experiencing

infertility exclusively due to a male factor, therefore suggesting a strong association between Fas signaling and male infertility (McVigar *et al.* 2004).

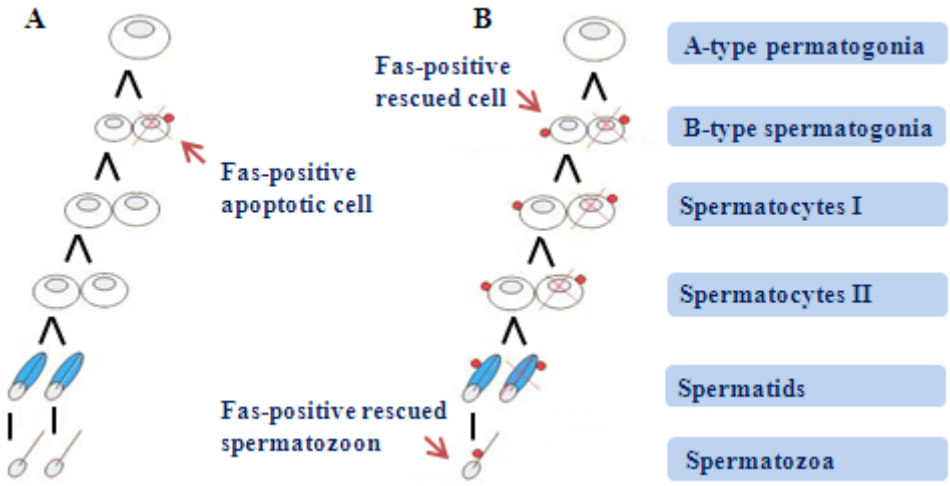

Figure 1.7. The role of Fas throughout spermatogenesis in men with normal (A) and poor (B) semen parameters.

An incorrect cell clearance via apoptosis may occur in men presenting abnormal sperm parameters leading to the production of spermatozoa with Fas positivity. These Fas-positive rescued cells suggest that 'abortive apoptosis' may have taken place (adapted from Sakkas *et al.* 1999a). Spermatocytes I - primary spermatocytes; spermatocytes II – secondary spermatocytes.

1.4.1.3 Oxidative stress

Several studies suggest that oxidative stress is the mechanism that most frequently causes defective sperm DNA (Bungum *et al.* 2012) and an association between sperm DNA damage and elevated ROS levels is well established (Twigg *et al.* 1998a; Barroso *et al.* 2000; Irvine *et al.* 2000; Henkel *et al.* 2005; Mahfouz *et al.* 2010).

Spermatozoa are particularly susceptible to ROS damage not only due to a plasma membrane rich in polyunsaturated fatty acids (PUFAs; Lewis & Agbage 2008) but also because they lack antioxidants and DNA repair systems, leaving to the oocyte the enormous task of repairing DNA and, with that, protect the offspring from anomalies that may arise from sperm DNA breaks (Bungum *et al.* 2012). Antioxidants within seminal plasma also protect sperm DNA following ejaculation (Twigg *et al.* 1998a;

Potts *et al.* 2000) but until then sperm must rely on testicular, epididymal and reproductive tract antioxidants (Tremellen 2008). However, when these antioxidants are not enough to overcome excessive ROS levels, cellular and DNA damage is frequently observed (Aitken & Krausz 2001).

Leukocytes and spermatozoa themselves, in particular immature gametes, are considered major sources of ROS in semen (Aitken & West 1990; Gomez *et al.* 1996; Ollero *et al.* 2001). Sperm presenting residual cytoplasmic droplets, known as a sign of immaturity (Aitken & West 1990; Gomez *et al.* 1996; Ollero *et al.* 2001), and samples exhibiting high concentration of leucocytes (>$1x10^6$/ml according to WHO 2010), have been associated with increased sperm DNA damage (Alvarez *et al.* 2002; Erenpreiss *et al.* 2002, Saleh *et al.* 2002b; Fischer *et al.* 2003) as a consequence of increased ROS levels (Zini *et al.* 2000, Saleh *et al.* 2002b). The association between sperm DNA damage and sperm-derived ROS proposes that DNA damage may be attributable to impaired spermiogenesis (Gomez *et al.* 1996), while the relationship between sperm DNA damage and leukocyte-derived ROS suggests that DNA damage may more likely arise from a post-testicular deficiency (e.g. epididymitis; Ochsendorf 1999).

1.4.2 Tests for the evaluation of sperm chromatin/DNA status

A multitude of assays has been developed to detect human sperm chromatin/DNA status. However, these techniques do not measure the same thing: while the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL; Gorczyca *et al.* 1993), the in situ nick translation (ISNT; Bianchi *et al.* 1993) or the single cell gel electrophoresis assay at neutral pH conditions (COMET; Haines *et al.* 1998) monitor DNA strand breaks directly, other tests such as the sperm chromatin structure assay (SCSA; Evenson *et al.* 1980), the acridine orange test (AOT; Tejada *et al.* 1984), the COMET at alkaline pH conditions (Hughes *et al.* 1996) or the sperm chromatin dispersion test (SCD; Fernandez *et al* 2003), measure the susceptibility of DNA to denaturation (indirect detection; Zini & Sigman 2009; Sakkas & Alvarez 2010). These latter assays rely on the premise that DNA of spermatozoa with a normal chromatin structure is resistant to denaturation while damaged DNA can more easily denature (Zini & Sigman 2009; Sakkas & Alvarez 2010). Other tests that depend on the

differential binding of colorimetric/fluorescent stains to detect loosely packaged chromatin have also been described (Schulte *et al.* 2010).

Initially used in somatic cells, the TUNEL assay was further adjusted to detect the incorporation of the fluorescent labeled deoxyuridine triphosphate nucleotide (dUTP) in the 3' terminus hydroxyl group of single and double stranded sperm DNA breaks using the enzyme terminal deoxynucleotidyl transferase (TdT; Gorcyza *et al.* 1993). This technique has produced consistent results but the many inter-laboratory protocol variations have limited the introduction of a consensual threshold beyond which one can infer that Assisted Reproductive Technology (ART) success might be compromised (Zini & Sigman 2009). Similarly to TUNEL, the ISNT assay detects single-stranded DNA breaks (Bianchi *et al.* 1993) by identifying the incorporation of fluorescent dUTP of single-stranded nicks in a reaction catalyzed by the template-dependent enzyme DNA polymerase I. Although it was considered a simple test, it has been shown to lack sensitivity (Twigg *et al.* 1998b). Another available test is the COMET assay, which performed at neutral pH detects double stranded DNA breaks (Singh & Stephens 1998; Erenpreiss *et al.* 2006), whereas at a higher pH senses both single and double stranded DNA breaks following denaturation (Erenpreiss *et al.* 2006; Simon & Carrell 2013). In this assay sperm cells are suspended in an agarose matrix, subjected to horizontal electrophoresis and stained with a fluorescent DNA-binding dye prior imaging analysis by dedicated software (Hughes *et al.* 1996). While short fragments of both single and double stranded DNA migrate during electrophoresis giving rise to a characteristic comet tail observable under fluorescence microscopy, high-molecular weight intact DNA segments remain in the comet head. Consequently, sperm with high levels of DNA damage present increased comet tail length and increased tail fluorescence (Hughes *et al.* 1996). Although this test is able to characterize DNA damage in a heterogeneous sperm population by measuring this parameter within each cell instead of giving the common overall measure of damaged spermatozoa in the total sperm population (Lewis & Agbaje 2008; Simon *et al.* 2010), it is expensive and labor-intensive.

In contrast, the SCD test has been described as an inexpensive method for the indirect analysis of sperm DNA fragmentation (Fernandez *et al.* 2003). In this case spermatozoa are initially immersed in an agarose matrix and exposed to acid-denaturing conditions followed by removal of nucleoproteins using a lysis solution. As an end-

product, structures termed nucleoids, with a central core and a peripheral halo of dispersed DNA loops, are formed. Sperm with no DNA damage release their DNA loops producing large halos while spermatozoa presenting a small or no halo possess fragmented DNA (Fernandez *et al.* 2003). As this test is relatively recent, few reports have addressed its predictive value on ART success (Muriel *et al.* 2006a,b; Velez de la Calle *et al.* 2008; Tavalaee *et al.* 2009; Sharbatoghli *et al.* 2012) and only one has suggested a threshold value predictive of fertilization in IVF/ICSI couples (Velez de la Calle *et al.* 2008).

Along with TUNEL, SCSA is a widely used technique and also the most studied one. Developed by Evenson and coworkers in 1980 (Evenson *et al.* 1980) this assay relies on the metachromatic shifts of the fluorochrome acridine orange (AO) following a mild acid- or heat-denaturing treatment. AO emits a green fluorescence when intercalated with intact double-stranded DNA whereas in the presence of single stranded DNA AO changes its fluorescence to red (denatured DNA; Evenson *et al.* 1999). DNA damage is measured by flow cytometry and is expressed as the DNA fragmentation index (DFI), which represents the ratio between the percentage of spermatozoa displaying red fluorescence and the total sperm population presenting fluorescence (red plus green; Evenson *et al.* 2002). SCSA is a highly reproducible test (Bungum *et al.* 2012) with useful thresholds for predicting the likelihood of ART success (Sakkas & Alvarez 2010). However, it requires specialized equipment to analyze the data (SCSAsoft®; Bungum *et al.* 2012) and it may be of no use in cases involving the assessment of DNA damage in testicular sperm or in severe oligozoospermic men (Lewis & Agbaje 2008). Similarly to SCSA, the AOT test is based on the metachromatic properties of AO to detect denatured sperm DNA. It is a simpler and less expensive method than SCSA since DNA damage can be detected by fluorescence microscopy (Tejada *et al.* 1984). Nevertheless, this technique has been shown to be heterogeneous, and rapid fading of the staining hampers visual interpretation, thus making the technique untrustworthy (Chohan *et al.* 2006).

Among the tests that detect chromatin packaging defects, Chromomycin A3 (CMA3) is able to identify sperm protamine deficiency (Bianchi *et al.* 1993; Bizzaro *et al.* 1998) either by fluorescence microscopy (Bianchi *et al.* 1993) or flow cytometry (O'Flaherty *et al.* 2008). CMA3 competes for the same DNA binding site as protamines (Berman *et al.* 1985; Gao & Patel, 1989; Bianchi *et al.* 1993), proportionally increasing

its fluorescence with decreasing protamine levels (Bianchi *et al.* 1993; Bizzaro *et al.* 1998). On the other hand, the Aniline Blue test (AB) relies on the properties of this acidic stain to bind histones present in the sperm nucleus, indicating an abnormal condensation status when sperm samples present increased blue staining (Terquem & Dadoune 1993; Dadoune *et al.* 1988; Auger *et al.* 1990). Finally, another simple colorimetric assay is the one that involves the use of Toluidine Blue (TB). This basic dye binds to DNA, increasing its staining with the increase of DNA availability, facilitated either by DNA damage or poor chromatin packaging (Erenpreiss *et al.* 2001; Erenpreisa *et al.* 2003). Notwithstanding their simplicity, the relevance of such tests in the ART context is not known.

Despite the plethora of tests available, no consensus exist on which test should be used in clinical practice (Zini & Sigman 2009; Avendaño & Oenhinger 2011), probably because of the absence of valid cut-offs and the often involvement of extensive protocols and/or expensive reagents and equipment (e.g. flow cytometer, fluorescence microscope, software) that do not exist in many standard andrology laboratories. Though specific cases may guarantee more detailed information about sperm chromatin/DNA status, it is unlikely that most laboratories can routinely introduce this parameter in semen analysis (Perreault *et al.* 2003). Furthermore, simpler methods such as the AB, TB (Erenpreiss *et al.* 2001) or SCD (Fernandez *et al.* 2003, 2005) may require specific stains and other reagents that are not usually present in standard laboratories.

Recently, we have described a simple, inexpensive and even quicker method than all mentioned above to analyze sperm chromatin status in both feline and human sperm using a simple modification of the Diff-Quik stain, a stain already implemented in most standard laboratories worldwide to evaluate sperm morphology under a standard bright-field microscope (Mota & Ramalho-Santos 2006; Sousa *et al.* 2009). In fact, due to the high correlation observed between the proportion of sperm with dark nuclei and TUNEL-positive cells and the significant increase of spermatozoa exhibiting this same staining when exposed to DNAse I, hydrogen peroxide and heat, all conditions known to promote DNA fragmentation and chromatin decondensation *in vitro*, we suggest that this staining also distinguishes sperm with abnormal/damaged sperm chromatin (either decondensed or with fragmented DNA; darker stain) from those presenting normal chromatin integrity (lighter stain; Figure 1.8). Chromatin/DNA anomalies may modify

interactions between the thiazin dye present in the Diff-Quik kit and the DNA molecule, creating more stain-binding sites and thus increase the percentage of darker sperm nuclei (Sousa *et al.* 2009).

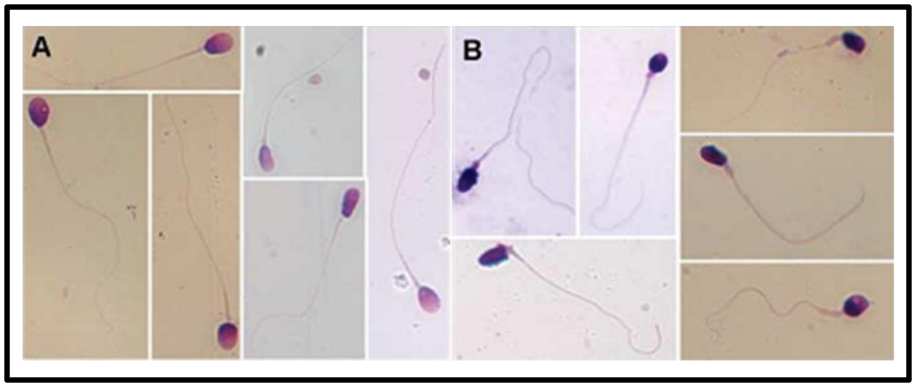

Figure 1.8. Human sperm chromatin status assessed by the modified Diff-Quik staining assay.
After fixation and coloration, sperm cells are visualized by standard bright-field microscopy. While spermatozoa with normal chromatin integrity exhibit lighter nuclear staining patterns (A), sperm with abnormal/damaged chromatin show darker staining patterns (B). This darker staining may reflect a higher amount of thiazin-bound sites generated by damaged chromatin/DNA. Different microscope/camera settings can lead to different backgrounds (from Sousa *et al.* 2009).

As formerly observed with other techniques, abnormal chromatin status assessed by the Diff-Quik staining assay was negatively correlated with embryo development rate and higher levels were associated to lower quality embryos and negative clinical pregnancies among IVF/ICSI couples. A threshold of 32% was proposed given the strong decline in embryo development and pregnancy rates when the percentage of abnormal dark staining was above this value (Sousa *et al.* 2009).

1.4.3 Influence of sperm chromatin/DNA damage on ART success

Chromatin/DNA damage has been often related to a wide variety of poor reproductive outcomes. Regardless, the existing data is controversial, particularly in what concerns *in vitro* ART treatments.

Negative relationships between sperm DNA damage and fertilization rates in both ART treatments have been described in several reports (Sun *et al.* 1997; Lopes *et al.* 1998; Benchaib *et al.* 2003; Huang *et al.* 2005; Muriel *et al.* 2006a; Velez de la Calle *et al.* 2008; Simon *et al.* 2011). In a large prospective multicenter study using the SCD assay, a DNA fragmentation value below 18% could be considered a significant predictor of oocyte fertilization among couples undergoing IVF/ICSI (Velez de la Calle *et al.* 2008). However, many others failed to observe any connection between these two parameters using several assays, including our own Diff-Quik staining (Larson *et al.* 2000; Larson-Cook *et al.* 2003; Tomlinson *et al.* 2001; Tomsu *et al.* 2002; Henkel *et al.* 2003, 2004; Lin *et al.* 2008; Sousa *et al.* 2009). A meta-analysis performed by Li *et al.* corroborated these findings by observing no differences in the fertilization rates among couples undergoing ICSI and IVF displaying high and low levels of DNA damage detected by TUNEL (Li *et al.* 2006). These results suggest that spermatozoa containing DNA damage are still capable of fertilizing an oocyte. This is not totally surprising as it is argued that the paternal genome is not expressed until the four to eight-cell stage and thus fertilization may not be dependent on sperm DNA status (Braude *et al.* 1988).

It may be predicted though that sperm DNA damage may interfere with embryo quality and development as well as pregnancy success. In fact, although no differences have been reported in some studies (Lopes *et al.* 1998; Tomlinson *et al.* 2001; Benchaib *et al.* 2003; Larson-Cook *et al.* 2003; Lin *et al.* 2008), lower embryo quality and/or impaired embryo development have been associated with sperm DNA damage assessed by a variety of methods (Sun *et al.* 1997; Tomsu *et al.* 2002; Virant-Klun *et al.* 2002; Muriel et al. 2006a; Benchaib *et al.* 2007; Velez de la Calle *et al.* 2008; Sousa *et al.* 2009; Simon *et al.* 2010, 2011). However, the relationship between sperm DNA damage and pregnancy success following IVF or ICSI is more conflicting. While some failed to detect any association between these parameters (Huang *et al.* 2005; Zini *et al.* 2005; Bungum *et al.* 2007; Lin *et al.* 2008; Velez de la Calle *et al.* 2008), others have observed that sperm DNA damage negatively influences the achievement of pregnancy following IVF and/or ICSI (Tomlinson *et al.* 2001; Benchaib *et al.* 2003, 2007; Henkel *et al.* 2003, 2004; Larson-Cook *et al.* 2003; Virro *et al.* 2004; Sousa *et al.* 2009; Simon *et al.* 2010, 2011). Benchaib and coworkers reported no pregnancies in IVF/ICSI cycles when DNA fragmentation measured by TUNEL was above 20% (Benchaib *et al.* 2003) and the same was observed by another study with a DFI value ≥27% (Larson-Cook *et*

al. 2003). Nonetheless, it has been demonstrated that full-term pregnancies can be achieved in assisted reproduction with higher levels of sperm DNA damage (Gandini *et al.* 2004; Boe-Hansen *et al.* 2006) although the chances of success may be reduced. Virro *et al.* reported decreased IVF/ICSI pregnancy rates when DFI≥30% (Virro *et al.* 2004) and others have detected the same outcome among IVF couples when TUNEL-positive spermatozoa exceeded 36.5% (Henkel *et al.* 2003). When 56% and 44% were respectively used as sperm DNA damage thresholds for both native and processed ART samples assessed by the COMET assay a similar decline in pregnancy rates after IVF was observed (Simon *et al.* 2010). Later on, comparable COMET threshold values were also found to be predictive of IVF pregnancy success by the same group (>52% and >42%, respectively; Simon *et al.* 2011). With the increasing number of reports evaluating DNA damage and its link to reproductive outcomes, meta-analyses have been performed with the purpose of increasing statistical power. According to Evenson and Wixon, couples experiencing IVF were about two times more liable to become pregnant if their DFI was below 30% (Evenson & Wixon 2006). Nevertheless, others reported that SCSA was not predictive of pregnancy rates among couples undergoing IVF and ICSI treatments (Li *et al.* 2006). In this same study, the authors found a significant reduction in pregnancy rate in IVF, but not in ICSI, in couples with high levels of sperm DNA damage assessed by TUNEL (Li *et al.* 2006). One should note that these meta-analyses involved a small subset of studies and when a larger meta-analysis was carried out a significant association between sperm DNA integrity assay outcomes and pregnancy success in both ART cycles was observed (Collins *et al.* 2008).

Several studies have addressed the impact of sperm chromatin/DNA status on ART fertility outcomes in native samples instead of after sperm selection whenever IVF and ICSI treatments are required, as it has been suggested (Tomlinson *et al.* 2001). Density gradient centrifugation (DGC) and swim up, the most common techniques performed in Andrology laboratories, either alone or in combination, allow the selection of motile spermatozoa with normal morphology to carry out ART treatments (Bungum *et al.* 2008). Additionally, they have also been shown to improve chromatin/DNA integrity levels as detected by a wide range of assays (Spanò *et al.* 1999; Tomlinson *et al.* 2001; Gandini *et al.* 2004, Marchesi *et al.* 2010). Since theoretically only the best sperm are recovered after DGC and/or swim up selection, it is argued that a certain degree of homogenization occurs (Tomlinson *et al.* 2001). Consequently, while some

DNA integrity tests, such as SCSA or TUNEL, have shown to predict ART fertilization and pregnancy in raw heterogeneous samples, some authors have reported the loss of this predictive ability in both IVF and ICSI outcomes when using homogeneous populations (Larson *et al.* 2000; Gandini *et al.* 2004; Seli *et al.* 2004; Bungum *et al.* 2008), possibly due to the "normalizing" effect promoted by the preparation techniques. Therefore this raises the question on whether our Diff-Quik staining assay and other tests actually predict ART success in processed samples that are used for ART cycles.

1.5 Male reproductive dysfunction - the role of environmental endocrine disruptors

In 1992 a meta-analysis published by Carlsen et al. revealed a decline of human sperm concentration from an average of 113 to 66 millions of spermatozoa *per* ml of semen over a 50-year period in men with no history of infertility (Carlsen *et al.* 1992). During the same phase, a similar decrease in mean semen volume was reported, thereby signaling a decrease in total sperm count (Carlsen *et al.* 1992). Subsequently, other studies have reported reduced sperm quality (concentration, motility and/or morphology; Auger *et al.* 1995; Zheng *et al.* 1997; Swan *et al.* 2000; Toft *et al.* 2004) paralleled by an increasing trend in testicular cancer and abnormalities in the male reproductive organs, such as hypospadias (abnormal localization of the male external urethral orifice) and criptorchidism (undescended testis; Toppari *et al.* 1996; Toft *et al.* 2004). Although several factors have been suggested to affect male reproductive health, cumulating evidence attribute to environmental toxicants, particularly to substances that possess (anti)oestrogenic- or antiandrogenic-like activities termed endocrine disruptors (EDs), an important role in this aetiology.

1.5.1 Environmental Endocrine disruptors - definition and sources of exposure

Due to structural similarities, certain environmental toxicants can mimic endogenous steroid hormones such as testosterone and oestradiol (Figure 1.9) and

interfere with their synthesis, binding and/or action by interacting with their receptors (Phillips & Tanphaichitr 2008) present in the testis and in many cells of the body. Of these EDs, organochlorines such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated biphenyls (PCBs) and the major and most stable dichlorodiphenyl trichloroethane (DDT) metabolite, p,p′-dichlorodiphenyldichloroethylene (p,p'-DDE), are potentially serious hazards to the general human and animal populations given their ubiquitous distribution, persistency and bioaccumulation in the food chain (WHO 2002). In fact, as these substances can be stored preferentially in body fat for long time periods, species at the top of the food chain are the most vulnerable because in addition to being directly exposed to them, they feed on animals with accumulations of these harmful compounds in their body tissue (Guillette 1994).

The representative compound of the PCDDs family, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (hereafter, TCDD), has been originated from industrial sources as an unintended by-product in the production of certain chlorophenols or chlorophenoxy acid herbicides (Saracci *et al.* 1991), waste incineration, metal production and fossil-fuel and wood combustion (Harnly *et al.* 1995). PCBs, on the other hand, besides being generated through industrial processes (Kimbrough 1995), were also used in hydraulic fluids, capacitors, lubricants, plasticizers and electrical insulators (Hauser *et al.* 2002). Although their use and manufacture has been banned in most industrialized countries together with the pesticide DDT, which was used to control malaria-bearing mosquitoes (De jager *et al.* 2006, 2009; Aneck-Hahn *et al.* 2007), the general human population continues to be exposed to PCBs, p,p'-DDE and TCDD given their presence in the air, soil, water and food (e.g. fish, meat, milk; Hauser *et al.* 2002) and strong resistance to biodegradation. Although they accumulate preferentially in the fat tissue, substantial amounts of these organochlorines have also been found in body fluids such as those associated with reproduction (e.g. follicular and seminal fluids; Heinrich-Hirsch *et al.* 1997; Tsutsumi *et al.* 1998; Dallinga *et al.* 2002; Younglai *et al.* 2002; Drbohlav *et al.* 2005; Kunisue *et al.* 2005) and also in serum (Rignell-Hydbom *et al.* 2004, 2005a; Spano *et al.* 2005; Stronati *et al.* 2006; De Jager *et al.* 2006, 2009; Mocarelli *et al.* 2008), breast milk and urine (Krauthacker *et al.* 1986; Chikuni *et al.* 1991; Schecter *et al.* 2002; Mocarelli *et al.* 2011).

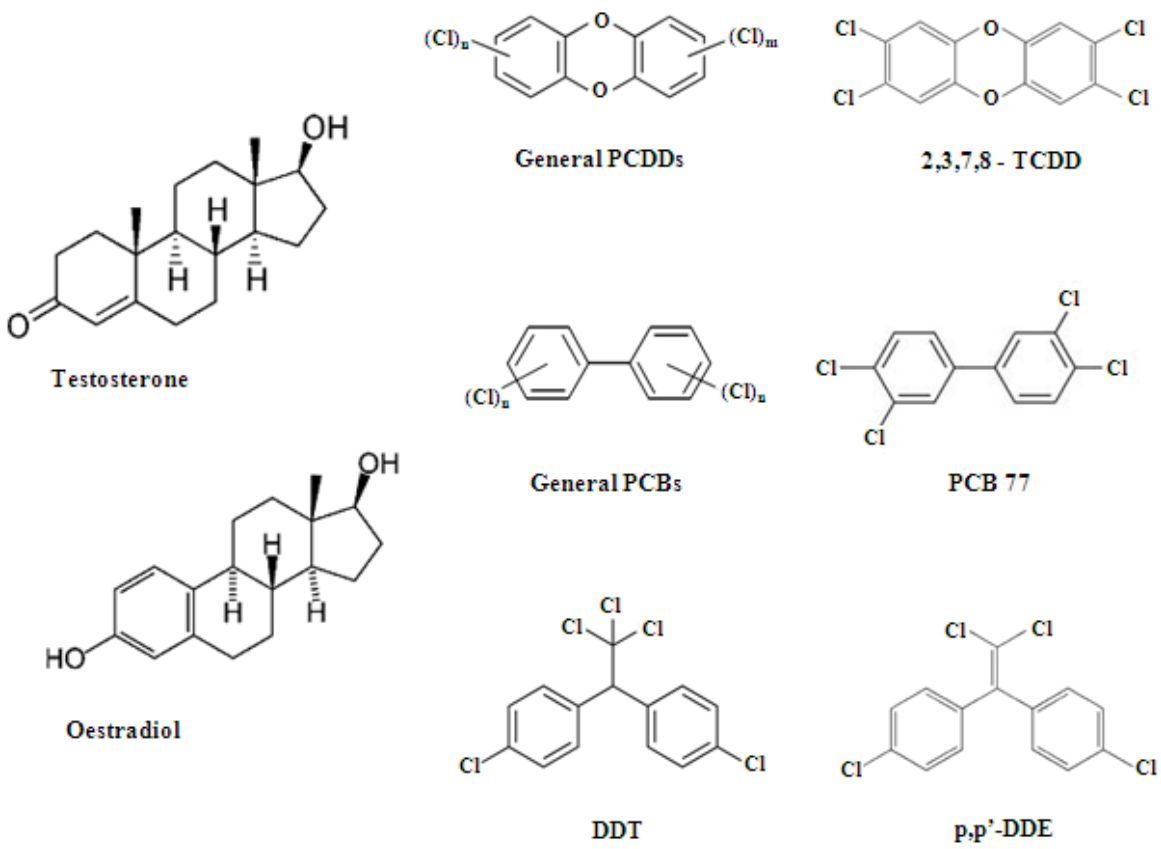

Figure 1.9. Structural representations of reproductive hormones and selected organochlorines.
Chemical structures of representative ED classes including polychlorinated dibenzo-p-dioxins (e.g. TCDD), polychlorinated biphenyls (e.g. PCB 77) and the pesticide DDT and its main metabolite p,p'-DDE are similar to the major reproductive hormones, testosterone and oestradiol (adapted from Phillips & Tanphaichitr 2008).

1.5.2 Influence of organochlorines on male reproductive status

1.5.2.1 TCDD

TCDD is the most powerful biological agent ever made by man and its interaction mostly with the aryl hydrocarbon receptor (AhR; Fernandez-Salguero *et al.* 1996; Schmidt *et al.* 1996; Mimura *et al.* 1997; Buchanan *et al.* 2000), a cytosolic-ligand transcription factor that is also expressed in testicular germ and somatic cells (Bidgoli *et al.* 2011) has shown to elicit (anti)oestrogenic responses in many tissues (Buchanan *et al.* 2000; Boverhof *et al.* 2006). Most of the research performed so far has focused on the toxicity of TCDD, on the basis that other related congeners will show the same

toxicity but with altered potency, determined by their relative agonism of the AhR and pharmacokinetics (Van den Berg *et al.* 2006).

Experimental animal data have shown adverse effects in the male reproductive system after exposure to TCDD, particularly (but not exclusively) when they were exposed during the prenatal and perinatal periods, considered critical windows of development. Although refuted by some studies (Ikeda *et al.* 2005; Bell *et al.* 2007), TCDD exposure *in utero* and throughout lactation has led to a broad spectrum of effects at low concentrations (ng/kg body weight), including decreased daily sperm production, decreased epididymal sperm count (Mably *et al.* 1992; Gray *et al.* 1995, 1997; Faqi *et al.* 1998a; Hurst *et al.* 2000), concentration, motility and viability (Arima *et al.* 2009), reduced epididymis weight and accessory glands weight (Mably *et al.* 1992; Gray *et al.* 1995, 1997; Faqi *et al.* 1998a) and altered anogenital distance, a measure of fetal androgen action (Jin *et al.* 2008).

Furthermore, besides affecting steroidogenesis and altering reproductive hormone levels (Kleeman *et al.* 1990; Adamsson *et al.* 2009; Dhanabalan *et al.* 2013), TCDD-induced alterations may also occur through other pathways, as Simanainen and colleagues have suggested after observing the same testosterone levels but variable levels of spermatogenesis defects in three different strains of rats with different susceptibilities to TCDD-induced toxicity (Simanainen *et al.* 2004). In fact, exposure to TCDD has been shown to cause oxidative stress in male rat testis by decreasing the activity of several antioxidant enzymes and increasing ROS levels and lipid peroxidation, whether rats were exposed during lactation or in adulthood (Al-Bayati *et al.* 1988; Latchoumycandane & Mathur 2002; El-Tawil & Elsaieed 2005; Jin *et al.* 2010). TCDD-induced oxidative stress has also been reported in the epididymis of adult rats (Latchoumycandane *et al.* 2003) as well as in other non-reproductive tissues (Slezak *et al.* 2000; Aly & Domenech *et al.* 2009; Pereira *et al.* 2013). Furthermore, exposure of male mice to TCDD for 24 hours caused a dose-dependent loss of MMP in epididymal sperm due to increased ROS levels, an effect that was not perceptible in spermatozoa from AhR knockout mice (Fisher *et al.* 2005). Interestingly, these authors further reported the same loss of MMP in mouse epididymal spermatozoa exposed to TCDD *in vitro*. Taken together, these findings showed not only the involvement of AhR in mediating TCDD-induced oxidative stress in the male reproductive system but also that even though many of AhR-mediated effects involve alterations at the gene

expression level, AhR is also capable of mediating non-genomic TCDD-induced effects (Fisher *et al.* 2005). This latter notion arose from the fact that spermatozoa, which also express this receptor (Khorram *et al.* 2004), presented mitochondrial dysfunction *in vitro,* although all the transcription and translation processes cease in the end of spermiogenesis (Matsuda *et al.* 1989; Lewis & Agbage 2008). Also consistent with a role of mitochondrial deregulation in TCDD toxicity is the up-regulation of the expression of a pro-apototic Bcl-2 family member, BAX, in rat testis after treatment (Jin *et al.* 2010).

In humans, the available data is limited and most of the information on the effects of TCDD originates from studies performed with the Seveso population (Italy) after an explosion in a trichlorophenol manufacturing plant that released high amounts of TCDD to the environment in 1976 (Di Domenico *et al.* 1990; Needham *et al.* 1997, 1999). In this population, TCDD exposure was associated with a lower male/female sex ratio at birth, which is a hallmark of endocrine disruption (Mocarelli *et al.* 1996), and the likelihood of fathering a female child was augmented with increasing serum TCDD concentrations from the fathers, particularly when they were younger than 19 years old at the time of the accident (Mocarelli *et al.* 2000). This sex ratio reduction was further observed in the progeny of Russian pesticide workers exposed to TCDD (Ryan *et al.* 2002) and in the offspring of rats exposed *in utero* and through lactation (Ikeda *et al.* 2005). Nevertheless, this finding has not been observed in men working in the factories that produced the TCDD-contaminated Agent Orange (Schnorr *et al.* 2001) nor in American Vietnam veterans who sprayed it during the war (Michalek *et al.* 1998). These men were exposed to TCDD at levels hundreds of times greater than the ones reported in the general population (Schecter *et al.* 1996; Schnorr *et al.* 2001). More recently, Mocarelli and co-workers have investigated sperm quality and hormone concentrations in men born between 1977 and 1984 to Seveso mothers exposed to TCDD. The breast-fed sons whose mothers had a serum dioxin concentration as low as 19 pg/g fat at conception presented lower sperm count, concentration and motility than controls and altered serum FSH and inhibin B levels, showing that *in utero* and lactational exposure of children to reasonably low TCDD concentrations can permanently decrease sperm quality (Mocarelli *et al.* 2011). Furthermore, the same authors have also found that men exposed to TCDD during childhood presented abnormal sperm concentration, motility and hormone levels even 22 years after

exposure, whereas men exposed during puberty or adulthood showed an increase or no differences in relation to controls, respectively. However, exposure during either infancy or puberty led to a permanent alteration of FSH and oestradiol levels. As semen parameters outcomes were divergent in both groups despite the same abnormalities in hormone levels (Mocarelli *et al.* 2008), other pathways rather than hormonal deregulation are probably involved in these TCDD-induced effects. These permanent alterations occurred at serum TCDD concentrations below 68 pg/g fat, which was within one order of magnitude of those in the industrialized countries in the 1970s and 1980s, leading to the hypothesis that TCDD may be responsible, at least in part, for the reported decrease in sperm quality, particularly in younger men (Mocarelli *et al.* 2008). Esquenazi et al have also found dose-related increases in the time taken to conceive (commonly dubbed as time to pregnancy) and infertility associated with individual serum TCDD levels in the Seveso women directly exposed to the organochlorine. Every 10-fold increase in serum TCDD levels corresponded to a 25% increase in time to pregnancy and approximately doubled the chances of infertility (Esquenazi *et al.* 2010). In a different study reporting an occasional episode of food poisoning, adult men exposed to TCDD presented decreased testosterone levels and reduced semen volume, probably as a result of the functional deregulation of the heavily androgen-dependent accessory glands, the secretions of which primarily constitute the seminal fluid (Dhooge *et al.* 2006). Occupational exposure to TCDD have also been shown to alter testosterone, LH and FSH levels in adult men (Egeland *et al.* 1994) but no significant associations between paternal exposure and pregnancy outcomes were detected (Townsend *et al.* 1982; Schnorr *et al.* 2001).

1.5.2.2 PCBs

PCBs are a class of compounds that comprise a total of 209 different congeners presenting varying patterns of toxicity. A small group of congeners with one or no chlorine atoms in their ortho-positions on the biphenyl molecule (i.e, mono- ortho and non-ortho, respectively) resembles the TCDD molecular conformation, binding to the same receptor, AhR, and presenting the greatest toxicity of all PCBs (Figure 1.9; Safe 1994). Nevertheless, evidences from *in vivo* studies have also shown that the non-

dioxin-like congeners are also able to induce toxic effects on diverse systems (Hansen 1999).

To determine whether environmental levels of PCBs were associated with lower sperm quality, Hauser and co-workers selected a study population without specific exposure to these organochlorines. They found that individuals presenting sperm samples below normal concentration, motility, and morphology tended to have higher serum PCBs concentrations than normozoospermic subjects (Hauser *et al.* 2002). A number of human studies have also shown inverse relationships between serum or seminal plasma PCBs within the range of current levels for most European populations and standard sperm parameters, particularly with sperm motility, which was found to be more vulnerable to PCB exposure than sperm concentration or morphology (Bush *et al.* 1986; Hauser *et al.* 2003; Ritchthoff *et al.* 2003). The negative impact of PCB congeners on this sperm parameter is suggested to be, at least in part, caused by post-testicular mechanisms, involving a reduced epididymal function (Elzanaty *et al.* 2006).

Concomitant with a decrease in sperm motility, a decline in the percentage of morphologically normal spermatozoa and more importantly, reductions in both sperm binding and penetration of hamster oocytes two hours after *in vitro* insemination were observed in men exposed *in utero* to contaminated rice oil containing high levels of PCBs and their pyrolytic products, mainly polychlorinated dibenzofurans (PCDFs; Guo *et al.* 2000). Supporting these findings, others have found impaired sperm quality and reduced sperm fertilizing ability in men postnatally exposed to this accidental contamination (Hsu *et al.* 2003). As in TCDD studies (Mocarelli *et al.* 2008, 2011), the fact that these latter outcomes were found 20 years after PCBs/PCDFs exposure indicates long-lasting damage to the male reproductive system (Hsu *et al.* 2003). Besides affecting conventional sperm parameters, chromatin/DNA integrity which is critical for the full expression of an individual fertility potential has been found to be decreased with increasing serum concentrations of PCBs in European populations (Spano *et al.* 2005; Rignell-Hydbom *et al.* 2005a; Stronati *et al.* 2006). A potential mechanism whereby PCBs may produce chromatin/DNA damage is through oxidative stress, which has been already reported to occur in animal models exposed to Aroclor 1254, a commercial mixture of PCBs (Krishnamoorthy *et al.* 2007).

Among the dioxin-like PCB congeners, the AhR-agonist non-ortho coplanar 3,3',4,4'-tetrachlorobiphenyl (PCB 77; Figure 1.9) is indisputably one of the most toxic congeners (Van Den Berg *et al.* 2006). Although scarcely studied, PCB 77 exposure has been shown to reduce male fertility in laboratory animals. Adult rats exposed *in utero* to a single dose of 100 µg/kg body weight showed altered serum testosterone levels, daily sperm production and testis weight (Faqi *et al.* 1998b). Furthermore, acute postnatal exposure to PCB 77 was found to induce several other anomalies (Hsu *et al.* 2004). Besides altering testis weight, rats exposed to 20 mg/kg PCB 77 presented decreased sperm counts and motility and increased AR rates. Moreover, sperm-oocyte penetration rates were significantly reduced at this or even lower PCB 77 concentrations (Hsu *et al.* 2004), suggesting reduced sperm fertilizing ability, a finding already reported by others in mice exposed throughout their entire development (Huang *et al.* 1998). Interestingly, Kholkute and co-workers have also reported an inhibition of fertilization rates when capacitated sperm and oocytes obtained from superovulated female mice were cultured in a PCB 77-containing medium (Kholkute *et al.* 1994). This is especially relevant as PCB 77 has been described in follicular fluid of infertile women undergoing ART (Drbohlav *et al.* 2005). Nevertheless, studies on the effects of PCB 77 on human male gametes are virtually inexistent with only one study reporting no effects on motility and viability when spermatozoa were exposed *in vitro* to this compound (Pflieger-Bruss *et al.* 2006a).

1.5.2.3 p,p'-DDE

Reproductive abnormalities attributed to p,p'-DDE exposure have been reported in a variety of wildlife species (Guillette *et al.* 1994, 1996; Fry 1995; Lundhorn 1997; Edwards *et al.* 2006) and laboratory animals (You *et al.* 1998; Loeffler & Peterson 1999). In rats, exposure to p,p'-DDE *in utero* and through lactation significantly decreased cauda epididymal sperm counts (Loeffler & Peterson 1999) and affected anogenital distance and nipple retention, both accurate indicators of endocrine disruption (You *et al.* 1998; Loeffler & Peterson 1999). p,p'-DDE was also identified as a cause of egg shell thinning in birds (Lundhorn 1997) and related to poorly organized testes, abnormally small phalli and altered testosterone levels in a population of male

juvenile alligators living on Lake Apopka, Florida, an heavily p,p'-DDE-contaminated area (Guillette *et al.* 1994, 1996). Although these alterations could be mediated by the ER, they are consistent with inhibition of androgen receptor (ARec)-mediated events. In fact, Kelce and co-workers reported that p,p'-DDE powerfully inhibits testosterone action by binding to the ARec and concomitantly suppresses testosterone-induced transcriptional activity in developing, pubertal and adult male rats, therefore suggesting that abnormalities in male sex development induced by p,p'-DDE might be mediated by interaction with the ARec (Kelce *et al.* 1995, 1997).

In humans, epidemiological studies on the effects of p,p'-DDE exposure on adequately contrasted populations have been performed. Both outdoor and indoor annual DDT spraying of dwellings as a strategic plan to control malaria vectors in several countries has put at risk male reproductive health, given the extremely high DDT and p,p'-DDE concentrations to which men were exposed to, not only through inhalation but also by dermal contact (soil and house dust) and ingestion of contaminated food and water (Aneck-Hahn *et al.* 2007). In these non-occupationally exposed individuals, whose serum p,p'-DDE concentrations were far superior than that reported for non-exposed populations (De Jager *et al.* 2006), semen quality was deeply decreased. In South African young males living in the malaria-endemic area of Limpopo Province, semen volume, sperm count, concentration, motility, morphology (Aneck-Hahn *et al.* 2007) and chromatin integrity (De Jager *et al.* 2009) were adversely affected by p,p'-DDE detected in serum. These findings were further corroborated by other studies performed in Mexican men living in the same circumstances (Ayotte *et al.* 2001; De Jager *et al.* 2006). In parallel, p,p'-DDE exposure was negatively correlated with the bioavailable/total testosterone ratio (Ayotte *et al.* 2001). These findings clearly showed that a high *p,p′*-DDE body burden is associated with poor semen parameters and altered androgen status, culminating in decreased testicular function.

Despite these results, contradictory reports have been published so far, and particular attention has been paid to an European Union-supported large-scale research project aimed at estimating the impact of organochlorines such as p,p'-DDE on human reproductive health in different populations, including Greenlandic Inuits, Swedish fishermen and Kharkiv (Ukraine) and Warsaw (Poland) men. All these studies reported no correlations between p,p'-DDE serum concentrations and semen volume (Rignell-Hydbom *et al.* 2004), sperm concentration (Rignell-Hydbom *et al.* 2004; Toft *et al.*

2006), morphology (Toft *et al.* 2006), chromatin/DNA damage (Rignell-Hydbom *et al.* 2005a; Spanò *et al.* 2005; Stronati *et al.* 2006) and markers of epididymal and accessory glands function (Rignell-Hydbom *et al.* 2005b; Elzanaty *et al.* 2006). However, sperm motility and serum testosterone and inhibin B levels in the Inuit population were altered by p,p'-DDE exposure (Giwercman *et al.* 2006; Toft *et al.* 2006). Although serum p,p'-DDE concentrations were considered elevated due to the high intake of contaminated food, which was the primary source of p,p'-DDE exposure in these men, they were at least 1000-fold lower than the ones reported in subjects from malaria-endemic regions, possibly explaining the conflicting results. In agreement, other studies have failed to detect any relationship between p,p'-DDE concentrations in individuals from the general population (with no obvious exposure to p,p'-DDE) recurring to fertility clinics and standard semen parameters (Hauser *et al.* 2003). Regardless, higher p,p'-DDE concentrations were found in the semen of infertile patients when compared to fertile men (Pant *et al.* 2004).

Overall, this means that while mostly relying on the evaluation of conventional semen parameters (e.g. sperm count, concentration, motility and morphology) to address the exposure of this and other environmental EDs (including TCDD and PCBs) at low concentrations, studies may be underestimating their potential damaging effects in human male reproductive function and fertility. Thus, the analysis of more accurate sperm functional markers such as capacitation, AR, mitochondrial function and Ca^{2+} levels, among others, formerly referred as of great importance for spermatozoa to fertilize an oocyte, is mandatory in these men. Furthermore, the fact that during their journey towards the oocyte, human spermatozoa steep in reproductive fluids that contain important amounts of these organochlorines represents an important direct route of exposure that should be considered. As observed in animals, individual EDs (e.g. the pesticide atrazine) and mixtures containing p,p'-DDE and PCBs may interact with pre-existing signaling pathways in spermatozoa and produce undesirable effects, possibly contributing to a decrease in male fertility (Campagna *et al.* 2002, 2009; Maravilla-Galván *et al.* 2009). p,p'-DDE concentrations found in human reproductive fluids have been also linked to failed fertilization (Younglai *et al.* 2002), suggesting that it may play a role in infertility.

1.6 Objectives

The present work aimed at evaluating the effects of specific organochlorines on human sperm function by using a direct approach that allowed mimicking the continuous exposure of spermatozoa to these compounds in the female reproductive tract *in vivo* (chapters 2 and 3). This study distinguishes itself from others by allowing the evaluation of more accurate functional sperm parameters, which are crucial for male fertility, than standard sperm parameters and for more than a few hours of *in vitro* exposure. Our purpose allowed using a more controlled environment to gain new insightful information on the non-genomic mechanisms of action by which each organochlorine may affect human spermatozoa. Furthermore, we intended to determine if our recently developed Diff-Quik staining assay was clinically useful in evaluating sperm chromatin status and predicting ART fertility outcomes after sperm selection (chapter 4). This assay might be practical in assessing this important marker of male fertility in large-scale EDs studies.

Chapter 2 Exposure to Persistent Environmental Organochlorines: Effects on Sperm Motility, Mitochondrial function, Energy status, Capacitation and Viability

Some of the contents of this chapter have been published in:

Mota PC*, Tavares RS*, Cordeiro M, Pereira SP, Publicover SJ, Oliveira PJ & Ramalho-Santos J. (2012) Acute effects of TCDD administration: special emphasis on testicular and sperm mitochondrial function. *Asian Pacific Journal of Reproduction* 1(4): 269-276. *Co-first authors of the paper.

Abstract

Organochlorines such as TCDD, PCB 77 and p,p'-DDE can be found at the site of fertilization *in vivo*. However, whether they are able to directly target human spermatozoa and affect important functional parameters remains to be explored. Thus, the present work was undertaken to evaluate their effects, at several concentrations, using a 3-day incubation approach that better mimic the *in vivo* putative exposure of spermatozoa to toxicants in the female reproductive tract.

While TCDD failed to induce changes in any sperm parameter studied ($p>0.05$), p,p'-DDE treatment significantly decreased both sperm MMP ($p<0.05$) and cellular ATP levels ($p<0.05$), which combined resulted in a remarkable decline of sperm motility ($p<0.05$). PCB 77 equally affected mitochondrial functionality and sperm motility following at least 2 days of exposure ($p<0.05$ and $p<0.01$, respectively) but contrarily to p,p'-DDE, it primarily caused a reduction in sperm motility followed by mitochondrial dysfunction, highlighting different mechanisms of action. Furthermore, p,p'-DDE, but not PCB 77, strongly inhibited capacitation after 24 hours of incubation to the highest concentration ($p<0.05$). Higher levels of PCB 77 and p,p'-DDE promoted cell death after at least 48 hours of exposure ($p<0.05$ and $p<0.01$, respectively).

In summary, individual exposure to p,p'-DDE or PCB 77 directly targeted spermatozoa *in vitro,* ultimately causing cells to die prematurely and thus compromising male fertility. On the other hand, TCDD treatment was found to be ineffective. It seems therefore that the reported effects of TCDD on human sperm parameters may rather result from alterations at the level of spermatogenesis and/or during the transit through the male reproductive tract. Lastly, one should also keep in mind that more relevant concentrations than the ones employed here may perhaps hamper human sperm function and further compromise male fertility due to their potential synergistic effects *in vivo*.

2.1 Material and methods

2.1.1 Materials

All reagents were provided by Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. 99% chemically pure p,p'-DDE, PCB 77 and TCDD (LGC Standards, Barcelona, Spain) were dissolved in dimethyl sulphoxide (DMSO) to final stock concentrations of 62.88 mM, 3.42 mM and 0.207 mM, respectively.

2.1.2 Human Biological samples

Fresh sperm samples from healthy subjects undergoing routine semen analysis or fertility treatments in the Human Reproduction Service at University Hospitals of Coimbra were used in agreement with the appropriate ethical and Internal Review Board of the participating Institution. All individuals signed informed consent forms. Samples were obtained by masturbation after 3 to 5 days of sexual abstinence and seminal analysis was performed according to the WHO guidelines (WHO 2010). All samples used were normozoospermic and had no detectable leukocytes or any other round cells. Spermatozoa were prepared by DGC (Isolate® Sperm Separation Medium, Irvine Scientific, CA, USA) and were allowed to capacitate in Sperm Preparation Medium (SPM; Origio, Medicult, Jyllinge, Denmark) for at least 3 hours at 37°C and 5% CO_2 prior exposure to environmental endocrine disruptors (except for the capacitation status assay as mentioned below). All samples used in this study had more than 80% viable spermatozoa after processing.

2.1.3 Exposure to p,p'-DDE, TCDD and PCB 77

In order to mimic sperm continuous exposure to p,p'-DDE, TCDD and PCB 77 in the female reproductive tract *in vivo*, sperm cells were independently exposed to p,p'-DDE (1-100 µM), TCDD (1 nM and 1 µM) or PCB 77 (1- 4 µg/ml = 3.4-13.7 µM) for 3 days at 37°C and 5% CO_2. 10 million cells/ml were used for each condition and kept in a phosphate buffered saline medium (PBS, GIBCO - Invitrogen, Paisley, UK) containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 1.0 mM Na-pyruvate, 10.0

mM Na-lactate, 0.3% (wt/vol) bovine serum albumin (BSA) and 1% (v/v) penicillin/streptomycin, pH 7.2-7.4, previously described as optimal to keep spermatozoa for longer time periods (Amaral *et al.* 2011). Proper controls were performed by adding 0.3%, 0.5% or 0.4% (v/v) DMSO to the medium, according to the endocrine disruptor used (p,p'-DDE, TCDD or PCB 77, respectively). Several sperm parameters were assessed daily and the medium were changed every day after a 10-minute centrifugation at 528 x g.

2.1.3.1 Viability and mitochondrial function

In order to evaluate membrane integrity and MMP, indicative of mitochondrial function, spermatozoa were incubated with 100 nM SYBR14 and 240 nM propidium iodide (PI; LIVE/DEAD Sperm Vitality kit, Molecular Probes, Eugene, OR, USA) coupled with 2 µM JC-1 (5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; Molecular Probes) for 20 minutes at 37°C in the dark (Amaral & Ramalho-Santos 2010; Baptista *et al.* 2013). SYBR14 is a cell membrane-permeant fluorescent dye that stains all sperm nuclei green whereas PI only stains red the sperm nuclei with compromised membrane integrity, overpowering the SYBR14-signal. The JC-1 dye exhibits potential-dependent accumulation in mitochondria, shifting its fluorescent emission from orange/reddish to green according to the high or low MMP, respectively. Therefore, this assay distinguishes between highly and less active mitochondria, respectively (Figure 2.1). Sperm cells were classified according to the staining patterns of the head (green or red) and midpiece (green or reddish) and results were expressed as percentage of viable spermatozoa or with highly active MMP. For each slide, at least two hundred spermatozoa were observed in different fields using a Zeiss Axioplan 2 Imaging fluorescence microscope equipped with a triple band pass filter (Carl Zeiss, Göttingen, Germany).

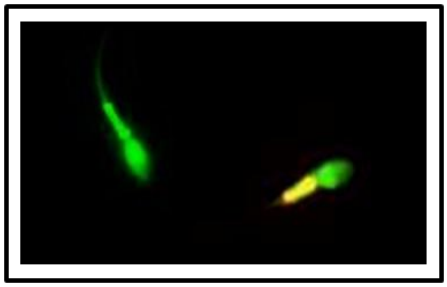

Figure 2.1. Sperm MMP assessed by the JC-1 fluorescent marker.

The spermatozoon on the left present low MMP (green midpiece) while the spermatozoon with a orange/reddish midpiece exhibit highly active mitochondria (high MMP; right side). The image was taken using a fluorescence microscope under a 100x objective magnification.

2.1.3.2 Motility

Motility was assessed by phase contrast microscopy (Nikon Instruments Inc, Melville, NY, USA). Two hundred spermatozoa were scored in four different fields and results were expressed as total motility, i.e. the percentage of spermatozoa displaying progressive motility in addition to cells commonly identified as motile but that do not progress (*in situ* motility).

2.1.3.3 ATP content levels

Sperm intracellular ATP levels were determined accordingly to the previous described methodology (Amaral *et al.* 2006; Sousa *et al.* 2013). After exposing spermatozoa to 0.6 M perchloric acid and 25 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na), sperm cells were centrifuged at 18 470 x g for 2 minutes at 4°C. The supernatants were neutralized on ice with a drop wise addition of 3 M KOH in 1.5 M Tris and analyzed for ATP levels in a Beckman System Gold high-performance liquid chromatography (HPLC) system that included a 126 Binary Pump Model and a 166 Variable UV model detector. Detection was performed at 254 nm and the column used was a Lichrosphere 100 RP-18 (5 mm; Merck, Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH_2PO_4; pH 6.5) and 1.0% methanol was

carried out with a constant flow rate of 1 ml/minute. Peak identity was established by following the retention time of standards.

2.1.3.4 Capacitation status

To further analyse if these organochlorines interfered with the capacitation process, spermatozoa were allowed to capacitate under p,p'-DDE, TCDD or PCB 77 exposure in the above PBS-based medium supplemented with 25 mM $NaHCO_3$ for the entire set of experiments. Capacitation was evaluated daily through the detection of phosphorylated tyrosines (Ramalho-Santos *et al.* 2007). Briefly, spermatozoa were fixed with 2% (v/v) formaldehyde in PBS for 40 minutes, permeabilized [1% (v/v) Triton X-100 in PBS; 20 minutes] and blocked with 0.1% (wt/v) BSA and 100 mM glycine in PBS for 30 minutes at room temperature. Afterwards, cells were incubated with a rabbit anti-human phosphotyrosine polyclonal antibody (1:10; Zymed, CA, USA) overnight at 37°C and washed with 0.1% Triton X-100 in PBS for 30 minutes. Spermatozoa were then exposed to an anti-rabbit secondary antibody (Texas Red®-X Goat Anti-Rabbit IgG; 1:200; Molecular Probes) for 1 hour at 37°C followed by a 15-minute wash with 0.1% Triton X-100 in PBS. A final labeling with the DNA-binding dye 4,6-diamino-2-phenyl-indole (DAPI; Molecular Probes) was used to counterstain spermatozoa nuclei. Only fully capacitated spermatozoa, i.e spermatozoa whose tails were entirely labeled, were considered positive (Figure 2.2). For each slide, at least two hundred spermatozoa were observed in different fields using a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss).

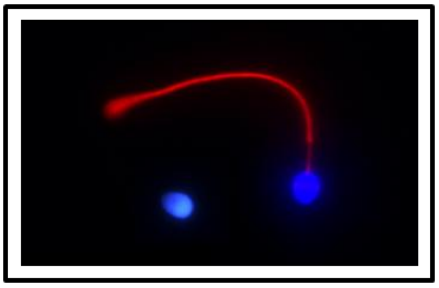

Figure 2.2. Tyrosine phosphorylation assessed by immunocytochemistry.

While a fully capacitated spermatozoon exhibited red fluorescence in both the flagellum and midpiece, the non-capacitated spermatozoon only displayed the blue DAPI counterstain. The image was taken using a fluorescence microscope under a 100x objective magnification.

2.1.4 Statistical analysis

Statistical analysis was carried out using the SPSS version 20.0 software for Windows (SPSS Inc., Chicago, IL, USA). Values are expressed as mean percentage ± standard error of mean (SEM) relative to control (except for ATP levels). All variables were checked for normal distribution using the Shapiro-Wilk test and comparisons between concentrations and their respective controls were performed by paired t-test. Further comparisons between all concentrations within each endocrine disruptor set of experiments were performed by one-way analysis of variance (ANOVA). $p \leq 0.05$ was considered significant.

2.2 Results

2.2.1 p,p'-DDE and PCB 77 compromise sperm survival

Although no effect on sperm viability was noticed during TCDD treatment ($p>0.05$ Figure 2.3A), both 100 and 50 µM p,p'-DDE significantly reduced sperm survival when compared to control groups (Figure 2.3B). In fact, while 50 µM p,p'-DDE compromised cell viability only after 3 days of exposure ($p<0.05$), 100 µM p,p'-DDE markedly decreased sperm viability earlier in time, as observed by the decreased percentage of spermatozoa with green head after 2 days of treatment ($p<0.01$, Figure 2.2B). Nevertheless, its maximal effect was only reached after 3 days of exposure ($p<0.001$, Figure 2.3B). Comparisons among concentrations revealed that both 100 and 50 µM p,p'-DDE reduced cell survival more drastically than smaller doses (25, 10 and 1 µM) after 2 and 3 days of exposure ($p<0.05$, Figure 2.3B). Furthermore, the effect promoted by 100 µM p,p'-DDE was so great that it even promoted higher cell death than 50 µM p,p'-DDE at both days 2 and 3 ($p<0.05$, Figure 2.3B).

Finally, exposure to 13.7 µM PCB 77 revealed a significant decline in sperm survival after 2 and 3 days of exposure, when compared to their respective controls ($p<0.05$, Figure 2.3C). No effect was observed at lower doses ($p>0.05$, Figure 2.3C). Following 3 days of exposure, cell viability significantly differed between the highest and the lower (3.4 and 6.8 µM) PCB 77 concentrations ($p<0.05$; Figure 2.3C).

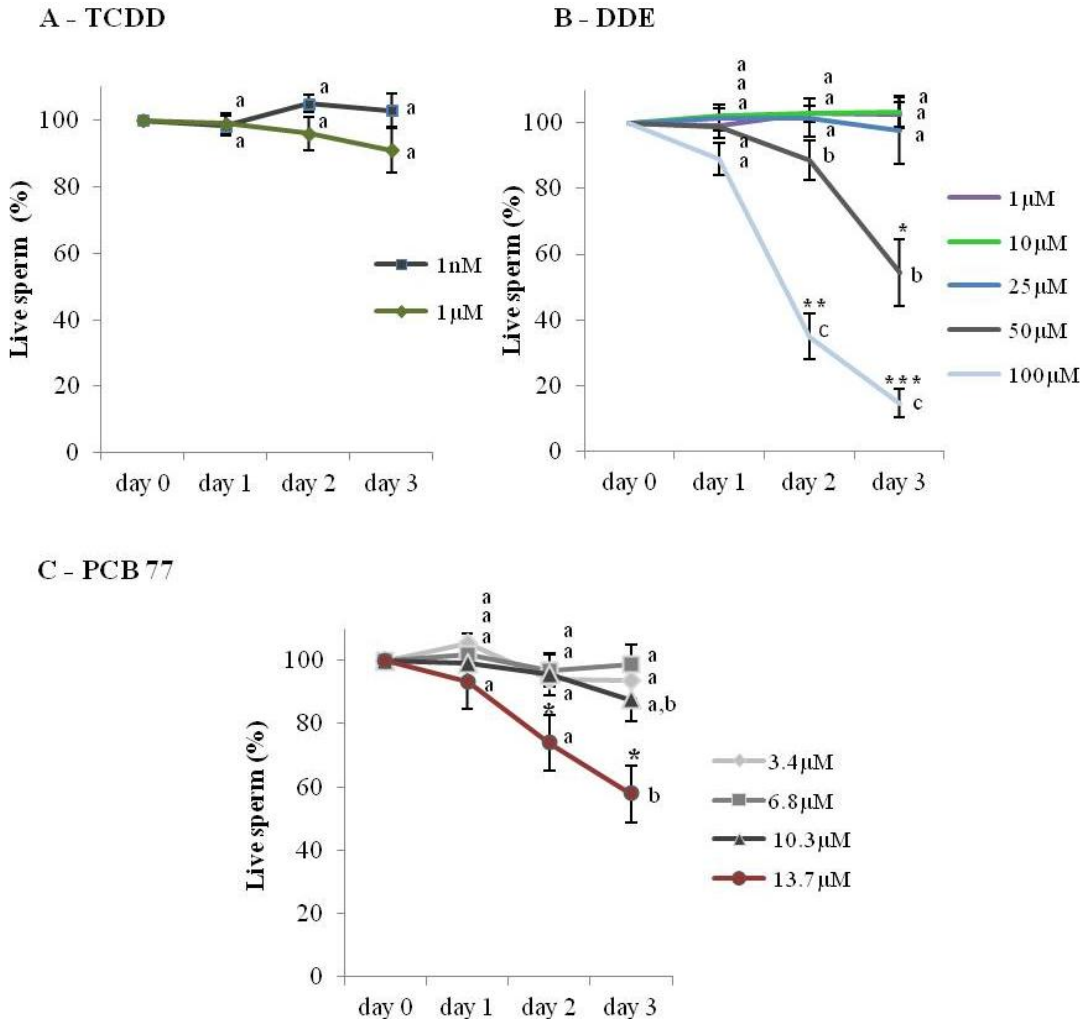

Figure 2.3. Daily percentages of viability in spermatozoa continuously exposed to **A)** TCDD (n=6), **B)** p,p'-DDE (n=5) or **C)** PCB 77 concentrations (n=6) for 3 days at 37ºC and 5% CO_2.

Results represent mean percentage ± SEM relative to control (% viable cells / % viable cells in DMSO x 100). * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) symbolize significant differences when compared to control. Different letters denote statistical differences between concentrations within the same day of exposure ($p<0.05$).

Longer incubation periods were not carried out since the percentages of viable sperm in control groups were below 50% after 4 days of exposure (data not shown).

2.2.2 p,p'-DDE and PCB 77 diminish both mitochondrial function and motility

TCDD induced no effects on both MMP and motility ($p>0.05$, Figure 2.4). Yet, in

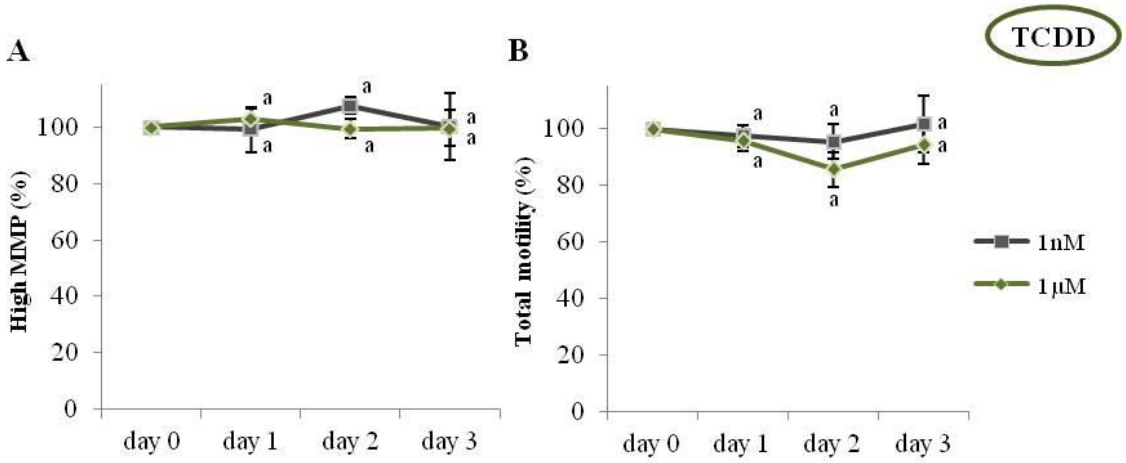

Figure 2.4. Daily percentages of **A)** high MMP and **B)** total motility in spermatozoa continuously treated with different TCDD concentrations for 3 days at 37ºC and 5% CO_2 (n=6).

Results represent mean percentage ± SEM relative to the respective control (% sperm parameter / % sperm parameter in DMSO x 100). No significant differences were observed when compared to controls ($p>0.05$). Similar letters denote no statistical differences between concentrations within the same day of exposure ($p>0.05$).

cells exposed to p,p'-DDE both parameters were significantly decreased when higher concentrations were used (Figure 2.5). While 24 hours of exposure to the maximal p,p'-DDE concentration was enough to adversely affect sperm motility ($p<0.01$, Figure 2.5B) and dramatically decrease the proportion of sperm with highly functional mitochondria ($p<0.001$, Figure 2.5A), 50 µM p,p'-DDE was only able to reduce both parameters following 48 hours of exposure (Figure 2.5). Moreover, the effects promoted by 100 and 50 µM p,p'-DDE treatments were found to be more severe in subsequent days (Figure 2.5).

Although sperm viability became compromised at these p,p'-DDE concentrations, both the proportions of sperm motility and MMP had already decreased, showing that the alterations in both parameters preceded cell death. A 3-day incubation with 25 µM p,p'-DDE promoted a significant decrease in the proportion of spermatozoa with highly functional mitochondria ($p<0.01$; Figure 2.5A); however, no difference was observed in total motility ($p>0.05$; Figure 2.5B). Comparisons between concentrations revealed significant differences in both the percentages of spermatozoa with high MMP and total motility already at day 1 ($p<0.05$, Figure 2.5).

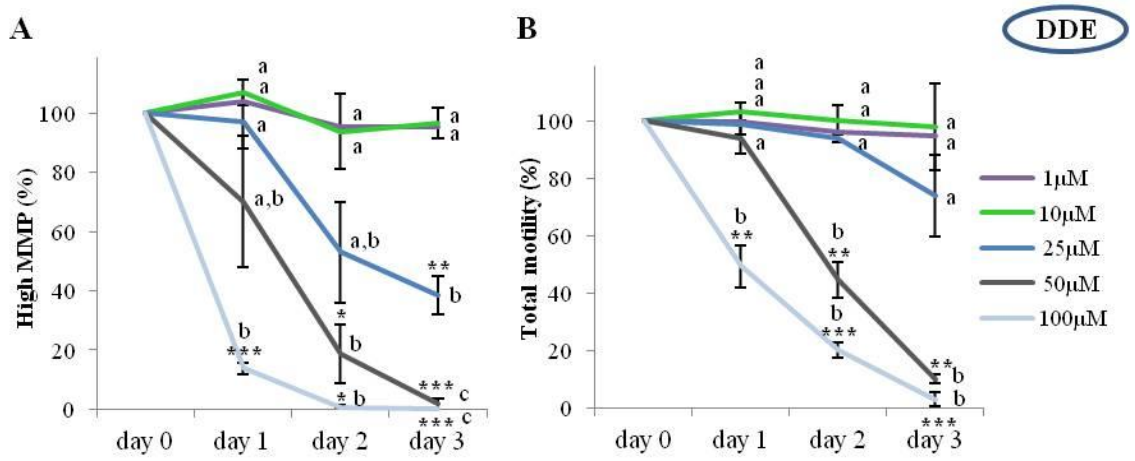

Figure 2.5. Daily percentages of **A)** high MMP and **B)** total motility in spermatozoa continuously exposed to different p,p'-DDE concentrations for 3 days at 37ºC and 5% CO_2 (n=5).

Results represent mean percentage ± SEM relative to the respective control (% sperm parameter / % sperm parameter in DMSO x 100). * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) symbolize significant differences when compared to controls. Different letters denote statistical differences between concentrations within the same day of exposure ($p<0.05$).

Exposure to 13.7 µM PCB 77 clearly provoked a significant decline in both motility and high MMP following 2 and 3 days of exposure, when compared to controls (Figure 2.6). However, contrarily to p,p'-DDE exposure, 13.7 µM PCB 77 seemed to primarily induced a severe decrease in motility ($p<0.01$, Figure 2.6B) and then adversely affect MMP ($p<0.05$ and $p<0.001$ after 2 and 3 days of treatment, respectively, Figure 2.6A). In fact, while less than 20% of spermatozoa were motile, 64.7±10.8% and 36.5±10.6% of spermatozoa still presented high MMP when exposed to 13.7 µM PCB 77 at both days 2 and 3, respectively (Figure 2.6).

Though sperm survival was significantly reduced at the same time periods in which motility and MMP were affected, the greater percentages of viable sperm using the same samples indicate that both motility and MMP were impaired before cell death (Figures 2.3C and 2.6). Comparisons between concentrations have shown that the percentage of spermatozoa with highly functional mitochondria at the highest dose tested was significantly lower than the one observed in the 6.8 µM PCB 77-exposed group following 2 days of incubation and from all the others after 3 days of exposure ($p<0.05$, Figure 2.6A). Finally, spermatozoa incubated with 13.7 µM PCB 77 presented lower motility when compared to all other concentrations following 2 and 3 days of

exposure ($p<0.05$, Figure 2.6B). Due to the observed effects on sperm MMP, motility and viability only the highest PCB 77 concentration was used in further studies.

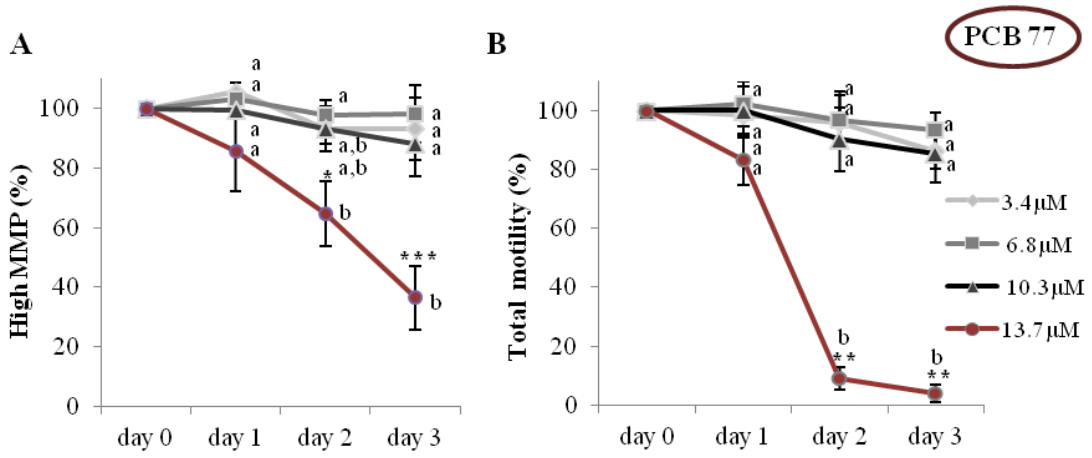

Figure 2.6. Daily percentages of **A)** high MMP and **B)** total motility in spermatozoa continuously exposed to different PCB 77 concentrations for 3 days at 37ºC and 5% CO_2 (n=6).

Results represent mean percentage ± SEM relative to the respective controls (% high MMP or total motility / % high MMP or total motility in DMSO x 100). * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) symbolize significant differences when compared to the controls. Different letters denote statistical differences between concentrations within the same day of exposure ($p<0.05$).

2.2.3 p,p'-DDE reduces cellular ATP levels

Individual exposure to p,p'-DDE adversely affected both motility and MMP before compromising sperm viability (Figures 2.3B and 2.5). Considering that ATP, which is mainly produced by mitochondria, is required for several purposes including sperm motility and viability maintenance (Ramalho-Santos *et al.* 2009), we hypothesized that ATP levels might be affected. Detection of cellular ATP levels by HPLC was only performed in spermatozoa exposed to p,p'-DDE at time points where viability was unaffected and at concentrations found to negatively influence sperm mitochondrial function and motility. Exposure to 100 µM p,p'-DDE significantly decreased sperm ATP levels after 24 hours of exposure when compared to control ($p<0.001$, Figure 2.7). Additionally, ATP levels were diminished in sperm treated with 50 µM p,p'-DDE after 1 and 2 days of exposure ($p<0.001$ and $p<0.05$, respectively; Figure 2.7) but not when exposed to 25 µM p,p'-DDE ($p>0.05$, Figure 2.7). Comparisons between concentrations

revealed that after 24 hours of incubation ATP levels from spermatozoa exposed to 25 µM p,p'-DDE were significantly higher than in those exposed to 100 µM p,p'-DDE ($p<0.05$, Figure 2.7). Accordingly, ATP levels from spermatozoa treated with 25 µM p,p'-DDE were significantly higher than the ones detected in spermatozoa exposed to 50 µM p,p'-DDE following 2 days of treatment ($p<0.05$, Figure 2.7).

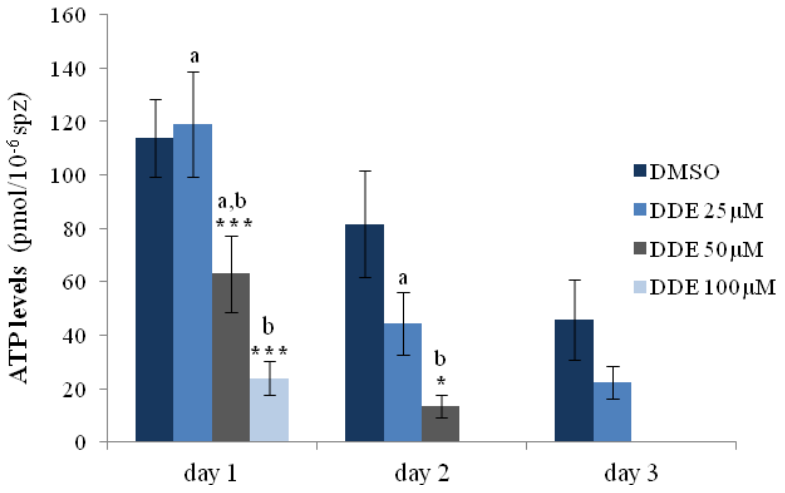

Figure 2.7. Daily ATP levels in spermatozoa continuously exposed to different p,p'-DDE concentrations for 3 days at 37ºC and 5% CO_2 (n=4).

Results represent mean levels of ATP (in picamols/ million of spermatozoa) ± SEM. * ($p<0.05$) and *** ($p<0.001$) symbolize significant differences in comparison to the respective controls. Different letters denote statistical differences between concentrations within the same day of exposure ($p<0.05$).

2.2.4 p,p'-DDE hampers sperm capacitation

Sperm capacitation entails numerous physiological and functional alterations, including the phosphorylation of tyrosine residues (Ramalho-Santos *et al.* 2007). The detection of such phosphotyrosines is therefore broadly accepted as a good marker of capacitation.

TCDD and PCB 77 concentrations failed to induce significant changes in sperm tyrosine phosphorylation under capacitating conditions ($p>0.05$, Figure 2.8A). Furthermore, comparisons between TCDD concentrations showed no differences within each day of exposure and the same was observed when the highest concentration of the dioxin-like PCB 77 was compared to both doses of TCDD ($p>0.05$, Figure 2.8B). On

the other hand, continuous exposure of non-capacitated spermatozoa to 100 µM p,p'-DDE significantly inhibited sperm capacitation after 24 hours of exposure ($p<0.05$, Figure 2.8B). Furthermore, this inhibition still persisted after 2 and 3 days of exposure ($p<0.01$, Figure 2.8B) despite the loss of viability (Figure 2.3B). Exposure to lower concentrations failed to affect the proportion of capacitated sperm ($p>0.05$, Figure 2.8B). Differences between p,p'-DDE concentrations were observed at both days 2 and 3 ($p<0.05$; Figure 2.8B).

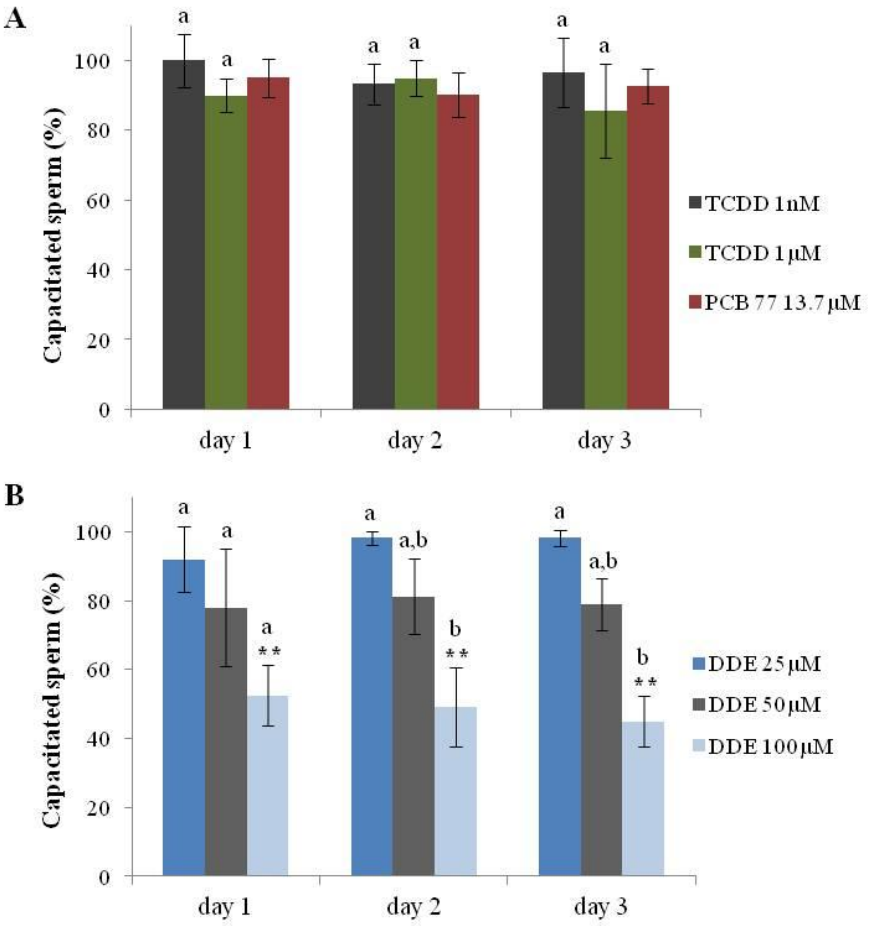

Figure 2.8. Daily percentage of capacitated spermatozoa continuously treated with **A)** TCDD or PCB 77 (n=6) and **B)** p,p'-DDE (n=5) concentrations for 3 days at 37ºC and 5% CO_2.

Results represent mean percentage ± SEM relative to the respective controls (% capacitated cells / % capacitated cells in DMSO x 100). ***** ($p<0.05$) and ****** ($p<0.01$) symbolize significant differences when compared to controls. Different letters denote statistical differences between concentrations within the same day of exposure ($p<0.05$). Comparisons between TCDD and PCB 77 concentrations retrieved no differences ($p>0.05$).

2.3 Discussion

In recent years there has been an emerging concern regarding the putative decline of male reproductive health induced by environmental organochlorines. As these organochlorines can be found in reproductive tissues and secretions, it is imperative to analyse whether compounds such as TCDD, PCB 77 and p,p'-DDE may directly target human spermatozoa altering important functional parameters that may jeopardize further spermatozoa-oocyte interactions and fertilization. Moreover, since spermatozoa are transcriptionally inactive, they are excellent models for the analysis of non-genomic effects promoted by environmental pollutants/endocrine disruptors. Herein, using a long-term protocol we were able to pinpoint effects that had been undetected so far.

Contrarily to previous reports that established a decline in sperm motility following a long-term exposure to TCDD in different stages of life (El-Sabeawy *et al.* 1998; Mocarelli *et al.* 2008, 2011; Arima *et al.* 2009), we failed to detect any *in vitro* alterations in motility as well as in viability, mitochondrial function and capacitation, even at 1 μM TCDD, a concentration far greater than background levels (Schecter *et al.* 1996; Tsutsumi *et al.* 1998). Yet, our results are in accordance with that of Hanf and colleagues who also reported a lack of effect on human sperm motility *in vitro* after 60 hours of incubation (Hanf *et al.* 1992). In the only study that addressed the effect of TCDD in sperm mitochondria, Fisher et al. described an increased proportion of C57BL/6 mouse epididymal sperm with low MMP when the animals were injected with TCDD for 24 hours, and this alteration was AhR-dependent. The same effect on MMP was further observed by the same authors in spermatozoa exposed to 1 or 5 nM TCDD for 45 minutes *in vitro*, suggesting that sperm cells are direct targets of TCDD (Fisher *et al.* 2005). In this case, either human sperm are more resistant to TCDD, as suggested by the characteristics of the human AhR, more closely related to the allele present in TCDD resistant DBA/2J mice (Ema *et al.* 1994), or mitochondria from human sperm differ greatly from their mouse sperm counterparts. The dioxin-like PCB 77, on the other hand, was able to promote a decrease in both motility and high sperm MMP, finally leading to cell death at the highest concentration tested. The doses employed here were higher than those chosen for TCDD given that the PCB 77 toxicity equivalency factor (TEF), which is an estimate of its toxicity in relation to the reference dioxin TCDD in assumed AhR-mediated events, is 0.0001 meaning that PCB 77 is 10

000 times less potent than TCDD (Van der Berg *et al.* 2006; U.S. Environmental Protection Agency 2010). In light of our findings it seems unlikely that the effects promoted by PCB 77 are mediated by the AhR in spermatozoa.

In vitro studies regarding the direct impact of PCBs on spermatozoa are scarce and evidence suggest that they may not affect sperm function greatly, although all studies have used fairly shorter incubation periods than we did. In fact, exposure to 5 and 10 µg/ml PCB 77 failed to produce any effect on both human sperm flagellar movement and viability, despite the high doses tested, and the same lack of effects was observed in single or combined exposure to the dioxin-like PCB congeners 118 (2,3',4,4',5-pentachlorobiphenyl) and 126 (3,3',4,4',5-pentachlorobiphenyl), and the di-ortho PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) for 5 hours (Pflieger-Bruss *et al.* 2006a,b). It was only when porcine sperm cells were treated with an organochlorine mixture containing several PCB congeners (e.g. PCB 77) and a panoply of other compounds, including p,p'-DDE, that both sperm parameters were found reduced (Campagna *et al.* 2002, 2009). The idea that sperm motility is particularly susceptible to PCBs action reported in both human and animal *in vivo* studies (Bush *et al.* 1986; Hauser *et al.* 2003; Ritchthoff *et al.* 2003; Hsu *et al.* 2004) is further strengthened in this study.

Mitochondria play a central role in sperm function due to their participation in numerous events/pathways crucial for fertilization (Ramalho-Santos *et al.* 2009; Amaral *et al.* 2013a). To date, no study has addressed the effect of PCB 77 on sperm or even testis mitochondria. Taking advantage of isolated rat liver mitochondria, which is normally used as a general toxicological indicator, Nishihara et al. found no obvious effects in mitochondrial functionality following PCB 77 treatment (Nishihara *et al.* 1985), which clearly contradicts our findings. Nonetheless, this is not surprising as we have previously reported that liver mitochondria differ from their testis counterparts in sensitivity, thus indicating that the former is not the best toxicological model to evaluate the possible effects of environmental toxicants on male fertility (Mota *et al.* 2011). In parallel, this study also showed that p,p'-DDE promotes mitochondrial dysfunction in the testis (Mota *et al.* 2011). Accordingly, p,p'-DDE was able to reduce the percentages of spermatozoa with highly functional mitochondria and also cellular ATP levels in the present study. The combined decrease of both parameters was associated with a clear decline in motility. As pinpointed in the introduction section, numerous reports have

established the important contribution of mitochondria in supplying energy required for sperm motility. Additionally, mitochondrial function has been extensively correlated with several parameters including sperm viability (Papaioannou *et al.* 1997; Spinaci *et al.* 2005) and fertilizing ability (Kasai *et al.* 2002; Marchetti *et al.* 2002; Gallon *et al.* 2006). When considering our results it is tempting to suggest that proper mitochondrial function is disrupted by p,p'-DDE exposure leading to a severe impairment of motility. Although this is not untrue, we may also hypothesize that p,p'-DDE may disturb other ATP-generator processes and thus contribute for the observed decline in motility. Interestingly, exposure to 13.7 µM PCB 77 induced a more prominent decrease in motility than in MMP at the same time points which clearly indicates a different mechanism of action from that of p,p'-DDE. Similarly to p,p'-DDE, other organochlorine pesticides including alachlor were able to decrease human sperm motility and affect mitochondria by promoting a decline in MMP *in vitro* (Grizard *et al.* 2007), further supporting our results.

To become functionally competent cells spermatozoa must undergo capacitation. However, little is known about the effects of environmental toxicants in such process. By incubating human spermatozoa in a capacitating medium containing our organochlorines we found that neither TCDD nor PCB 77 influenced sperm capacitation, as determined by tyrosine phosphorylation. Yet, p,p'-DDE significantly inhibited capacitation at the highest concentration. As ejaculated spermatozoa cannot immediately fertilize an oocyte, requiring a preparatory period in the female reproductive tract to capacitate, one may hypothesize that inhibition of this process by p,p'-DDE may prevent all further key steps, i.e. AR, sperm-oocyte fusion and fertilization. Our results are in agreement with other studies that have reported the same inhibition in spermatozoa exposed to atrazine *in vitro* (Maravilla-Galván *et al.* 2009) and to the pesticide fenvalerate *in vivo* (Shi *et al.* 2011). Conversely, Campagna and coworkers described the opposite effect when testing a mixture of several toxicants including PCBs and p,p'-DDE in spermatozoa for 2 and 4 hours (Campagna *et al.* 2009).

Taken together, individual exposure to PCB 77 or p,p'-DDE was shown to promote non-genomic effects, affecting sperm functional parameters important for fertilization, some of which often ignored in both *in vivo* and *in vitro* studies on this topic. Furthermore, using a 3-day incubation approach we observed that all

organochlorines act differently. In contrast to both PCB 77 and p,p'-DDE, that directly targeted human spermatozoa and compromised male fertility, no alterations were found in this study using TCDD. Therefore, the effects described for TCDD in human sperm reported by others seem to be indirect, i.e. they may arise from alterations at the spermatogenesis level and/or during the transit through the male reproductive tract. Finally, although the concentrations used here were not environmentally-relevant, one should also keep in mind that spermatozoa are directly exposed to a wide broad of environmental endocrine disruptors (Schecter *et al.* 1996; Kumar *et al.* 2000; Dallinga *et al.* 2002; Younglai *et al.* 2002; Pant *et al.* 2004; Drbohlav *et al.* 2005; Kunisue *et al.* 2005) and little is known about possible synergistic effects of these and other compounds on their functional parameters, an issue that should be considered in further studies. Thus, when acting together, more relevant concentrations may eventually hamper human sperm function and compromise male fertility.

Chapter 3 Exposure to Persistent Environmental Organochlorines: Effects on Intracellular Ca^{2+} levels and AR

Some of the contents of this chapter have been published in:

Tavares RS, Mansell S, Barratt CL, Wilson SM, Publicover SJ & Ramalho-Santos J. (2013) p,p'-DDE activates CatSper and compromises human sperm function at environmentally-relevant concentrations. *Human Reproduction* 28:3167-3177.

Mota PC*, Tavares RS*, Cordeiro M, Pereira SP, Publicover SJ, Oliveira PJ & Ramalho-Santos J. (2012) Acute effects of TCDD administration: special emphasis on testicular and sperm mitochondrial function. *Asian Pacific Journal of Reproduction* 1(4): 269-276. *Co-first authors of the paper.

Abstract

The environmental organochlorines TCDD, PCB 77 and p, p′-DDE may promote non-genomic actions and interact directly with pre-existing signaling pathways, as already reported in other cell types. However, although dioxins, PCBs and pesticides are often found in both male and female reproductive fluids, their effects in gamete function, particularly in what concerns Ca^{2+} homeostasis and AR remain to clarify.

While both TCDD and PCB 77 did not affect $[Ca^{2+}]_i$ greatly, p,p'-DDE was able to rapidly increase $[Ca^{2+}]_i$ ($p<0.05$) in most cells with magnitudes of response up to 200%, without affecting sperm viability (chapter 2). Strikingly, even at extremely low doses such as 1 pM and 1 nM p,p'-DDE affected intracellular Ca^{2+} homeostasis in 21.1±3.0% and 28.0±10.65% of cells, respectively ($p<0.05$). Furthermore, experiments performed in a low Ca^{2+} medium demonstrated that extracellular Ca^{2+} influx was responsible for this Ca^{2+} elevation ($p<0.01$). Mibefradil and NNC 55-0396, both inhibitors of the sperm-specific Catsper channel, reversed the p,p'-DDE-induced $[Ca^{2+}]_i$ rise, suggesting the participation of Catsper in this process ($p<0.05$). In fact, whole cell patch-clamp recordings confirmed Catsper as a target of p,p'-DDE action by monitoring an increase in CatSper currents of more than 100% ($p<0.01$). Finally, taking advantage of our previously described extended incubation protocol we found that acrosomal integrity was adversely affected after 2 and 3 days of exposure to p,p'-DDE, suggesting that $[Ca^{2+}]_i$ rise may cause premature acrosome reaction ($p<0.05$). On the other hand, PCB 77-induced acrosomal loss at both days 2 and 3 ($p<0.05$) may have resulted from the increased cell death previously observed at these specific time points (chapter 2). TCDD failed to induce any change in acrosomal status ($p>0.05$).

In conclusion, a novel non-genomic p,p'-DDE mechanism specific to sperm is shown in this study. p,p'-DDE was able to induce $[Ca^{2+}]_i$ rise in human sperm through the opening of Catsper consequently compromising sperm parameters important for fertilization and ultimately, male fertility. The promiscuous nature of CatSper activation may turn human sperm more vulnerable to the action of some persistent endocrine disruptors.

3.1 Material and methods

3.1.1 Materials

All reagents were provided by Sigma-Aldrich unless stated otherwise.

3.1.2 Human biological samples

Fresh normozoospermic sperm samples from both human healthy donors recruited at the Biosciences School, University of Birmingham, and Medical Research Institute, University of Dundee; as well as healthy patients undergoing routine semen analysis or fertility treatments in the Human Reproduction Service at University Hospitals of Coimbra were used accordingly to the proper ethical and Internal Review Board of the participating Institutions. All individuals signed informed consent forms. Samples were obtained by masturbation after 3 to 5 days of sexual abstinence and seminal analysis was performed according to the WHO guidelines after liquefaction (WHO 2010). All samples used in this study had no detectable leukocytes (or any other round cells) and presented more than 80% viable sperm after processing.

3.1.3 Single-cell Ca^{2+} imaging experiments

Spermatozoa were prepared by swim up and allowed to capacitate in a supplemented Earle's balanced salt solution (sEBSS) containing 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.81 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 116.4 mM NaCl, 5.5 mM D-glucose, 2.5 mM Na-pyruvate, 41.8 mM Na-lactate and 0.3% (w/v) BSA for at least 3 hours at 37°C under 5% CO_2. $[Ca^{2+}]_i$ measurements were then carried out after loading 4 million sperm/ml with the Ca^{2+} fluorescent marker Oregon Green BAPTA-1AM (10 µM; Molecular Probes) for 1 hour at 37°C under 5% CO_2 in a purpose-built, perfusable, imaging chamber composed of a coverslip previously coated with 1% (wt/v) air-dried poly-D-lysine solution on the lower surface. Cells adhered to this coated area and were observed under a Nikon TE200 inverted microscope (Nikon Instruments Inc.). The chamber was connected to a perfusion apparatus and any loose cells and extracellular dye were removed by perfusion of the chamber with sEBSS prior to start recording. All tests

were carried out in a dark room at 25ºC with a steady perfusion rate of 0.4 ml/minute. Real time recordings were performed at intervals of 2.5 seconds using an acquisition software platform (IQ Andor Technology, Belfast, UK).

To evaluate the effects of p,p'-DDE, TCDD and PCB 77 on $[Ca^{2+}]_i$, spermatozoa were exposed to a wide range of concentrations diluted in standard sEBSS. Furthermore, experiments using a low-Ca^{2+} sEBSS medium ($CaCl_2$ was adjusted to 5 mM with the addition of 6 mM ethylene glycol tetraacetic acid (EGTA; final $[Ca^{2+}]$<500 nM) were performed to further assess the contribution of the internal Ca^{2+} stores on the p,p'-DDE-induced $[Ca^{2+}]_i$ rise. Finally, inhibition studies were performed using 30 µM mibefradil and 10 µM NNC 55-0396 (Brenker *et al.* 2012). These drugs have been shown to effectively block CatSper currents at these concentrations. When a plateau in the p,p'-DDE-induced $[Ca^{2+}]_i$ rise was reached, either mibefradil or NNC 55-0396 was added, allowing the amplitudes of agonist and antagonist effects to be compared in each cell. Before finishing each experiment, spermatozoa were washed with standard sEBSS and exposed to 3.2 µM progesterone to determine if they were responding properly to the physiological stimuli (positive control). Solvent controls were carried out with 0.3%, 0.5% or 0.4% (v/v) DMSO, respectively.

Analysis of images, background correction and normalization of data was performed as described in previous studies with minor modifications (Kirkman-Brown *et al.* 2000). The region of interest was drawn around the head and neck region (midpiece) 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 % change in intensity at time t, 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 differed significantly from the mean of 10 determinations of normalized F during control (or inhibitor) treatment ($p<0.05$). Mean amplitudes and percentage of responsive cells were calculated for each concentration in each sperm sample analyzed.

3.1.4 Whole-cell patch-clamp experiments

Cells were prepared by swim up in an artificial human tubal fluid solution containing 98.0 mM NaCl, 4.7 mM KCl, 0.3 mM KH_2PO_4, 2.0 mM $CaCl_2$, 0.2 mM $MgSO_4$, 21.0 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), 3.0 mM D-glucose, 21.0 mM lactic acid, 0.3 mM Na-pyruvate, pH 7.4 (Lishko *et al.* 2011). Spermatozoa were then resuspended and allowed to capacitate in a HEPES-buffered solution (HS; 130.0 mM NaCl, 5.0 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM D-glucose, 1.0 mM Na-pyruvate, 10.0 mM lactic acid and 20.0 mM HEPES, pH=7.4) supplemented with 20% (w/v) fetal bovine serum and 25.0 mM $NaHCO_3$ (pH 7.4) for at least 3 hours at 37°C and 5% CO_2 (Lishko *et al.* 2010).

Whole cell currents were evoked by 1 second voltage ramps from -80 mV to +80 mV from a holding potential of 0 mV (before correction for junction potential). Seals between the patch pipette and human spermatozoa were formed in standard HS either at the human sperm cytoplasmic droplet or, if the cytoplasmic droplet was not clear, in the neck/midpiece region (Lishko *et al.* 2011). Pipettes were filled with a Cs^+-based solution comprising 130 mM $CsMeSO_3$, 40 mM HEPES, 1 mM Tris-HCl, 3 mM EGTA, 2 mM EDTA, pH adjusted to 7.4 with CsOH. A divalent-free caesium (Cs^+)-based medium (DVF) comprising 140 mM $CsMeSO_3$, 40 mM HEPES and 3 mM EGTA (pH 7.4 adjusted with CsOH) was used to allow proper recordings of CatSper monovalent currents. Besides seal formation, HS was used to record baseline current as Ca^{2+} contained in this solution inhibits monovalent CatSper currents and causes Ca^{2+}-dependent inactivation of CatSper channels (Lishko *et al.* 2011). 5µM p,p'-DDE was added to DVF at specific time points. All experiments were performed at 25ºC.

3.1.5 Exposure to p,p'-DDE, TCDD and PCB 77

Spermatozoa were isolated by density gradient centrifugation (Isolate® Sperm Separation Medium, Irvine Scientific) and allowed to capacitate for at least 3 hours at 37°C under 5% CO_2. As described in chapter 2, spermatozoa (10 million/ml) were independently exposed to several concentrations of p,p'-DDE (1 - 50 µM), TCDD (1 nM and 1 µM) and PCB 77 (13.7 µM) for 3 days at 37°C and 5% CO_2. Acrosomal status was assessed daily and appropriate controls were performed with the vehicle, DMSO.

3.1.5.1 Acrosomal integrity

Acrosomal integrity was evaluated using PSA, a lectin that binds to the acrosomal contents, particularly to glycoproteins present in the matrix (Liu & Baker 1988), conjugated with fluorescein isothiocyanate (PSA-FITC). Briefly, spermatozoa were fixed with 2% (v/v) formaldehyde in PBS for 40 minutes, permeabilized [1% (v/v) Triton X-100 in PBS; 20 minutes] and blocked with 0.1% (wt/v) BSA and 100 mM glycine in PBS for 30 minutes at room temperature. Afterwards cells were incubated with PSA-FITC (1:200) for 1 hour at 37°C followed by washing with 0.1% (v/v) Triton X-100 in PBS for 15 minutes. A final labeling with DAPI (Molecular Probes) was used to counterstain sperm nuclei. At least 200 sperm cells were scored in different fields in each slide using a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss). Only acrosomes presenting a homogeneous green fluorescence were considered intact (Figure 3.1).

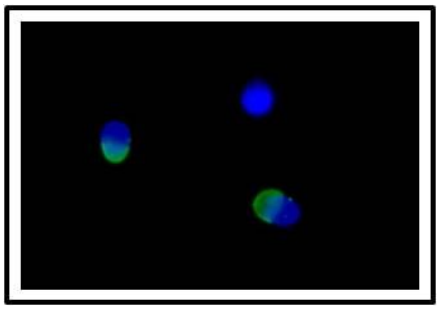

Figure 3.1. Acrosomal integrity evaluated by PSA-FITC.

Spermatozoa presenting homogenous green fluorescence on the posterior region of the head possess intact acrosomes whereas acrosome-reacted cells exhibit blue fluorescence resulting from the DAPI nuclear counterstain. The image was taken using a fluorescence microscope under a 100x objective magnification.

3.1.6 Statistical analysis

Statistical analysis was carried out using the SPSS version 19.0 software for Windows (SPSS Inc., Chicago, IL, USA). All variables were checked for normal distribution using the Shapiro-Wilk test (or the Kolmogorov-Smirnov test for n>25) and multiple comparisons were performed by the paired t-test or ANOVA for normal variables. Correlations were performed by the Spearman non-parametric test. Results are

expressed as mean percentage ± SEM. Statistical significant differences were considered when p values ≤0.05.

3.2 Results

3.2.1 p,p'-DDE, but not PCB 77 or TCDD, promotes a relevant $[Ca^{2+}]_i$ rise

Single cell assessment of $[Ca^{2+}]_i$ showed that within seconds of exposure a wide range of p,p'-DDE concentrations (1 pM - 50 µM), and to a lesser extent PCB 77 and TCDD doses, caused an increase in Ca^{2+} levels, reversible upon sEBSS media washout (Figures 3.2A and 3.3A). In reality, after adjusting the effect of DMSO exposure throughout time (by performing the same kind of experiment in figure 3.2A but with the purpose of analyzing the sole effect of the solvent DMSO for the 7 minutes of exposure and ensure that $[Ca^{2+}]_i$ rise in the last 3.5 minutes was due to the endocrine disruptor used; data not shown) we found that only small fractions of cells exposed to either TCDD or the dioxin-like PCB77 increased their $[Ca^{2+}]_i$ significantly above the control (4.90±2.40%, 7.50±3.70% and 5.22±2.37% for 1 nM TCDD, 1 µM TCDD and PCB 77, respectively, $p<0.05$; Figure3.2B), showing the low biological relevance of this finding. In sharp contrast, p,p'-DDE was able to induce a remarkable increase in sperm intracellular Ca^{2+} levels when compared to the respective control (Figure 3.3A). At 25 µM and 50 µM p,p'-DDE >91% of sperm showed a significant increase in $[Ca^{2+}]_i$ ($p<0.05$, Figure 3.3B) and similar mean Ca^{2+} response amplitudes (55.6±6.7% and 55.5±8.1% respectively; Figure 3.3C). p,p'-DDE was found to be so effective that even at concentrations as low as 1 pM and 1 nM, we observed elevated Ca^{2+} levels in 21.1±3.0% and 28.0±10.65% of cells ($p<0.05$, Figure 3.3B), with mean amplitudes of response of 16.7±2.8% and 15.4±3.6%, respectively (Figure 3.3C). Whereas the dose-effect curve for the proportion of responsive cells was roughly sigmoidal, the curve for $[Ca^{2+}]_i$ response amplitudes appeared biphasic, with markedly greater responses at 25 µM and 50 µM p,p'-DDE (Figure 3.3B,C). When we examined the amplitude distribution of the single cell responses we observed that from 1 pM to 10 µM p,p'-DDE most responsive cells showed an increase in fluorescence intensity of up to 20%, but at higher doses the shape of the distribution was completely different, with 'enhanced' response amplitudes ranging between 20% and 100% and occasional responses of up to 200% (Figure 3.3D).

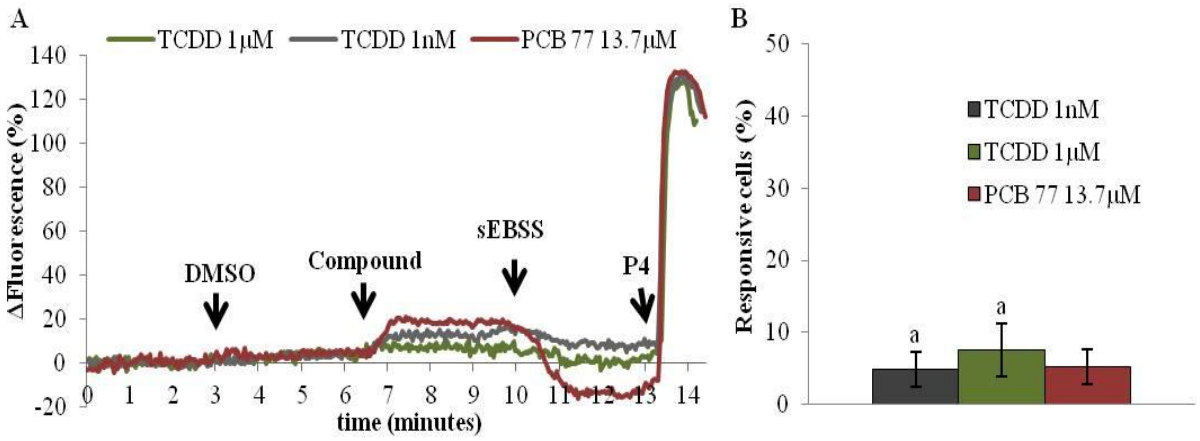

Figure 3.2. Intracellular Ca^{2+} levels during TCDD or PCB 77 exposure in human sperm.

A) Fluorescence-time traces representing intracellular Ca^{2+} changes in 3 individual cells exposed to different conditions. DMSO was added after 3 min of perfusion with sEBSS followed by exposure to 1nM TCDD (dark grey trace), 1µM TCDD (dark green trace) or 13.7µM PCB 77 (dark red trace). After a further 3.5 min exposure TCDD or PCB77 were washed out by perfusion with fresh sEBSS. Arrows indicate the exact time points at which spermatozoa were bathed with different solutions. P_4– 3.2µM progesterone. B) Proportion of cells responsive to TCDD or PCB 77. Results represent mean percentage ± SEM from 500 cells analyzed individually in a total of 5 independent experiments for each TCDD or PCB 77 concentration. Similar letters denote no statistical differences between TCDD concentrations ($p>0.05$). Comparisons between the percentages of responsive cells to TCDD and PCB 77 concentrations retrieved no differences ($p>0.05$).

A positive control was included in all experiments by adding the physiological stimulus progesterone, which causes increased Ca^{2+} levels and triggers AR, to ensure that all samples were responding normally.

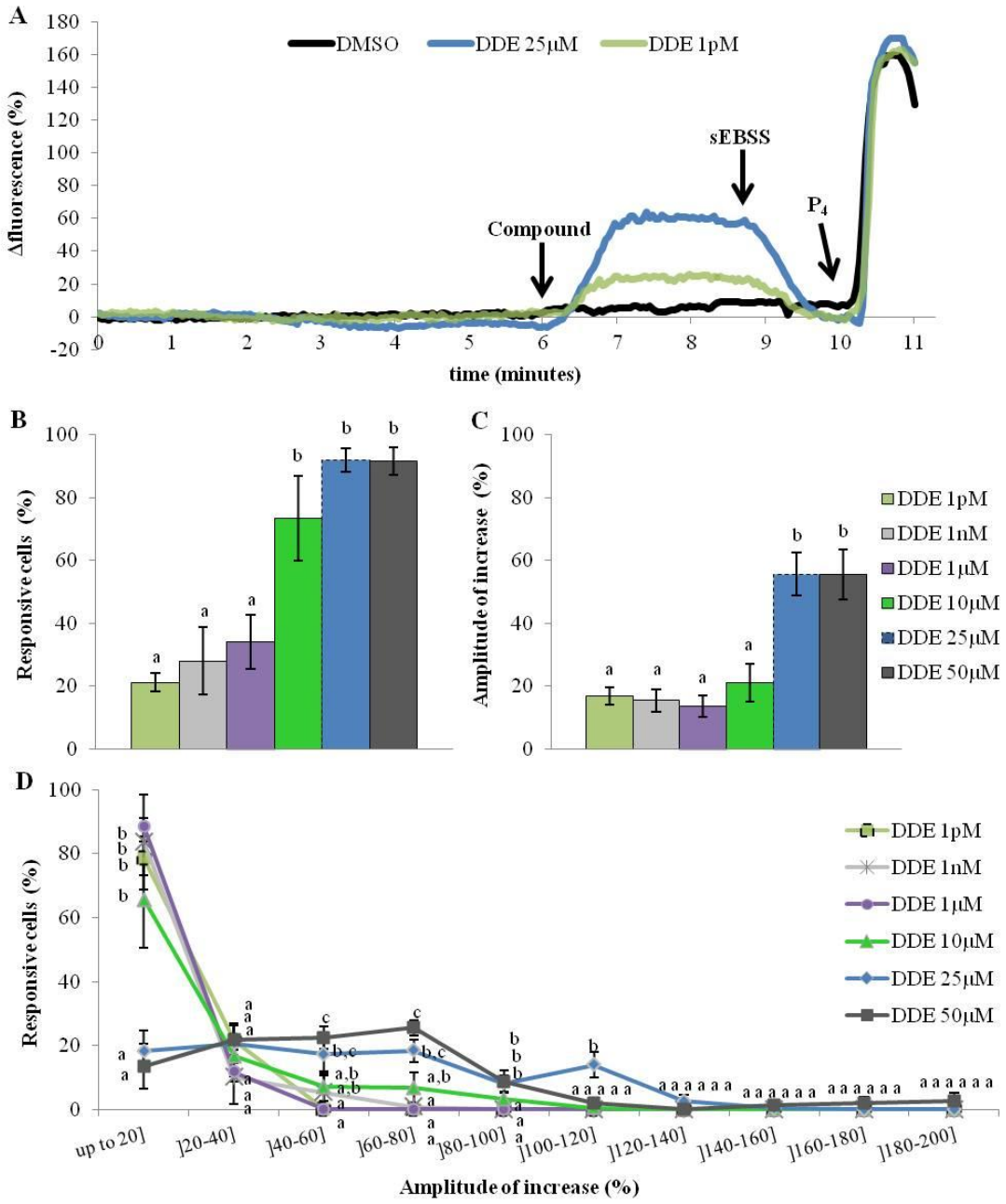

Figure 3.3. Intracellular Ca^{2+} levels during p,p'-DDE exposure in human sperm.

A) Fluorescence-time traces representing intracellular Ca^{2+} changes in 3 individual cells exposed to different conditions. DMSO (black trace), 1pM (green trace) or 25µM p,p'-DDE (blue trace) were added after 6 min of perfusion with sEBSS. After a further 3 min p,p'-DDE was washed out by perfusion with fresh sEBSS. Arrows indicate the exact time points at which spermatozoa were bathed with different solutions. P_4– 3.2µM progesterone. B) Proportion of cells responsive to p,p'-DDE. C) Magnitude of Ca^{2+} response in responsive cells. D) Amplitude distribution of $[Ca^{2+}]_i$ increase (significant increase in fluorescence) at each dose tested. Results represent mean percentage ± SEM from 500 cells analyzed individually in a total of 5 independent experiments for each p,p'-DDE concentration. Different letters denote statistical differences between concentrations ($p<0.05$).

3.2.2 The effect of p,p'-DDE on $[Ca^{2+}]_i$ is abolished in low Ca^{2+} medium

Given the striking results obtained with p,p'-DDE we next evaluated whether its effect on human sperm could be due to a Ca^{2+} influx from the medium or the mobilization of intracellular Ca^{2+} stores present in sperm (Jimenez-Gonzalez *et al.* 2006; Costello *et al.* 2009). These and subsequent Ca^{2+} imaging experiments were performed with 1 pM and 1 nM p,p'-DDE, the concentrations within the range often found in human reproductive fluids (mean values ranging from 47 pM to 111 nM according to Kumar *et al.* 2000; Dallinga *et al.* 2002; Younglai *et al,* 2002; Pant *et al.* 2004) and also at 25 µM, the minimal saturating concentration for the observed effects on $[Ca^{2+}]_i$.

Perfusion of the recording chamber with low Ca^{2+} medium (<500 nM) caused an immediate decrease in sperm $[Ca^{2+}]_i$ that stabilized at a new level within 3 minutes of exposure and remained unaltered when p,p'-DDE was added (Figure 3.4A). At 1 pM and 1 nM no cells showed $[Ca^{2+}]_i$ responses ($p<0.01$ compared to experiments in standard sEBSS, Figure 3.4B) and at 25 µM p,p'-DDE only 2.0±1.2% of cells responded with an increase in Ca^{2+} levels ($p<0.01$ when compared to the 91.9±3.7% of cells in standard sEBSS; Figure 3.4B). Moreover, the magnitude of response provoked by 25 µM p,p'-DDE

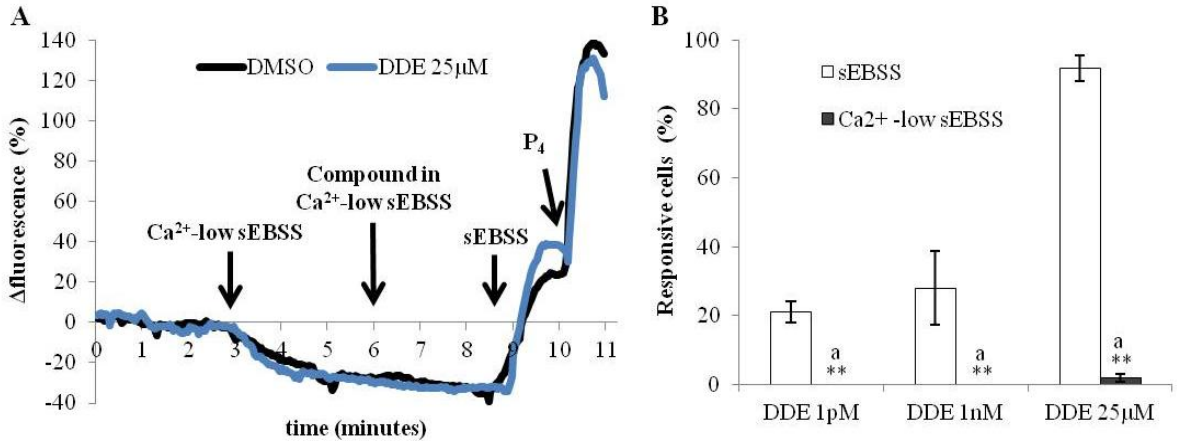

Figure 3.4. Effect of p,p'-DDE in a low Ca^{2+} sEBSS medium (<500 nM).

A) Fluorescence-time traces representing intracellular Ca^{2+} changes in 2 individual cells exposed to different conditions. DMSO (black trace) or 25µM p,p'-DDE (blue trace), both diluted in low-Ca^{2+}-sEBSS medium, were added after 6 minutes of perfusion. Arrows represent the exact time points in which spermatozoa were bathed with different solutions. P_4– 3.2µM progesterone. B) Percentage of p,p'-DDE responsive cells. Results represent mean percentage ± SEM from 500 cells evaluated individually in a total of 8 independent experiments for each p,p-DDE dose. ** corresponds to statistical differences between concentrations subjected to different conditions ($p<0.01$). Similar letters represent lack of statistical significance ($p>0.05$).

was only of 22.8±10.7% compared to the 55.6±6.7% observed in standard sEBSS ($p<0.05$). When we analyzed the distribution of the single cell response we found that 72.2±14.7% of cells responded with an increase in fluorescence intensity of up to 20%, resembling the response observed in spermatozoa exposed from 1 pM to 10 μM p,p'-DDE in standard sEBSS medium. In all these experiments, when standard sEBSS was returned to the chamber $[Ca^{2+}]_i$ levels increased as expected, and responded normally to the progesterone stimulus (Figure 3.4A).

3.2.3 p,p'-DDE effect on $[Ca^{2+}]_i$ is reversed by CatSper blockers

CatSper, the only Ca^{2+} conductance channel that has been detected in patch clamped human sperm so far (Kirichok & Lishko 2011) is highly promiscuous, activating in response to a wide range of small organic molecules (Brenker *et al.* 2012). In order to investigate whether activation of CatSper might mediate p,p'-DDE-induced Ca^{2+} influx, we used 30 μM mibefradil and 10 μM NNC 55-0396, both of which inhibit Catsper currents in human sperm (Lishko *et al.* 2011; Strünker *et al.* 2011). Cells were first exposed to p,p'-DDE (1 pM, 1 nM and 25 μM) to establish Ca^{2+}-influx and after a delay of 2.5 minutes the inhibitors were added in separate experiments (Figure 3.5A). Both drugs caused a transient increase in fluorescence, as previously illustrated (Strünker *et al.* 2011; Brenker *et al.* 2012) which also occurred in control experiments in the absence of p,p'-DDE (Figure 3.5A DMSO trace). However, within few minutes $[Ca^{2+}]_i$ significantly decreased and stabilized at a new, lower level (Figure 3.5A). 30 μM mibefradil strongly reversed the effect of p,p'-DDE in >90% of cells (Figure 3.5A-C). This effect was observed at all doses and when mibefradil was applied during 1 pM or 1 nM p,p'-DDE exposure $[Ca^{2+}]_i$ decreased below control conditions ($p>0.05$; Figure 3.5A), therefore showing a reversal effect higher than 100% (Figure 3.5C). Examination of individual cell responses showed that the magnitudes of the rise in fluorescence caused by p,p'-DDE and the subsequent decrease upon application of mibefradil were clearly correlated ($p<0.05$; Figure 3.5D), confirming that mibefradil was acting by blocking the effect of p,p'-DDE.

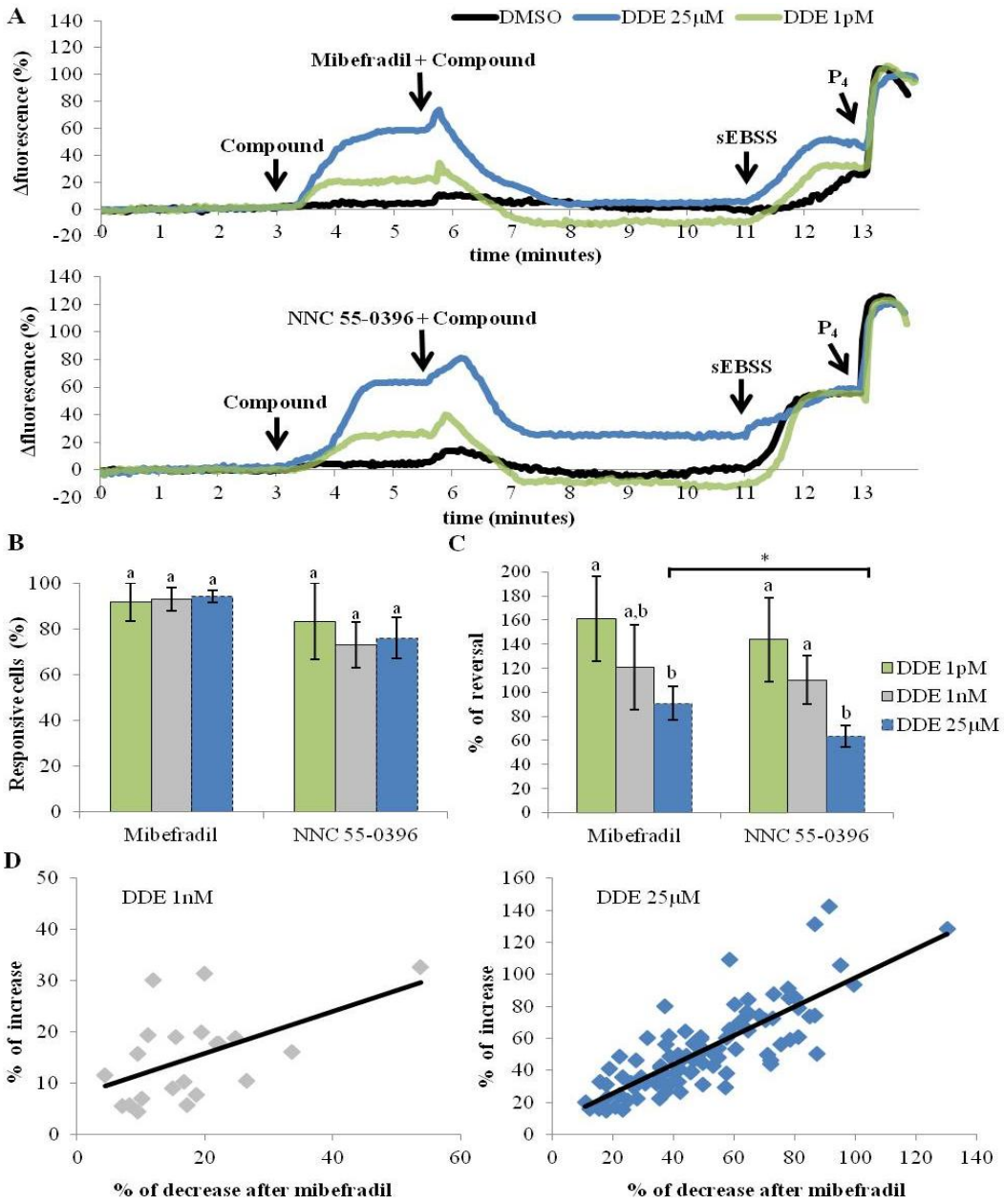

Figure 3.5. Mibefradil and NNC 55-0396 effects following p,p'-DDE-induced $[Ca^{2+}]_i$ rise.

A) Fluorescence-time traces representing intracellular Ca^{2+} changes in 6 individual cells exposed to different conditions. DMSO (black traces), 1pM (green traces) or 25µM p,p'-DDE (blue traces) were added after 3 min of perfusion with sEBSS. 30µM mibefradil or 10µM NNC 55-0396 were applied after a further 2.5 min when effects on $[Ca^{2+}]_i$ had stabilized. Arrows represent the exact time points at which sperm were bathed with different solutions. P_4 – 3.2µM progesterone. B) Proportion of responsive cells. C) Percentage of reversal by mibefradil and NNC 55-0396 of the preceding p,p'-DDE-induced increase. Mibefradil and NNC 55-0396 alone had no effect (not shown). Results represent mean percentage ± SEM from 500 cells analyzed in a total of 5 independent experiments for each concentration. Different letters denote statistical significance between doses within each inhibitor experiments ($p<0.05$) and * represent statistical differences between the same dose exposed to both inhibitors ($p<0.05$). D) Correlation between amplitudes of the p,p'-DDE-induced $[Ca^{2+}]_i$ rise and the subsequent fall in $[Ca^{2+}]_i$ upon mibefradil application in individual sperm exposed to 1nM (left panel) or 25µM p,p'-DDE (right panel). Significant correlations were found for both 1nM (rho=0.492, $p<0.05$) and 25µM p,p'-DDE (rho=0.804, $p<0.001$). Each panel shows all cells from a single experiment.

10 µM NNC 55-0396 also reversed the p,p'-DDE-induced $[Ca^{2+}]_i$ rise in most cells ($p<0.05$; Figure 3.5A-C). However, NNC 55-0396 reversal of the $[Ca^{2+}]_i$ rise caused by 25 µM p,p'-DDE was only partial when compared to mibefradil ($p<0.05$; Figure 3.5C). Analysis of individual spermatozoa responses showed that, similarly to the effect of mibefradil, the amplitude of the effect of NNC 55-0396 was correlated with the amplitude of the preceding rise induced by p,p'-DDE ($p<0.05$; Figure 3.6).

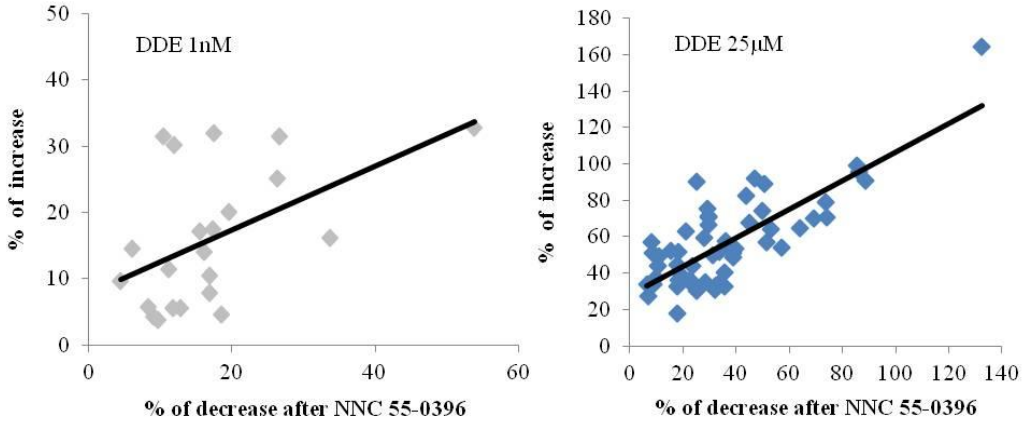

Figure 3.6. Correlation between amplitudes of the p,p'-DDE-induced $[Ca^{2+}]_i$ rise and subsequent fall in $[Ca^{2+}]_i$ upon NNC 55-0396 application in individual sperm cells.

Spermatozoa were exposed to 1 nM (left panel) or 25 µM p,p'-DDE (right panel). Significant correlations were found for both 1 nM (rho=0.506, $p<0.05$) and 25 µM p,p'-DDE (rho=0.635, $p<0.001$). Each panel shows all cells from a single experiment.

3.2.4 p,p'-DDE enhances CatSper currents in human sperm

The action of p,p'-DDE on $[Ca^{2+}]_i$ is mediated by Ca^{2+} influx and can be reversed by CatSper antagonistic drugs, suggesting that this DDT metabolite activates CatSper. To confirm this we investigated the effect of 5 µM p,p'-DDE (a concentration that gave detectable $[Ca^{2+}]_i$ responses in 50.0% of cells but where response amplitude was not 'enhanced' as suggested by Figure 3.3) on CatSper in sperm held under whole cell clamp. Using divalent free conditions and $CsMeSO_3$-based bath and pipette media, large CatSper currents, carried by Cs^+, were induced by 1 second voltage ramps from -80 mV to +80 mV (Lishko *et al.* 2011). 5 µM p,p'-DDE increased CatSper current by 116.0±10.0% (n=5,

p<0.01) without changing reversal potential or the characteristic outward rectification of the current (Figure 3.7A), similarly to the agonistic effect of 3.2 µM progesterone (Figure 3.7B). Analysis of the time-course of the action of p,p'-DDE showed that showed that currents increased slowly over a period of 10-20 seconds and then stabilized (Figure3.7C).

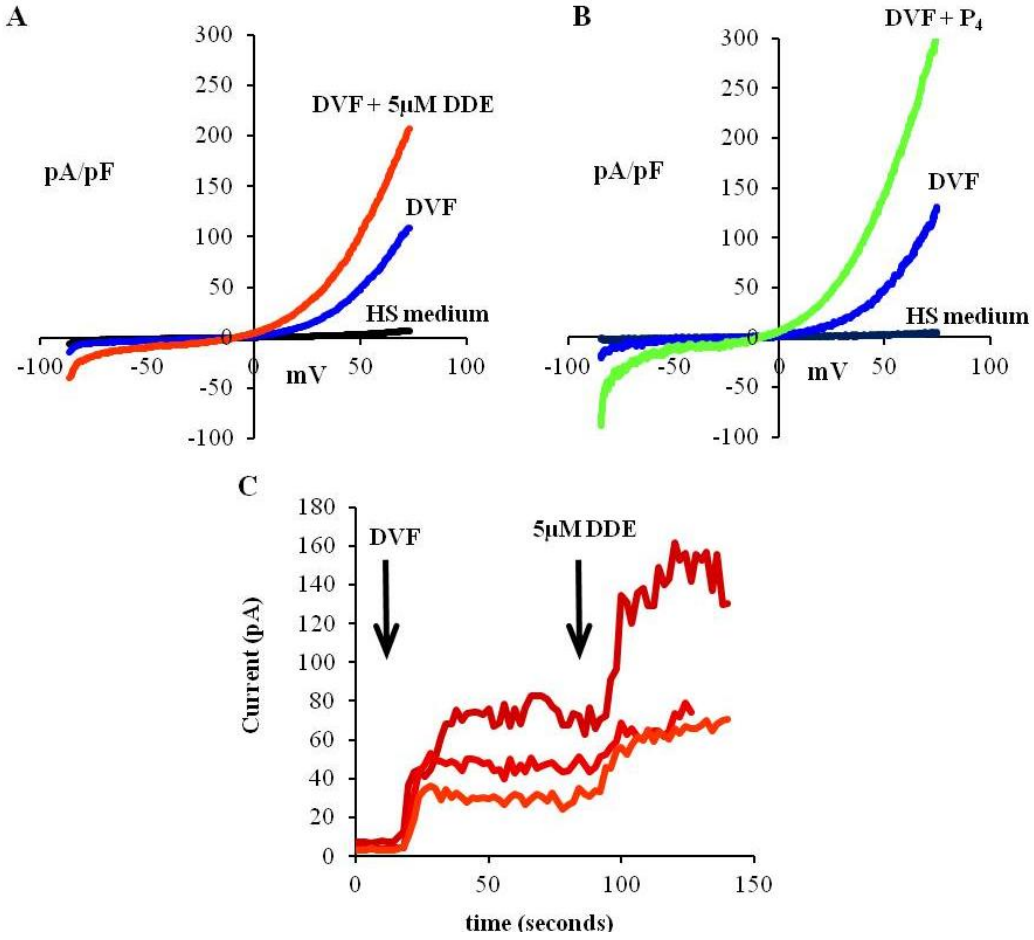

Figure 3.7. Effect of 5 µM p,p'-DDE on monovalent CatSper currents in human sperm.

A) Example of currents induced by applying a 1 sec voltage ramp from -80 mV to 80 mV to a cell bathed in divalent cation-containing medium (black trace), after superfusion with divalent-free Cs^+-based medium (DVF; dark blue trace) and then after application of 5µM p,p'-DDE (red trace). B) Example of a similar experiment in which the current was recorded first in divalent cation-containing medium (black trace), then after superfusion with DVF (dark blue trace) and finally in the presence of 3.2µM progesterone (P_4; green trace). C) Time-course of changes in current induced by 5µM p,p'-DDE. Current amplitude was quantified using the average current over the last 3 mV of the voltage ramp (77-80mV). Traces show responses of 3 different cells. The first arrow shows superfusion with DVF and the second shows application of 5µM p,p'-DDE.

In most cells seals became unstable after 1-2 minutes and recordings were lost abruptly or after a second rapid rise in current.

3.2.5 p,p'-DDE causes acrosomal loss

To evaluate if changes in $[Ca^{2+}]_i$ could affect sperm function, acrosomal integrity was assessed (Figure 3.8). In accordance to the negligible TCDD-induced $[Ca^{2+}]_i$ rise, no differences in the proportion of spermatozoa displaying intact acrosomes were detected with TCDD ($p>0.05$; Figure 3.8A). Yet, 13.7 µM PCB 77 was able to induce acrosomal loss following 2 and 3 days of exposure ($p<0.05$; Figure 3.8A), albeit the minor proportion

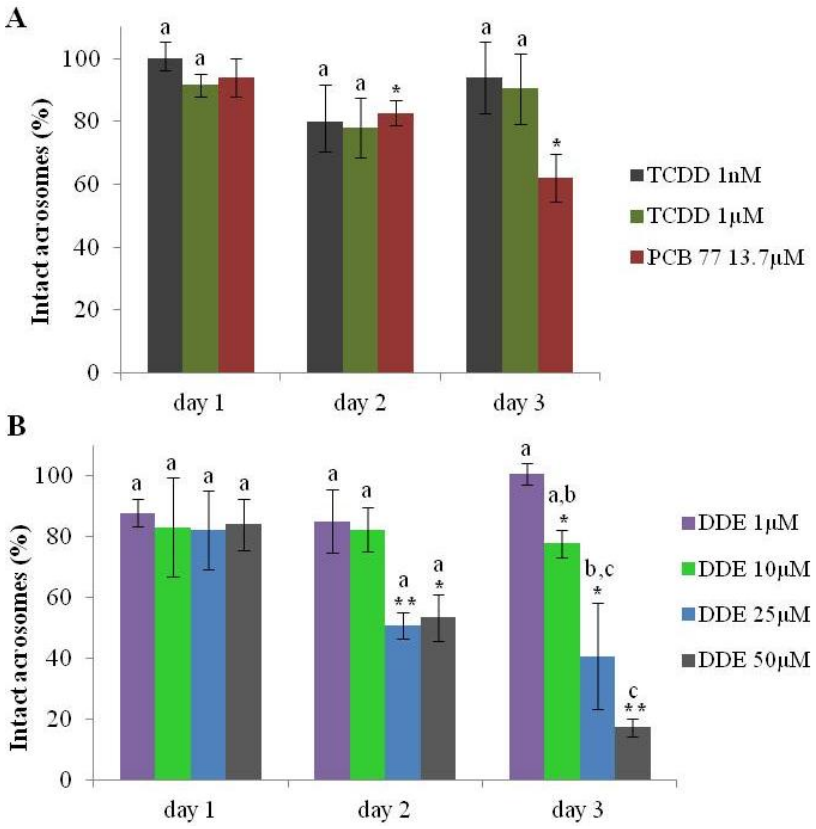

Figure 3.8. Daily acrosomal integrity during the continuous exposure to different concentrations of A) TCDD, PCB 77 or B) p,p'-DDE at 37ºC and 5% CO_2.

Results represent mean percentage ± SEM relative to the control (% acrosome intact / % acrosome intact in DMSO x 100), n=6. * ($p<0.05$) and ** ($p<0.01$) denote significant differences towards control and different letters between concentrations ($p<0.05$). Comparisons between TCDD and PCB 77 concentrations retrieved no differences ($p>0.05$).

of spermatozoa with increased $[Ca^{2+}]_i$ upon exposure (Figure 3.2B). This significant loss may, nonetheless, merely reflect the strong decrease in viability previously reported in chapter 2 at these specific time points. Alternatively, 25 μM and 50 μM p,p'-DDE were able to significantly reduce acrosomal integrity after 2 days of exposure ($p<0.01$ and $p<0.05$, respectively, Figure 3.8B), without affecting viability (chapter 2). This effect was also observed at day 3 for both 25 μM and 10 μM p,p'-DDE-exposed cells ($p<0.05$). Following 3 days of treatment with 50 μM p,p'-DDE acrosomal loss was further enhanced, though, in this case, this might be the consequence of cell death (chapter 2). No differences among 1 μM p,p'-DDE-exposed spermatozoa and control were observed in this 3-day incubation approach ($p>0.05$). Furthermore, comparisons between concentrations showed significant differences in acrosomal integrity only at day 3 ($p<0.05$, Figure 3.8B).

It should be noted that, while subtle changes in Ca^{2+} levels were detected in the above experiments, acrosomal integrity monitored here reflects an all-or-nothing measurement, and relevant changes in the sperm secretory vesicle may occur much earlier without being detected by this less sensitive assay. Further experiments are warranted to clarify this difference in time between acrosomal loss and Ca^{2+} levels.

3.3 Discussion

Several studies have focused on the likely genomic effects of TCDD, PCB 77 and p,p'-DDE on male fertility (El-Sabeawy *et al.* 1998; Faqi *et al.* 1998a,c; Gray *et al.* 1997; Huang *et al.* 1998; You *et al.* 1998; Loeffler & Petersen 1999; Hsu *et al.* 2004; Rignell-Hydbom *et al.* 2005a,b; Dhooge *et al.* 2006; De Jager *et al.* 2006; Stronati *et al.* 2006; Aneck-Hahn e*t al.* 2007; Choi *et al.* 2008; Mocarelli *et al.* 2008), without exploring possible rapid non-genomic actions on human sperm. This is especially relevant as sperm can be directly exposed to organochlorines as previously mentioned. We found that p,p'-DDE, but not TCDD or PCB 77, promoted a remarkable $[Ca^{2+}]_i$ rise in human sperm, with high concentrations causing a large and rapid rise in $[Ca^{2+}]_i$ fluorescence by up to 200%, and low concentrations (1 pM and 1 nM) also producing significant responses.

Effects on cytosolic Ca^{2+} levels after exposure to several toxicants, including p,p'-DDE, have been reported in many cell types, apparently mimicking the action of steroids

(Ruehlmann *et al.* 1998; Nadal *et al.* 2000; Younglai *et al.* 2004, 2006; Wu *et al.* 2006), but dose dependence and magnitude of the effect vary greatly. p,p'-DDE and other pesticides such as kepone, methoxychlor and the isomer o,p-DDE were found to increase cytosolic Ca^{2+} levels in human umbilical vein endothelial and granulosa-lutein cells (Younglai *et al.* 2004; Wu *et al.* 2006), although in the latter cells Ca^{2+} changes induced by methoxychlor and o,p-DDE were not as clear or consistent as those induced by kepone (Wu *et al.* 2006). Furthermore, methoxychlor at high concentrations (2.8-280 µM) failed to induce changes in Ca^{2+} levels (Wu *et al.* 2006). In fact, contrary to the sigmoid curve of dose-response found in this study, the effect of methoxychlor is another example of a non-classical response, showing an inverse U-shaped curve (Wu *et al.* 2006). The traditional dose-response effect observed in many toxicological studies is not always applicable, especially when environmental toxicants acting as endocrine disruptors are involved (Krimsky 2001).

In mouse β pancreatic cells bisphenol A (BPA), diethylstilbestrol and o,p'-DDT increased the frequency of glucose-provoked $[Ca^{2+}]_i$ fluctuations (Ruehlmann *et al.* 1998). A similar response was observed at pico- and nanomolar concentrations in a GH3/B6 pituitary cell line exposed to o,p-DDE (Wozniak *et al.* 2005), showing the concerning extensive range of action of these environmental endocrine disruptors. Although 10 µM p,p'-DDE failed to affect $[Ca^{2+}]_i$ in rat myometrial smooth muscle cells, 50 and 100 µM p,p'-DDE induced $[Ca^{2+}]_i$ rise by 586% and 921%, respectively (Juberg *et al.* 1995), effects far greater than those reported here. In 2005, Wróbel and Kotwica also demonstrated that nanomolar concentrations of PCB 77 were able to increase basal $[Ca^{2+}]_i$ in bovine myometrial cells although, in this case, after 48 hours of exposure (Wrobel & Kotwica 2005). This same effect was later reported by Yilmaz and coworkers in mouse thymocytes following treatment with 5 µM or 10 µM PCB 77, albeit for smaller periods of time (5, 15, 30 and 60 minutes; Yilmaz *et al.* 2006). This was nevertheless refuted by others that have found no effect on Ca^{2+} homeostasis in rat cerebellar granule cells at similar concentrations (Kodavanti *et al.* 1993; Tan *et al.* 2004) or with 100 nM PCB 77 in rat insulinoma cells (RINm5F cells; Fischer *et al.* 1999). However, in these latter cells pure non-coplanar PCB congeners [PCBs 47 (2,2',4,4'-tetrachlorobiphenyl) and 153] and the commercial PCB mixture Aroclor 1254 produced a meaningful sustained $[Ca^{2+}]_i$ rise within 20-30 seconds of exposure (Fischer *et al.* 1999), similarly to what we observed with p,p'-DDE.

TCDD effect on Ca^{2+} levels is also variable. While TCDD at 15.5, 31.1 and 46.6 µM increased cytosolic Ca^{2+} in mouse B6C3F1 neutrophils and monocytes (Levin *et al.* 2007) 100 nM was enough to provoke a statistically significant increase in Ca^{2+} levels in human CD19+ B cells but not in CD3+ T cells or monocytes, inevitably showing differences in the responses of subsets of lymphoid cells (Mounho *et al.* 1997). In contrast, $[Ca^{2+}]_i$ was significantly elevated in a 10 nM TCDD-treated rat microglial cell line (HAPI cells; Xu *et al.* 2013) and this result was consistent with the observation of a rapid concentration-dependent rise in Ca^{2+} levels in primary cultures of rat hippocampal cells treated with 10-100 nM TCDD (Hanneman *et al.* 1996). Nevertheless, 30 nM TCDD failed to alter Ca^{2+} homeostasis in a human mammary epithelial cell line (MCF-10A cells) after 2-, 6-, and 18 hours of exposure (Tannheimer *et al.* 1999).

To further assess the p,p'-DDE mechanism of action in human sperm we exposed cells to the compound in a low Ca^{2+} medium. Under these conditions the effect was largely abolished, showing that p,p'-DDE mainly promotes Ca^{2+} influx at the plasma membrane. Intriguingly, although higher concentrations of p,p'-DDE resulted in larger $[Ca^{2+}]_i$ signals (Figure 3.2C), this effect apparently occurred by 'recruitment' of a larger 'type' of Ca^{2+} signal (Figure 3.2D). This may possibly reflect a secondary release of stored Ca^{2+} downstream of Ca^{2+} influx (Harper *et al.* 2004).

To further explore which plasma membrane Ca^{2+} channel(s) was involved, a pharmacological approach was used. In mouse and human sperm, CatSper is believed to be the principal plasma membrane Ca^{2+} channel (Kirichok *et al.* 2006; Qi *et al.* 2007; Smith *et al.* 2013). Using the Catsper blockers mibefradil and NNC 55-0396 (Lishko *et al.* 2011; Strünker *et al.* 2011), we observed a strong suppression of the p,p'-DDE-induced Ca^{2+} increase in the large majority of cells. NNC 55-0396, the putatively more potent Catsper inhibitor (Lishko *et al.* 2011) induced a lower decrease of Ca^{2+} levels at 25 µM p,p'-DDE when compared to mibefradil, but this may mirror the significant rise in $[Ca^{2+}]_i$ caused by NNC 55-0396 itself (Strünker *et al.* 2011).

We further confirmed p,p'-DDE action on Catsper using whole cell patch-clamp recordings with divalent cation-free bath and pipette solutions containing Cs^{+}, conditions under which the large monovalent currents show CatSper activity (Kirichok *et al.* 2006; Lishko *et al.* 2011; Strünker *et al.* 2011). Treatment with p,p'-DDE caused instability and ultimately loss of the seal within 1-2 minutes, an effect that is apparently related to patch

formation and/or the recording conditions used, since cell viability was not affected. It has been shown by patch clamp that human sperm Catsper currents are powerfully potentiated by progesterone (Kirichok & Lishko 2011; Strünker *et al.* 2011), whereas the steroid had no effect on currents in sperm from an infertile CatSper-deficient patient (Smith *et al.* 2013) suggesting that CatSper is central to the non-genomic action of the steroid. The high potency of p,p'-DDE in elevating $[Ca^{2+}]_i$ in human sperm may therefore reflect a steroid-like effect and p,p'-DDE might even bind the same activating site as progesterone and thus promote Ca^{2+} influx, although the sustained nature of the p,p'-DDE-induced signal does not resemble the biphasic $[Ca^{2+}]_i$ elevation induced by progesterone. Alternatively, this action of p,p'-DDE may reveal a more general feature of CatSper. In addition to progesterone the channel is activated by membrane potential, internal pH, prostaglandins, odorants and other small organic molecules (Lishko *et al.* 2011; Strünker *et al.* 2011; Brenker *et al.* 2012), apparently acting as a polymodal sensor upon which diverse stimuli converge to generate $[Ca^{2+}]_i$ signals in sperm. The promiscuous nature of the channel, though apparently important for detection of cues in the female reproductive tract (Brenker *et al.* 2012), may render sperm sensitive to some EDs such as p,p'-DDE.

After observing the intracellular Ca^{2+} changes promoted by p,p'-DDE we hypothesized that AR, a strongly Ca^{2+}-dependent event, might be compromised. Furthermore, although the other organochlorines caused negligible effects on sperm Ca^{2+} levels we further decided to analyse TCDD- and PCB 77-induced AR as well. In fact, by mimicking the female reproductive tract conditions, where sperm can be maintained for several days, potentially with constant exposure to organochlorine pollutants, we found decreased acrosomal integrity suggesting the induction of spontaneous AR following 2 and 3 days of p,p'-DDE exposure. While this outcome was also noted during PCB 77 exposure, possibly due to the loss of sperm viability, TCDD treatment did not differed from control.

Although other pathways may certainly be involved, and further studies are warranted, we hypothesize that p,p'-DDE effect on acrosomal status was possibly achieved by the continuous Ca^{2+} entry and sustained Ca^{2+} overload. Elevated p,p'-DDE concentrations not only promoted $[Ca^{2+}]_i$ rise in a higher percentage of cells with higher magnitudes of response but also induced acrosomal loss earlier in time. In contrast, since 10 μM p,p'-DDE induced smaller magnitudes of response a decrease in acrosomal integrity was only detected after 3 days of exposure. In accordance, a mixture containing

p,p'-DDE was found to increase $[Ca^{2+}]_i$ and potentiate spontaneous AR rates in porcine sperm *in vitro* (Campagna *et al.* 2009). Although the authors did not explore which was the source responsible for the observed higher Ca^{2+} levels, they suggested that this mixture could modify sperm plasma membrane, allowing non-regulated Ca^{2+} entry that would finally lead to AR, thus lowering sperm survival, among other effects (Campagna *et al.* 2009). On the contrary, the organochlorine pesticide lindane was found to inhibit spontaneous AR in human sperm *in vitro* (Silvestroni & Pallesch 1999). This compound was able to quickly depolarize sperm plasma membrane, opening Ca^{2+} channels and causing an increase in intracellular Ca^{2+} levels, but probably by altering sperm membrane dipole potential, AR was found reduced (Silvestroni *et al.* 1997; Silvestroni & Pallesch 1999). Conversely, others failed to detect differences in acrosomal integrity after an *in vitro* exposure to lindane for 5 hours (Pflieger-Bruss *et al.* 2006a). The same outcome was reported by these authors when spermatozoa were individually exposed to a number of PCBs including the congener 77 using the same settings (Pflieger-Bruss *et al.* 2006a,b). Regarding the effect of TCDD on acrosomal status the only study available reported a decrease in AR rate in the presence of heparin in rat epydidimal spermatozoa treated during puberty (El-Sabeawy *et al.* 1998).

In general, all these data clearly support the involvement of different mechanisms of action through which endocrine disruptors exert their effects, but the highly promiscuous nature of CatSper may cause sperm sensitivity to some compounds that interact with key site(s) on the channel. p,p'-DDE-induced $[Ca^{2+}]_i$ rise may prematurely trigger acrosomal loss long before they reach the oocyte, thus adversely affecting male fertility. p,p'-DDE concentrations in follicular fluid have already been correlated with failed fertilization (Younglai *et al.* 2002) and described as being higher in semen from infertile patients (Pant *et al.* 2004), suggesting an important role of p,p'-DDE in human (in)fertility.

Concluding, even at concentrations found in reproductive fluids, p,p'-DDE was able to induce a rise in $[Ca^{2+}]_i$ in human sperm through a novel non-genomic mechanism involving the opening of the sperm-specific cation channel Catsper and ultimately compromising male fertility.

Chapter 4 Sperm abnormal nuclear staining after selection and *in vitro* ART fertility outcomes

The contents of this chapter have been published in:

Tavares RS*, Silva AF*, Lourenço B, Almeida-Santos T, Sousa AP & Ramalho-Santos J. (2013) Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization. *Andrology* 1, 830-837. *Co-first authors of the paper.

Abstract

Sperm chromatin/DNA damage can be measured by a large number of assays. However, it has been reported that some tests may lose prognostic value in ART when assessed in post-processed samples, possibly due to the "normalizing" effect promoted by the sperm preparation procedures before performing ART treatments. Recently, we have implemented a modified version of the Diff-Quik staining assay that allows the evaluation of human sperm chromatin status in native samples. However, its relationship with both IVF and ICSI fertility outcomes after sperm selection required further clarification.

Sperm chromatin integrity from 138 couples undergoing IVF or ICSI cycles was significantly improved after density gradient centrifugation and swim-up techniques ($p<0.001$), but no correlations were found with fertilization or embryo development rates ($p>0.05$). Nonetheless, sperm samples presenting lower percentages of damaged chromatin were associated to better quality (grade I) embryos in both ART treatments ($p<0.05$) and clinical pregnancy success among IVF couples ($p<0.05$). Moreover, regression analysis confirmed the clinical value of Diff-Quik staining in predicting IVF clinical pregnancy (OR 0.927, 95% CI: 0.871-0.985, $p=0.015$) and a threshold value of 34.25% was further established. The proportion of IVF couples achieving a clinical pregnancy was reduced 1.9-fold when the percentage of abnormal dark staining was $\geq$34.25% ($p=0.05$).

In conclusion, the Diff-Quik staining assay provides useful information about ART success, particularly in IVF cycles, where some degree of "natural" sperm selection may occur; but not in ICSI, where spermatozoa are chosen by the operator. This quick and low cost assay is suggested as an alternative method to detect sperm chromatin status in minimal clinical settings, when no well-implemented and robust assays (e.g. SCSA, TUNEL) are available.

4.1 Material and methods

4.1.1 Materials

All chemicals were supplied by Sigma-Aldrich unless otherwise described.

4.1.2 Patients

A total of 138 cycles (57 IVF and 81 ICSI) from 138 couples experiencing infertility for at least 1 year, with no viral infections, and whose female partners were <40 years old and presented baseline FSH levels below 12 IU/l were included in this study. Couples with normal standard semen parameters according to the WHO criteria (WHO 2010) were referred to IVF, while ICSI was performed either on couples with poor sperm quality (e.g low concentration and/or motility) or with a previous IVF history of failed fertilization or low fertilization rates.

Sperm samples were used in agreement with the appropriate ethical and Internal Review Board of the Institution, who approved the experimental work. All subjects signed informed consent forms and samples were obtained by masturbation after 3-5 days of sexual abstinence. Sperm cells were treated in accordance to the WHO guidelines (WHO 2010).

4.1.3 Sperm preparation for ART procedures

After sample collection and liquefaction, spermatozoa were sequentially isolated by DGC and swim up techniques (Amaral *et al.* 2007; Sousa *et al.* 2011). Briefly, sperm were placed on top of a density gradient medium containing the 50% upper and 90% lower layers (Isolate® Sperm Separation Medium) and centrifuged at 528 x g for 10 minutes. After discarding both the seminal fluid and the 50% upper layer that usually contains dead sperm, white cells, and miscellaneous debris, spermatozoa from the 90% lower layer were washed with SPM (Origio Medicult) and centrifuged at 528 x g for another 10 minutes. Motile cells were then allowed to swim up for 30 minutes at 37°C and 5% CO_2 in the latter medium. The motile sperm fraction was used in ART procedures.

4.1.4 Sperm chromatin status assessment from both unprocessed samples and motile sperm fractions

Sperm chromatin status from both native samples and their respective motile sperm fractions after sperm selection (n=138) was evaluated by the Diff-Quik® (Dade Behring Inc, Newark, NJ, USA) staining as stated elsewhere (Ramalho-Santos *et al.* 2007; Sousa *et al.* 2009). This commercially available kit is composed by a fixative (methanol), a dye that stains basic proteins red (eosin) and a thiazin which stains sperm DNA blue. Briefly, 10 µl of the sample was dragged with a coverslip and allowed to air dry. Slides were then sequentially dipped in each kit solution for no longer than 10-20 seconds each and finally rinsed in water to remove excess dye. This is crucial in order to avoid a uniformly dark staining on all sperm, useful to assess morphology, but which does not provide any chromatin status information. Slides were observed under a bright-field microscope (Nikon Instruments Inc.) and staining features were constantly evaluated within each slide. Both light- and darkly stained sperm heads were visible in each slide, the latter representing abnormal chromatin status (Mota & Ramalho-Santos 2006; Sousa *et al.* 2009). The proportion of sperm with abnormal dark nuclei representing abnormal/damaged chromatin was established after scoring 200 cells in four different fields in each slide. Counts were performed blindly by at least two observers, and intra- and inter-observer variability was negligible.

4.1.5 Ovarian stimulation

Individualized ovarian stimulation protocols were performed after evaluation of each patient reproductive status (e.g. ovarian reserve and hormone levels). Long and short protocols of pituitary desensitization with GnRH agonists and antagonists, respectively, were performed. Follicular growth was stimulated by recombinant FSH (37.5-325 IU/day GONAL-f® [Merck Serono, London, UK] or Puregon® [N.V. Organon, Oss, the Netherlands]) or human menopausal gonadotropin (hMG; 50-300 IU/day Menopur®, Ferring Pharmaceuticals, West Drayton, UK) and when at least one leading follicle reached a 18-mm-diameter (monitored by ultrasound), ovulation was induced with human chorionic gonadotropin (hCG; 5 000 IU Pregnyl®, N.V. Organon). Ultrasound-guided vaginal oocyte aspiration was performed 35-36 hours post-hCG administration.

4.1.6 IVF and ICSI Protocols

Following sperm preparation and oocyte retrieval, IVF and ICSI procedures were performed (Santos *et al.* 2006). For ICSI cycles, cumulus cells were removed from the aspirated cumulus-oocyte-complexes (COCs) by incubation with 50 µl of hyaluronidase (SynVitro® Hyadase; Origio Medicult) and intermittent pipetting for a maximum of 30 seconds. Sperm suspensions were then placed in SpermSlow™ medium (Origio Medicult) and only free and motile spermatozoa were immobilized and injected into the oocytes with holding and microinjection pipettes. For IVF, each COC insemination was carried out with 100 000 spermatozoa. Injected and inseminated oocytes were cultured at 37ºC and 6% CO_2 in IVF medium (Origio Medicult). Fertilization was assessed after 17-20 hours.

4.1.7 Fertility outcome parameters

Fertilization and embryo development rates were scored as the number of 2PN oocytes/number of inseminated or injected oocytes and the number of embryos/number of inseminated or injected oocytes, respectively (Sousa *et al.* 2009). 48 hours post-fertilization, embryos were graded from I to IV in accordance to the number, form and symmetry of blastomeres and the presence of blastomere fragmentation (Elder & Dale 2000). Grade I embryos, i.e embryos with regular blastomere shape and simetry, light cytoplasmic appearance and blastomere fragmentation up to 10%, from couples only displaying this high embryo quality were included in the "G1" group whereas couples having at least one embryo classified differently were included in the "other grade" group (Sousa *et al.* 2009). Embryo transfer rate was determined as the number transfers performed/number of cycles which obtained embryos. Finally, clinical pregnancies were determined by ultrasound detection of the gestational sac(s). Clinical pregnancy rate was scored as the number of couples with positive clinical pregnancy/number of transfers.

4.1.8 Statistical analysis

Statistical analysis was carried out using the SPSS version 20.0 software for Windows (SPSS Inc., Chicago, IL, USA). Values are expressed as mean ± SEM. All

variables were checked for normal distribution by the Kolmogorov-Smirnov test (or the Shapiro-Wilk test for n<25) and the independent t-test for normal variables were performed to compare abnormal dark staining before and after sperm selection, between the two embryo quality groups and between pregnancy outcome groups in both IVF and ICSI cycles. As fertilization and embryo development rates presented a non-normal distribution, Spearman's non-parametric correlation coefficient test was performed to determine if there were any correlations with the abnormal dark staining. The sample sizes used in this study provided power values equal or greater than 80% to detect a 10-15% difference in the proportion of abnormal dark staining between groups in each condition assessed. Demographic data comparisons between ART treatments and pregnant versus non-pregnant couples were performed by the independent t-test or the related Mann-Whitney test for non-normal variables and the χ^2-test for categorical data. Logistic regression analysis was carried out to assess the predictive value of several factors in ART pregnancy outcomes. Receiver-operating characteristic (ROC) curve analysis allowed the determination of a significant threshold for IVF clinical pregnancy and the χ^2-test was performed to find a possible threshold effect. $p \leq 0.05$ was considered significant.

4.2 Results

4.2.1 Characteristics of the study population

Demographic data from 57 IVF and 81 ICSI cycles are displayed in Table 4.1. Although the female factor (e.g. tubal anomalies, endometriosis, polycystic ovary syndrome) was the major cause of infertility in both IVF and ICSI couples (64.91% and 51.90%, respectively), the proportion of couples experiencing infertility exclusively due to a male factor (e.g. varicocele) was significantly higher among couples referred to ICSI cycles (22.78% and 1.75%, p=0.001). Since one of the criteria used to perform ICSI is poor semen quality (e.g. low concentration and/or motility), the decrease observed in the sperm concentration of these patients was not surprising (74.21±6.88 $x10^6$/ml and 93.40±6.5 $x10^6$/ml, p<0.05). However, total motility did not differ between treatments (p>0.05). Among IVF couples, the number of inseminated/injected (11.03±0.88 and 4.07±0.32, p<0.001) and fertilized oocytes (6.11±0.62 and 2.49±0.20, p<0.001), as well as

Table 4.1 Background information on both IVF and ICSI cycles.

		IVF	**ICSI**	p-value
Cycles/couples included (n)		57	81	
Female age (years±SEM)		33.00±0.49	33.14±0.36	>0.05
Male age (years±SEM)		35.16±0.73	35.37±0.57	>0.05
Diagnosis of infertility (%)				
	unexplained	21.05	12.66	>0.05
	male factor	1.75	22.78	0.001
	female factor	64.91	51.90	>0.05
	male and female factors	12.28	12.66	>0.05
Type of infertility (%)				
	primary	72.73	72.15	>0.05
	secondary	27.27	27.85	>0.05
Duration of infertility (years±SEM)		5.16±0.45	6.09±0.35	>0.05
No. of inseminated/injected oocytes (mean±SEM)		11.03±0.88	4.07±0.32	<0.001
No. of 2PN oocytes (mean±SEM)		6.11±0.62	2.49±0.20	<0.001
Fertilization rate (%)		57.22±3.77	64.33±3.53	>0.05
No. of embryos (mean±SEM)		4.85±0.50	2.09±0.13	<0.001
Embryo development rate (%)		50.00±3.44	56.70±3.37	>0.05
No. of transferred embryos (mean±SEM)		1.95±0.07	2.05±0.01	>0.05
Embryo transfer rate (%)		86.00	94.20	>0.05
Clinical pregnancy rate (%)		41.86	27.69	>0.05
Mean sperm concentration (10^6/ml±SEM)		93.40±6.50	74.21±6.88	0.018
Sperm motility (mean %±SEM)		60.89±2.52	56.68±2.46	>0.05

2PN – 2 pronuclei; Fertilization rate - number of 2PN oocytes / number of inseminated or injected oocytes; Embryo development rate - number of embryos / number of inseminated or injected oocytes; Embryo transfer rate - number of transfers performed / number of cycles that obtained embryos; Clinical pregnancy rate - number of pregnant couples /number of transfers.

the number of embryos retrieved (4.85±0.50 and 2.09±0.13, p<0.001) were significantly higher than the ones obtained by couples undergoing ICSI treatments. No differences were observed regarding male and female age, type and duration of infertility, and fertilization, embryo development, embryo transfer and clinical pregnancy rates (p>0.05, Table 4.1).

4.2.2 Sperm chromatin damage after DGC and swim up selection

To potentially maximize ART outcomes, and thus achieve pregnancy, sperm cells are traditionally processed by DGC and/or swim up procedures worldwide. Sperm chromatin integrity was significantly improved after this selection, as observed by the decreased proportion of abnormal dark staining in the motile sperm fraction when compared to their unprocessed counterparts (41.13±2.15 and 51.40±1.92, p<0.001, Figure 4.1). It seems thus that the Diff-Quik staining efficiently detects an enrichment of sperm cells with chromatin integrity in post-prepared samples, as others have reported using different assays (Spanò *et al.* 1999; Tomlinson *et al.* 2001; Gandini *et al.* 2004; Marchesi *et al.* 2010).

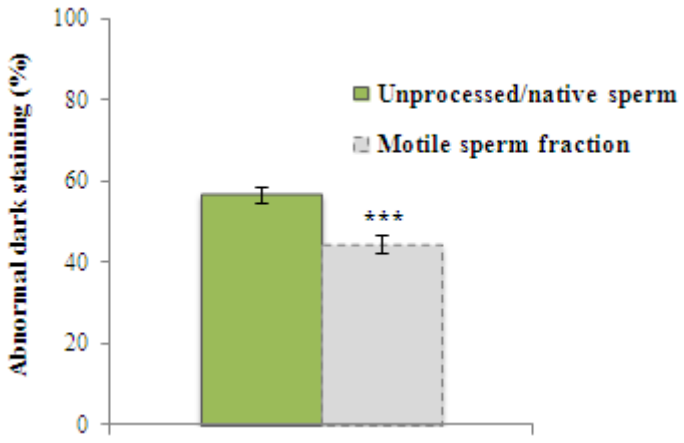

Figure 4.1. Proportion of abnormal dark staining in both native samples and respective motile sperm fractions after sperm selection.

Bars represent mean percentage ± SEM of a total of 138 samples analyzed. *** symbolizes p<0.001.

4.2.3 Chromatin damage, fertilization and embryo development rates

No correlation was detected between the percentage of sperm with dark nuclei and IVF (rho=-0.105, n=57, p>0.05) or ICSI fertilization rates (rho=-0.123, n=81, p>0.05). Moreover, the same lack of association was found between abnormal dark staining and embryo development rates in both IVF and ICSI cycles (rho=-0.029, n=51 and rho=-0.067, n=74, respectively, p>0.05).

4.2.4 Chromatin damage, embryo quality and clinical pregnancy

To further analyse if sperm dark nuclei had any effect on embryo quality, embryos were graded from I to IV according to several embryo features (Elder & Dale 2000) and couples with only good quality embryos, commonly classified as Grade I, were included in the "G1" group, while couples that exhibited at least one embryo with a different grade were involved in the "other grade" group. The two embryo quality groups in IVF cycles were produced by sperm with distinct proportions of dark nuclei, with samples that generated the "G1" group showing a significant lower proportion of sperm with dark staining (32.41±4.03%, n=22, and 47.04±5.77%, n=22, respectively, p<0.05). Similarly, ICSI couples included in the "G1" group (n=38) presented a significantly lower percentage of abnormal dark staining than the ones (n=30) in the "other grade" group (35.26±3.97% and 47.8±4.69%, respectively, p<0.05).

Several factors may affect pregnancy success in both IVF and ICSI treatments (Table 4.2). Pregnant IVF couples presented a higher number of inseminated (11.22±0.95 and 9.00±1.39, p<0.05) and fertilized oocytes (6.78±0.70 and 4.89±0.60, p<0.05) and a decreased proportion of spermatozoa with dark nuclei (36.89±4.52% and 51.75±5.63%, p<0.05) than their non-pregnant counterparts. Conversely, only the number of embryos obtained (2.60±0.19 and 2.09±0.14, p=0.05) and transferred (2.40±0.16 and 1.89±0.12, p<0.05) were significantly increased among pregnant couples following an ICSI cycle. No difference was detected between the percentages of abnormal dark staining when pregnant and non-pregnant ICSI groups were compared (35.45±4.40% and 44.93±4.15%, p>0.05, Table 4.2). From all variables listed in Table 4.2, only female age (OR 0.632; 95% CI: 0.431 - 0.926, p<0.05, Table 4.3), total motility (OR 1.092; 95% CI: 1.016-1.174, p<0.05,

Table 4.2 Comparison of several variables among pregnant and non-pregnant groups that underwent IVF or ICSI treatments.

	IVF			ICSI		
	pregnant	non-pregnant	p-value	pregnant	non-pregnant	p-value
No. of couples/cycles included	18	25		18	47	
Female age (years±SEM)	31.67±0.79	33.72±0.93	>0.05	34.27±0.62	32.59±0.51	>0.05
Male age (years±SEM)	33.33±0.67	35.65±1.50	>0.05	35.91±1.22	35.17±0.88	>0.05
No. of inseminated/injected oocytes (mean±SEM)	11.22±0.95	9.00±1.39	0.023	3.93±0.37	4.61±0.50	>0.05
No. of 2PN oocytes (mean±SEM)	6.78±0.70	4.89±0.60	0.031	3.13±0.34	2.77±0.28	>0.05
Fertilization rate (mean %±SEM)	63.87±5.68	60.57±5.29	>0.05	81.00±5.07	68.80±4.06	>0.05
No. of embryos (mean±SEM)	5.17±0.54	4.44±0.59	>0.05	2.60±0.19	2.09±0.14	0.05
Embryo development rate (mean %±SEM)	50.70±6.07	53.36±4.75	>0.05	71.33±6.05	58.21±4.13	>0.05
No. of transferred embryos (mean±SEM)	2.11±0.08	1.89±0.11	>0.05	2.40±0.16	1.89±0.12	0.023
Mean sperm concentration (10^6/ml±SEM)	112.22±9.55	85.39±12.45	>0.05	89.14±18.26	72.25±8.76	>0.05
Motility (mean %±SEM)	68.83±3.30	58.67±4.51	>0.05	63.47±3.83	55.55±3.74	>0.05
Abnormal dark staining (mean % ±SEM)	36.89±4.52	51.75±5.63	0.047	35.45±4.40	44.93±4.15	>0.05

2PN – 2 pronuclei; Fertilization rate - number of 2PN oocytes / number of inseminated or injected oocytes; Embryo development rate - number of embryos / number of inseminated or injected oocytes.

Table 4.3) and abnormal dark staining (OR 0.927, 95% CI: 0.871-0.985, p<0.05, Table 4.3) were found to be predictors of clinical pregnancy among IVF cycles. Though female age seems to be the factor that most contributes to pregnancy success in IVF treatments in this particular study, our staining foresees a decrease in pregnancy chances of 7.3% *per* each 1% increase of abnormal dark staining observed. Contrary to this, the number of embryos obtained (OR 4.054; 95% CI: 1.308-12.561, p<0.05, Table 4.3), but not the abnormal dark staining (OR 1.01, 95% CI: 0.957-1.106, p>0.05), was predictive of clinical pregnancy for ICSI cycles.

Considering the prognostic value of the Diff-Quik staining in IVF pregnancy success, a ROC analysis was performed in an attempt to identify a threshold value for abnormal dark staining beyond which clinical pregnancy would be compromised. With an area under the curve of 0.700 cm^2 ($p<0.05$), a threshold value was set at 34.25% with a sensitivity of 77.8% and a specificity of 52.9%. The proportion of pregnant couples having an abnormal dark staining ≥34.25% was 1.9-fold reduced when compared to the fraction of pregnant couples with sperm dark nuclei below this cut-off (p=0.05).

Table 4.3 Odds ratio and 95% confidence intervals from several predictors of clinical pregnancy in both IVF and ICSI cycles.

		OR (95% CI)	p-value
IVF	Female age	0.632 (0.431-0.926)	0.018
	Motility	1.092 (1.016-1.174)	0.017
	Abnormal dark staining	0.927 (0.871-0.985)	0.015
ICSI	No. of embryos	4.054 (1.308-12.561)	0.015

OR – Odds Ratio; CI – Confidence Interval

4.3 Discussion

Routine semen analysis does not include the evaluation of sperm DNA damage, despite the multitude of available assays. This lack of assessment has been extensively criticized, as it has become more evident that men with normal standard semen parameters may possess abnormal levels of DNA damage (Agarwal & Allamaneni 2004), therefore potentially contributing to the limited success of ART.

Recently, we modified the Diff-Quik staining method to allow the assessment of sperm morphology and abnormal chromatin status in the same slides (Mota & Ramalho-Santos 2006; Sousa *et al.* 2009). Despite its clinical value when used in unprocessed

samples, its relationship with ART fertility outcomes after sperm selection was further probed in this study. Since many tests may lose their predictive value when assessed in post-prepared samples (Larson *et al.* 2000; Gandini *et al.* 2004; Seli *et al.* 2004; Muriel *et al.* 2006b; Bungum *et al.* 2008), it has been suggested that the evaluation of sperm DNA integrity must be carried out in the whole ejaculate when it concerns *in vivo* conception and after sperm selection when ART treatments are needed (Tomlinson *et al.* 2001).

Sperm preparation techniques such as DGC and swim up favor the selection of live, highly motile and morphologically normal spermatozoa that will be used in ART cycles (Bungum *et al.* 2008). However, some conflicting results exist on whether these techniques, alone or in combination, select sperm with lower levels of DNA damage. Nevertheless, as previously reported by several authors (Spanò *et al.* 1999; Tomlinson *et al.* 2001; Gandini *et al.* 2004; Marchesi *et al.* 2010), a significant improvement of sperm chromatin integrity following sperm preparation procedures was found in this study, thus suggesting the use of better quality sperm in ART procedures. Furthermore, these results support the clinical usefulness of the Diff-Quik staining assay.

Although many reports have indicated an obvious influence of sperm DNA damage on fertilization rates (Sun *et al.* 1997; Lopes *et al.* 1998; Benchaib *et al.* 2003; Velez de la Calle *et al.* 2008; Simon *et al.* 2011), we did not observe any relationship between abnormal chromatin status, as monitored by this assay, and fertilization rates in both IVF and ICSI treatments. These findings are, however, in agreement with several other studies involving both IVF (Tomlinson *et al.* 2001; Tomsu *et al.* 2002; Henkel *et al.* 2003, 2004; Lin *et al.* 2008) and ICSI cycles (Henkel *et al.* 2003; Lin *et al.* 2008). As pinpointed in the introduction section, the activation of the paternal genome is thought to only occur at 4-8-cell stage embryo (Braude *et al.* 1988; Borini *et al.* 2006) which may explain why paternal chromatin status might not affect fertilization significantly (Ahmadi & Ng 1999). However, abnormal levels of DNA damage may affect later stages of development (Virro *et al.* 2004; Borini *et al.* 2006; Simon *et al.* 2010). In the current study we failed to observe any relationship between the percentages of abnormal dark staining and embryo development rates in post-prepared samples in both IVF and ICSI cycles, but embryo quality and pregnancy success were adversely affected by abnormal chromatin status monitored by this assay. Abnormal dark staining was found to have a small but significant prognostic value in IVF pregnancy success. Obviously one may not exclude that other

factors besides sperm chromatin/DNA damage (e.g. female age) may also influence these reproductive parameters.

Reports focusing on the relationships between DNA damage and embryo or pregnancy outcomes in ART are conflicting probably because the type and degree of DNA injury differ among studies, as do the DNA integrity assays employed. For instance, single stranded DNA breaks are thought to be easier to repair by the oocyte than double-stranded nicks (Sakkas & Alvarez 2010) but the large majority of tests cannot tell which spermatozoon possesses what. Furthermore, the ability of the oocyte to mend such damage is limited (Ahmadi & Ng 1999) and the efficiency of repair relies on oocyte quality which, in turn, is influenced by numerous factors including female age (Alvarez 2005).

Similarly to what is described here, Simon and co-workers have also found a decrease in embryo quality and pregnancy success with increased sperm DNA fragmentation assessed by the Comet assay after DGC among IVF couples, but not between pregnant and non-pregnant groups after ICSI cycles (Simon *et al.* 2010). However, contrary to our data, they failed to observe any relationship with embryo quality after ICSI treatments (Simon *et al.* 2010). Additionally, an inverse correlation between embryo quality and Comet sperm DNA damage among IVF couples was detected in processed samples by Tomsu and colleagues; however, no correlation with pregnancy outcomes was shown (Tomsu *et al.* 2002). In other studies positive clinical pregnancies and lower pregnancy loss rates were observed among ICSI couples that presented a lower proportion of sperm with fragmented DNA detected by TUNEL after DGC (Benchaib *et al.* 2003; Borini *et al.* 2006). Conversely, others failed to observe any relationship between DNA damage, evaluated in post-prepared spermatozoa by SCSA, TUNEL or ISNT, and embryo quality or clinical pregnancies in IVF and/or ICSI cycles (Sun *et al.* 1997; Larson *et al.* 2000; Tomlinson *et al.* 2001; Benchaib *et al.* 2003; Gandini *et al.* 2004; Seli *et al.* 2004; Borini *et al.* 2006; Bungum *et al.* 2008).

Although the percentage of sperm with DNA damage may considerably decrease after sperm preparation techniques, as we demonstrated here, there is still a reasonable likelihood of the technician choosing a spermatozoon with damaged chromatin (i.e. decondensed chromatin and/or DNA damage) when performing ICSI, which may explain our findings. This is particularly worrisome given that DNA damage may not be fully repaired by the oocyte machinery, but still allow for embryo development, increasing the

risk of conceiving a child with genetic anomalies (Marchetti & Wyrobek 2005; Aitken & De Iullis 2010). On the other hand, our data suggests that there may be some degree of "natural" selection in IVF cycles, thus favoring sperm with no or less damage to the chromatin/DNA to successfully achieve pregnancy.

Clinical pregnancy success was severely reduced in IVF couples having at least 34.25% of abnormal dark staining in this report. Interestingly, several other studies showed similar cut-offs for IVF. Lower pregnancy rates were observed among couples presenting samples with at least 35% (Frydman *et al.* 2008) or more than 36.5% TUNEL-positive spermatozoa (Henkel *et al.* 2003, 2004). The same outcome was found by Virro and colleagues when DFI ≥30% using SCSA (Virro *et al.* 2004).

Taken together, the modified Diff-Quik staining assay provides useful information about ART success in post-prepared samples, particularly in IVF treatments where an operator does not choose the sperm that will fertilize the oocytes. However, despite its low cost and simple methodology, this staining displays a certain degree of subjectivity and exposure to the thiazin dye for longer periods than those described here will produce a uniformly dark staining that will compromise chromatin damage assessment. Proper training, nevertheless, allows the achievement of consistent and reproducible results, with minimal variability. Although SCSA is a very robust assay that analyzes 5 000 - 10 000 sperm cells, using objective, machine-defined criteria and with high levels of repeatability (Evenson *et al.* 1999, 2002), it is not used in most Andrology laboratories as a routine procedure, nor are many other chromatin/DNA integrity tests. The need of extensive protocols and/or expensive reagents and equipment (e.g. a flow cytometer, fluorescence microscope and/or dedicated software) are limiting factors when the goal is to routinely implement DNA damage analysis worldwide. Based on our present and previous results (Sousa *et al.* 2009) we therefore suggest that the modified Diff-Quik staining may be used as an alternative method to detect sperm chromatin damage, in the absence of more robust tests.

Chapter 5 Concluding Remarks and Future Directions

5.1 Exposure to environmental organochlorines

Many studies regarding the effects of environmental endocrine disruptors (EDs) have particularly centered their attention on long-term influences on the testis and male reproductive tract, with reproductive tract anomalies and standard semen parameters frequently being assessed as biologically important endpoints.

Despite the importance of these studies, we now provide evidence that p,p'-DDE concentrations produce direct (non-genomic) effects in mature sperm function. We reported that through the opening of Catsper p,p'-DDE primarily promotes Ca^{2+} influx which, due to its high and sustained nature, impairs cell function, ultimately compromising sperm fertilizing capacity and male fertility. In accordance with others, non-regulated Ca^{2+} entry may trigger acrosome reaction (Campagna *et al.* 2009) and since Ca^{2+} is involved in several other signaling pathways (Jimenez-Gonzalez *et al.* 2006; Costello *et al.* 2009), alterations in its homeostasis may also be implicated in the effects reported in the present study.

Keeping in mind the role of mitochondria not only as an important cellular energy source but also in regulating Ca^{2+} homeostasis, the extracellular Ca^{2+} uptake by these cells may lead to a mitochondria Ca^{2+} overload that will culminate in a general mitochondrial dysfunction and cellular ATP depletion, which will further compromise sperm motility and ultimately lead to cell death (Crompton 1999; Halestrap 2005; Orrenius *et al.* 2007). However, we cannot exclude that other pathways might be involved. Given the comparable Ca^{2+} responses produced by 25, 50 and also 100 μM p,p'-DDE (data not shown) and the increasing time periods required by the lower concentrations to produce such effects, we hypothesize that besides Ca^{2+} other component(s) might be affected by p,p'-DDE exposure, which under the action of lower doses need(s) more time to induce observable effects. As only 100 μM p,p'-DDE inhibited the process of capacitation we imagine that p,p'-DDE may also affect other aspects of the capacitation signaling pathway that at lower concentrations may not be as affected despite the extended incubation periods. These assumptions, nevertheless, require confirmation in future studies.

Considering our results and taking into account that spermatozoa can survive in the female reproductive tract for more than 3 days, continuous exposure to decreasing p,p'-

DDE doses might still affect sperm function later on. Spermatozoa may acrosome-react, lose motility and die before reaching the oocyte, further losing their fertilizing capacity.

Direct exposure to PCB 77 was also found to impair sperm function at the highest dose tested. Although this concentration is non-physiological we cannot assume that human spermatozoa are resistant to PCB 77. In fact, as previously mentioned, we should always consider that spermatozoa are exposed to a wide range of environmental EDs that can act synergistically at the site of fertilization *in vivo*, producing effects even at smaller and more relevant concentrations than the ones found to independently affect spermatozoa. Importantly, PCB 77 acted through a different mechanism when compared to p,p'-DDE, yet it culminated in cell death, possibly attributable to mitochondrial dysfunction. The observation that motility was compromised before any detectable alteration in mitochondrial function may seem odd; however, there might be several explanations for this. PCB 77 might be affecting metabolic pathways other than OXPHOS that were not addressed in the present work and/or induce alterations in sperm motor machinery, similarly to what has been suggested with sildenafil citrate (Sousa *et al.* 2013). Further studies are warranted to determine how PCB 77 affects flagellar propulsion.

On the other hand, our *in vitro* approach failed to detect any effect of TCDD in human spermatozoa. Overall, although all the compounds used here are organochlorines, our results clearly point to different mechanisms of action. Further *in vivo* validation of our results is, nevertheless, required.

Importantly, recent reports concerning the potential transgenerational effects induced by environmental EDs in rodents retrieved worrisome conclusions: EDs may change the DNA methylation programming of the male germ line which will subsequently transmit this altered epigenome in an imprinted-like manner across generations to promote adult onset disease (Manikkam *et al.* 2012, 2013). These studies showed that although pregnant dams (F0 generation) were the only ones transiently exposed to EDs at the time of male sex determination, F3 and F4 males presented transgenerational inheritance of testis pathology which included increased germ cell apoptosis (Anway *et al.* 2006, 2008; Guerrero-Bosagna *et al.* 2012), decreased motility and reduced spermatid (Anway *et al.* 2006) and epididymal sperm numbers (Anway *et al.* 2006, 2008; Guerrero-Bosagna *et al.* 2012). This represents a totally new research field that remains largely unexplored and so far few EDs have been screened. As neither p,p'-DDE nor PCB 77 were studied, it is our

intention to evaluate their potential epigenetic transgenerational effects and analyze sperm epigenome which will allow the identification of DNA methylation regions that can be potentially used as epigenetic biomarkers for transgenerational exposure and disease. This could be done using human sperm samples.

Furthermore, to determine *in vivo* effects more accurately it would be interesting to analyze animals living in the area of Souselas (district of Coimbra), suggested as being under the action of environmental EDs formed as byproducts of incineration processes (Cardoso 2002; Nunes & Matias 2003). This was actually one of our initial goals; however this has proven difficult to carry out.

5.2 Assessment of sperm chromatin/DNA damage

The influence of sperm chromatin/DNA fragmentation on achieving pregnancy either naturally or through ART has being recognized as a valuable addition to the conventional semen analysis but no test has been routinely implemented worldwide. The Diff-Quik staining is neither time-consuming nor requires expensive reagents and equipment and has proved to be a reliable and useful assay to detect sperm chromatin/DNA damage in a standard clinical ART setting. Furthermore, a clear cut-off was established for IVF pregnancy success that was similar to what has been described for other assays. Although Diff-Quik staining was not predictive of pregnancy success in ICSI couples, further studies are required to address its possible prognostic value in ICSI pregnancy loss as other studies have shown that sperm DNA damage may also affect ongoing pregnancies. Accordingly, increased miscarriage rates significantly differed between patients with high and low sperm DNA damage undergoing ICSI (Borini *et al.* 2006; Benchaib *et al.* 2007) and a meta-analysis involving a total of 741 ICSI cycles have confirmed the predictive value of sperm DNA damage in pregnancy loss (Zini *et al.* 2008).

On the same vein, it is also crucial to evaluate the impact of chromatin/sperm DNA damage, determined by our method, on intrauterine insemination (IUI) pregnancy success. In fact, IUI is not only the less invasive of all ART procedures but also the first line of treatment in couples that cannot achieve pregnancy naturally, despite the lack of severe infertility problems (Muriel *et al.* 2006b). So far, few studies have actually addressed the

effect of sperm DNA damage on IUI fertility outcomes and the large majority used SCSA (Bungum *et al.* 2004, 2007, 2008). A meta-analysis published by Evenson and Wixon concluded that IUI couples whose male partners presented a DFI below 30% were 7.3 times more prone to achieve a pregnancy/delivery than the ones exhibiting higher values (Evenson & Wixon 2006). Thus, it will be essential to tackle if, as SCSA, the Diff-Quik staining may also be useful in predicting IUI pregnancy success and establish a threshold that may help the clinician to decide whether IUI is the best treatment option for a given couple or if, on the contrary, more invasive techniques should be carried out.

Although some degree of subjectivity can be attributed to our method proper training allows reliable results, as discussed previously. Furthermore, this subjectivity could also be easily reduced if an automated image analysis software could be developed for the quantification of sperm chromatin damage in any sample.

Given its consistency and minimal variability observed during this and an earlier study by our group (Sousa *et al.* 2009), we consider that our simple, fast and low-cost technique is suitable for further large-scale studies, such as those concerning the impact of environmental EDs on sperm DNA damage in human populations.

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