DEPARTAMENTO DE CIÊNCIAS DA VIDA

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# Effects of Nicotine, Anandamide and Sildenafil Citrate in Sperm Function: The Role of the Mitochondria

Maria Inês Ramalho de Almeida e Sousa

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# Effects of Nicotine, Anandamide and Sildenafil Citrate in Sperm Function: The Role of the Mitochondria

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor João Ramalho-Santos (Universidade de Coimbra) e coorientação da Doutora Sandra Amaral (Universidade de Coimbra)

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## Abbreviations:

Δ-9-THC - delta-9-tetrahydtocannabinol

AcHR - acetylcholine receptors

ADP - adenosine diphosphate

AEA - anandamide

AEC - adenylate energy charge

AMP - adenosine monophosphate

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ATP - adenosine triphosphate

BSA - bovine serum albumin

cAMP - cyclic adenosine monophosphate

CB1R cannabinoid receptor 1

CB2R - cannabinoid receptor 2

cGMP - cyclic guanosine monophosphate

COX - cytochrome c oxidase

CSE - cigarette smoke extracts

DMSO – dimethyl sulfoxide

DNA - deoxyribonucleic acid

EDTA-Na - sodium ethylenediaminetetraacetic acid

EMT - endocannabinoid membrane transporter

ETC - electron transport chain

FAAH - fatty acid amide hydrolases

FCCP - p-trifluoromethoxy carbonyl cyanide phenyl hydrazone

FDA - food and drug administration

FSH - follicle stimulating hormone

GABA - γ-aminobutyric acid

GnRH - gonadotropin releasing hormone

$H_2O_2$ - hydrogen peroxyde

HPLC - high performance liquid chromatography

I - immotile sperm

$K_{ATP}$ - ATP-sensitive potassium channel

KOH - potassium hydroxide

LH - luteinizing hormone

MMP - mitochondrial membrane potential

mtDNA - mitochondrial DNA

NADH - nicotinamide adenine dinucleotide

NAT - N-acyltranferase

NArPE - N-arachidonoyl-phosphatidylethanolamina

NO - nitric oxide

NP - non-progressive motility

NRS - reactive nitrogen species

O2 - molecular oxygen

$O2^{\bullet-}$ - superoxide radical

$OH^-$ - hydroxide ion

$ONOO^-$ - peroxynitrite

OXPHOS - oxidative phosphorylation

PBS - Phosphate buffered saline

PDE5 - 5-phosphodiesterase

Pi - inorganic phosphate

PI - propidium iodide

PKG - phosphokinase G

POLG - DNA polymerase subunit gamma

Pr - progressive motility

QH - ubisemiquinone radical intermediate

ROS - reactive oxygen species

RPM - rotations per minute

RT-PCR - real time reverse transcriptase-polymerase chain reaction

SOD - superoxide dismutase

TFAM - transcription factor A mitochondrial

TRPV1 - vanilloid receptor type 1

WHO - world health organization

## Resumo

A infertilidade é diagnosticada como a incapacidade de um casal conceber após pelo menos um ano de relações desprotegidas e é um dos mais sérios problemas sociais com que as nações mais desenvolvidas se estão a deparar. Estima-se que aproximadamente metade dos casos de infertilidade de um casal está associada a problemas na fertilidade masculina e que estes poderão estar relacionados com a sua exposição a diversos tóxicos ambientais aliados às escolhas e estilos de vida de um individuo, como o ato de fumar drogas recreativas ou de tomar certos fármacos que poderão conter substâncias que afetam negativamente a fertilidade masculina. A nicotina, a anandamide e o sildenafil citrato são três substâncias relacionadas com os hábitos mencionados anteriormente para além de já terem sido confirmadas como sendo três substâncias que afetam negativamente a fertilidade masculina e que alteram a funcionalidade mitocondrial de outros tipos celulares. Uma vez que a atividade mitocondrial dos espermatozóides está intimamente relacionada com a fertilidade masculina e estas substâncias têm efeitos adversos na mitocôndria de outras células será interessante estudar o efeito da nicotina, da anandamida e do sildenafil na função espermática, tal como na bioenergética celular do espermatozoide e na sua função mitocondrial.

Tendo isso em consideração, o objetivo deste trabalho é estudar o efeito da nicotina, da anandamide e do sildenafil nos parâmetros seminais, mobilidade e viabilidade, tal como na bioenergética celular por determinação do conteúdo celular de nucleótidos de adenosina, e na função mitocondrial através do estudo de possíveis alterações no potencial membranar mitocondrial ou na produção de espécies reativas de oxigénio. Para isso, amostras de sémen foram individualmente incubadas com as substâncias em estudo por 12 e 24 horas.

Os resultados demonstraram que todas as três substâncias diminuem a mobilidade espermática sem alterarem a viabilidade dos espermatozoides. Da mesma forma foi verificada uma diminuição na quantidade de ATP presente nas amostras expostas às três substâncias individualmente, que muitas das vezes s traduziu numa diminuição da carga energética das amostras. Estas alterações na bioenergética espermática podem ser a causa para as alterações verificadas na mobilidade. No entanto, as alterações no conteúdo celular de nucleótidos nas amostras incubadas com nicotina e anandamide não poderão ser justificadas por

alterações na actividade mitocondrial uma vez que não se verificaram alterações no potencial membranar mitocondrial nem na produção de espécies reactivas de oxigénio. Relativamente ao efeito do sildenafil na função mitocondrial dos espermatozóides não foram conduzidos ensaios. Para complementar este trabalho e aprofundar o conhecimento sobre o efeito das substâncias na função mitocondrial, deveriam ser conduzidos mais ensaios tanto para o sildenafil como para as outras duas substâncias em análise no potencial mitocondrial, na produção de espécies reativas de oxigénio e também na atividade dos complexos enzimáticos da cadeia de transporte de eletrões na mitocôndria.

## Abstract

Infertility is one of the most serious social problems facing advanced nations and it is estimated that approximately half of all cases of infertility are caused by factors related to the male partner. Male infertility can be due to exposure to environmental toxicants as several lifestyle-related and environmental factors appear to negatively affect male fertility. Smoking recreational drugs and taking therapeutic drugs are some of the lifestyle-related activities commonly associated with male infertility. Nicotine, anandamide and sildenafil citrate are three substances linked to the practice of these ordinary personal habits that are already proven to have negative effects on sperm function and to alter mitochondrial function in several cell types. Furthermore, knowing that sperm mitochondrial dysfunction is intimately related with male infertility it would be interesting to study the effects of these three substances on sperm function and more precisely on sperm mitochondrial function.

Having this in consideration, the aim of this work is to study the effect of nicotine, anandamide and sildenafil citrate in the sperm function focusing on sperm bioenergetics and mitochondrial function. In order to do this, human sperm samples were incubated with nicotine, anandamide and sildenafil citrate separately for 12 and 24 hours, then basic sperm parameters, namely motility and viability were evaluated. Sperm bioenergetics was assessed by adenosine nucleotides content and energy charge calculation and mitochondrial function was evaluated by mitochondrial membrane potential and mitochondrial reactive oxygen species determination.

Results show that each of the three substances decreases sperm cell motility without altering cell viability. They also show that all the three substances decrease cellular content of ATP and eventually cellular energy charge. These bioenergetical alterations can be responsible for the reduction observed in sperm motility and could be due to induced mitochondrial dysfunction. However, results showed that neither nicotine nor anandamide provoked any changes in both mitochondrial membrane potential and mitochondrial ROS production. Regarding

to sildenafil citrate, no assays on mitochondrial function were performed and it would be interesting in the future to assess this topic.

Nevertheless, more studies should be conducted to further investigate the role of nicotine, anandamide and sildenafil citrate on mitochondrial function and more precisely on mitochondrial oxidative phosphorylation.

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## Chapter 1



## Chapter 2

# Chapter 1

## Introduction

## 1.1. The sperm cell

Spermatozoa are extremely specialized and unique cells, indispensable for the reproductive process, ensuring the contribution of a paternal genome to the zygote. Spermatozoa are produced in the testes through a highly regulated and complex process, called spermatogenesis. In man spermatogenesis starts at puberty and continues until old age (De Kretser & Kerr, 1988; Kerr et al., 2006; Kopera et al., 2010). It takes place in the male testes, more precisely in the seminiferous tubules, in a step-wise fashion and in humans takes approximately 74 days. Three major phases can be distinguished in this process: spermatogoniogenesis, spermiogenesis and spermiation. Spermatogoniogenesis is characterised by continuous mitotic divisions of the spermatogonial cells, namely type A spermatogonia, that will not only produce a pool of stem-like cells, that will remain at the basal membrane, but also mature into type B spermatogonia, that subsequently differentiate into primary spermatocytes (Holstein et al., 2003; Lie et al., 2010). Subsequently, primary spermatocytes enrol in two meiotic divisions resulting in four haploid cells called round spermatids. The condensation of the nuclear chromatin to about one tenth of the volume of an immature spermatid, the formation of the enzyme filled acrosome, the development of flagellum structures, as well as the reorganization of the organelles, such as the mitochondria at the mitochondrial sheath, are the hallmarks of spermiogenesis, a process that will result in the differentiation of round spermatids into mature elongated spermatids. This process ends when the cells are released from the germinal epithelium; in a process called spermiation. At this point, the free cells are called spermatozoa (Holstein et al., 2003; Johannisson et al., 2003; Fauser et al., 1999).

The whole process of spermatogenesis is under the strictly regulation of a rather simple hormone network, the hypothalamus-hypofisis-gonad axis. At the paracrine level, spermatogenesis is controlled by the secretion of hypothalamic gonadotropin releasing hormone (GnRH) that stimulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland (McLachlan, 2000). LH is thought to be primarily responsible for stimulating the secretion of testosterone by Leydig cells. Testosterone is necessary for normal sperm development as it activates genes in Sertoli cells that promote differentiation of spermatogonia cells, and therefore the continuation of the

spermatogenic process, that finishes with the release of spermatozoa in the seminiferous tubules lumen. (Jonge et al., 2006).

The sperm cell is unique, and three major parts can be immediately distinguished: head, midpiece and tail. According to Mortimer and Menkveld normal human spermatozoa must have a head with a smooth oval configuration, with a regular outline, and a well-defined acrosome constituting 40%-70% of the anterior sperm head. The head length is between 3 and 5 μm, and its width ranges between 2 and 3 μm (Mortimer and Menkveld et al., 2001). The sperm head is also composed of a nucleus, in which histones, have been partially replaced during spermiogenesis by protamines that convey the hypercondensation of the sperm DNA causing the nucleus to form a compact, hydrodynamic shape (Brewer et al., 2002; Dadoune, 2003).

The midpiece is slender, less than one third of the width of the head; it is aligned with the longitudinal axis of the head and is approximately 7 to 8 μm long. It is in the midpiece where all mitochondria are stored. It is believed that sperm mitochondria are responsible, at least in part, for the energy production required for sperm cell function. The tail is thinner, uncoiled and should present a regular outline. It is at least 45 μm in length and provides a motile force for the spermatozoon, which is based upon a unique arrangement of microtubules within the sperm flagellar axoneme (Figure 1) ; (Sun et al., 2006; Wu et al., 2008).

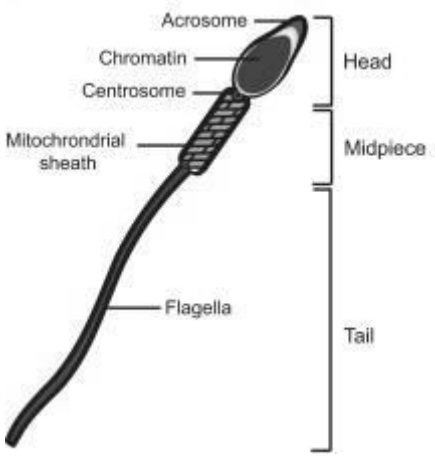



**Figure 1** -Schematic of a human sperm cell, which is composed of three regions. The head region containing the highly compacted sperm DNA and the acrosome, the mid-piece region that contains the paternal mitochondria, used for energy generation and the flagellar tail that provides motility (Wu et al., 2008).

Sperm are present in huge quantities in any ejaculate; up to half a billion cells. Because of the many steps required, variability and meiotic assortment among relevant reproductive genes provide limitless ways to affect sperm function. The huge numbers of spermatozoa produced by spermatogenesis represent the results of such recombinations. For that reason an intense selection is mandatory, and occurs in the female tract. (Holt and Van Look et al. 2004).

Even after all of the changes in spermatogenesis, spermatozoa are still not capable to successfully fertilize an oocyte as they lack progressive motility and other attributes. Nevertheless, during the epididymal transit, sperm undergo maturation processes necessary for them to acquire these functions, and the final maturation is completed in the female reproductive tract, where capacitation and the binding to the zona pelucida and subsequent acrosomic reaction take place. Capacitation, a process that involves multiple metabolic, biochemical, membrane and ionic changes (de Lamirande et al., 1997; Yanagimachi, 1994), is also associated with the development of hyperactivated motility, where the relatively linear and progressive swimming pattern of spermatozoa in seminal plasma is modified to motility patterns qualified as non-progressive, vigorous, frantic with high amplitude. Furthermore, the acrosome reaction is triggered by the binding of the sperm cell with the oocyte zona-pelucida and is characterized by release of the acrosomic vesicles that contain numerous enzymes such as acrosin that will digest the oocyte zona-pelucida. (Burkman, 1991; de Lamirande et al., 1997; Ho and Suarez, 2001).

## 1.2. Mitochondrial function vs dysfuntion

Mitochondria are cytoplasmic organelles that have a crucial role in cellular metabolism and homeostasis, regulation of the cell signaling network, and in programmed cell death. Besides the production of the majority of cellular Adenosine Triphosphate (ATP) in the presence of molecular oxygen ($O_2$), mitochondria also regulate the cytoplasmic redox state and $Ca^{2+}$ balance, catabolize fatty acids and synthesize heme, nucleotides and aminoacids (Khvorostov et al., 2008). Mitochondria are also essential for stereidogenesis, that occurs in Leydig cells. Additionally mitochondria also have a central role in the

xenobiotic metabolism that can also affect several other pathways (Brookes et al., 2004).

Mitochondria are composed of two layers of membranes that divide the organelles into four different compartments, the outer membrane, inner membrane, intermembrane space and matrix; each with different biochemical properties. The respiratory enzyme complexes, responsible for the production of ATP in an aerobic regimen are located in the inner membrane (Detmer et al., 2007).

Aerobic production of ATP in the mitochondria is a complex process which implies combined metabolism of different substances through glycolysis, fatty acid oxidation, the citric acid cycle and Oxidative Phosphorylation (OXPHOS) trough the Electron Transport Chain (ETC). Resulting substrates of glycolisis and Krebs cycle, nicotinamide adenine dinucleotide (NADH) and succinate are oxidized by the ETC complexes located in the mitochondrial inner membrane with the final goal of producing ATP by ATP Synthase (Zhang et al., 2006).

The ETC is composed of five multiprotein complexes: Complex I or NADH Dehydrogenase, Complex II or Succinate Dehydrogenase, Complex III or Cytochrome C Reductase, Complex IV or Cytochrome C Oxidase, Complex V or ATP Synthase and two electron carriers, coenzyme Q and cytochrome C. The high energy electrons derived from NADH and succinate are transferred into Complexes I and II, respectively. The electrons are then transferred from Complexes I and II to the lipid-soluble carrier coenzyme Q which in turn donates electrons to Complex III. Cytochrome c is the next electron acceptor and donates electrons to Complex IV. In the final step, the electrons are accepted by $O_2$ leading to the production of $H_2O$. The energy released from the electron transfer through these complexes is utilized to pump protons from the matrix to the intermembrane space, creating the electrochemical gradient verified. The protons then return to the mitochondrial matrix through Complex V, which uses the energy stored in the electrochemical gradient to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) ; (Bayir et al., 2008).

However, 1–4% of the oxygen is incompletely reduced to $O_2^{\bullet-}$, which can originate other reactive oxygen species (ROS) via various enzymatic or non enzymatic reactions. The ETC generates $O_2^{\bullet-}$ primarily at complexes I and III.

Complex III produces $O_2^{\bullet-}$ by autoxidation of the ubisemiquinone radical intermediate (QH), formed during the Q cycle in this complex. However, Complex III has the capacity to release $O_2^{\bullet-}$ to both sides of the mitochondrial inner membrane. The mechanism of $O_2^{\bullet-}$ production by Complex I is still unclear but it is believed that it may occur by reverse electron transfer from complex II upon succinate oxidation and that this mechanism may account for most of the physiologically relevant ROS produced in the mitochondria (Figure 2) ; (Fogg et al., 2011).

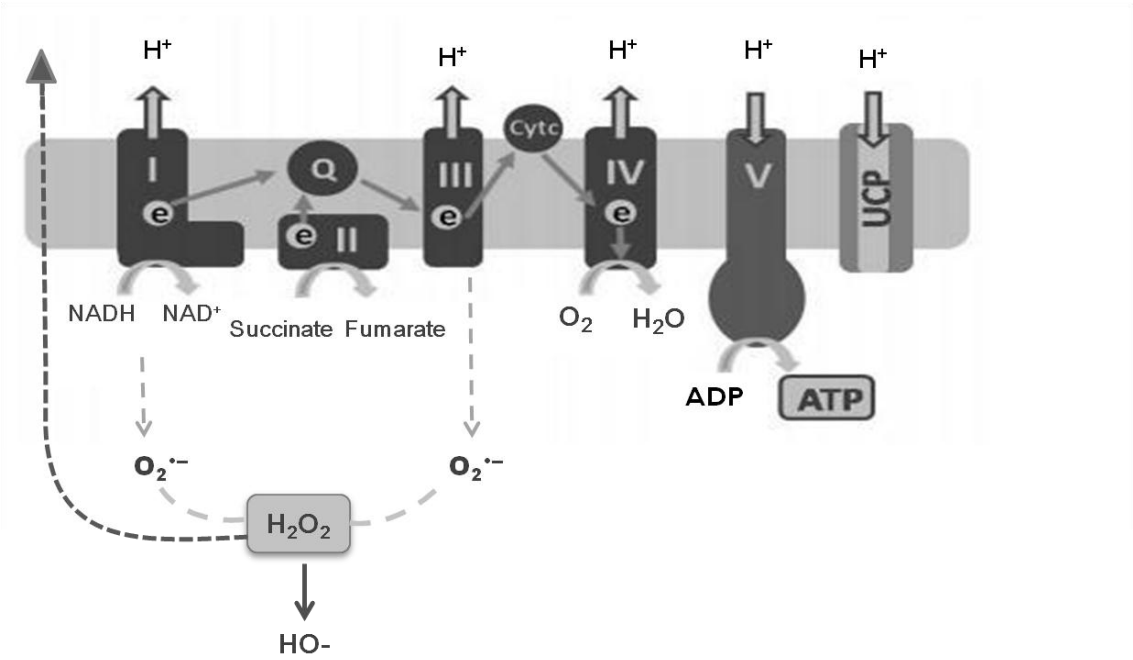



**Figure 2 -** Production of Reactive Oxygen Species in the mitochondria. Superoxide (O2) is mainly generated at complexes I and III of the electron transport chain located in the mitochondrial inner membrane. (adapted from Ramalho-Santos et al., 2009).

Each mitochondrial site of superoxide generation may play a distinct role during different stimuli. The primary ROS produced by mitochondria is $O_2^{\bullet-}$, either in the matrix and the intermembrane space. As it is charged, $O_2^{\bullet-}$is not readily diffusible across mitochondrial membranes. There are two main ways to transport $O_2^{\bullet-}$through the outer mitochondrial membrane and into the cytosol. The first is a voltage-dependent mitochondrial anion channel. The second and more important mechanism for transmembrane movement of reduced oxygen involves conversion to Hydrogen Peroxide ($H_2O_2$) by both enzymatic and non-enzymatic antioxidant

defenses. Once generated, the uncharged ROS $H_2O_2$ can easily move across the membrane (Zhang et al., 2006). Other metabolic pathways also generate ROS as by-products. One example is during the fatty acid oxidation that occurs in peroxisomes, where high-potential electrons can be transferred to $O_2$, which yields $H_2O_2$, with the initial reaction being catalyzed by acyl-CoA oxidase. Another is through the inhibition of Complex IV by nitric oxide (NO) or the direct reaction of this molecule with $O_2$ leading to the production of peroxynitrite ($ONOO^-$) (Hernández-García et al., 2010).

A number of physiological factors modulate mitochondrial ROS generation. Among those, three factors have been extensively studied, including mitochondrial membrane potential, intracellular $Ca^{2+}$, and NO. The generation of mitochondrial ROS is dependent on membrane potential. High membrane potential seems to favor the production of ROS, particularly at complex III. This is thought to be due to the slowed electron transport and the prolongation of QH occupancy in the complex III (Hernández-García et al., 2010). High concentrations of ROS are highly toxic to the cell, because they non-specifically damage nucleic acids, proteins, lipids, and other cellular components. Therefore organisms developed enzymatic (superoxide dismutase, catalase, glutathione peroxidise) and non-enzimatic (ubiquinol and cytochrome c) defences to lower their concentration to the needed levels.

## 1.3. The importance of mitochondria for sperm function

Mitochondria are responsible for the production of ATP that, in sperm, is required mainly for motility, as well as for the cellular events involved in capacitation, hyperactivation and acrosome reaction. Nonetheless, the dominant pathway of ATP production in the sperm cell has been a topic in discussion for some time. It is now believed that the extension of both glycolitic and OXPHOS pathways are species-specific (Ramalho-Santos et al., 2009) and that sperm can use both depending on substrate availability. One consequence of OXPHOS in sperm is the production of ROS (Aitken et al., 1995). Though low and controlled levels of ROS take part in sperm capacitation, acquisition of hyperactivated motility, acrosome reaction and oocyte interaction (de Lamirande et al., 1997), spermatozoa are especially susceptible to high levels of ROS because they possess

a plasma membrane that is extremely rich in unsaturated fatty acids and these are particularly vulnerable to lipid peroxidation. Such an abundance of unsaturated lipids is necessary to create the membrane fluidity required by the membrane fusion events associated with fertilization (acrosomal exocytosis and sperm–oocyte fusion); however their presence leaves these cells open to peroxidative attack (Jones et al., 1979).

In 1979 Jones and collaborators observed a correlation between the lipid peroxide content of human spermatozoa and severe motility loss (Jones et al., 1979). This data was subsequently corroborated by several independent studies (Aitken and Fisher, 1994; Sharma and Agarwal, 1996). Furthermore, it was also observed that human sperm cell exposure to extracellular ROS induces a loss of motility that is directly correlated with the level of lipid peroxidation experienced by the spermatozoa (Gomez et al., 1998). For that reason it is important for the sperm cell to be able to regulate and control ROS levels. In sperm this regulation possibly occurs through the activity of a large range of antioxidant defenses namely the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase/ reductase system and the non-enzymatic substances ascorbic acid and glutathione among others (Tremellen, 2008).

Loss of sperm function can also be associated with the accumulation of multiple mitochondrial DNA (mtDNA) rearrangements and the relevance of mitochondrial activity in sperm function has been studied at the gene level, namely the significance of sperm mtDNA integrity in male (in)fertility. Some reports have suggested that low quality sperm present abnormal mtDNA content (May-Panloup et al., 2003; Amaral et al., 2007), and that the expression of Transcription factor A, mitochondrial (TFAM) and of the catalytic subunit of DNA polymerase gamma (POLG), both of which are implicated in the regulation of mtDNA copy number, are both lower in poorer quality sperm (Amaral et al., 2007).

Summing up, mitochondria-based events regulate different aspects of reproductive function and alterations in mitochondrial homeostasis in sperm could lead to male infertility.

## 1.4. Environmental and endogenous factors that may affect mitochondrial sperm function

Sperm and mitochondrial function comprise an assembly of delicate and complex processes that can easily be affected by several life-style and environmental factors.

As mentioned before the mitochondrion is an extremely important organelle as it is involved in the production of energy, in the regulation of cell signaling network and in programmed cell death. Proof of that is the fact that functional disruption of the mitochondria is associated with several disorders such as Charcot-Marie Tooth disease, autossomic optic atrophy or even Alzheimer's disease (Alikhani et al., 2011; Detmer et al., 2007). As mitochondrial homeostasis is so important for cell function and survival, it is not surprising that mitochondrial function became a target for the study of general cellular status (Brand et al., 2011). Moreover, nowadays we are exposed to huge amounts of known and unknown substances; most of them with negative impact in one's health. For that reason, in the last years, numerous reports have been published describing the hazardous effects of some life-style related substances that are harmful at a cellular level due to induced mitochondrial dysfunction (Lim et al., 2009). As mitochondria-based events regulate different aspects of reproductive function, alterations in mitochondrial homeostasis in sperm can lead to male infertility.

Infertility is defined as the inability to conceive after frequent and unprotected sexual intercourse for more than a year, a condition that currently affects 15% of couples worldwide. Approximately 50% of the cases are attributed to the male partner (Juul, 1999; Miyamoto et al., 2012). Pathophysiological conditions (e.g. varicocele and urogenital infection) are directly linked to only 23% of all male infertility cases, with environmental factors, such as exposure to environmental toxicants, being one of the major causes of the remaining cases. Numerous environmental toxicants have been shown to adversely affect spermatogenesis in human, which can lead to low sperm count, abnormal morphology and poor semen quality (Wong et al., 2011). Smoking recreational drugs is the most commonly lifestyle factor suspected to have adverse effects on health (Sharpe et al., 2010). Besides smoking, other curious pharmacological drugs

seem to affect male fertility, namely Viagra. Nicotine, anandamide (AEA) and Sildenafil citrate are three substances associated with the habits of smoking recreational drugs and taking Viagra. All of them were proven to interfere in human fertility and to alter mitochondrial function in several cell types, as will be described below.

## 1.5. Nicotine, anandamide and sildenafil on mitochondria and on male fertility

### 1.5.1. Effects of nicotine on mitochondria and on male fertility

#### 1.5.1.1. Nicotine

Tobacco combustion holds about 4000 compounds. Smoke can be divided in two phases; one gaseous and one composed of solid particles. The main components of the gaseous phase are carbon monoxide, nitrogen oxide, ammonia and hydrocarbons. The particle phase is mainly constituted by nicotine. Nicotine is a volatile natural liquid alkaloid that is the primary addictive agent of tobacco smoke (Figure 3) ; (Jana et al., 2010; Benowitz et al., 2009).


**Figure 3** – Molecular structure of the liquid alkaloid nicotine, the primary psycho-active component of tobacco smoke (Benowitz et al., 2009).

Nicotine is distilled from burning tobacco. Its absorption across biological membranes depends on pH. Higher pHs stimulate nicotine absorption (Gori et al., 1986). For that reason, when smoke reaches the lungs (pH=7.4) it is quickly absorbed and enters circulation. Nicotine distribution trough the organism is not homogeneous and there's a higher accumulation in liver, lungs, kidney and in the brain (Breese et al., 1997). When nicotine reaches the brain it binds to nicotine

acetylcholine receptors (AcHR) in dopaminergic neurons. These receptors are ligand-gated ion channels that, when opened, allow the influx of calcium ions. This intake will then trigger a voltage-dependent release of dopamine in dopaminergic neurons. Dopamine then releases pleasure evocating signals, experience which are responsible for the known addictive properties of tobacco (Figure 4) (Benowitz et al., 2009).

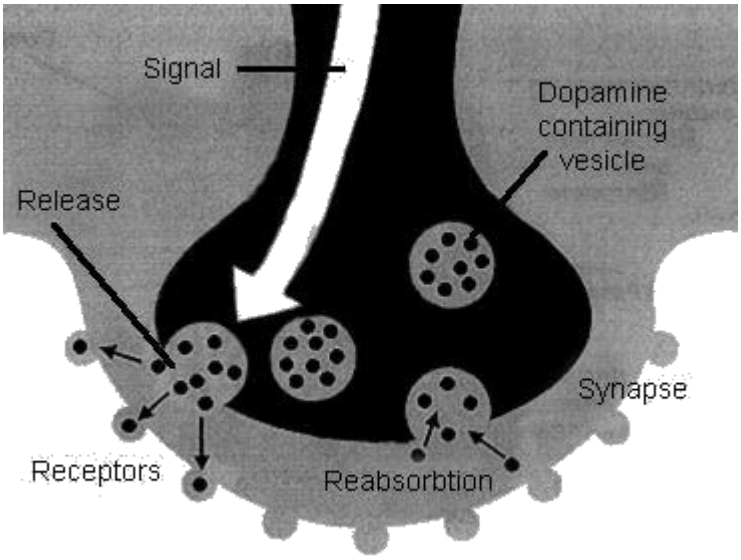



**Figure 4 –** Illustration of the mechanism through which nicotine induces the sensation of pleasure in tobacco smokers. Nicotine binds to nicotinc receptors in dopaminergic neurons and transmits a signal through the axon that ends in the release of dopamine in the synaptic cleft (Center for Substance Abuse Treatment, 1999).

On the other hand, when nicotine reaches the liver it is metabolized in six different products according to the pathway triggered. Its main metabolites are cotinine, 3-hidroxycotinine and nicotine isomethonium. Some of these exert similar effects as those exerted by nicotine, namely cotinine and 3-hidroxycotinine (Benowitz et al., 2009).

### 1.5.1.2. Effects of nicotine on mitochondrial function

Some of the negative effects of nicotine in the mitochondrial function of several cell types have already been described. For example, nicotine causes reduction in the electron transport chain enzymes activity (namely NADH dehydrogenase) of mitochondria isolated from mouse brain and consequently on

oxygen consumption, which is also associated with ROS production (Cormier et al., 2001; Das et al., 2009). Interestingly, it was recently shown by microarray and RT-PCR that nicotine modulates mitochondrial enzyme expression in the rat brain, especially the expression of NADH dehydrogenase and cytochrome c oxidase sub-units (Wang et al., 2009). In human esophageal cell carcinoma cell-lines, cigarette smoke extract was shown to reduce mitochondrial membrane potential and ATP production. It was also observed an increase in glycolitic gene expression by RT-PCR. The results suggest that cigarette exposure can modulate mitochondrial function to a more glycolitic state, favourable to cancer cells (Kim et al., 2010). In agreement with these observations, Zhang and co-workers showed that nicotine induces resistance to chemotherapy by modulating mitochondrial signalling and activity in lung cancer cell-lines (A549) (Zhang et al., 2009). Additionally, it had also been described that in isolated rat pancreatic islets nicotine also reduced the activity of respiratory chain complexes and increased pancreatic oxidative stress, mitochondria-mediated apoptosis and glucose intolerance, that the authors suggested to be implicated in the reduction of insulin secretion (Bruin et al., 2008). All these alterations at the mitochondrial level may be explained by the presence of nicotinic acetylcoline receptors in the mitochondria, whose existence was proven in isolated mitochondria from mouse liver (Gergalova et al., 2012).

### 1.5.1.3. Effects of nicotine on male fertility and sperm parameters

The first studies related to the effects of nicotine in testicular and sperm function started in 1994, when seminal plasma concentrations of nicotine, cotinine and hidroxycotinine were evaluated, through HPLC, in different populations of smoking men. According to the number of cigarettes smoked per day and to the degree of exposure of non-smoking men to tobacco smoke, groups of men were separated and their seminal plasma concentrations of these substances were assessed. The nicotine and cotinine concentration in seminal plasma of active smokers was about 70ng/ml and 303ng/ml, respectively (Pacifici et al., 1993; Sofikitis et al., 1995) while in passive smokers only exposed to tobacco smoke the concentration of these substances was significantly lower (10,7 and 4,4 ng/ml). In non-smoking men these substances were not detected at all (Pacifici et al., 1995).

The first *in vivo* studies where the sperm function of populations of smoking men were compared to that of non-smoking men showed a significant reduction in sperm viability (Zavos et al., 1998) and motility (Taszarek-H et al., 2005) and an increase in the number of leukocytes present in semen (Taszarek-H et al., 2005), in sperm with abnormal morphology (Wong et al., 2000) and in DNA fragmentation (Sepaniak et al., 2006). More recently, population-based studies show a higher percentage of infertile men in smoking sub-populations when compared to non-smoking sub-populations in the different parts of the world (Daling et al., 2010; Millet et al., 2006). However, there is still some controversy around this issue since the more recent works report no alterations in the sperm function of smoking men (Cohan and Badawy et al., 2010; Aghamohammadi et al., 2011).

However, as already referred, tobacco smoke is a collection of hazardous substances and none of the above reported studies specifies the nicotine alterations on sperm parameters. Keeping in mind that nicotine crosses through the hemato-testicular barrier and that there are nicotinic acetylcoline receptors in the sperm plasma membrane (Bray et al., 2005; Meizel, 2004); subsequent studies were developed to determine if the already observed increase in seminal plasma concentration of nicotine would influence sperm function. For that reason, *in vitro* studies were conducted where exposure of spermatozoa to nicotine and cotinine (found in active smokers) was observed to lead to a reduction on sperm motility, namely progressive motility, while maintaining viability (Gholam et al., 2008). However there are reports that imply that this effect is only verified with high non physiological concentrations of nicotine. This means that nicotine may not be the primary responsible agent of the effects observed *in vivo* (Gandini et al., 1997). Other described effects of nicotine in sperm function include an increase on sperm DNA fragmentation (Sepaniak et al., 2006), alterations of ionic permeability through the plasma membrane of spermatozoa due to a decrease on membrane status (Calzada et al., 1992; Arabi, 2004) and changes in the number and arrangement of axonemal microtubules that may be responsible for a decrease in the fertilizing capacity (Zavos et al., 1998).

Regarding to mitochondrial activity; *in vivo* assays developed by Colagero and collaborators report that the rate of sperm respiration in smokers is significantly lower of that in non-smockers. The authors suggested nicotine may

also be the cause of an observed increase apoptotic signal in sperm that is accompanied by a decrease in mitochondrial membrane potential (MMP) when the samples were exposed to cigarette smoke extracts (CSE); (Calogero et al., 2009). In concordance with this study, it was observed that smoking sub-populations present a decrease in testicular antioxidant content and an increase in lipid peroxidation and ROS production (Jana et al., 2010). However there is no solid evidence about the actual effect of nicotine on mitochondrial sperm function.

### 1.5.2. Effects of anandamide on mitochondria and on male fertility

#### 1.5.2.1. Anandamide

Canabinoids are the main constituents of the marijuana plant. Its primary psychoactive cannabinoid is delta-9-tetrahydtocannabinol (Δ-9-THC) that has already been described to have an adverse effect on male fertility (Morgan et al., 2012). Beside cannabinoids, a family of unsaturated fatty acids with cannabinoid-like properties have been identified in a number of different biological systems; they are called endocannabinoids (Howlett, 1995). Endocannabinoids are unsaturated fatty acids that are endogenous ligands for plasma membrane receptors cannabinoid receptor 1(CB1R) and cannabinoid receptor 2 (CB2R) that belong to the superfamily of G-protein-coupled receptors. Moreover, endocannabinois binding to its receptors mimics some of the effects caused by delta-9-tetrahydrocannabinol, which include the suppression of glutamate and GABA release in glutamatergic or GABAergic neurons, respectively (Figure 5); (Basavarajappa, 2007). Arachidonoylethanolamine commonly known as AEA was the first endocanabinoid isolated from the brain (Figure 6); (Devane et al., 1992).

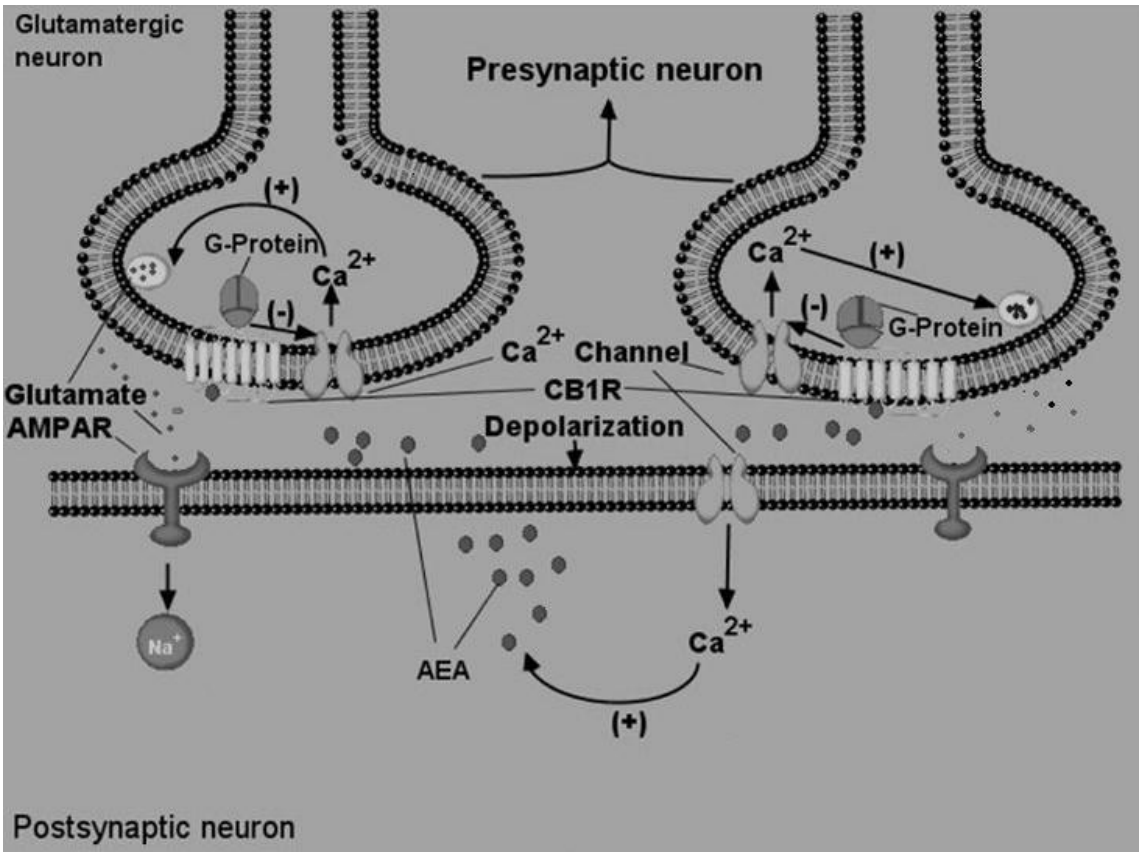
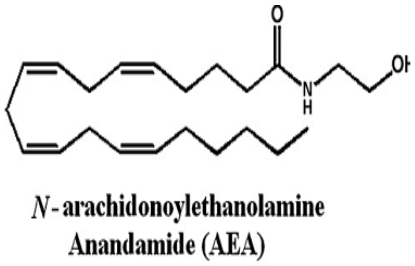



**Figure 5** – Schematic diagram to illustrate the role of AEA and CB1 receptors on excitatory neurotransmission. Presence of AEA in the synaptic cleft activates CB1 pre-synaptic neurons and suppresses the release of glutamate (Basavarajappa, 2007).

AEA synthesis may occur via multiple biosynthetic pathways. The most extensive pathway starts with the transacylation of phospholipid precursors by $Ca^{2+}$-dependent N-acyltranferase (NAT) to produce N-arachidonoyl-phosphatidylethanolamina (NArPE) ; (Jin et al., 2007). Then NArPE is converted into AEA trough the activity of a specific phospholipase D (Okamoto et al., 2004). When the transduction signal finishes, AEA response terminates by re-uptake through the endocannabinoid membrane transporter (EMT) and hydrolysis via fatty acid amide hydrolases (FAAH) ; (Kaczocha et al., 2009). Additionally, AEA also behaves as an "endovanilloid", and can bind to the type 1 vanilloid receptor (TRPV1), a non-selective ion channel that belongs to the vanilloid-type transient receptor family (Lewis et al., 2009).




*N*-arachidonoylethanolamine
Anandamide (AEA)

**Figure 6 -** Chemical structure of the main endocannabinoid, anandamide (Lewis et al., 2009)

### 1.5.2.2. Effects of anandamide on mitochondrial function

AEA is proved to induce alterations in mitochondria from several cell types. In isolated mitochondria from mouse liver AEA was observed to increase the swelling and the membrane fluidity. Furthermore, it decreased mitochondrial membrane potential and cytochrome c release induced by calcium ions (Catanzaro et al., 2009). In a different study, using a Clark-type electrode Zaccagnino and collaborators showed that AEA inhibits oxidative phosphorylation by strongly inhibiting respiratory state III, though there were no significant alterations on oxygen consumption. Moreover, AEA was also shown to decrease ATP synthase activity (Zaccagnino et al., 2011). Similar alterations were observed in isolated rat heart mitochondria where a decrease in mitochondrial membrane potential, analysed by Rhodamine 123 fluorescence was observed, and in oxygen consumption, determined polorographically using an oxygen electrode chamber. However, the authors did not observe significant alterations in ROS production (Athanasiou et al., 2007). Recently, Anandamide was also shown to be involved in the inhibition of mitochondrial biogenesis, as it was observed to induce a reduction in mtDNA expression simultaneously with the reduction of cytochrome c oxidase (COX IV) and citrate synthase activity. These last parameters were analysed for mitochondria isolated from adipose, skeletal muscle and liver tissue from mice (Tedesco et al., 2010).

### 1.5.2.3. Effects of anandamide on male fertility and sperm parameters

In 2005 Rossato et al. identified the CB1receptor on human sperm, by RT-PCR and Western Blot (Rossato et al., 2005). The characterization and location of this receptor was promptly done. Since then, growing evidence has been accumulating to show the central role of the endocannabinoid system in controlling reproductive function in mammals and humans. It was already shown that AEA decreases LH and testosterone levels in the mice testis (Wenger et al., 2001) and that it is probably due to reduced gonadotropin release from GnRH-secreting neurons (Farkas et al., 2010). Furthermore, AEA was observed to induce Sertoli cell apoptosis by activation of TRPV1 receptors that can be rescued by AEA binding to CB2R in the presence of FSH (Maccarrone, 2003; Lewis et al., 2009). Following that, the next step was to figure out what happened to sperm function after activation of this receptor. According to the literature, CB1R activation by AEA reduces sperm motility without reducing viability. Moreover, AEA was shown to inhibit the acrosome reaction and also the capacitation process (reducing protein phosphorylation and cAMP production) (Rossato et al., 2005; Aquila et al., 2009), probably via activation of the receptors CB1R and TRPV1 as has been demonstrated in boar (Gervasi et al., 2011). Also, recent reports show that anandamide also alters sperm mitochondrial function. In fact, reduction of MMP was shown by the determination of mitochondrial membrane potential, by fluorescent microscopy using Rhodamine-123 and by flow-cytometry using the fluorescent dye JC-1 (Rossato et al., 2005, Barbonetti et al., 2010).

Despite all of the work done, there is still no information reggarding ROS production and energy charge and alterations of sperm exposed to AEA.

## 1.5.3. Effects of sildenafil on mitochondria and on male fertility

### 1.5.3.1. Sildenafil citrate

Sildenafil Citrate is a white crystalline powder, water soluble citrate salt synthesized by Pfizer in the United Kingdom (Figure 7). It was first developed to treat pulmonary hypertension and angina pectoris as it reduces right ventricular systolic pressure and increases blood supply to the lungs. Interestingly, sildenafil

exhibited an unexpected pharmacological effect; marked penile erection on consumers that lead to its use in the treatment of erectile dysfunction (Zhao et al., 2001 and 2003).

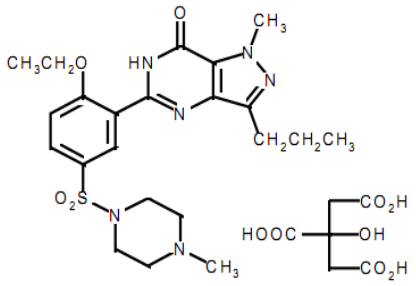



**Figure 7** - Chemical structure of sildenafil citrate commercialized by Pfizer.

Sildenafil citrate is absorbed after oral administration and is eliminated by hepatic metabolism where mainly cytochrome P450 converts it to an analogue molecule by N-demethylation. This is the major circulating sildenafil metabolite and it triggers the same response as its parental molecule (Pfizer, 2010). However its activity is only 50% that of sildenafil citrate. Despite hepatic metabolism, sildenafil citrate continues to be present in blood circulation and especially in seminal fluid (Aversa et al., 2000).

Sildenafil exerts its effects enhancing the downstream effects of NO mediated smooth muscle relaxation. NO is a labile gas produced by NO synthase in vascular endothelial cells that can diffuse trough capillary membranes. It interacts with the enzyme guanilate cyclase in the cytoplasm to produce cGMP that will activate cyclic nucleotide-dependent protein kinases such as phosphokinase G (PKG). This activation begins a cascade of protein phosphorylation that ends up in the phosphorylation of the actin-myosin system and subsequently on smooth muscle relaxation (Figure 8). More importantly, sildenafil directly inhibits phosphodiesterase-5 leading to an increase in cGMP amplifying NO muscle relaxation and consequently increasing penile erectile function (Dimitriadis et al., 2008; Ramani et al., 2010).

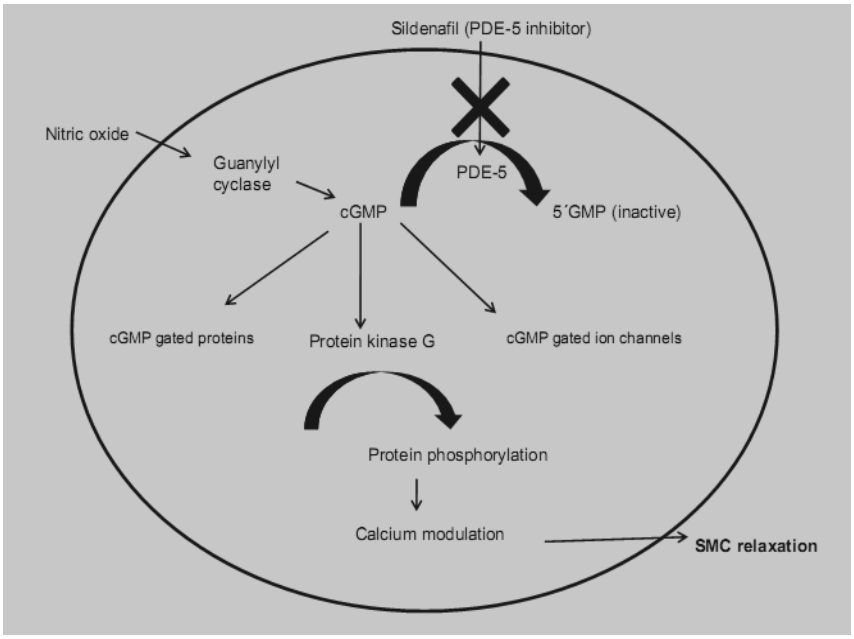



**Figure 8 –**Schematic diagram presenting the mechanism of action of Sildenafil citrate. Sildenafil directly inhibits PDE-5 increasing plasma levels of cGMP that ultimately lead to smooth- muscle relaxation (Ramani et al., 2012).

### 1.5.3.2. Effects of sildenafil on mitochondrial function

The effects of sildenafil on mitochondrial function are still not well understood. Due to its pharmacological use the two main cellular targets for mitochondrial study are the sperm cell and cardiomyocites.

For instance, it was observed that sildenafil has an anti-apoptotic effect in perfused mouse myocardial tissue by the activation of the expression of several factors including anti-apoptotic genes and members of the BCl-2 family, that are major regulators of cytocrome c release from mitochondria during apoptosis (Vidavalum et al., 2009). Salloum and co-workers also show that sildenafil protects rabbit myocardial tissue against injury through a mechanism dependent on cGMP-induced mitochondrial ATP-sensitive potassium channel ($K_{ATP}$) channel opening. The authors suggest that this mitochondrial channel opening enables additional protons to be pumped out of the mitochondrial matrix to enhance the gradient in the intermembrane space and that this will result in increased ATP production (Salloum et al., 2007). Interestingly, in Leydig cell mitochondrial ultrastructure was observed to be altered by the exposure of mice to sildenafil. Mitochondria

presented large vacuoles scattered through the cytoplasm, enlarged mitochondria with discontinue cristaes and membranes with vesicles at the periphery, characteristic of more active steiroid-secreting cells (Saraiva et al., 2009).

### 1.5.3.3. Effects of sildenafil on male fertility and sperm parameters

Since its approval by the FDA as a treatment for erectile dysfunction in 1998 (Dimitriadis et al., 2008), many studies have been developed to better understand the effects of Sildenafil citrate on sperm parameters.

An important step was taken when the concentration of Sildenafil citrate in the seminal fluid of healthy men treated with 100mg of sildenafil for at least 3 months was found to be 0.1-0.3μM and that this seminal concentrations did not change seminal and erection parameters (Aversa et al., 2000)

Later, in a randomized, placebo controlled, cross-over study, sperm motility and morphology parameters of 16 healthy volunteers that took single doses of 100mg of sildenafil, were evaluated. After four hours of the administration semen samples were collected for the measurement of sildenafil seminal plasma concentration and sperm parameters evaluation. The authors reported no significant alterations on sperm motility, count, viability and percentage of abnormally shaped sperm (Purvis et al., 2002).

According to this report in 2003 du Plessis developed a similar study in which the effect of *in vivo* sildenafil citrate (50mg) administration and *in vitro* 8-bromo-cGMP treatment on sperm parameters, were evaluated. No significant differences were reported in the percentage of spermatozoa with progressive motility although a statistically significant increase in total percentage of motile sperm was observed (du Plessis et al., 2003). Also in 2003 Jannini and collaborators found that sildenafil administration had no effect on sperm motility, sperm concentration and in the total number of spermatozoa ejaculated. Similarly to the results obtained by du Plessis and collaborators, no effect of sildenafil administration was observed in the percentage of nonlinear progressive motile spermatozoa. However, a significant increase was seen in the linear progressive motility due to sildenafil administration (Jannini et al., 2004).

These results contrast with other studies where oral administration of Sildenafil (50mg) was observed to result in a significant increase in sperm

progressive motility (contrary to the effects of the treatment with tadalafil (20mg) which is another 5-phosphodiesterase inhibitor that leads to a decrease in sperm motility (Pomara et al., 2007)). More recent studies report an increase on sperm motility caused by treatment with sildenafil (Kanakas et al., 2001; Ali et al., 2007). In a different experiment in mice a decrease of the fertilization rate of male mice taking sildenafil was observed, associated with a reduction in the number of embryos developped, as consequence of the impairment in cleavage rates within those embryos (Glenn et al., 2009).

In order to evaluate if sildenafil affects the in the steroidogenesis process, the effect of this drug in Leydig cells was assessed. According to Saraiva and colleagues Leydig cells exposed to sildenafil presented ultrastructure alterations characteristic of activated steroid-secreting cells. In addiction sildenafil-treated mice showed significant increased levels of total testosterone which are consistent with the hypothesis that the accumulation of cGMP by PDE5 inhibition could be involved in androgen biosynthesis stimulation (Saraiva et al., 2009).

In parallel with the *in vivo* studies there were also studies done *in vitro* to attest the same parameters. In 2000 Lefiévre and co-workers developed a study to figure out whether PDE activity in human spermatozoa changes when sildenafil is present in the medium. The authors have shown an increase in sperm cAMP levels and capacitation, which are associated with an increase in the levels of tyrosine phosphorylation but did not trigger the acrosome reaction in capacitated spermatozoa. Additionally, an increase in curvelinear velocity after 30 minutes of incubation with 100-200μM of sildenafil was also shown, which was then suggested by the authors to trigger human sperm motility (Lefiévre et al., 2000).

Other studies from the same year using much lower concentrations than those verified on the seminal plasma of men taking Viagra (0.19-1.12nM comparing to 0.1-0.3μM) had different outcomes; some did not show any changes in mobility, viability, nor membrane integrity, (Burger et al., 2000) while others reported an increase in sperm motility and hyperactivation in the first 4 hours of incubation (Cuadra et al., 2000). In studies using a micromolar range of concentrations, it was also observed that 30 minutes of incubation with 4μM causes a 50% reduction on sperm motility that was suggested to be the consequence of a possible decrease in the medium pH (Andrade et al., 2000). More

recent *in vitro* studies are also still contradictory as some of them report significant increase in motility (Glenn et al., 2007) and others report different alterations in motility according to the different concentrations used on the incubations. An example is the study enrolled by Mostafa and co-workers in which incubations with 6, 3, 1.5, 0.7, 0.15 μM showed decrease in sperm motility for the incubations with the higher and the lower concentrations and a significant increase in motility for the intermediate concentrations (Mostafa, 2007).

Although there is no consensus regarding the effects of Viagra in sperm function, namely on its motility, it is possible that sildenafil exerts its effects through a mitochondria-mediated mechanisms akin to what happens in other cells. So far there are still no reports regarding possible alterations caused by sildenafil in sperm mitochondria that could explain the observed changes in motility.

Altogether, nicotine, anandamide and sildenafil can disrupt and alter mitochondrial function through different pathways in different cell types and all these substances are known to affect sperm function. As sperm function is deeply dependent on mitochondrial function it will be interesting to study the effects of these three substances in the mitochondrial sperm function and clarify possible associations between disrupted sperm function and mitochondrial function, which is something that has not been explored yet.

## 1.6. Objectives

The evidence for declining sperm counts in recent decades points to an environmental/lifestyle impact on the sperm cell and on spermatogenesis and is an important health issue. Several lifestyle-related (obesity, smoking) factors appear to negatively affect both the adult testes and specially the sperm cell, emphasizing the importance of environmental/lifestyle impacts throughout the life course. Having that in mind, in this work we propose to study the effect of nicotine, anandamide and sildenafil citrate in sperm function, focusing on the sperm cell bioenergetics and mitochondrial function.

Nicotine, anandamide and sildenafil are three life-style related substances that are already proven to have an impact on male fertility, namely in sperm quality parameters. They are also implicated in the alterations on the mitochondrial functions of other cell-types. Having this in consideration, the aim of this study is to evaluate the effect of the three mentioned substances on sperm quality parameters namely motility and viability, to study possible alterations on sperm cell bioenergetics and to evaluate the mitochondrial status of the sperm cells exposed to this substances by assessing possible alterations on MMP and ROS production.

# Chapter 2

# Materials and methods

## 2. Materials and methods

### 2.1. Materials

The experimental drug anandamide was purchase from Santa Cruz Biotecnology, sildenafil citrate was obtained from Pfizer and nicotine was purchased from Sigma-Aldrich.

The fluorescence probes SYBR-14, PI for the LIVE/DEAD assay and the fluorescence dye MitoSOX-Red were acquired from Invitrogen Molecular Probes.

Any other reagents were purchased from Sigma-Aldrich.

### 2.2. Sperm collection and processing

The present work was developed using human sperm samples provided by the Laboratório de Citogenética do Departamento de Medicina Materno Fetal, Genética e Reprodução Humana dos Hospitais da Universidade de Coimbra. Samples were collected from healthy men that frequented the clinic for rotine check-ups and fertility treatment. Written informed consents were obtained from all the donors involved in this study and all of the biological material was processed according to Hospitais da Universidade de Coimbra's approved procedures. The samples were collected by masturbation, after a 3-5 day period of sexual abstinence and standard semen analyses were carried out according to the world health organization (WHO) standard procedure (WHO, 2010).

### 2.3. Study design

To determine sample concentration, spermatozoa were immobilized by osmotic shock (by adding water) and sperm count was done using a Neubauer hematocytometer. After that, separate aliquots containing $10x10^6$ spermatozoa/ml were incubated in the presence of 10mM nicotine, 2,5 µM anandamide and 0.3 and 3 µM sildenafil in a PBS-based medium: PBS-Glucose-BSA medium ( PBS, 1% P/S, 0.1g/L $Ca^{2+}$, 0.1g/L $Mg^{+}$, 5mM glucose, 1mM piruvate, 10mM lactate and 3g/L BSA, pH=7.4) for 12 and 24 hours. These concentrations were chosen taking in consideration the physiological concentrations of all the substances in seminal plasma and the ones used in previous studies. After this, several parameters (viability, motility, ATP content, mitochondrial membrane potential and ROS production) were evaluated as described below.

### 2.3.1 Evaluation of Sperm motility

According to WHO a simple system for grading motility is recommended that distinguishes spermatozoa with progressive motility (Pr), non-progressive motility (NP) and immotile sperm (I). Total motility is composed by Pr+NP (WHO standard procedure; WHO, 2010). While progressively motile spermatozoa move actively, either linearly or in a large circle, regardless of speed, non-progressive motile spermatozoa move in-situ without progressing. Sperm motility was accessed using a Nikon Eclipse E200 microscope (20x, phase contrast).

### 2.3.2. Evaluation of sperm viability

To analyze the effects of the substances in cell viability the percentage of sperm live cells was determined through a LIVE/DEAD assay. In this assay, cells are doubly-stain using the fluorescent dyes SYBR-14 and propidium iodide (PI), both prepared in DMSO. SYBR-14 is a green nuclear dye that is membrane permeable. Hence, it stains all spermatozoa in a sample. On the other hand, PI is a red nuclear dye that it is not membrane permeable which means it will only stain spermatozoa that have a compromised plasma membrane; and are therefore dead. In the end, live cells will only present green fluorescence and dead cells will present both green and red fluorescence (Garner et al, 1994).

For this assay a small volume (50µL) of the sperm sample ($10x10^6$ spz/ml) were incubated in the above described PBS-Glucose-BSA medium with the two stains SYBR (100nM) and IP (240nM) for 20 minutes at 37°C. The percentage of live and dead sperm cells were assessed using a Leica fluorescence microscope and a total of 100 spermatozoa were counted for every sample.

### 2.3.3. Evaluation of sperm mitochondrial membrane potential

Sperm mitochondrial membrane potential (MMP) was evaluated by flow-cytometry using the fluorescent dye JC-1 as it possesses the unique ability to differentially label mitochondria according to its membrane potential. In the intermembrane mitochondrial space there is the accumulation of $H^+$ ions that are responsible for the electrogradient that drives ATP production. In high concentrations of $H^+$ JC-1 forms multimeric aggregates that emit orange-redish

light, when excited at 488 nm. In low concentrations of $H^+$ JC-1 forms monomers that emit green light, when excited at 488 nm. This way mitochondria with high MMP will emit orange-redish light and mitochondria with lower MMP will emit green light. A decrease in mitochondrial membrane potential is associated with a decrease in the red/green fluorescence intensity ratio.

Sperm samples of $2.5x10^6$ spermatozoa/ml were stained. For each assay two controls were done, one where the sample was incubated with no dye and another where the sample was incubated with the dye and FCCP (50μM), an uncoupler that lowers MMP. Samples were incubated with JC-1 (2 μM) at 37° C, in the dark for 15 minutes. Then samples were centrifuged at 1800rpm for 5 minutes to wash excessive staining and pellets were ressuspended in PBS. Samples were then analyzed using a BD FACSCalibur flow cytometer. For each sample 50000 events were recorded at a flow rate of 300-400 cells/sec. Based on the light scatter characteristics of spermatozoa, debris and aggregates were gated out by establishing a region around the population of interest in the forward scatter/side scatter dot plot on a log scale. Compensation between FL1 and FL2 was carefully adjusted according to the manufacturer's instructions. Green fluorescence (480–530nm) was measured in theFL-1 channel and orange-red fluorescence (580–630 nm) was measured in the FL-2 channel. Data analysis was performed using the Cell Quest Pro Acquisition program and the percentages of sperm with red/green fluorescence were calculated.

Every sample incubated in the presence or absence of nicotine was divided in three aliquots. One control that, during the assay, was not incubated with JC-1 in order to obtain the negative fluorescence control, other in which the sample was incubated with only JC-1 and a third one incubated with JC-1 and FCCP. FCCP is an uncoupler that depletes mitochondrial membrane potential, therefore sperm cells will present a major percentage of green fluorescent mitochondria. Using the referred controls, three gates were defined. One, where the major population of cells presented red fluorescent mitochondria (R5), other where they present mostly green mitochondria (R3) and an intermediate region (R4) (Figure 9).

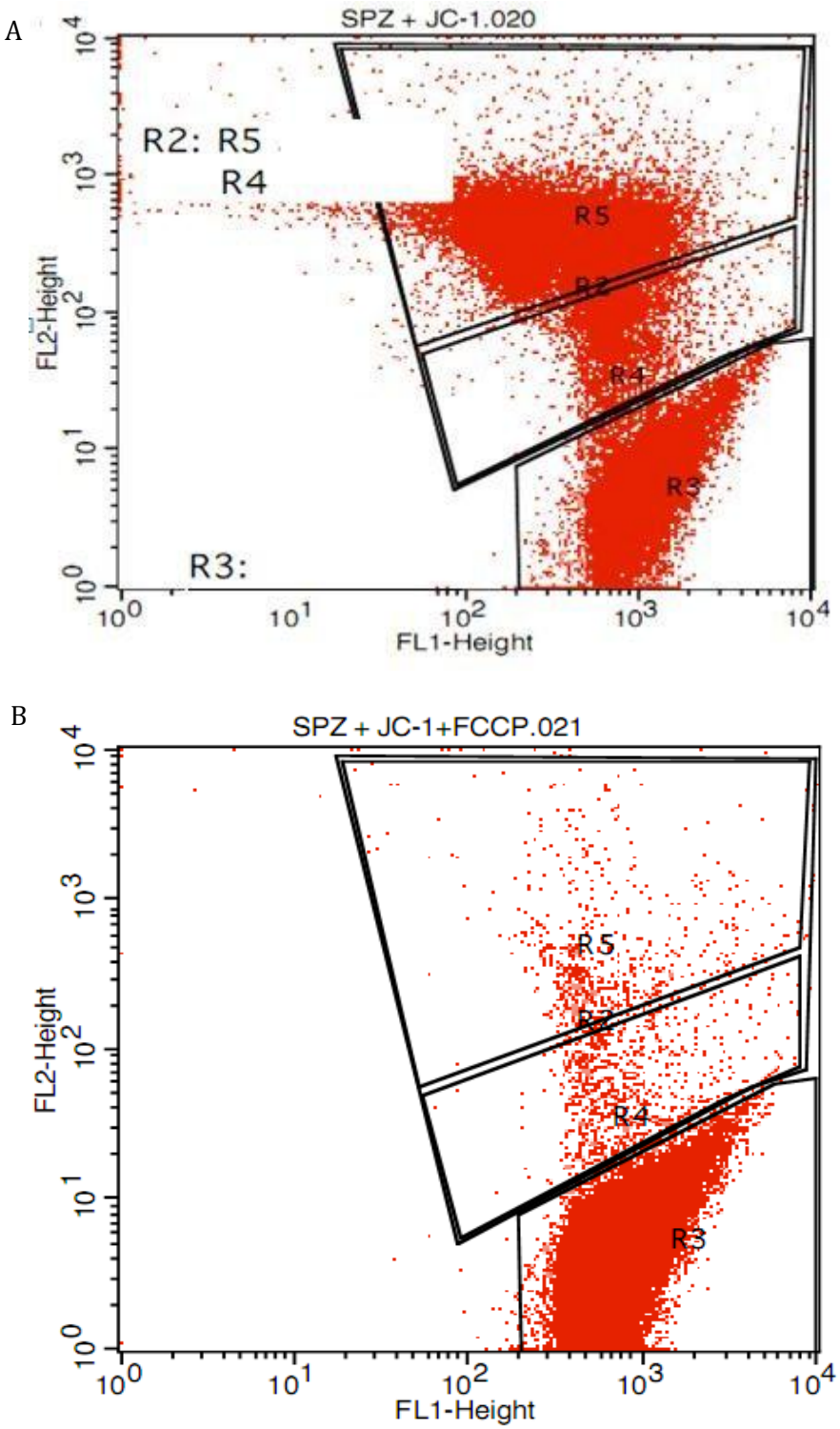



**Figure 9 –** (A) Flow-cytometry dot-plot chart of samples incubated with the fluorescent dye JC-1. (B) Flow-cytometry dot-plot chart of samples incubated with the fluorescent dye JC-1 and the uncoupler FCCP. R5 region is where the population of cells with red fluorescent mitochondria is located. R3 contains the population of cells that presented green fluorescent mitochondria. R4 is an intermediate region with cells presenting both types of mitochondrial staining.

### 2.3.4. Evaluation of sperm ROS production by flow-cytometry

ROS production, namely superoxide ion production, was evaluated by using the fluorescent probe MitoSOX-Red. MitoSOX–Red is a red reagent that is live-cell permeant and rapidly and selectively targets the mitochondria (Robinson et al., 2008). When the reagent arrives in the mitochondria it is oxidized by superoxide and subsequently exihibits red fluorescence. MitoSOX-Red is a selective reagent that can only be oxidized by superoxide but not by other ROS or Reactive nitrogen species (NRS). This way, mitochondria that present higher red fluorescence intensities produce higher amounts of superoxide.

Sperm suspensions of $2.5x10^6$ spermatozoa/ml were incubated with MitoSOX-Red (3µM), at 37° C in the dark for 15 minutes. To wash excessive staining, samples were centrifuged at 1800 rpm for 5 minutes and pellets were ressuspended in PBS. Finally samples were analyzed using a BD FACSCalibur flow cytometer. For each sample 50000 events were recorded at a flow rate of 300-400 cells/sec. Based on the light scatter characteristics of spermatozoa, debris and aggregates were gated out by establishing a region around the population of interest in the forward scatter/side scatter dot plot on a log scale. Red fluorescence (580–630 nm) was measured in the FL-2 channel. Data analysis was performed using the Cell Quest Pro Acquisition program.

Similarly to what was done for MMP evaluation, every sample was divided in 4 aliquots. One, that was not incubated with MitoSOX-Red to give us a negative fluorescence control, other incubated only with MitoSOX-Red, a negative control using the antioxidant enzyme superoxide dismutase (SOD) and a positive control using the ETC complex III inhibitor antimycin A. The controls described were used to define three regions (Figure 10), a region of a low production of ROS sperm population (R2), a region of high production of ROS population (R5) and an intermediate region (R4).

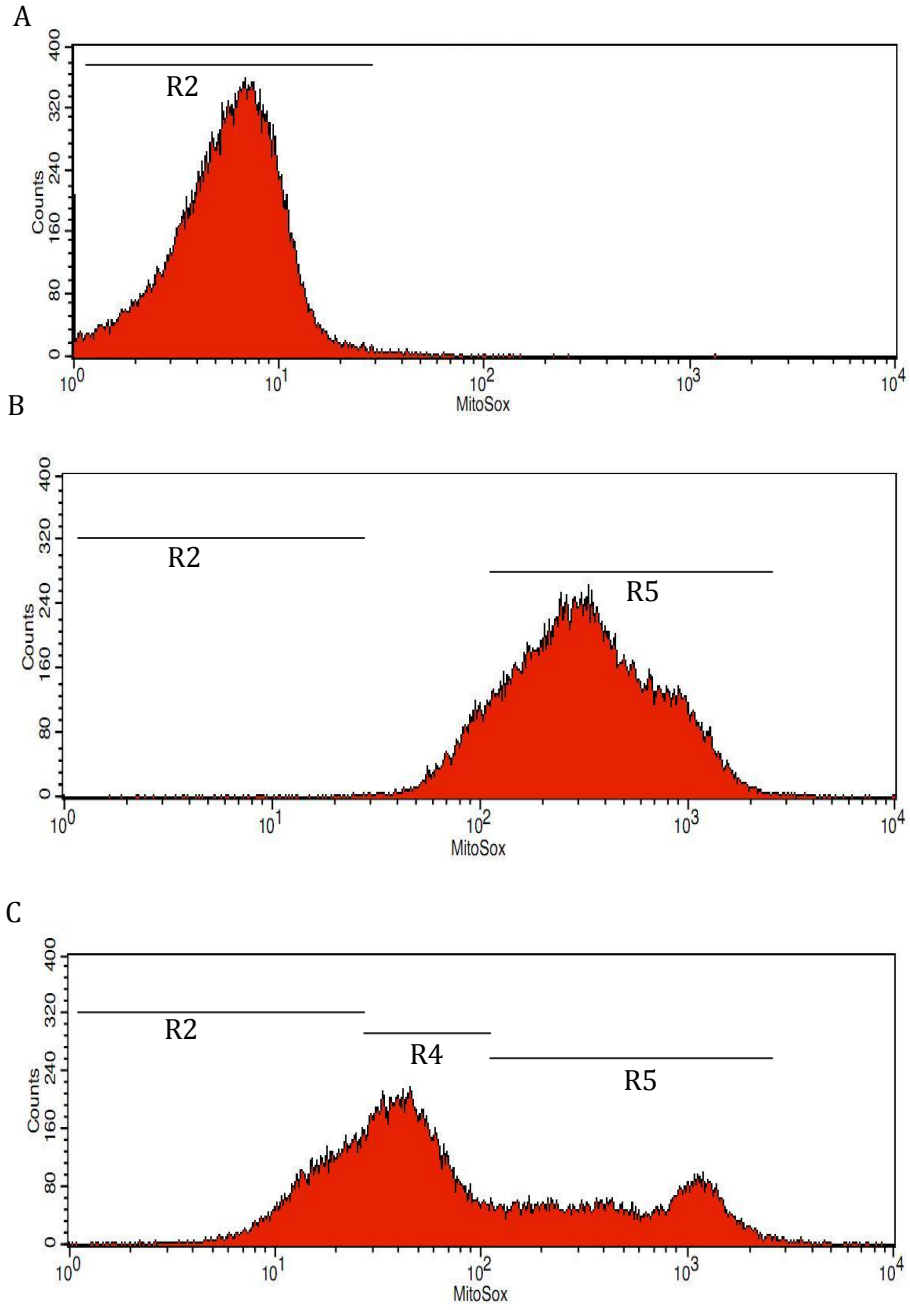



**Figure 10** - (A) Flow-citometry histogram of a control sample not incubated with MitoSOX-Red. (B) Flow-citometry histogram of a sample incubated with MitoSOX-Red and Antimycin A. (C) Flow-citometry histogram of a sample incubated only with MitoSOX-RED. R2 - Low Ros production region; R5 – high ROS production region; R4 - intermediate region.

### 2.3.5. Cellular adenosine nucleotides content evaluation by HPLC

Intracellular adenine nucleotides (ATP, ADP and AMP), from sperm cells were determined after cell extraction with 0.6M perchloric acid supplemented with 25mM EDTA-Na. Cells were centrifuged at 14,000 rpm in an Eppendorf Scanspeed 1730R centrifuge for 2 min, at 0–4°C. The supernatants were neutralized with drop wise addition of 3M KOH in 1.5M Tris and assayed for internal ATP, ADP and AMP by separation in a reverse-phase high-performance liquid chromatography (HPLC) according to a previously described procedure (Stocchi et al., 1985).The chromatography apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrosphere 100 RP-18 (5 mm) from Merck. An isocratic elution with 100mM phosphate buffer ($KH_2PO_4$; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. Peak identity was determined by following the retention time of standards. Adenylate energy charge (AEC) was determinate according the following formula: 0.5 x (ADP+2xATP) / (ATP + ADP + AMP).

### 2.3.6. Statistical analysis

Statistical analysis was performed using the SPSS software version 20.0 for Windows (SPSS Inc, Chicago, IL). The normality of the data was assessed by the Kolmogorov-Smirnov test and results are presented as mean±S.E.M. of the number of experiences indicated. Statistical significance between control samples and samples exposed to each substance was accessed using the Mann– Whitney test or the t-Test according to each parameter's normality and statistical correlations were performed using Pearson's test.

# Chapter 3

# Results

# 3. Results

## 3.1. Effects of Nicotine, Anandamide and Sildenafil in sperm function and bioenergetigics

The fastest and easiest way to determine if a substance is affecting sperm function is to test sample viability and motility.

According to seminal plasma concentrations of the substances in study and to the concentrations used in previous *in vitro* studies, samples were incubated with 10 mM of nicotine, 2,5μM of anandamide (AEA) or 0,3 and 3 μM of sildenafil for 12 and 24H and sperm motility and viability were evaluated and spermatozoa were classified according to the WHO parameters mentioned in chapter 2.

To determine if the three substances induce alterations in the bioenergetic status of the sperm cell, adenosine nucleotide content (ATP, ADP and AMP) and energy charge were determine by HPLC. In addition, we evaluated MMP and mitochondrial ROS production, by flow-cytometry. Flow-cytometry analysis was performed based in the definition of three regions. For JC-1 fluorescence analysis the defined regions gives us information about the percentage of sperm cells with higher (red, R5), intermediate (R4) and lower (green, R3) MMP, and for MitoSOX-Red fluorescence analysis the three regions correspond to higher (R5), lower (R2) and intermediate (R4) ROS production.

### 3.1.1. Nicotine

#### 3.1.1.1. Motility and Viability

Nicotine at a 10mM concentration clearly decreases sperm motility. After a 12 hour incubation there was a significant decrease in total motility (Pr + NP) (57,4% to 37,7% motile spermatozoa). The same pattern was also observed after 24 hour incubation with nicotine where a 24% decrease in total motility was observed (Figure 11).

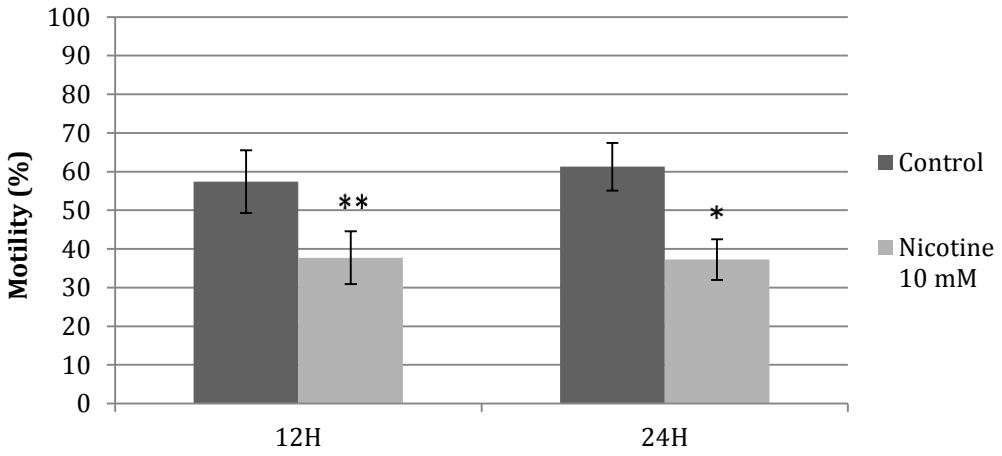



**Figure 11**– Effects of 10 mM nicotine in sperm total motility. Sperm motility was determined as described in the materials and methods section. Data are presented as mean ± S.E.M. and results are expressed as a percentage of the total motile sperm for 12H incubations (n=10) and 24H incubations (n=8). *,P≤0.05; **,P≤0.01

Regarding to sperm sample viability, after 12 hours of incubation with 10mM of nicotine, a significant 5% decrease in viability was observed when compared to the control. However, this difference is no longer verified in samples incubated in the presence of nicotine for 24 hours (Figure 12).

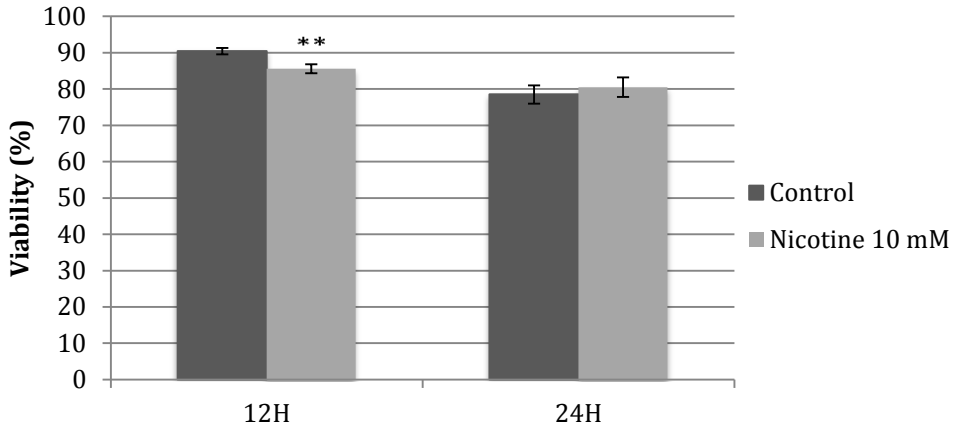



**Figure 12 –** Effects of 10mM nicotine in sperm viability. Sperm sample viability was determined as described in the materials and methods section. Data are presented as mean ± S.E.M percentage of 100 cells counted per sample. Samples were incubated in 10mM nicotine and viability was assessed after 12H (n=10) and 24H (n=8). **,P≤0.01

### 3.1.1.2. Adenosine nucleotides content and energy charge

A significant decrease in ATP content in samples exposed to nicotine after 12 hours was observed. However, this decrease in ATP levels was not significantly reflected in energy charge ratio. Interestingly, after 12 hour incubations a tendency to a decrease in ADP and a consequent increase in AMP levels were observed. At 24 hours of incubation no significant difference in ATP, ADP and AMP content was registered. Importantly, these non-significant differences are enough to significantly decrease the energy charge of the sample (Table 1). In addition, a positive correlation between motility and ATP sperm cell content (p=0.046, r= 0.820) was found in controls that was not verified for samples incubated with nicotine.

**Table 1** - Nicotine effect on adenosine nucleotide content and energy charge of sperm samples exposed for 12 and 24 hours.

| | ATP [PMOL/$10^6$ SPZ] | ADP [PMOL/$10^6$ SPZ] | AMP [PMOL/$10^6$ SPZ] | Energy Charge |
|---|---|---|---|---|
| **Control 12H** | **93.10** ±13.75 | **16.20**±3.61 | **28.31**±7.25 | **0.74**±0.04 |
| **Nicotine 10mM 12H** | **73.62**± 12.33 * | **13.87**±3.39 | **32.73**±7.09 | **0.66**±0.05 |
| **Control 24H** | **66.38** ±8.43 | **7.02**±1.26 | **17.69**±4.91 | **0.77**±0.05 |
| **Nicotine 10mM 24H** | **54.42**±14.11 | **7.74**±1.09 | **26.57**±5.32 | **0.63**±0.06 * |

Data is presented as mean ± S.E.M, for each condition studied; n=7 for 12 H incubations and n=6 for 24H incubations with nicotine. *p≤0.05

### 3.1.1.3. Mitochondrial function

#### 3.1.1.3.1. Mitochondrial membrane potential

No significant differences between samples incubated with nicotine and controls, for each of the three defined regions, were observed using JC-1. However, a decrease in higher MMP sperm populations is visible, for samples incubated with nicotine for 12 and 24 hours of incubation, though not statistically significant (Figure 13).

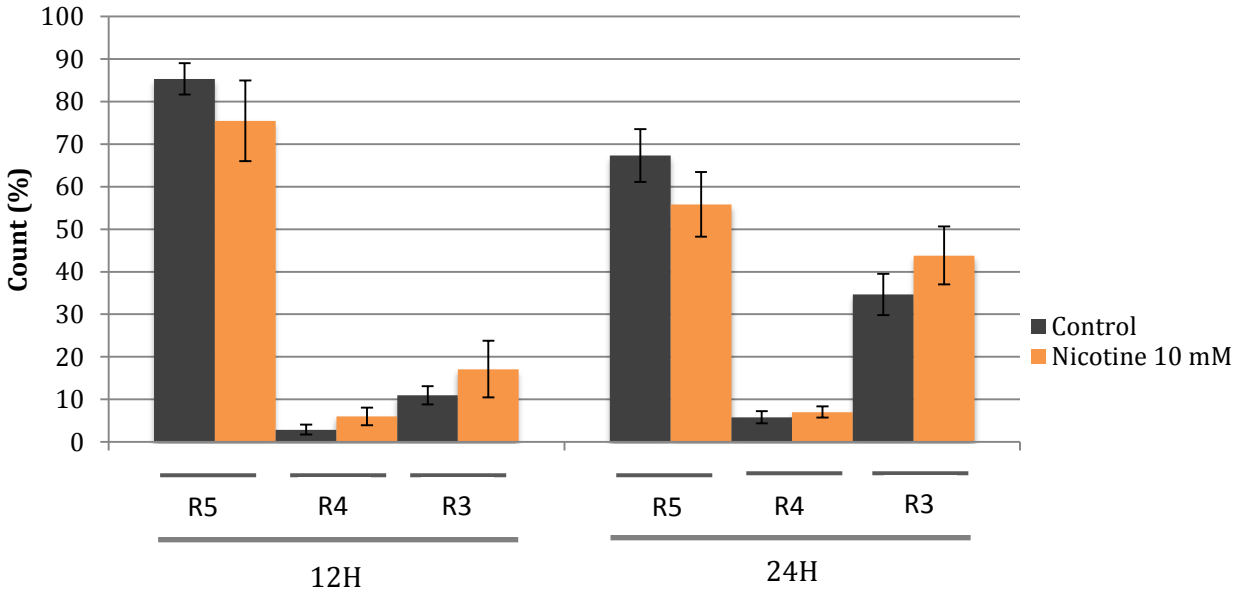



**Figure 13 -** Effects of nicotine (10mM) on sperm mitochondrial membrane potential. Flow-cytometry assay was performed as described in the materials and methods section. Data is presented as mean ± S.E.M percentage of 50000 events per sample incubated in the presence or absence of nicotine for 12H (n=2) and 24H (n=5).

### 3.1.1.3.2. ROS production

Results show no differences in ROS production between samples incubated with 10mM nicotine and controls for 24 hours, for each of the three defined regions (Figure 14).

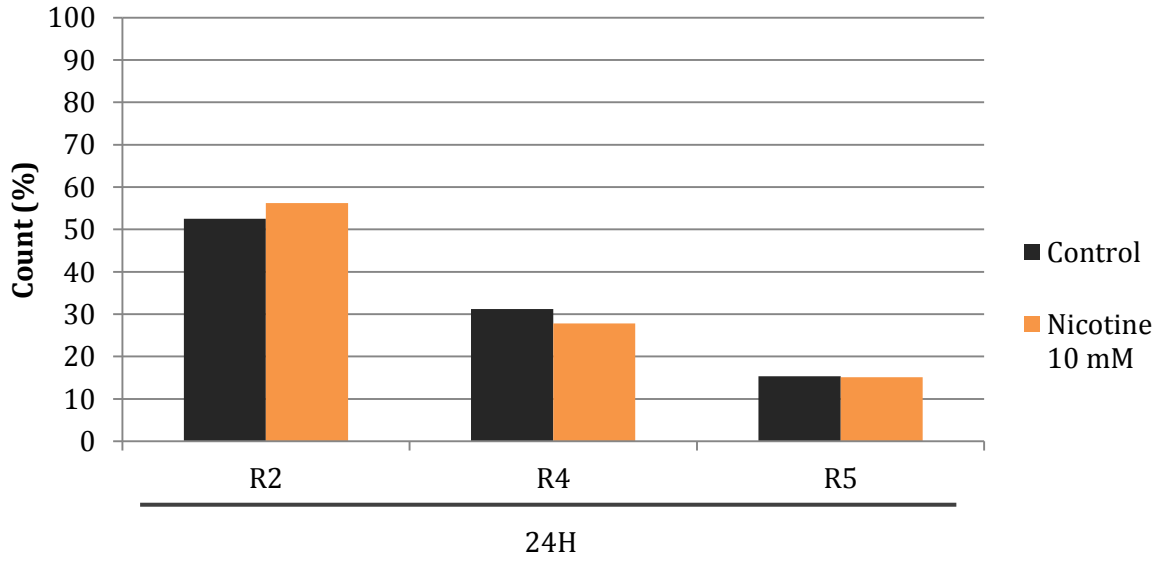



**Figura 13** - Effects of nicotine (10mM) on sperm mitochondrial ROS production. Data is presented as mean ± S.E.M percentage of 50000 events per sample incubated in the presence or absence of nicotine for 24H (n=2).

### 3.1.2. Anandamide

#### 3.1.2.1. Motility and Viability

Anandamide leads to a decrease in sperm motility without affecting sperm viability. A significant decrease of approximately 10% in sperm total motility (progressive motility and non-progressive motility) was observed for both 12 and 24 hours of incubation with AEA 2.5 µM (Figure 15).

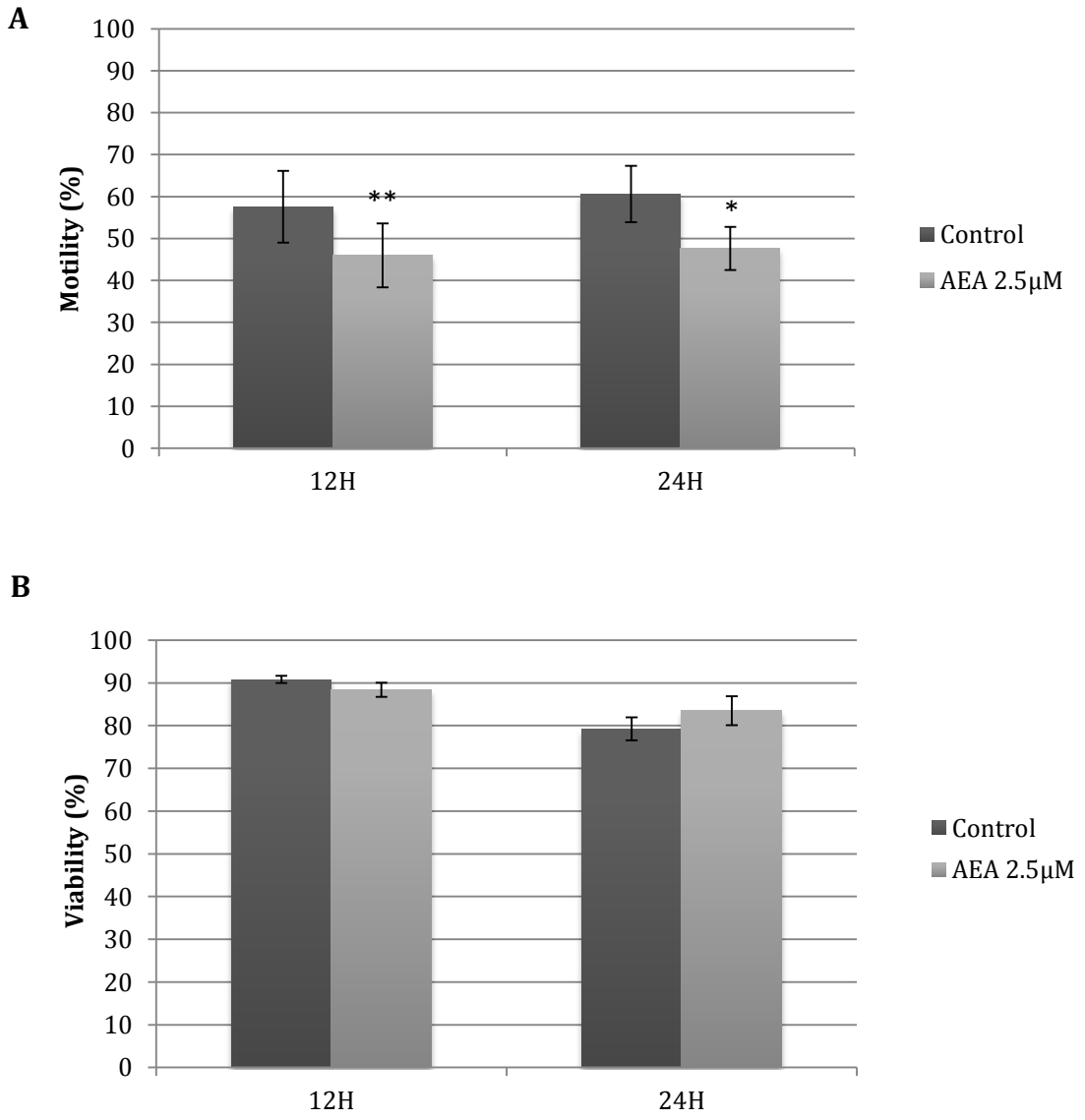



**Figure 15 –** Effects of 2.5 µM anandamide on sperm motility and viability. (A) Medium percentage of motile sperm cells. (B) Medium percentage of live sperm cells. Data is presented as mean ± S.E.M. for 12 hours (n=10) and 24 hours (n=8) of incubation. *,P≤0.05; **,P≤0.01.

### 3.1.2.2. Adenosine nucleotides content and energy charge

Sperm samples incubated with 2.5 µM AEA for 12 hours presented a significant decrease in ATP content (80.24 to 68.87 pMol ATP) that translates in a lower energy charge, when compared to the control group. This significant ATP level reduction is also verified after 24 hours of incubation (60.45 to 56.96 pMol ATP). However the extension of this decrease was not mirrored in the energy charge ratio after 24 hours of incubation (Table 2). Furthermore, through Pearson's analysis a correlation between motility and energy charge, was found for control samples (p=0.041, r=0.774), that was not present for samples incubated with anandamide.

**Table 2** - Anandamide effect on adenosine nucleotides content and energy charge of sperm samples exposed for 12 and 24 hours.

| | ATP [PMOL/$10^6$ SPZ] | ADP [PMOL/$10^6$ SPZ] | AMP [PMOL/$10^6$ SPZ] | Energy Charge |
|---|---|---|---|---|
| Control 12H | 80.23 ±7.76 | 12.87 ±3.13 | 27.97 ±.35 | 0.74 ±0.04 |
| AEA 2.5µM 12H | 68.89 ±7.59 ** | 12.77 ±2.92 | 30.60 ±7.41 | 0.69 ±0.03 * |
| Control 24H | 60.45 ±7.02 | 7.09 ±1.24 | 17.58 ±4.92 | 0.76 ±0.04 |
| AEA 2.5µM 24H | 56.96 ±5.89 * | 8.61 ±1.60 | 21.40 ±4.31 | 0.71 ±0.04 |

Data is presented as mean ± S.E.M, for each condition studied; n=7 for 12 H incubations and n=6 for 24H incubations with 2.5 µM of anandamide. *p≤0.05; **p≤0.01

### 3.1.2.3. Mitochondrial function

#### 3.1.2.3.1. Mitochondrial membrane potential

Results showed no differences in MMP between sperm samples incubated with 2.5 µM anandamide and controls for 24 hours, for each of the three defined regions (Figure 16).

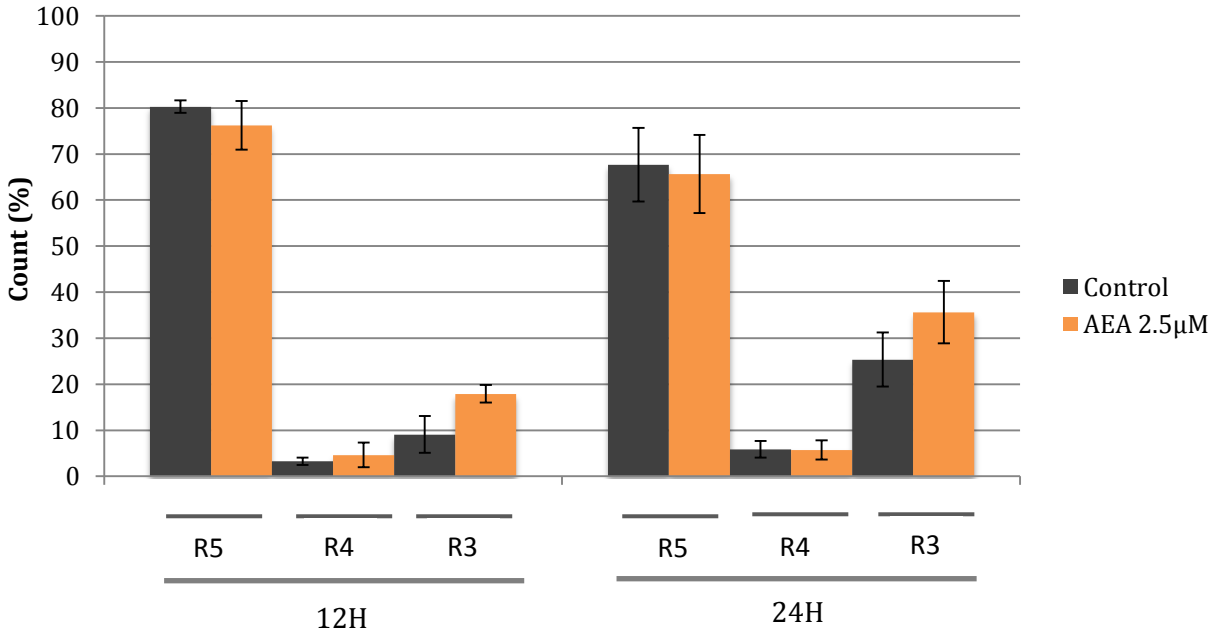



**Figure 16** - Effects of anandamide (2.5 µM) on mitochondrial membrane potential. Flow-cytometry assay was performed as described in the materials and methods section. Data is presented as mean ± S.E.M percentage of 50000 events per sample incubated in the presence or absence of anandamide for 12H (n=2) and 24H (n=5).

### 3.1.2.3.2. ROS production

Sperm samples incubated 24 hours with anandamide showed higher levels of ROS production, when compared to the control group. However results are not statistically significant (Figure 17).

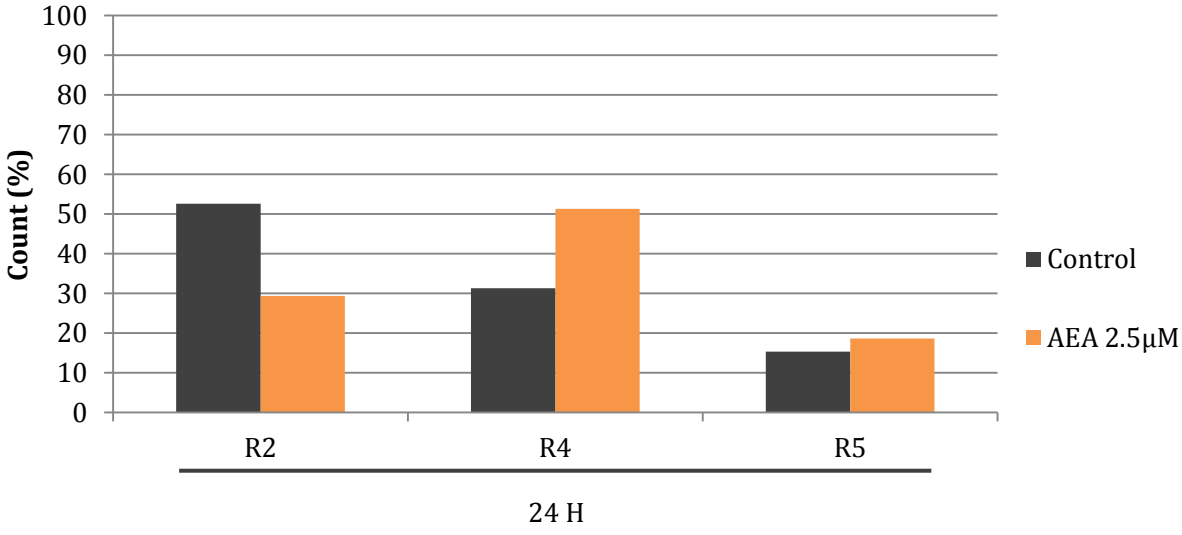



**Figure 17 -** Effects of anandamide (2.5µM) on sperm mitochondrial ROS production. Data is presented as mean ± S.E.M percentage of 50000 events per sample incubated in the presence or absence of anandamide for 24H (n=2).

### 3.1.3. Sildenafil Citrate

#### 3.1.3.1. Motility and Viability

A significant decrease in motility for both concentrations of sildenafil used, was observed. After 12 hours of incubation with 3µM of sildenafil, a reduction of approximately 15% in total motility was registered. Interestingly, sample incubation with a lower concentration of sildenafil (0.3µM), for the same amount of time induced a larger decrease in motility (30% decrease). No significant alterations in viability were verified for the conditions in study. After 24 hours of incubation with sildenafil, the same pattern was observed. Sperm sample incubation with 0.3 µM caused a higher decrease in total motility (40 % decrease in total motility) when compared to 3 µM sildenafil (20% decrease in total motility). Still, there were no observable alterations in sperm viability (Figure 18).

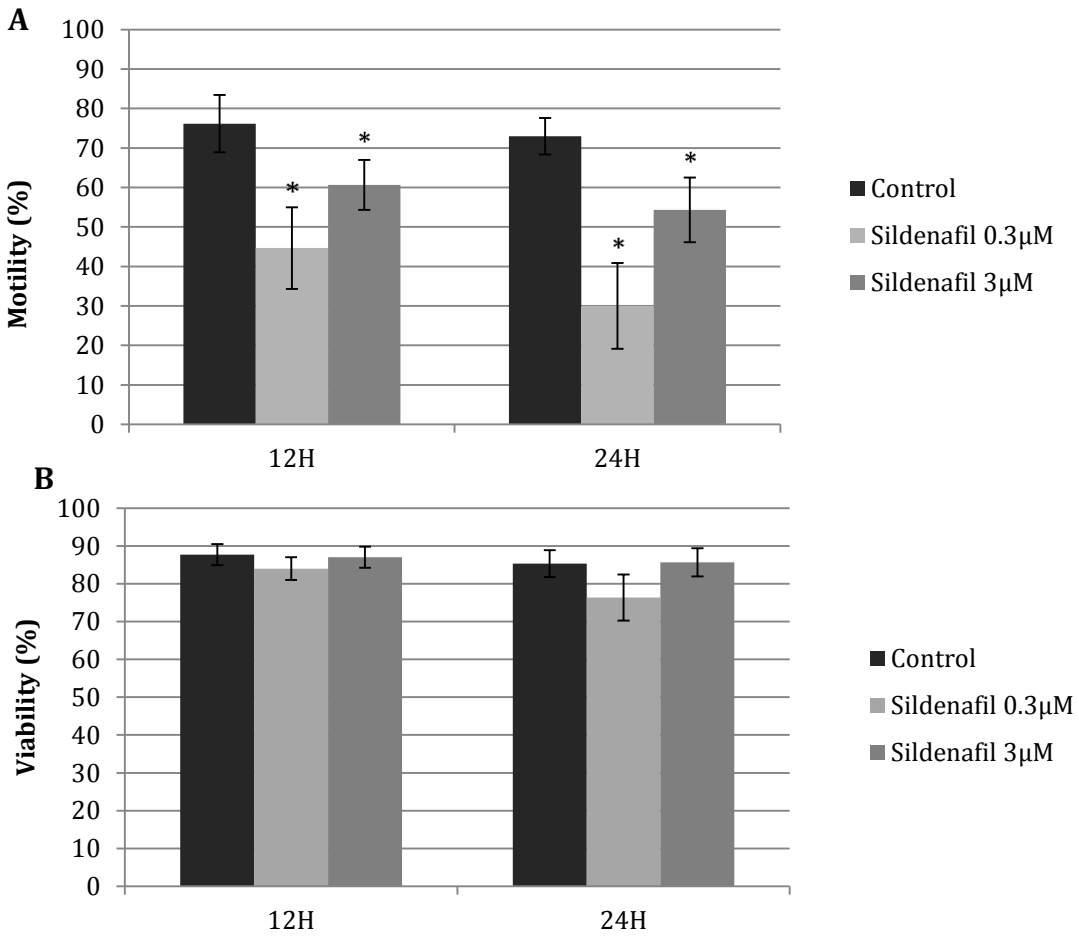



**Figure 18 –** Effects of 0,3 and 3 µM of sildenafil citrate in sperm motility and viability. (A)Medium percentage of motile sperm cells. (B) Medium percentage of live sperm cells. Data is presented as mean ± S.E.M. for 12 hours (n=7) and 24 hours (n=6) of incubation *,P≤0.05; **,P≤0.01.

### 3.1.3.2. Adenosine nucleotides content and energy charge

Sildenafil citrate starts affecting ATP production after at least 12 hours. After 24 hours of incubation, both concentrations of sildenafil citrate exerted observable effects. In fact, both decreased ATP content and energy charge in a similar way (Table 3).

**Table 3** - Sildenafil effect on ATP content and energy charge of samples exposed for 12 and 24 hours.

| | ATP [PMOL/$10^6$ SPZ] | ADP [PMOL/$10^6$ SPZ] | AMP [PMOL/$10^6$ SPZ] | Energy Charge |
|---|---|---|---|---|
| **Control 12H** | 91.89±13.25 | 13.22±1.14 | 54.66±7.35 | 0.61±0.05 |
| **Sildenafil 0.3µM12H** | 73.31±7.57 | 12.65±0.73 | 54.82±7.39 | 0.57±0.04 |
| **Sildenafil 3µM12H** | 77.35±11.99 | 12.53±0.89 | 55.74±6.92 | 0.57±0.05 |
| **Control 24H** | 107.27±20.76 | 13.89±1.00 | 48.02±6.87 | 0.66±0.02 |
| **Sildenafil 0.3µM 24H** | 87.07±15.32* | 13.94±1.49 | 49.52±6.90 | 0.62±0.01* |
| **Sildenafil 3µM 24H** | 87.91±15.88* | 13.93±1.30 | 49.67±7.05 | 0.62±0.03* |

Data is presented as mean ± S.E.M, for each condition studied; n=5 for both 12 and 24 hour incubations with 0,3 and 3 µM of sildenafil. *p≤0.05

# Chapter 4

# Discussion

## 4. Discussion

Mitochondria are central organelles controlling the life and death of the cell, that participate in key metabolic reactions, synthesize most of the ATP and regulate a number of signalling cascades, including apoptosis (Dimmer et al., 2006). In sperm, mitochondria are stored in the midpiece and their functionality is intimately associated with both sperm quality and fertilization ability (Ramalho-Santos et al., 2009). In fact, human ejaculates are very heterogeneous, composed by different subpopulations that have distinct biochemical and physiological characteristics and this heterogeneity is also reflected in differences in mitochondrial-related traits (Sousa et al., 2011). As mitochondrial function is intimately associated with sperm quality, it is to expect that induced alterations in the mitochondria by external life-style and environmental factors will interfere with male fertility.

Over the past years there has been substantial evidence that nicotine, anandamide and sildenafil citrate alter mitochondrial status in different cell types. Moreover these three substances are reported to exert negative effects on male reproductive function. Having this in mind, in the present work the effects of nicotine, anandamide and sildenafil on sperm cell parameters and bioenergetics were evaluated. However, due to the complexity of male fertility-related processes, we should keep in mind that other factors; can be involved in the reproductive impairments observed.

### 4.1. Nicotine

Smoking has always been associated with infertility and it was already shown, in several population-based studies, that smoking sub-populations presented a higher percentage of infertile men (Daling et al., 2010; Millet et al., 2006). One of the components of tobacco smoke is nicotine which is the main addictive substance in tobacco. Although Gandini and co-workers suggested that nicotine is not responsible for tobacco smoke effects in fertility (Gandini et al., 1997), their conclusions were drawn from assays of 1 hour exposure of sperm cells to nicotine which is not enough to cause the pejorative alterations reported by

other several studies, namely in motility (Calogero et al., 2009; Sofikitis et al., 1995). In the present work, exposure of sperm cells to 10mM nicotine was observed to cause a decrease in viability after 12 hours of incubation (5%) However, this pattern was no longer seen after 24 hours of incubation. These results may be justified by the effect of nicotine on dying sperm after 12 hours. Zavos and co-workers previously suggested that nicotine-induced alterations in viability could be mirrored in motility (Zavos et al., 1998). However, we observed a different pattern; a significant decrease of 20% in motility after 12 and 24 hours of incubation that seems to have no relation with the viability results, as reported recently by Gholam and colleagues (Gholam et al., 2008). Moreover, alterations in sperm motility can be a result of changes on energy status of the sperm cell (Ruiz-Pesini et al., 1998). In fact, we observed that sperm sample exposure to nicotine lead to a decrease in ATP content and energy charge. This is congruent with the results obtained from exposure of human epithelial cancer cell lines to cigarette smoke extract, in which a decrease in ATP was also observed and that was suggested to be due to a nicotine-mediated mitochondrial function shift to a more glycolitic state (Kim et al., 2010). In fact, as mitochondria are the main producers of cellular ATP, a decrease in ATP content can be a result of some type of mitochondrial dysfunction. Additionally, it was already known that MMP is one of the most important factors (contributing for more than 90% of total proton motive force) to well functioning OXPHOS, and any alteration on MMP can be reflected in mitochondrial functionality ( Ramalho-Santos et al., 2009). Accordingly, Calogero and colleagues that reported a significant decrease in MMP in sperm samples exposed to cigarette smoke extract (CSE) (Calogero et al., 2009). However, though in the present study a decrease in the number of cells presenting mitochondria with a higher MMP was observed, this decrease was not significant, which can suggest that nicotine is not the only component in CSE that exerts the previously described effects or alternatively that more assays should be ran in order to achieve statistical significance.

In this work ROS mitochondrial production, more specifically superoxide mitochondrial production, was also evaluated. As already referred in chapter 1, high levels of ROS can be detrimental for sperm cell function. Additionally, they can also interfere with mitochondrial function and consequently in cell

phosphorylative capacity. Although it was previously shown that there was a higher production of ROS in the testes of rats treated with nicotine (Jana et al., 2010), in this work no alterations could be observed at sperm level. In fact, as this study was done in vivo, spermatogenesis process could also be affected at several levels and results might reflect alterations in all cell-types at the seminiferous tubules, both germinative and non-germinative. Though these results seem to be in accord with the results obtained on the MMP assay, we have also to take in consideration that only two assays were performed.

It should also be noted that the physiological concentration of nicotine in active smokers is almost 10000 times lower than the concentration in use here (Pacifici et al., 1994; Sofikitis et al., 1995). However a series of studies both in sperm and in testicular cells use much higher concentrations in *in vitro* studies as a starting point to more physiological approaches (Arabi et al., 2004; Ghaffari et al., 2009).

Nonetheless, results obtained in the present study show that nicotine seem to impair sperm function and bioenergetics, as observed by the decrease in ATP sperm motility that may be one of the causes of decreased male fertility in smoking men. However, as no alterations in mitochondrial parameters were observed, it might be suggested that the nicotine-induced sperm alterations are mitochondria-independent. A possible intervention in the glycolitic pathway or in different ratio of ATP consumption by the sperm cells exposed to nicotine may also be involved.

## 4.2. Anandamide

Anandamide (AEA), a major endocannabinoid that binds to CB1 and TRPV1 receptors and affects many reproductive functions (for a review see Lewis et al., 2009). Results show that sperm cell exposure to physiological concentrations of anandamide, found in marijuana smokers (2.5 μM), significantly decreases sperm motility without interfering with cell viability, thus indicating that the effects of anandamide on sperm motility are specific and not due to cell death-related processes. These results, are in accordance with described in numerous previous reports (Rossato et al., 2005; Aquila et al., 2009). However, the mechanisms underlying the effects of anandamide in human sperm have not yet been

elucidated, although influences of cannabinoids on ion channels, adenylate cyclase activity, and protein phosphorylation had been suggested to be involved (Rossato et al., 2005).

Additionally, it was found in our study that AEA also interferes with sperm bioenergetics as it significantly decreased ATP sperm cell content after 12 and 24 hours of incubation and this was translated in a significant decrease in cell energy charge. This is in agreement with studies done, in isolated mitochondria from mouse liver, in which AEA not only lead to a decrease in ATP production by selective inhibition of the ATP synthase complex (Zaccagnino et al., 2011), but also decreased Cytochrome oxidase activity and citrate synthase activity (Tedesco et al., 2010), suggesting an AEA-mediated inhibition of the OXPHOS pathway. Despite the alterations observed in cell ATP content, no changes were observed in the mitochondria parameters evaluated, namely in MMP, after anandamide treatment. These results are not in agreement with previous *in vitro* reports on AEA effects on MMP in sperm samples, measured by both Rhodamine 123 fluorescence and JC-1 fluorescence assays (Barbonetti et al., 2010; Rossato et al., 2005), in which this endocannabinoid significantly decreases MMP of sperm samples. The contradictory results may be explained by the fact that, in the reported work, sperm samples incubated with anandamide were previously put through a swim-up assay. This selective assay separates the original total sperm sample in two populations according their migratory capacity. The subpopulation with the migrated sperm cells was the sample incubated with anandamide, resulting in a less scattered, more homogeneous and less representative population. Being a more selective, less heterogeneous sperm sample the effects of anandamide might be more noticeable. Reported results can also be explained by the time of exposure of sperm samples to AEA, as in the mentioned experiments, samples were incubated with AEA for only 30 minutes to 1 hour. Truthfully, anandamide's half-life is minutes *in vivo*, as it is quickly processed and metabolized in the brain, by the active enzyme FAAH (Fatty acid amide hydrolase), extensively distributed in the brain (Clement et al., 2003). However, in sperm samples this enzyme is not present in large amounts, which means that AEA has a much longer half-life. Having this in mind, it is suggested that reported results may describe an early

adaptative response to anandamide exposure, whereas the obtained results in this study represent the response to a long-termed exposure to AEA.

In addition, there seems to be an increase in mitochondrial ROS production. However, this increase is not statistically significant, which is in agreement with other studies. In fact, in isolated mitochondria from H460 cell line, AEA exposure did not affect ROS production. Yet, the results of the present study may have significant clinical implications as it is well known that marijuana smokers show an alteration in fertility (Howlett et al., 1995) and this could be due to a direct negative influence of cannabinoids on human sperm. Furthermore, cannabinoids may also alter sperm functions in vivo via indirect mechanisms due to alteration of the functionality of Leydig and Sertoli cells that express CB-Rs (for a review see Lewis et al., 2009).

### 4.3. Sildenafil

Sildenafil citrate, the first introduced phosphodiesterase-5 (PDE5) inhibitor, has proven effective in the treatment of male erectile dysfunction. The effect of sildenafil on human sperm motility is still debated. Nanomolar range concentrations were reported to exerted both an increase (Cuadra et al., 2000) and no effects in motility (Burger et al., 2000), when samples were exposed to sildenafil for 3 to 4 hours. However, for incubations of 24 hours, a significant decrease in motility was described (Cuadra et al., 2000). More recent reports, using micromolar (0.67-10 μM) concentrations of sildenafil, have been more consistent on describing an increase in sperm motility. However, in these experiments, sperm samples were incubated with sildenafil for up to 3 hours (Glenn et al., 2007; Mostafa et al., 2007). The results obtained in this work show an accentuated significant decrease on sperm motility, without altering viability with a 0.3 μM concentration showing a more extensive decrease in motility than a 3 μM concentration.

In accordance to our results, Mostafa and collegues described an interesting effect of a range of different concentrations of sildenafil in sperm motility. According to their results, concentrations of 0.7-1.5μM of sildenafil increase motility in a significant way, on the other hand, higher concentrations (3-6 μM) do not differ much from control samples and lower concentration (0.15-0.7 μM) seem

to further lower motility values. (Mostafa et al., 2007). Additionally, as motility of samples incubated with sildenafil citrate decreases in a time dependent manner (Cuadra et al., 2000), it is expected that at 24 hours of incubation the higher values on motility are lower than the ones from control samples.

Sperm cell ATP content and energy charge were significantly lower for sperm samples incubated with both 0.3 and 3 μM of sildenafil citrate for 24 hours and this decrease may account for the differences observed in motility. Surprisingly, both concentrations showed the same decrease in all adenine nucleotides levels when compared to the control. This may suggest that ATP production and availability is not the only contributor to the effects observed in sperm motility and further studies on the impact of sildenafil on the bioenergetic function of sperm cells should be carried out. It remains to be determined if mitochondrial functionality is affected by sildenafil.

## 4.4. General Considerations and future work

The evidence for declining sperm counts in recent decades mean that the environmental/lifestyle impact on the quality of the sperm cell is an important health issue. In this study the effects of three substances that have already been proven to alter male fertility on sperm quality parameters and bioenergetics were reported. Results presented in this work, show that both nicotine, anandamide and sildenafil citrate exert a negative effect on sperm motility without interfering with cell viability. Importantly, all the drugs seem to affect the sperm at an energetic level. Surprisingly, this energetic induced alterations, at least with the results obtained so far, seems to be mitochondria-independent, opening new avenues for research, namely exploring other ATP producing/consuming pathways.

In fact, it was shown that anandamide and nicotine do not alter MMP and may eventually alter ROS production. To complete this study, more experiments on the influence of the three substances on ROS production should be conducted. Also, experiments on the effect of sildenafil on MMP remain to be done. Additionally, for both nicotine and anandamide the same assays should be performed for more physiological concentrations. Finally, it would also be interesting to test for the

activity of mitochondrial electron transport chain complexes, to prove that OXPHOS is affected by the substances studied.

# Chapter 5

# Concluding Remarks

## 5. Concluding Remarks

The presented work helps in the elucidation on the role of nicotine, anandamide and sildenafil on sperm function, focusing on the sperm cell bioenergetics and on mitochondrial function. This is important as the three substances are related to ordinary life-style choices that are proven to have negative effects in male fertility, and can be responsible for the observed decrease in sperm quality observed throughout the years.

In vitro exposure of human sperm samples to nicotine; the major addictive substance in tobacco smoke; showed to negatively affect spermatozoa, by reducing sperm motility without altering cell viability. This reduced motility observed can be a consequence of a decrease in cellular ATP content and consequent decrease in cell energy charge. However, the bioenergetic alterations do not seem to be related to nicotine-induced mitochondrial dysfunction as the decrease in the number of cells presenting mitochondria with a higher MMP, after nicotine exposure was not significant and no alterations in ROS production could be observed at sperm level. With the results obtained we can conclude that nicotine does have a negative effect in sperm function and bioenergetics, but the effects of tobacco observed in sperm mitochondrial function are not alone caused by nicotine.

In the same way, anandamide; an analog of the active substance of marijuana (THC); decreases sperm cell motility without altering cell viability. Also, though the changes observed in motility could possibly be due to a decrease in ATP content and a decreased cell energy charge, no alterations on mitochondrial membrane potential were observed. However, there seems to be a tendency for an increase in ROS production after sperm cell exposure to anandamide. For this to be clarified more assays should be conducted.

Sildenafil or Viagra is a phosphodiesterase-5 inhibitor that increases intracellular levels of cGMP. There has been much controversy about the effects of this substance on the sperm cell. In this work we prove that sildenafil citrate decreases sperm motility without altering sperm viability and that there is a decrease in ATP content and energy charge at least after 24 hours of incubation. Unfortunately essays on mitochondrial function were not conducted in this study.

In the future it would be interesting to study the mitochondrial implications of sperm cell exposure to sildenafil. Furthermore, to complement the effects of the three life-style-related substances in sperm mitochondrial function and OXPHOS it would be interesting to study their effects on mitochondrial electron transport chain complexes activities.

# Chapter 6

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## 6. References

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