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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Insights into the reproductive dysfunction in diabetes: an *in vitro* approach

Joana Mafalda Diogo Portela

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Sandra Amaral (Instituto de Investigação Interdisciplinar, Universidade de Coimbra) e do Professor Doutor João Ramalho-Santos (Departamento de Ciências da Vida, Universidade de Coimbra).

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Index

Abbreviations

ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ANT4	Adenosine nucleotide translocase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumine
COX II	Cytochrome C oxidase II
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAPI	4', 6-diamidino-2-phenylindole
DGKK	Diacylglycerol kinase kappa
DM	*Diabetes Mellitus*
DNA	Deoxyribonucleic acid
ETC	Electron transfer chain
ED	Erectile dysfunction
FCCP	p-trifluoromethoxy carbonyl cyanide phenyl hydrazine
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GLUTs	Glucose transporters
HRP	Horseradish peroxidase
ICSI	Intracytoplasmic sperm injection
IRB	Internal Review Board
IVF	*In vitro* fertilization
JC-1	5,5', 6,6'- tetrachloro-1,1',3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide
KSR	Knockout serum replacement
LDH	Lactate dehydrogenase
LDHC	LDH type C
MCTs	Monocarboxylate transporters

MMP	Mitochondrial membrane potential
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
ODF	Outer dense fibers
PBS	Phosphate-buffered saline
PFK	Phosphofructokinase
PKC	Protein kinase K
PSA	*Pisum sativum* agglutinin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxigen species
rpm	Rotations per minute
RT	Room temperature
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences Program
SSC	Side scatter
TBS	Tris buffer solution
Tris-EDTA	Tris- Ethylenediaminetetraacetic acid
ZP	*Zona pelucida*
% (v/v)	Percentage volume/volume
% (w/v)	Percentage weight/volume
WHO	World Health Organization
α-MEM	Minimum essencial medium alpha

Abstract

Diabetes mellitus (DM) is a degenerative disease representing one of the greatest concerns to modern global health, and worryingly with its incidence increasing worldwide at epidemic rates. DM is associated with the emergence of a variety of clinical complications, including reproductive dysfunction.

Given the extension and multifactorial nature of diabetes-induced physiological changes, it remains unclear what are the mechanisms that may contribute for the reproductive dysfunction described in male diabetic patients.

Considering that hyperglycemia has been described as a major effector of the disease pathophysiology, two different *in vitro* approaches were used to address the isolated effect of high glucose conditions on sperm function and spermatogenesis, thus avoiding other *in vivo* confounding players.

A complete and integrated analysis, through a diversity of important indicators of spermatozoa functionality (motility, viability, capacitation status, acrosomal integrity, mitochondrial superoxide production and mitochondrial membrane potential) suggests that high glucose concentrations does not seem to directly affect spermatozoa, at least *in vitro*.

Organ culture experiments, mimicking the spermatogenic process, determined that high glucose levels increase Sertoli cell number while decreasing tubular luminal area, therefore suggesting an impairment of this somatic cell type with hub importance in spermatogenic control.

Taken together, this study suggests that high glucose levels *per se* seems to influence the male reproductive system only at the spermatogenesis level, stressing the importance of other factors involved in the disease.

Keywords: male reproductive function, Diabetes mellitus, spermatozoa, organ culture

Resumo

A *Diabetes mellitus* (DM) é uma doença degenerativa cuja incidência está a aumentar de forma galopante, sendo actualmente considerada um grave problema de saúde pública. A DM está ainda associada ao surgimento de uma grande variedade de complicações clínicas afectando todos os sistemas de órgãos, não sendo o sistema reprodutor masculino uma excepção.

Dada a extensão e natureza multifatorial das alterações fisiológicas induzidas pela DM, permanecem ainda por esclarecer quais os mecanismos responsáveis pela disfunção reprodutora frequentemente reportada em pacientes diabéticos do sexo masculino, incluindo alterações na espermatogénese ou em vários parâmetros seminais.

Considerando que a hiperglicémia tem sido descrita como um dos principais efectores das alterações associadas à DM, no presente projecto, foi estudado, através de duas abordagens *in vitro* distintas, o efeito isolado da hiperglicémia na função espermática e na progressão do processo espermatogénico. Estes sistemas permitiram assim excluir outros factores envolvidos na doença.

Uma análise completa e integrada, realizada através da avaliação de uma grande diversidade de importantes indicadores da funcionalidade espermática (mobilidade, viabilidade, estado de capacitação, integridade acrossomal, produção de superóxido mitocondrial e potencial membranar mitocondrial) sugere que, pelo menos *in vitro*, elevadas concentrações de glucose não afectam directamente o espermatozóide.

Os resultados obtidos com um sistema de cultura de órgãos permitiram verificar que elevados níveis de glucose levaram a um aumento do número de células de Sertoli e a uma diminuição da área luminal tubular. Estes resultados sugerem, portanto, uma disfunção neste tipo de células somáticas essenciais para o controlo da espermatogénese.

Através deste estudo foi possível verificar e sugerir que a disfunção reprodutora de pacientes do sexo masculino parece não resultar apenas da influência da glucose, apesar de terem sido detectadas alterações ao nível da espermatogénese, sublinhando a importância da natureza multifactorial da doença.

Palavras-chave: função reprodutora masculina, Diabetes mellitus, espermatózoide, cultura de órgãos.

Chapter 1

Introduction

1. Introduction

1.1. Overview

Infertility is already considered a major health problem, in both developed and developing countries, with one in six couples having fertility problems (Munster & Schmidt, 1995). In fact, approximately 80 million couples in reproductive age throughout the world are infertile, that is, are incapable to conceive after at least one year of regular and unprotected intercourse (Munster & Schmidt, 1995; Boivin *et al*., 2007).

Male infertility contributes to at least 50% of all cases of infertility, affecting approximately one in 20 men in the general population (McLachalan & Kretser, 2001; Tremellen, 2008). Male infertility diagnosis relies mainly on the assessment of 3 standard sperm parameters - concentration, morphology and motility - based on World Health Organization (WHO) established criteria of normality. Thus a normal sample (normozoospermic) contains ≥15 millions of spermatozoa *per* mL of semen, ≥40% of motile sperm and at least 4% of sperm with normal morphology (WHO, 2010). Compared with other conditions, male infertility has a high prevalence and, most worryingly, reports suggest that it will continually increase (Irvine, 1998; Skakkebaek *et al*., 2006). Besides the already known factors that may cause infertility such as congenital and acquired urogenital abnormalities, infections of the male accessory glands as well as occupational, environmental and lifestyle factors, systemic diseases such as *Diabetes mellitus* (DM) may further exacerbate this decline in male fertility (Irvine, 1998; Dohle *et al*., 2005).

DM is a degenerative disease with serious consequences in terms of human health that is progressing at epidemic rates. In fact, while in the year 2000 WHO reported 177 million people affected worldwide, this number is projected to rise to over 300 million by 2030, representing a 39% increase (Wild *et al*., 2004). This rising incidence of DM will unavoidably result in an increased prevalence in men prior to and during their reproductive age (Agbaje *et al*., 2007). Although it is already known that diabetes has deleterious effects on male reproductive function, the details remain undefined. Hence, a rigorous scientific analysis is needed in order to elucidate the mechanisms responsible for the reproductive impairment due to DM.

Understanding spermatogenesis, in particular the complex cellular interactions and metabolic networks entailed in this process, as well as the functionality of the sperm cell as its end product, is essential to ultimately deciphering, treating or preventing the male reproductive impairment in DM.

1.2. The male reproductive system: the testis

The male reproductive system comprises a series of organs – the testes, a network of excretory ducts and accessory glands - that jointly contribute towards the reproductive process (Fox, 2003).

The testes are paired organs that essentially perform two functions: production of male gametes, through a process defined as spermatogenesis, and synthesis of male sex hormones (Weinbauer *et al*., 2010). Each testis is encased by a fibrous capsule of dense connective tissue, the *tunica albuginea,* whose internal extensions (septa) divide the testicular parenchyma into about 250–400 conical lobules. The testicular lobules are composed of numerous seminiferous tubules and the surrounding interstitial space, in which groups of androgen-producing Leydig cells and additional cellular elements are present (Figure 1) (Weinbauer *et al*., 2010; Ježek *et al*., 2013).

Seminiferous tubules are highly convoluted structures with both ends opening into the *rete testis*, an anastomotic network of tubules located at the testicular *mediastinum* (Ježek *et al*., 2013). A *tunica propria*, composed of peritubular contractile myoid cells interposed between connective tissue layers of collagen and elastic fibers, envelop the seminiferous tubules. It is the occasional contraction of these myoid cells that promotes the transport of immature spermatozoa to the *rete testis* ultimately reaching the epididymis, an elongated organ overlying the posterior surface of the testis in which the process of sperm maturation will take place during several days before the sperm is released into the vas deferens (Figure 1a) (Weinbauer *et al*., 2010; Ježek *et al*., 2013).

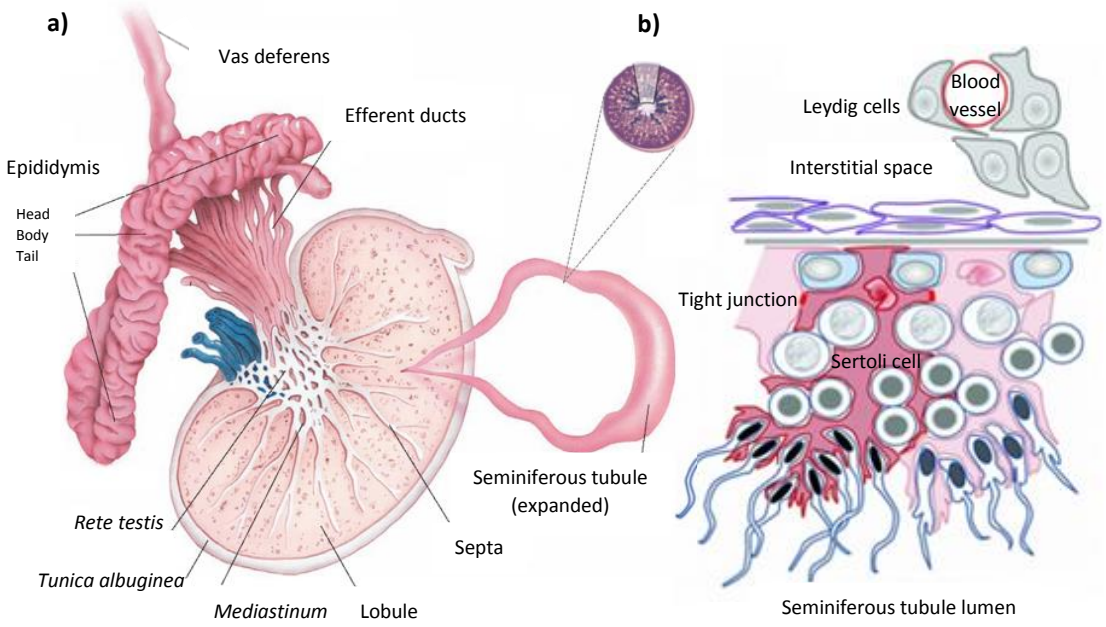

Figure 1-a) Schematic illustration of testicular morphology (Sagittal section). Adapted from Encyclopædia Britannica, Inc. (2010). **b)** Diagram of the essential structure of the seminiferous epithelium and its relation with Leydig cells and interstitial space. Adapted from Hunter *et al.,* 2012.

The seminiferous epithelium consists of concentric layers of germinal cells at different developmental stages, intimately associated with a single type of somatic cell – the Sertoli cell (Figure 1b) (Amann, 2008; Hess & França, 2008; Hermo *et al*., 2010; Ježek *et al*., 2013). Nearly 35–40% of the volume of the seminiferous epithelium is occupied by Sertoli cells (Weinbauer *et al*., 2010) that are located on the basal membrane, extending cytoplasmatic projections and ramifications to the lumen of the seminiferous tubules and around all the germ cells (Hess & França, 2008; Weinbauer *et al*., 2010). The morphology of Sertoli cells is strictly related to their multiplicity of physiological functions, supporting, guiding and nourishing germ cells. In fact, Sertoli cells coordinate the spermatogenic process, assuming a hub role in the regulation, maintenance and subsequent success of spermatogenesis (Holstein *et al*., 2003; Hermo *et al*., 2010; Weinbauer *et al*., 2010). The basolateral region of adjacent Sertoli cells are connected by a complex network of specialized tight junctions (Figure 1b), establishing the blood-testis-barrier that will further divide the seminiferous epithelium in two anatomically different compartments: the basal compartment, where early germ cells are located, and the adluminal compartment where later stages of maturing germ cells

can be found (Figure 2) (Fox, 2003; Holstein *et al*., 2003; Weinbauer *et al*., 2010). During their development germ cells are displaced from the basal to the adluminal compartment, and this is accomplished by a synchronized dissolution and reassembly of the tight junctions above and below the migrating germ cells (Holstein *et al*., 2003; Weinbauer *et al*., 2010). Besides the structural function, this barrier also has an important role in controlling the entrance of certain molecules to the adluminal compartment and providing immunological protection for the developing germ cells (Fox, 2003; Holstein *et al*., 2003; Johnson *et al*., 2008). Additionally, Sertoli cells produce and secrete the tubular fluid that enables sperm transport (Weinbauer *et al*., 2010).

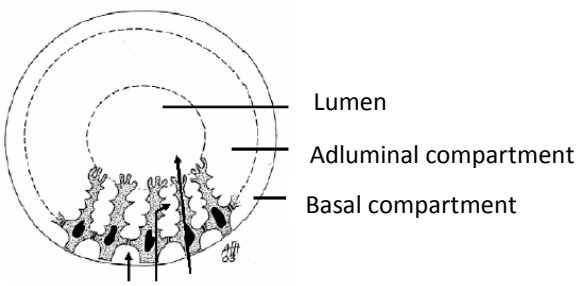

Figure 2- Division of the seminiferous epithelium in basal and adluminal compartment, by tight junctions between Sertoli cells. Arrows indicate the direct transport of substances to the basal compartment and via Sertoli cells to the adluminal compartment and lumen. Adapted from Holstein *et al*., 2003.

Outside the seminiferous tubules, the interstitial compartment represents about 12–15% of the total human testicular volume, 10–20% of which is occupied by Leydig cells (Figure 1b) (Weinbauer *et al*., 2010; Ježek *et al*., 2013). Leydig cells are prominent cells responsible for the production and secretion of testosterone in a complex process that involves the mitochondria and endoplasmatic reticulum – steroidogenesis (Holstein *et al*., 2003; Weinbauer *et al*., 2010; Ježek *et al*., 2013). Aside from Leydig cells, the intertubular space of testis also contains immune cells, microvasculature, lymphatic vessels as well as nerve fibers, fibroblasts and loose connective tissue (Holstein *et al*., 2003; Weinbauer *et al*., 2010).

Hence, the specific organization of the testis allows spermatogenesis and steroidogenesis to occur in two morphologically and functionally distinct compartments, the former event occurring in the seminiferous tubules and the latter in the interstitial space (Weinbauer *et al*., 2010).

1.3. Spermatogenesis

Spermatogenesis is an orderly, well-defined, dynamic and metabolic active process through which a complex and interdependent population of undifferentiated spermatogonial germ cells develop into male mature gametes, the spermatozoa (Holstein *et al*., 2003; Hermo *et al*., 2010). Several aspects of spermatogenesis are species specific, including its duration. In fact, while in men the spermatogenic process commences at puberty and continues throughout life, taking around 70 days (Misell *et al.,* 2006) in the mouse it starts soon after birth with a duration of approximately 35 days (Dym *et al*., 2009; Hermo *et al*., 2010).

The entire spermatogenic process can be divided in three specific functional phases: spermatogoniogenesis; meiotic division and spermiogenesis (Figure 3) (Holstein *et al*., 2003; Hermo *et al*., 2010; Weinbauer *et al*., 2010).

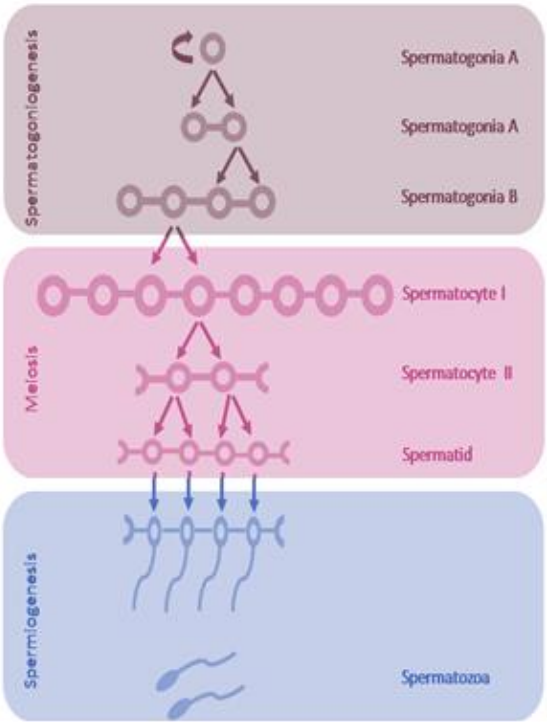

Figure 3- Phases of spermatogenesis - spermatogoniogenesis, meiosis and spermiogenesis- and the specific germ cells involved in which phase. Adapted from Amaral *et al*., 2009a.

Spermatogoniogenesis consists in the mitotic proliferation and differentiation of diploid immature germ cells, the spermatogonia, into spermatocytes (Fox, 2003; Hermo *et al*., 2010; Weinbauer *et al*., 2010). Spatially, this phase occurs in the basal compartment of the seminiferous tubules where spermatogonia lie and form

heterogeneous groups of cells that can be distinguished according to their morphology, physiology and nuclei staining as type-A or type-B spermatogonia in humans (Holstein *et al*., 2003; Ehmcke *et al*., 2006; Sutovsky & Manandhar, 2006; Hess & França, 2008; Hermo *et al*., 2010) and as type-A, intermediate and type-B spermatogonia in rodents (Ehmcke *et al*., 2006; Hess & França, 2008; Dym *et al*., 2009).

Regardless of the species, type-A spermatogonia represent the stem cell pool of the testis, while type-B spermatogonia are the precursors of the primary spermatocytes and the last cells of the line that undergo mitosis (Hess & França, 2008; Hermo *et al*., 2010; Ježek *et al*., 2013). Importantly, spermatogonial cell divisions are incomplete. Actually, daughter cells remain interconnected via cytoplasmic bridges forming a syncytium responsible for the coordinated and synchronous development of germ cells (Holstein *et al*., 2003; Weinbauer *et al*., 2010).

The longer phase of spermatogenesis is the meiotic phase in which diploid primary spermatocytes undergo the first meiotic division to yield secondary spermatocytes, which proceed through the second division of meiosis to produce round spermatids. Therefore, one primary spermatocyte produces four round spermatids still interconnected through cytoplasmic bridges and that are mitotically inactive cells. In this phase it is possible to observe great variations in cellular size, with the primary spermatocytes representing the largest cells in the seminiferous epithelium (Holstein *et al*., 2003; Hess & França, 2008; Hermo *et al*., 2010; Weinbauer *et al*., 2010; Ježek *et al*., 2013).

During the last phase of spermatogenesis, round spermatids undergo a remarkable change in their shape, that culminates in the production of elongated spermatids (Sutovsky & Manandhar, 2006; Hess & França, 2008; Hermo *et al*., 2010; Weinbauer *et al*., 2010; Ježek *et al*., 2013). The differentiation of round spermatids includes structural modifications in the nucleus shape with condensation of the chromatin to nearly one tenth of the original volume; formation of an acrosome and its attachment to the nucleus; development of a flagellum; exclusion of the majority of cytoplasm as well as reorganization of organelles, such as the mitochondria in the midpiece (Fox 2003; Holstein *et al*., 2003; Sutovsky & Manandhar, 2006; Hess & França, 2008; Auger, 2010; Weinbauer *et al*., 2010).

Because of its compact arrangement, mainly due to the partial replacement of histones with protamines (Brewer *et al.,* 1999), sperm chromatin is believed to be silent implying that the proteins necessary for spermiogenesis have to be transcribed before this time point and justifying the finding of RNA species with long half-life and RNA binding proteins in sperm (Sutovsky & Manandhar, 2006; Weinbauer *et al*., 2010; Amaral *et al*., 2013a). Spatially, this spermatogenic phase occurs in the adluminal compartment of the seminiferous tubules (Sutovsky & Manandhar, 2006).

Although throughout the process of spermiogenesis the germ cells remain associated with Sertoli cells via specialized junctions, and with each other via cytoplasmic bridges, in the last step of spermatogenesis the bridges broke and fully differentiated elongated spermatids detach from the apical surface of seminiferous epithelium into the seminiferous lumen in a process managed by the Sertoli cells called spermiation (Holstein *et al*., 2003; Sutovsky & Manandhar, 2006; Hess & França, 2008; Weinbauer *et al*., 2010). After their release into the lumen of seminiferous tubule, spermatozoa and tubular secretions are subsequently transported into the *rete testis*, the efferent ducts and the epididymis, where sperm undergo further maturation, acquire progressive motility and are stored between ejaculations (Figure 1a) (Fox 2003; Holstein *et al*., 2003; Sutovsky & Manandhar, 2006; Ježek *et al*., 2013).

Spermatogenesis is considered a redundant process with low efficiency due to particularly high germ cell loss and number of malformed sperm in the ejaculate. When compared with rodents, spermatogenic efficiency in men is lower (Holstein *et al*., 2003).

Additionally, spermatogenesis is highly dependent on the action of a complex and finely regulated hormone network that jointly works to the same end: spermatogenic success (Weinbauer *et al*., 2010). Overall, considering the complexity of the process it is not surprising that spermatogenesis can be disturbed at several levels and by a variety of factors, either environmental or pathophysiological factors, that can compromise its progression, efficiency and successful outcome (Holstein *et al.,* 2003).

Research towards the development of an authentic *in vitro* spermatogenesis system has been ongoing for nearly a century (Goldschmidt, 1915; Champy, 1920; Stukenborg *et al*., 2009). Although several *in vitro* methods have been developed varying from conventional to tree-dimensional cultures, the organ culture system provides the enormous advantage of closely mimicking and maintaining the spatial structure of the

seminiferous epithelium (Staub, 2001; Stukenborg *et al*., 2009; Hunter *et al*., 2012). In 1964, Steinberger and coworkers, developed an organ culture method employing a gas-liquid interphase, i.e. fragments were maintained in a manner that ensures the simultaneous contact with the gas atmosphere and the culture medium, avoiding the ischemia that hampers the previous organ culture methods (Steinberger *et al*., 1964; Reuter *et al*., 2012). However, in this experiment the authors did not observe the completion of meiotic division or the production of spermatids (Steinberger *et al*., 1964).

Due to the high complexity of the spermatogenic process, it was a major challenge to completely induce and simulate mammalian spermatogenesis *in vitro*, the first of such achievement occurred in 2011, when Sato and collaborators developed a based gas-liquid interphase organ culture system, that supported complete mouse spermatogenesis, from primitive spermatogonia to the formation of sperm, which functional competence was demonstrated by micro-insemination (Sato *et al*., 2011; Sato *et al*., 2013). It was the refinement of media constituents and conditions of culture, previously established by Steinberger and coworkers that allowed this remarkable result (Gohbara, *et al*., 2010; Sato *et al*., 2011).

Since the testicular architecture and cell–cell interactions are preserved in a comparable manner to the *in vivo* situation, as all principal testicular cell types are efficiently sustained, the organ culture method provide the possibility to study and dissect the mutual interplay between germ and somatic cells and, importantly, allow to define and control the surrounding environment, evaluating the effects of particular substances and drugs on spermatogenesis without the *in vivo* confounding existing factors (Gassei & Schlatt, 2007). Additionally, *in vitro* spermatogenesis may possible serve as a platform for future clinical applications, through the development of new diagnostic, fertility preservation and therapeutic techniques for male infertility (Sato *et al*., 2011; Reuter *et al*., 2012). Nevertheless, organ cultures using adult testicular tissue have not been successful (Gassei & Schlatt, 2007; Reuter *et al*., 2012).

1.4. Spermatozoa – the final player

Spermatozoa, the end-product of spermatogenesis, is a remarkable and highly specialized cell committed to carry the 23 paternal chromosomes as well as the centrosome, into a far-reaching target cell, the oocyte, ultimately achieving fertilization (Sutovsky & Manandhar, 2006).

Mature sperm has a unique shape, different from all other cells in the body, being composed of three morphologically and functionally distinct subcellular compartments: the head, the midpiece and the tail, all covered by a single plasma membrane (Figure 4) (Sutovsky & Manandhar, 2006; Barratt *et al*., 2009; Sathananthan, 2013). Additionally, this cell is characterized by a scarce amount of cytoplasm and consequent absence of some organelles common to somatic cells; lack of physiologically active transcription or translation and rapid motility (Sutovsky & Manandhar, 2006; Barratt *et al*., 2009).

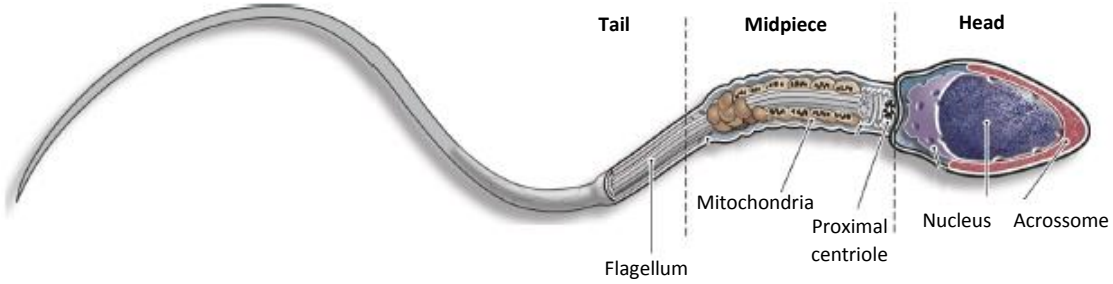

Figure 4- Schematic illustration of sperm morphology. Sperm is structurally divided into head, midpiece and tail. Adapted from du Plessis *et al*., 2011.

The sperm head is an extremely hydrodynamic structure permissive of sperm motility, comprising a condensed haploid nucleus with the paternal genome, surrounded anteriorly by the acrosome, a large specialized secretory vesicle, derived from the Golgi apparatus and containing numerous hydrolytic enzymes as acrosin (Sutovsky & Manandhar, 2006; Barratt *et al*., 2009; Amaral *et al*., 2013a; Sathananthan, 2013). In fact, during oocyte-*zona pelucida* (ZP) penetration the contents of this vesicle are released by exocytosis in the so-called acrosome reaction (de Lamirande *et al*., 1997; Sutovsky & Manandhar, 2006; Hess & França, 2008; Sathananthan, 2013). This is a calcium (Ca^{2+}) dependent event in which the outer acrosomal and the plasma membranes fuse at multiple sites (Florman *et al*., 2008). After binding to the ZP, a

signaling cascade is activated involving Ca^{2+} influx, second messengers, internal pH rise and activation of protein kinases (Abou-haila & Tulsiani, 2009). This event is critical for fertilization both *in vivo* and *in vitro*. In fact, sperm that suffer acrosome reaction prematurely or that lack the acrosome have no fertilizing capacity *in vivo* (Liu & Baker 1988; Albert *et al.,* 1992).

However, human spermatozoa are not able to perform acrosome reaction immediately after ejaculation. It is only after being in the female reproductive tract for some time that spermatozoa undergo a series of poorly understood maturation steps, collectively called capacitation, that confer sperm the ability to fertilize (Yanagimachi, 1994; Abou-haila & Tulsiani, 2009). Overall, capacitation is a process involving multiple structural, metabolic and biochemical changes of spermatozoa including increased plasma membrane permeability and fluidity due to cholesterol efflux, intracellular Ca^{2+} increase, internal pH rise and protein phosphorylation, typically on tyrosine residues (Figure 5) (Visconti & Kopf 1998; Abou-haila & Tulsiani, 2009).

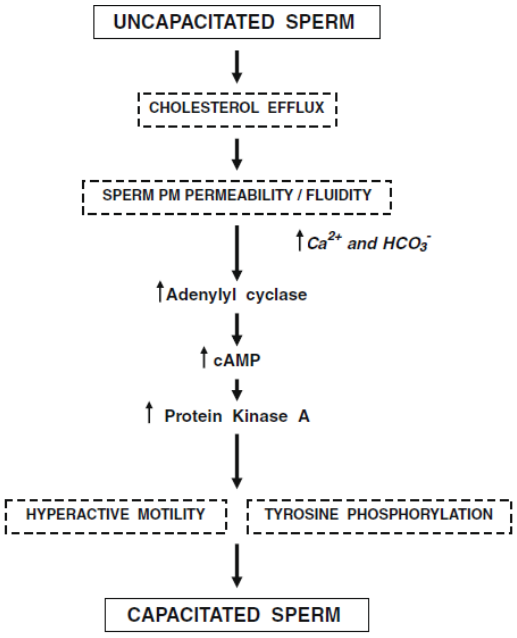

Figure 5- Schematic representation of events and intracellular signaling pathways involved in spermatozoa capacitation process. Adapted from Abou-haila & Tulsiani, 2009.

The importance of the increased membrane fluidity in capacitated spermatozoa is to facilitate fusion-related events during spermatozoa-oocyte interactions such as acrosome reaction and sperm-oocyte fusion (Primakoff & Miles, 2002). Furthermore,

capacitation is also associated with the development of hyperactivated motility that is believed to allow sperm progression through oviductal mucus and to penetrate both the cumulus complex and oocyte ZP, after acrosome reaction (Suarez, 2008; Abou-haila & Tulsiani 2009). *In vitro*, the capacitation process may also be achieved using chemically defined media, mimicking the electrolyte composition of the oviduct fluid. The media should essentially contain energy substrates such as lactate, pyruvate and glucose, ions (Ca^{2+} and HCO_3^-) and serum albumin, a cholesterol acceptor (Visconti & Kopf 1998; Abou-haila & Tulsiani 2009).

The midpiece is a compartment defined by the presence of an helicoidal mitochondria sheath encircling the outer dense fibers (ODF) and the axoneme, a structure composed of microtubules arranged in a characteristic 9 + 2 manner (nine peripheral doublet microtubules surrounding a central doublet) (Sutovsky & Manandhar, 2006; Turner, 2006; Sathananthan, 2013). The length and number of mitochondria in the midpiece varies according to species, with 22 to 75 mitochondria being described in human sperm (Olson & Winfrey, 1990; Ramalho-Santos & Amaral, 2013).

As in other cells, sperm mitochondria are double membrane organelles, composed of an outer and inner membrane separated by the intermembrane space and surrounding a matrix. Mitochondrial inner membrane contains the complexes of the electron transfer chain (ETC) that orchestrate the oxidative phosphorylation process (Detmer *et al*., 2007). The electron transport throughout ETC complexes generates a transmembrane electrochemical gradient that drives ATP synthase to catalyze the production of ATP from ADP and inorganic phosphate (Kakkar & Singh, 2007; Newmeyer & Ferguson-Miller, 2003). The transmembrane electrical potential is the main component of the electrochemical gradient, accounting for more than 90% of the total proton motive force (Moreira *et al*., 2005), and is thus crucial for good mitochondrial performance. Therefore, changes associated with mitochondrial membrane potential (MMP) are critically important in studies regarding mitochondrial function. Furthermore, mitochondria are implicated in several other processes, such as regulation of the intrinsic apoptosis pathway, Ca^{2+} storage, steroidogenesis, lipid metabolism and insulin secretion, among others (Ramalho-Santos *et al.* 2009). Importantly, during cellular respiration, electrons at several sites of the ETC may react with oxygen forming

reactive oxygen species (ROS) that can have both beneficial and adverse effects according to the concentration on the cellular systems (Inoue *et al*., 2003; Turrens, 2003).

The sperm tail, comprising the majority of the sperm length, is responsible for sperm progressive motility in which the major motor portion is the axoneme (Fox, 2003; Sutovsky & Manandhar, 2006; Turner, 2006; Inaba, 2011). The driving force for sperm propulsion is provided by dyneins, ATPase proteins that project from the outer microtubular doublets of the axoneme (Turner, 2006).

Besides all the species-specific variations in sperm morphology and structural details, human ejaculates are very heterogeneous. Spermatozoa have marked variations in size and shape and besides the specific criteria established by WHO to consider a sperm morphologically normal there is still some controversy regarding what should be considered a normal sperm. In fact, when considering WHO criteria, the number of "normal" sperm in an ejaculate is very low, even in normozoospermic fertile men. Moreover, human sperm can present a variety of malformations involving all of its components, both external and internal with which defective spermatogenesis and some epididymal pathologies are commonly associated (WHO, 2010; Sathananthan, 2013).

1.5. The testicular and sperm metabolism

It is unquestionable that spermatogenesis is a metabolically active process dependent on the cooperation between the several testicular cell types (Alves *et al.,* 2013a). Both somatic and developing germ cells of the testicular epithelium have distinct metabolic preferences and ATP demands (Bajpai *et al*., 1998).

Studies have shown that Sertoli cells actively metabolize glucose but the majority of it is converted to lactate rather than oxidized via Krebs cycle (Robinson & Fritz, 1981; Riera *et al*., 2002).

Germ cells are strictly dependent on carbohydrate metabolism, using both glycolysis and oxidative phosphorylation pathways to obtain energy. Their metabolic profile throughout development switch as a consequence of substrate availability, imposed by the compartmentalization of the seminiferous tubules by the blood-testis barrier. In fact, although glucose is readily available to the cells in the basal

compartment of the seminiferous tubules only low levels of glucose are present in seminiferous tubular fluid in the adluminal compartment (Robinson & Fritz, 1981; Bajpai *et al*., 1998). Actually, the transport of interstitial fluid glucose is highly controlled, being mediated by specific glucose transporters (GLUTs) present in the plasma membrane of Sertoli cells. In this way, spermatogonia, lying in the basal compartment take advantage of the easy access to glucose and exhibit high glycolytic activity (Bajpai *et al*., 1998; Boussouar & Benahmed, 2004; Ramalho-Santos *et al.,* 2009). Contrarily, spermatocytes and round spermatids developing in the adluminal compartment, produce energy mainly by oxidative phosphorylation. However, since these cells are unable or inefficient to metabolize glucose they are almost completely dependent on Sertoli cells for provision and maintenance of adequate levels of lactate and pyruvate (Boussouar & Benahmed, 2004; Rato *et al*., 2012; Alves *et al*., 2013a). Of note, spermatocytes are also endowed with glycolytic enzymes, and seem to have an intermediate metabolism, capable of using both glycolysis and oxidative phosphorylation (Bajpai *et al*., 1998). In accordance, Kokk and collaborators detected GLUT 1, GLUT 2 and GLUT3 in early mouse spermatocytes (Kokk *et al*., 2007).

Mature sperm seem to be the more versatile cells in terms of metabolism. Without any doubt, the male gamete is a cell with very high energy demands, with the axonemal dynein as the major ATP consumer (Turner, 2006; Mukai & Travis, 2012). Amazingly enough, the nature of the ATP required to maintain motility is still controversial (Ruiz-Pesini *et al*., 2007; Bucci *et al*., 2011; Piomboni *et al*., 2012). In order to understand this, many studies on sperm bioenergetics have been carried out for decades and usually focused on two metabolic pathways: oxidative phosphorylation and glycolysis (Piomboni *et al*., 2012). Although, both pathways seem to be involved in sperm energy supply for motility, recent findings showed that along with enzymes involved in glycolysis and oxidative phosphorylation, sperm are also endowed with enzymatic machinery known to be active in the fatty acids beta-oxidation pathway, suggesting that also other substrates can provide energy to fuel sperm motility (Amaral *et al.,* 2013a).

Therefore, sperm cells exhibit a great versatility in their metabolism taking advantage of different ATP-producing pathways, dependently on the substrates available (Ruiz-Pesini *et al.,* 2007; Storey, 2008; Piomboni *et al.,* 2012). Nonetheless, since the final objective of each pathway is to fuel sperm motility to further reach the

oocyte and achieve fertilization, the balance between different pathways must be dependent on the substrates available in the female genital tracts (Ford, 2006; Storey, 2008).

1.6. The role of mitochondria on male reproductive function

Such as is the case in other systems in the body, testicular mitochondria are involved in many functions while also producing ATP. Mitochondria functionality has important consequences in terms of reproductive function since it has specific roles in spermatogenesis, being also associated with sperm quality and fertilization potential, as will be further described (Agarwal *et al*., 2003; Tremellen, 2008).

First of all, several mitochondrial proteins are known to be expressed and synthesized during spermatogenesis and therefore used as markers of mitochondria and germ cell development (Meinhardt *et al*., 1999) such as cytochrome c oxidase II (COXII) that is mainly expressed in pachytene spermatocytes (Saunders *et al*., 1993). Additionally, and further supporting the importance of the mitochondria in reproductive function, specific isoforms of oxidative phosphorylation constituents are found in testicular mitochondria, such as cytochrome c and subunit VIb-2 of the COX (Hess *et al*., 1993; Hüttemann *et al*., 2003).

In terms of metabolism, and despite the association between testicular cells mitochondrial morphology and metabolic state (De Martino *et al*., 1979), the importance of mitochondrial ATP for spermatogenesis can be demonstrated by the meiotic arrest observed in mice that do not express a testis-specific adenine nucleotide translocase (ANT4), essential for the translocation of ADP and ATP across the inner mitochondrial membrane (Brower *et al*., 2009). Another essential process assumed by mitochondria is steroidogenesis that takes place in Leydig cells and that results in testosterone production (Wang & Stocco, 2005; Weinbauer *et al.,* 2010). Energized, polarized, and actively respiring mitochondria have been proven to be necessary for this process (Hales *et al*., 2005; Allen *et al*., 2006).

The regulation of apoptosis in the testis is an additional function attributed to mitochondria, ensuring a manageable number of germ cells that can be supported by existing Sertoli cells (Ramalho-Santos & Amaral, 2013). Accordingly, the cytochrome c testis isoform, with its high apoptotic activity, has been implicated in the elimination of

damaged germ cells, ensuring the fidelity and efficiency of sperm DNA transmission (Liu *et al.,* 2006).

Moreover, several mitochondrial parameters were positively correlated with sperm quality and function (Ruiz-Pesini *et al.,* 1998; Gallon *et al*., 2006; Nakada *et al*., 2006; Marchetti *et al*., 2012). Specifically, it has been shown that mitochondrial complexes activities were correlated with sperm motility (Ruiz-Pesini *et al.*, 1998), that the mitochondrial membrane potential is important for *in vitro* fertilization success (Marchetti *et al.*, 2012) and that there is an association between the mitochondrial function and the sperm samples quality (Gallon *et al.,* 2006; Sousa *et al*., 2011). In fact, electron microscopy from asthenozoospermic sperm samples (with reduced motility) revealed that sperm have several mitochondrial anomalies such as shorter midpieces and fewer mitochondrial gyres when compared to normozoospermic (Mundy *et al*., 1995). In the same vein, several reports described that alterations in the mitochondrial genome (mtDNA) can compromise sperm quality, motility and function (St. John *et al*., 2001; Nakada *et al*., 2006). Furthermore, sperm mitochondria have the ability to accumulate calcium, suggesting also a role in the several sperm signaling pathways dependent on calcium (Publicover *et al*., 2007; Publicover *et al*., 2008).

Due to its important and wide range functions in the male reproductive system it is not surprising that mitochondrial dysfunction has been associated with male reproductive impairment and infertility (Amaral & Ramalho-Santos, 2010). Several studies have stated an involvement of oxidative stress, arising when ROS production overwhelms the antioxidant scavenging system, in the development of infertility (Agarwal *et al*., 2003; Cocuzza *et al.,* 2007; Gharagozloo & Aitken, 2011). In fact, high ROS levels have been detected in semen samples of 25% to 40% of infertile men (Agarwal *et al*., 2003; Cocuzza *et al.,* 2007; Aitken *et al*., 2010; Gharagozloo & Aitken, 2011). Mitochondria are the major cellular ROS generator as a result of oxidative metabolism, with 0.2–2% of the oxygen uptake by the cells converted by the mitochondria to ROS (Cocuzza *et al.,* 2007; Orrenius *et al.,* 2007). In the male reproductive tract, ROS are constantly produced in small controlled amounts by a variety of semen components being leukocytes and spermatozoa the major endogenous sources (Agarwal *et al*., 2003; Aitken & Baker, 2006; Cocuzza *et al.,* 2007; Gharagozloo & Aitken, 2011).

While at low and controlled levels, ROS are essential for the regulation of many sperm physiological process, such as capacitation, acquisition of hyperactivated motility, acrosome reaction and oocyte interaction, increased ROS levels in semen have been associated with compromised sperm quality and function (Agarwal *et al*., 2003; Cocuzza *et al.,* 2007). Spermatozoa are especially vulnerable to ROS-induced damage not only because their plasma membrane is richly endowed with polyunsaturated fatty acids, highly susceptible to oxidation, but also because their cytoplasm contains low concentrations of antioxidants and sperm lack DNA repair systems (Agarwal *et al*., 2003; Cocuzza *et al.,* 2007; Gharagozloo & Aitken, 2011). At the membrane level, ROS lipid peroxidation disrupts fluidity leading to severe motility loss and impaired membrane fusion events, such as the acrosome reaction, ultimately compromising fertilization (Agarwal *et al*., 2003; Aitken & Baker, 2006).

In addition, both the mitochondrial (mtDNA) and nuclear (nDNA) genomes of human spermatozoa might be directly damaged by ROS, causing base modifications, deletions, single and double strand breaks, chromatin cross-linking and chromosomal rearrangements (Aitken & Baker, 2006; Cocuzza *et al*., 2007; Gharagozloo & Aitken, 2011; Lavranos *et al*., 2012). In fact, strong evidence suggests that the high levels of ROS are important contributors to the nDNA damage commonly observed in spermatozoa of infertile men (Aitken *et al*., 1998; Agarwal *et al*., 2003). This damage has been strongly associated to an increase of germ cell apoptosis that will ultimately be mirrored in a decrease in sperm counts and deterioration of sperm quality and motility resulting in poor reproductive outcomes (Agarwal *et al*., 2003; Cocuzza *et al*., 2007; Gharagozloo & Aitken, 2011; Lavranos *et al*., 2012)**.** Moreover, mtDNA has been shown to be much more susceptible to oxidative damage than nDNA. Therefore, spermatozoa with defective mitochondria are less efficient in ATP production and may also generate more oxidative stress, which may further damage mitochondria and mtDNA, in a vicious cycle-like manner, conducing to a state of energy crisis and a decline of motility and fertility (Kao *et al*., 1998).

1.7. Diabetes

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action, or both (American Diabetes Association (ADA), 2012). DM is considered a multifactorial disease with a variety of etiologies, including genetic and environmental factors (Wild *et al*., 2004). According to WHO criteria, the vast majority of diabetes cases fall into two broad etiopathogenetic categories: type 1 and type 2 diabetes (WHO, 1999; ADA, 2012).

Type 1 diabetes, the less prevalent form of the disease, onsets at an early age, occurring mainly in childhood and adolescence (WHO, 1999). It is the consequence of a cellular-mediated autoimmune destruction of pancreatic β-cells, leading to absolute, or near absolute, deficiency of insulin secretion, in such way that daily insulin therapy is required (Emilien *et al.*, 1999; WHO, 1999). However, knowledge regarding the causes of autoimmune destruction of β-cells is scarce. Several factors were suggested to be involved such as genetic predisposition and exposure to common viruses or other substances early in life. Untreated type 1 diabetes is characterized by hyperglycemia, hypoinsulinemia, ketonuria, and hyperlipidemia, resulting from a general metabolic failure (Emilien *et al.*, 1999; ADA, 2012).

Type 2 diabetes represents 90–95 % of all diagnosed cases and typically develops in adults over 40 year manifesting primarily by insulin resistance and/or inadequate compensatory insulin secretion response, rather than absolute insulin secretory deficiency (WHO, 1999; Fujimoto, 2000; Marx, 2002; ADA, 2012). Despite the ample pathophysiology of type 2 diabetes, β-cell dysfunction is a hallmark of the pathogenesis of this form of diabetes (Emilien *et al.*, 1999; Marx, 2002). In normal conditions, insulin action essentially provides an integrated set of signals that balances nutrient availability and demand: it promotes carbohydrate uptake at key sites, stimulates the conversion of carbohydrate and protein to lipids and suppresses hepatic glucose production (Fujimoto, 2000; Samuel & Shulman, 2002). The impaired insulin secretion and action in type 2 diabetes will lead to a wide range of metabolic anomalies, including hyperglycemia, due to impaired glucose uptake and overproduction of hepatic glucose, and dyslipidemia (Fujimoto, 2000; Samuel & Shulman, 2002).

Genetic abnormalities in proteins of the insulin signaling cascade and increased ectopic lipid accumulation have been suggested as possible mechanisms involved in the development of insulin resistance (Fujimoto, 2000; Amaral & Ramalho-Santos, 2014). In fact, the chronic high levels of both glucose and free fatty acids (FFA) in the blood seems to be involved in the insulin resistance and the deterioration of β-cell function through a mechanism termed glucolipotoxicity, an oxidative stress-mediated process that damages exposed cells and tissues (Fujimoto, 2000; Evans *et al*., 2003; Amaral & Ramalho-Santos, 2014). Therefore, type 2 diabetes may affect several systems in the body, especially the retina, kidneys, nerves, heart, and blood vessels, limiting longevity and life quality (WHO, 1999; Evans *et al*., 2003; ADA, 2012).

Aging, increased caloric intake, sedentary life and adiposity increase the risk of developing this form of diabetes (Fujimoto, 2000; Evans *et al*., 2003; ADA, 2012) and while until recently type 2 diabetes rarely affected people before middle age, even children are no longer free from developing type 2 diabetes (Marx, 2002). The treatment does not usually involve insulin administration, but only alterations in lifestyle, including exercise and decrease caloric consumption (Alves *et al*., 2013b). Noteworthy, the clinical symptoms may be present for a long period of time before diabetes is detected, allowing the progression of functional changes in cells and tissues that may not be reverted even when a correct therapy is carried out (ADA, 2012; Alves *et al*., 2013a).

Taken together, the study of DM implications in human health is a challenge.

1.7.1. Diabetes pathophysiology

Growing evidence suggests that oxidative stress is implicated in the pathophysiology of DM, particularly in the impairment of pancreatic β-cell function and in the insulin resistance syndrome, thus also contributing to the development of several clinical complications (Evans *et al*., 2003; Brownlee, 2005). Hyperglycemia has been described as one of the major responsible for diabetes associated-complications and four metabolic pathways have been suggested as the major effectors of hyperglycemia-induced damage that result in general increase of oxidative stress: i) increased polyol pathway flux; ii) increased advanced glycation end products (AGEs) formation; iii) activation of protein kinase C (PKC) isoforms and iv) increased hexosamine pathway flux (Brownlee, 2001). Althogether, and also due to the general decrease in antioxidant

levels reported in diabetic conditions (Tabak *et al*., 2011), these pathways result in a general increase in oxidative stress, making it difficult to clarify if ROS production is a cause or consequence of DM (Evans *et al*., 2003; Amaral *et al*., 2008b).

Moreover mitochondrial dysfunction has also been associated with diabetes, which is not surprising due their multitasking properties. Accordingly, the production of mitochondrial ROS due to hyperglycemia is widely recognized as the trigger of the diabetes-related complications resultant from the above described pathways (Tremellen, 2008; Sivitz & Yorek 2010). Of note is the fact that increased ROS production will not only affect macromolecules and membranes but also mitochondria itself, contributing to further dysfunction in a vicious cycle of damage (Amaral & Ramalho-Santos, 2014). In fact, under hyperglycemic conditions higher levels of ROS are generated, as a consequence of mitochondrial hyperpolarization and increased respiration due to increased input of substrates in the respiratory chain (Yu *et al*., 2006). Accordingly, several changes in mitochondrial bioenergetics parameters, such as oxygen consumption, transmembrane electric potential, phosphorylative efficiency, calcium loading capacity, resistance or susceptibility to oxidative stress, and the mitochondrial permeability transition have been observed in diabetic animal models (Palmeira *et al*., 2001; Ferreira *et al*., 2003; Oliveira *et al*., 2003; Sivitz & Yorek 2010). Additionally, several alterations in mitochondria morphology, number, biogenesis and fusion/ fission mechanism had been described in diabetic tissue (Rolo & Palmeira, 2006; Sivitz & Yorek, 2010). As an example, changes in mitochondria morphology were observed to be associated with hyperglycaemia- induced ROS overproduction (Yu *et al*., 2006). Importantly, mitochondrial functionality is also essential for insulin action being involved in insulin secretion and signaling (Sivitz & Yorek 2010).

Altogether, growing evidence supports that mitochondrial dysfunction and related increase in oxidative stress are intrinsically related with DM despite it remains to be determined if these factors are a cause, a consequence of diabetes or both (Dumas *et al*., 2009).

1.8. Diabetes and male reproductive function

It is well known that diabetes is associated with the development of a wide range of long-term systemic complications (Marx, 2002). In the same vein, increasing evidence suggests that DM also has an adverse effect on reproductive function both in men and women, as can be inferred by the reduced fertility and increase in reproductive losses (Miller *et al*., 1981; Lucas *et al*., 1989; Mulholland *et al.*, 2011; la Vignera *et al*., 2012). This is not unexpected, considering diabetes-associated vascular and endothelial complications, as well as the increased oxidative stress, potentially affecting the reproductive system at several levels (Giugliano *et al*., 1996). In fact, it has been reported that a great majority of diabetic male patients have alterations in sexual function, including a decrease in libido, impotence and reduced fertility (Penson *et al*., 2009). Yet the real impact of DM on male reproductive function and the mechanisms responsible for the low fertility rates remain unclear (Alves *et al*., 2013b).

Despite the conflicting results, in general semen analyses of diabetic individuals revealed a decrease sperm motility (Barták *et al*., 1975; Delfino *et al.*, 2007) and concentration (Handelsman *et al*., 1985), presence of abnormal morphologic forms with defects in the acrosome, nucleus, mitochondria and plasma membrane (Barták *et al*., 1975; Vignon *et al.*, 1991; Delfino *et al.*, 2007) and generally increased semen plasma abnormalities (Amaral *et al.*, 2006; Agbaje *et al.*, 2007; Tremellen, 2008; Alves *et al*., 2013b). Interestingly, diabetics that have normal semen parameters showed significantly higher levels of damage in sperm nuclear and mitochondrial DNA, probably due to supra-physiological levels of glucose and related oxidative stress (Agbaje *et al.*, 2007). Additionally, AGEs that result from and in oxidative damage and that have been implicated in an increasing number of diabetic complications had been detected at higher levels in the reproductive tract and seminal plasma of type 1 and 2 diabetic males (particularly in sperm) (Wautier & Schmidt, 2004; Mallidis *et al*., 2007; Mallidis *et al*., 2009) and were suggested to be involved in the ROS initiated sperm DNA damage.

Additionally, some studies reported several structural and morphological changes in the testicular histology of diabetic individuals, such as decreased tubule diameters, altered Sertoli-germ cells connections, degenerating Sertoli cell apical membranes and vacuolization of Sertoli cells (Sexton & Jarow, 1997; Amaral *et al*., 2006). Overall the existing literature suggests that diabetic conditions might disrupt

spermatogenesis and therefore, sperm quality and/or functioning can be compromised, ultimately resulting in infertility (Alves *et al*., 2013b).

At a pos-testicular level, erectile dysfunction (ED) has been described as quite frequent in diabetes, with 50–75 % of diabetic men having some degree of ED, a condition that greatly contributes to the reduced fertility among diabetic patients (Musicki & Burnett, 2007). Additionally, an effect of diabetes at the pre-testicular level (hypothalamus-hypofyse) had also been suggested although the existing reports are often controversial. However the general believe is that hormonal alterations resultant from the disease might also have repercussions at the testicular level (Sexton & Jarrow, 1997; Bacceti *et al*., 2002; Schoeller *et al*., 2012).

Concluding, as DM may affect male reproductive function at multiple levels (Agbaje *et al*., 2007) is crucial to clarify the mechanisms by which diabetes influence testicular and sperm function.

1.9. Objectives

In order clarify the existing questions regarding the influence of DM on male reproductive impairment it was proposed to uniquely study the high glucose factor, the foremost recognized signature of this pathology. Using different *in vitro* approaches previously established, the aim was to investigate the influence of high glucose conditions particularly at two distinct physiological levels: the functionality of spermatozoa and at the spermatogenesis progression.

We wanted to evaluate a variety of spermatozoa functional parameters, using not only conventional procedures but also by means of techniques already implemented in our group. Moreover, we proposed to decipher the effect of different glucose conditions on spermatogenetic process and testicular epithelium taking advantage of a recently developed organ culture method.

Chapter 2

Materials and methods

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all chemicals and reagents were provided by Sigma-Aldrich (St. Louis, MO, USA). The fluorescent probes MitoSOX-Red and 5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) were acquired from Molecular probes (Eugene, OR, USA).

2.2. Spermatozoa functionality evaluation

2.2.1. Human biological material

The present study was developed using human semen samples obtained at the Human Reproduction Service of University Hospitals of Coimbra (Coimbra, Portugal) from healthy men undergoing routine semen analysis or fertility treatments involving either *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

All donors signed informed consents and all biological material was used in accordance with the proper ethical and Internal Review Board (IRB) guidelines provided by the University Hospitals of Coimbra.

2.2.2. Semen samples preparation

Semen samples were obtained by masturbation, after 3 to 5 days of sexual abstinence, and routine seminal analysis was performed according to the WHO guidelines (WHO, 2010).

Spermatozoa were separated from other semen constituents (e.g. seminal plasma, debris and other cell types) via density-gradient centrifugation using SupraSperm (Medicult-Origio, Jyllinge, Denmark) with a 40% (v/v) density top and an 80% (v/v) density lower layers. Briefly, liquefied semen was layered on top of the gradient and centrifuged at 1500 rpm for 10 minutes. Purified sperm population that remained at the high density medium was recovered and posteriorly resuspended in Sperm Preparation Medium (Medicult-Origio, Jyllinge, Denmark).

Sperm concentration of each semen sample was assessed through a Neubauer haemocytometer after spermatozoa immobilization by osmotic shock. During the

course of this study only normozoospermic samples with no detectable leucocytes were used.

2.2.3. Experimental design

Sperm samples, with an adjusted concentration of $10x10^6$ spermatozoa/mL, were incubated at 37°C and 5% CO_2 in phosphate-buffered saline (PBS) supplemented with 0.9 mM $CaCl_2$; 0.5 mM $MgCl_2$; 0.3% (w/v) BSA; 1 mM sodium pyruvate; 10 mM sodium lactate and 1% (v/v) penicillin/streptomycin, pH≈ 7.4 (Amaral *et al*., 2011; Sousa *et al*., 2014). For the experimental purpose 3 different concentrations of D-glucose were used: i) 5 mM representing the physiological concentration; ii) 25 mM to mimic a diabetic condition and iii) 50 mM, a condition to evaluate cells response to massive doses of glucose. Osmotic controls were also performed with equal concentrations of L-glucose. The medium was daily renewed and incubations maintained over a maximal period of 2 days.

The influence of glucose on sperm functionality, particularly sperm motility, viability, capacitation status, acrosomal integrity, mitochondrial superoxide production and mitochondrial membrane potential were assessed daily.

2.2.4. Motility and viability evaluation

Sperm motility and viability were daily monitored by phase-contrast optical microscopy (Nikon Eclipse E200, Nikon Instruments Inc., Melville, NY, USA). For each condition, a total of 100 spermatozoa were evaluated in different fields.

A WHO established and recommended simple system for grading spermatozoa motility that distinguishes spermatozoa with progressive motility, non-progressive motility and immotile sperm was followed (WHO, 2010). Sperm motility was assessed at 200X magnification and results were expressed as the percentage of total motility (progressive plus non-progressive motility).

Sperm viability was determined using 0.5% (w/v) Eosin Y, according to WHO procedures (WHO, 2010). This method is based on the principle that damaged plasma membranes, such as those found in non-viable cells, allowing the entrance of membrane-impermeable stains. Consequently, whereas dead spermatozoa heads stained pink, live spermatozoa remain unstained. Viability examination was performed

at 400X magnification and results were expressed as the percentage of live spermatozoa.

2.2.5. Capacitation status: assessment of tyrosine phosphorylation

For evaluation of capacitation status, samples were processed as previously described in section 2.2.2, excepting that after density-gradient centrifugation sperm *pellets* were ressuspended in non-capacitation medium. Selected sperm samples ($10x10^6$ spermatozoa/mL) were then allowed to capacitate in PBS-based medium described in section 2.2.3 supplemented with 25mM $NaHCO_3$ for at least 3 hours at 37°C and 5% CO_2.

Capacitation status was assessed daily through the detection of phosphorylated tyrosines, a crucial capacitation-associated event broadly accepted as a good capacitation status marker (Ramalho-Santos *et al*., 2007). Briefly, a 100 µL sperm sample aliquot was centrifuged at 1800 rpm during 5 minutes. Spermatozoa were fixed with 2% (v/v) formaldehyde in PBS during 40 minutes. For sperm membranes permeabilization, samples were incubated for 20 minutes with 1% (v/v) Triton-X in PBS. Posteriorly, non-specific antibody reactions were blocked with 0.1% (w/v) BSA + 100mM glycine in PBS. Subsequently, spermatozoa were incubated overnight at 37°C with a rabbit anti-phosphotyrosine polyclonal antibody (1:10; Zymed, CA, USA). After washing steps, spermatozoa were exposed to an anti-rabbit secondary antibody (1:200; IgG; Molecular Probes) for 1 hour. Samples were mounted on glass slides with Vectashield Mounting Media containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Labs, CA, USA) to counterstain spermatozoa nuclei.

For each slide, at least 100 spermatozoa were observed in different fields by means of a Leica fluorescence microscope (Leica DM4000B) at 1000X magnification. While capacitated spermatozoa exhibited fluorescence in both flagellum and midpiece, non-capacitated spermatozoa were not stained, only displaying the blue DAPI counterstain. Results were presented as the percentage of capacitated sperm.

2.2.6. Acrosomal integrity evaluation

Acrosomal integrity was evaluated using *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC) (Magargee, 1988; Kohn *et al*., 1997). Since

PSA binds to the acrossomal contents, particularly to glycoproteins present in the matrix (Liu & Baker, 1988), this method allows the distinction between intact and reacted acrosomes: intact acrosomes exhibit bright green homogeneous fluorescent staining, while acrosome-reacted spermatozoa show no fluorescence signal in the acrosomal region, heterogeneous spots of fluorescence or only a fluorescing band at the equatorial segment of the sperm head (Ramalho-Santos *et al*., 2007). Briefly, a 100 µL sperm sample aliquot was centrifuged at 1800 rpm during 5 minutes. Samples were fixed with 2% (v/v) formaldehyde in PBS during 40 minutes. For sperm membranes permeabilization an incubation for 20 minutes with PBS contained 1% (v/v) Triton-X was performed. Posteriorly, samples were blocked with a solution containing PBS + 0.1% (w/v) BSA + 100mM glycine. Lastly, samples were incubated with PSA-FITC (1:200) for 1 hour at 37°C and, after washing procedures, mounted on glass slides with Vectashield Mounting Media containing DAPI.

At least 100 spermatozoa were examined in different fields by means of a Leica fluorescence microscope (Leica DM4000B, Wetzlar, Germany) at 1000X magnification. Results were presented as the percentage of spermatozoa with intact acrosome.

2.2.7. Flow cytometric analysis

During this study, sperm mitochondrial function, particularly MMP and mitochondrial superoxide production, was monitored by flow cytometry.

Following incubation with specific fluorescent products (JC-1 and MitoSOX-red, respectively) as described below, sample analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with an argon laser operating with an excitation wavelength of 488 nm coupled with the following emission filters: 530/30 band pass (FL-1 / green), 585/42 band pass (FL-2 / red) and >620 nm long pass filter (FL-3 / far red). MitoSOX-Red emitted fluorescence was detected on the FL-2 channel and JC-1 dual emitted fluorescence was detected on FL-1/2 channel.

Based on distinct light scatter characteristics of spermatozoa (forward and side scatter), non-sperm specific events were gated out of the analysis. A total of 20000 sperm-specific events *per* condition were recorded and data acquirement and analysis performed using the BD Cell Quest Pro Acquisition program.

2.2.7.1. Evaluation of sperm mitochondrial superoxide production

Following exposure to different experimental conditions, sperm mitochondrial superoxide generation was determined using the fluorescence probe MitoSOX-Red.

MitoSOX-Red is a cationic dye derived from dihydroethidum able to permeate cells being selectively targeted to mitochondria matrix. Once in the mitochondria, it is rapidly oxidized by superoxide but not by other ROS, nor reactive nitrogen species (RNS). The oxidation product of MitoSOX-Red exhibits red fluorescence upon binding to nucleic acids (Koppers *et al.*, 2008; Aitken *et al.,* 2013). Thus, mitochondrial superoxide generation might be determined by the fluorescent intensity of this probe – mitochondria with higher red fluorescence intensities have produced higher amounts of superoxide.

Sperm suspensions ($2.5x10^6$ spermatozoa/mL) were incubated with 3 μM of MitoSOX-Red at 37°C for 15 minutes, in the dark. In order to remove excessive probe, samples were centrifuged at 1800 rpm for 5 minutes and *pellets* resuspended in PBS-supplemented medium described in section 2.2.3. Additionally, for each sample appropriate controls were prepared: a negative fluorescence control consisting of spermatozoa incubated without MitoSOX-Red and a positive control by simultaneous incubation with 80 μM of antimycin A - a mitochondrial complex III inhibitor known to produce a burst of mitochondrial superoxide (Amaral *et al*., 2013c). These controls allowed us to define flow cytometry histogram regions of non-labeled sperm cells (region M1) and of superoxide producing cells, respectively (Figure 6).

Results were expressed as the percentage of MitoSOX positive cells, i.e. percentage of events in the M2 region of the histogram.

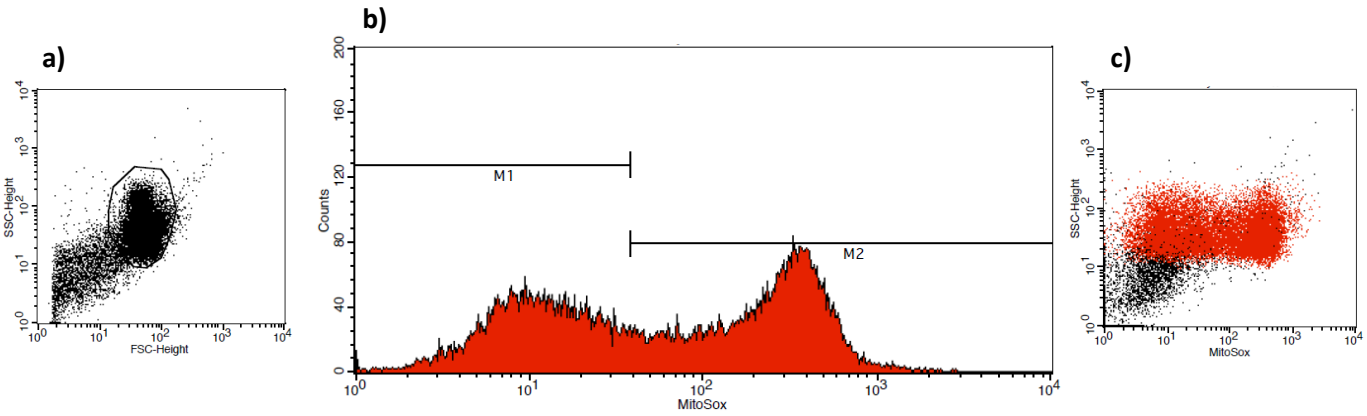

Figure 6 – Mitochondrial superoxide production in spermatozoa. **a)** Dot-plot chart showing selected sperm population. FSC- forward scatter; SSC- side scatter. **b)** Flow cytometry histogram for sperm labeled with MitoSOX-Red. M1- region that comprises non-labeled sperm cells; M2- region that comprises labeled sperm population producing superoxide. **c)** Flow cytometry dot-plot obtained with the same sample. It is possible to observe the two populations referred in b).

2.2.7.2. Evaluation of sperm mitochondrial membrane potential

Sperm MMP was assessed using the fluorescent probe JC-1 as it possesses the unique ability to differentially label mitochondria according to its membrane potential by reversibly shift its fluorescent emission from green to red (Amaral & Ramalho-Santos, 2010). Whereas high MMP leads to the spontaneous formation of JC-1 multimeric aggregates that emit red fluorescence, when the MMP is low JC-1 remains in its monomeric form displaying green fluorescence (Amaral & Ramalho-Santos, 2010). Therefore, this assay allows the distinction between cells with functional mitochondria.

Sperm suspensions ($2.5x10^6$ spermatozoa/mL) were stained with JC-1 at a final concentration of 2 µM for 20 minutes at 37°C, in the dark. To remove excessive probe, samples were centrifuged at 1800 rpm for 5 minutes and *pellets* resuspended in the PBS-based medium previously described. Additionally, for each sample appropriate controls were prepared: a negative fluorescence control consisting of spermatozoa incubated in the absence of the probe and a positive control by simultaneous incubation with 50 µM of p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP), an uncoupler that collapses MMP (Amaral *et al*., 2013c). Using the referred controls, two regions of flow cytometry analysis were defined: region 2 (R2) where the major population of sperm cells presented red fluorescent mitochondria and region 3 (R3) where sperm mostly presented green fluorescence indicative of low MMP (Figure 7).

Both red and green JC-1 fluorescence were measured and results presented as the percentage of sperm-specific events with high MMP, following exposure to different concentrations of glucose.

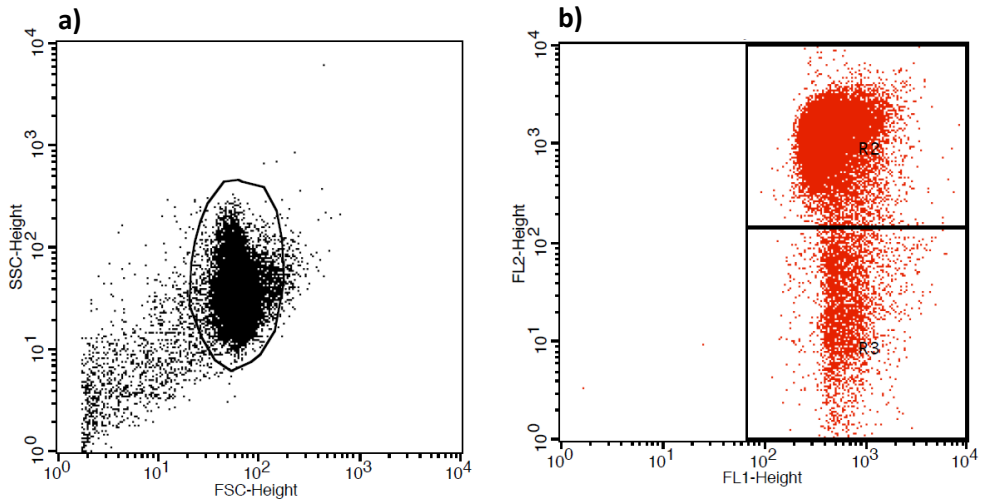

Figure 7 – Spermatozoa mitochondrial membrane potential analysis. **a)** Flow-cytometry dot-plot chart showing the selected sperm population. FSC- forward scatter; SSC- side scatter. **b)** Dot-plot chart for sperm labeled with the fluorescent dye JC-1, with regions comprising the population of cells that presented high (R2) and low (R3) MMP.

2.3. *In vitro* spermatogenesis evaluation under high glucose conditions

To study the influence of glucose in spermatogenesis progression, organ culture experiments were performed according to the interphase gas-liquid method previously described by Sato *et al*. This method allows the *in vitro* development of spermatogenesis at least until the formation of round spermatids or even spermatozoa (Sato *et al*., 2011).

2.3.1. Animals

For organ culture experiments C57BL/6 mice pups with 5 postnatal days were obtained from CNC/FMUC animal facility. During all procedures the ‘‘Principles of Laboratory Animal Care’’ (NIH publication 83-25, revised 1985) were followed. Animals were sacrificed by cervical dislocation.

2.3.2. Agar gel stands for organ culture

To produce the agar gel stands, 3% (w/v) agar (Becton, Dickinson & Company, USA) was dissolved in distillated water. After autoclaving, 10 mL of agar solution were

poured into 10 cm Petri dishes. Once cooled, the agar gel was cut into hexahedrons and 2 to 3 pieces of the agar stands were placed, *per* well, in a six-well plate. Agar gel stands were then soaked in the different culture media described below for more than 24 hours before use and incubated at 35°C and 5% CO_2 (Sato *et al*., 2011).

2.3.3. Organ culture procedure

Testes of C57BL/6 mice pups were removed, decapsulated and gently separated into 3 to 4 fragments to a Petri dish containing 2 mL of cold α-Minimum Essential Medium (α-MEM) (Gibco, Molecular probes). Testis tissue fragments were then placed on 3% (w/v) agar gel stands previously soaked in α-MEM (5mM D-glucose) + 10% (v/v) Knockout Serum Replacement (KSR) (Gibco, Molecular probes) medium supplemented with either 20 or 45mM of D-glucose (Figure 8). KSR was previously demonstrated to be indispensable to induce spermatogenesis in organ culture experiments (Sato *et al*., 2011). Osmotic controls were performed in the same conditions with L-glucose. The amount of medium in each well was then adjusted to the superior limit of the agar stands so that fragments of testis tissue were positioned on the surface of the medium, allowing the easy exchange of oxygen while also permitting full access to nutrients (Sato *et al*., 2011).

Testis fragments were incubated during 3 weeks at 35°C and 5% CO_2 and medium renewed weekly. For evaluation of testicular tissue progression, cultured tissues were weekly observed (Leica DMI3000B Microscope, total magnification 50X), photographed and representative fragments were removed of the culture for fixation and posterior histological examination.

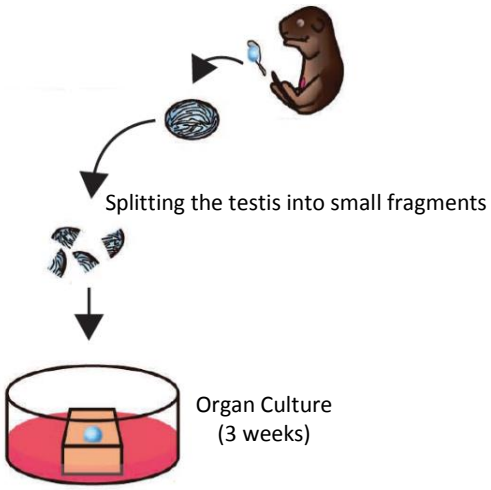

Figure 8 – Schematic diagram of the organ culture experimental procedure. Adapted from Sato *et al.*, 2013.

2.3.4. Histological Procedures

Testis fragments cultured in the different described conditions were fixed in Bouin's solution during 24 hours at room temperature (RT), dehydrated in 70% ethanol and afterwards embedded in paraffin using routine histology procedures. Paraffin-embedded testis fragments were cut into 5 µm transverse sections (Leica Rotary Microtome), collected onto slides with adherent coating (Thermo Fisher Scientific Inc., MA, USA) and dried for 48-72 hours at 50°C (Amaral *et al.*, 2009b). For posterior histological analysis, sections were deparaffinized in xylene and rehydrated in graded series of ethanol solutions (99-70%) (Wistuba *et al.*, 2003). All sections were stained with hematoxylin, dehydrated through graded series of ethanol solutions (70-99%) and finally mounted with Eukitt mounting medium.

2.3.4.1. Immunohistochemistry

With the purpose of distinguishing germ cells from Sertoli cells, testis fragments sections were immunohistochemically stained.

After deparaffination and rehydration, sections underwent antigen retrieval in tris-EDTA pH= 9, at 100°C for 10 minutes. Subsequently, endogenous peroxidases were inactivated with 3% (v/v) H_2O_2 for 15 minutes at RT. To suppress non-specific antibody binding, samples were blocked with 25% goat serum in 0.5% BSA + Tris buffer solution (TBS) for 30 minutes at RT (Taylor, 2009).

Primary antibody specific for Sertoli cells (rabbit anti SOX-9 antibody [1:150]) was then added to the sections and incubated overnight at 4°C in a dark humidified chamber. Negative control reactions for unspecific labeling were performed on a sequential tissue section whether by omitting primary antibody (negative reagent control) or by using an antibody for a target molecule that do not exist in testis tissue (negative tissue control) (Taylor, 2009). Particularly, a negative tissue control was performed with rabbit anti Diacylglycerol kinase kappa (DGKK) antibody (1:100). Secondary antibody (anti rabbit-HRP linked [1:200]) was then applied for 1 hour at RT.

The immunoreactions were revealed by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 15 minutes and nuclei were counterstained with hematoxylin for 10 minutes. Positive staining appeared as a brown precipitate in the cells (Figure 9). After dehydration in ethanol, histological sections of testis fragments were mounted with Eukitt mounting medium.

Stained histological sections of testis fragments were evaluated on a Leica microscope (Leica DM4000B) at 100-630X magnification.

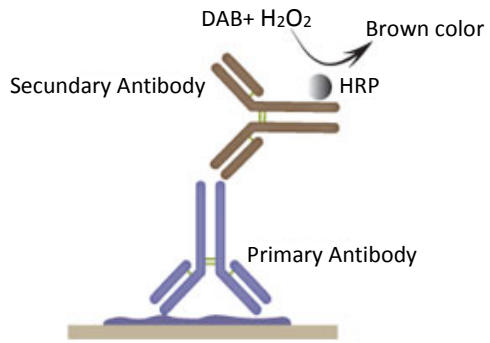

Figure 9 – Schematic representation of immunohistochemical staining with DAB. The secondary antibody is covalently coupled to the horseradish peroxidase (HRP) enzyme that, on the presence of H_2O_2, oxidize the chromogenic substrate DAB into a brown colored product.

2.3.4.2. Morphometric analysis

Images of the testis cross sections were captured at 100-630X magnification using Leica´s microscope and histomorphometric analysis, specifically germ and somatic cell numbers and both tubular and luminal areas (µm) were determined using the NIH ImageJ free software (Amaral *et al.*, 2009b).

2.4. Statistical analysis

Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences Program) version 20.0 for Windows (SPSS Inc, Chicago, IL, USA). All variables were evaluated for normal distribution through Shapiro-Wilk test and the homogeneity of variances assessed by Levene's test.

Statistical comparison between conditions were performed using the t-test or the related Mann–Whitney test and Wilcoxon tests for non-normal variables. Values of $p \leq 0.05$ were considered significant. Results are presented as mean± standard error of the mean (SEM) and the number of experiments indicated.

Chapter 3

Results

3. Results

The goal of this project was to investigate, using two different *in vitro* approaches, the mechanisms involved in the putative male reproductive impairment due to DM on i) sperm cell function and bioenergetics and at ii) spermatogenesis progression.

3.1. Effects of glucose in spermatozoa functionality

3.1.1. Motility and viability

The primary and routine procedures for spermatozoa quality determination involve the assessment of motility and viability (WHO, 2010). Evaluation of these parameters allows a fast and easy way to determine if a particular substance is affecting sperm cells.

Our results show no significant differences, neither in motility nor in viability, between samples incubated with glucose at physiological (5mM) and high concentrations (25 or 50mM) at any day of incubation (Figure 10). Nevertheless, statistical significant differences were found when comparing D-glucose *versus* L-glucose. After day 1, L-glucose-incubated spermatozoa showed significantly lower motility, although the viability was not affected. Viability of spermatozoa incubated with non-metabolizable substrate was only significantly decreased after 2 days of incubation (Figure 10-b).

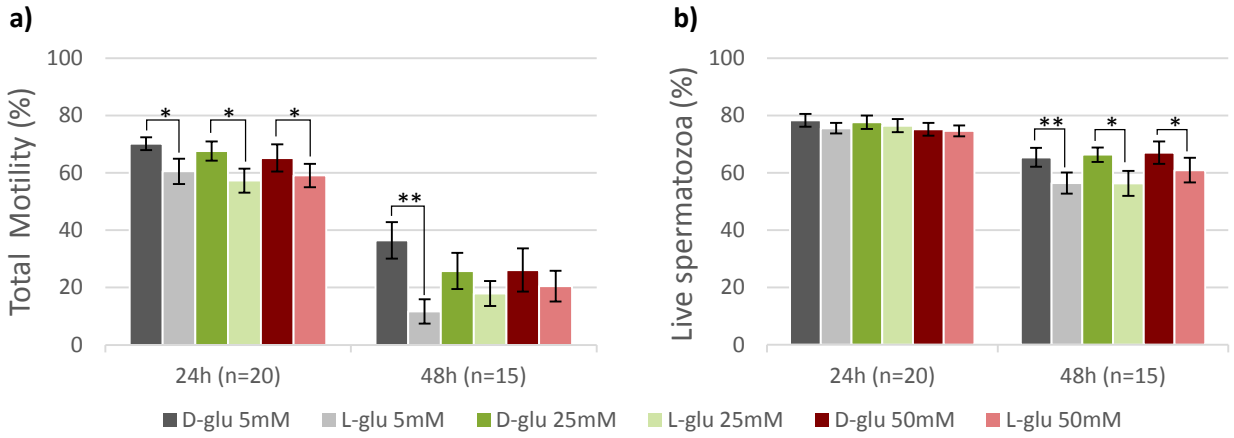

Figure 10 - Spermatozoa motility and viability throughout 2 days incubation in different glucose levels. **a)** Percentage of total motility. **b)** Percentage of live spermatozoa. Samples were incubated in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu). Results are presented as mean ±SEM and the number of experiments specified. *p≤0.05 and **p≤0.01 were considered significant.

3.1.2. Capacitation and acrosomal status

Capacitation and subsequent acrosomal reaction are essential occurring processes, as only capacitated sperm with an intact acrosome have fertilizing ability (Ramalho-Santos *et al.*, 2007). Therefore, these important indicators of spermatozoa functionality were evaluated daily.

3.1.2.1. Capacitation status

As the phosphorylation of tyrosine residues is a characteristic event of capacitation, the influence of glucose concentration on capacitation status was assessed trough the immunocytochemical detection of phosphorylated tyrosines (Figure 11).

The high amount of D-glucose appears to influence the capacitation process but only at day 1 as the higher concentration (50mM) induced a significantly higher percentage of capacitated spermatozoa (43±10.62%) than 25mM (32.20±10.85%). Yet no differences were observed between the physiological level of D-glucose and the other concentrations (Figure 12).

In accordance with the previous results (section 3.1.1), glucose availability seems also to be important for sperm capacitation. This starts to be particularly evident at day 1, when the percentage of capacitated spermatozoa incubated in 50mM L-glucose-

containing media is significantly lower (12±3.77%) than that obtained when exposed to 50mM D-glucose media (43±10.62%) and became more pronounced at day 2, when the differences between D an L-glucose are significant for all concentrations (Figure 12).

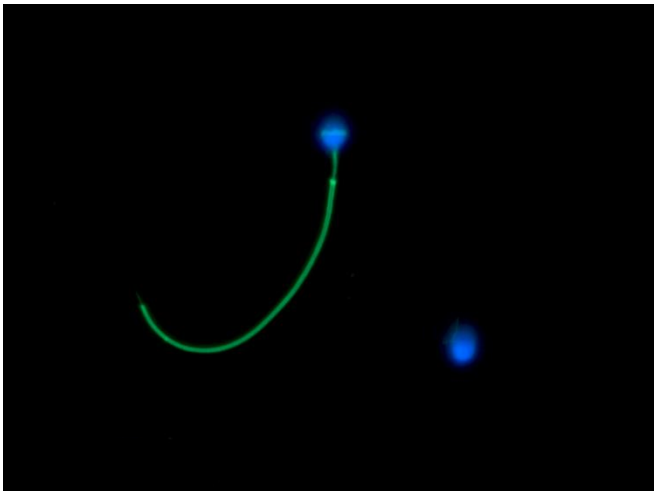

Figure 11 - Capacitation status assessment by immunocytochemical identification of tyrosine phosphorylation. While capacitated spermatozoa exhibited green fluorescence in both flagellum and midpiece, the non-capacitated spermatozoa only displayed the blue DAPI counterstain on the nucleus. 1000X magnification.

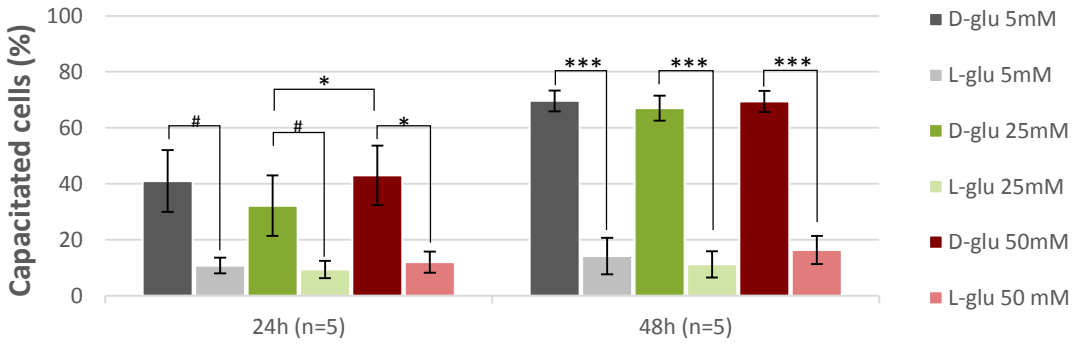

Figure 12 - Percentage of capacitated spermatozoa incubated under different glucose levels. Samples were incubated in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu). Results are presented as mean ±SEM and the number of experiments specified. *p≤0.05 and ***p≤0.001 were considered significant; # represents a statistical trend.

3.1.2.2. Acrossomal integrity

The influence of glucose on sperm acrosomal integrity was assessed using PSA-FITC, a specific acrosomal content marker as previously described (Figure 13).

At day 1, no differences in the percentage of intact acrosomes were noticed between spermatozoa incubated with the physiological concentration and high glucose

levels. However, after 2 days of incubation, the high level of D-glucose (50mM) significantly affected acrossomal integrity (Figure 14).

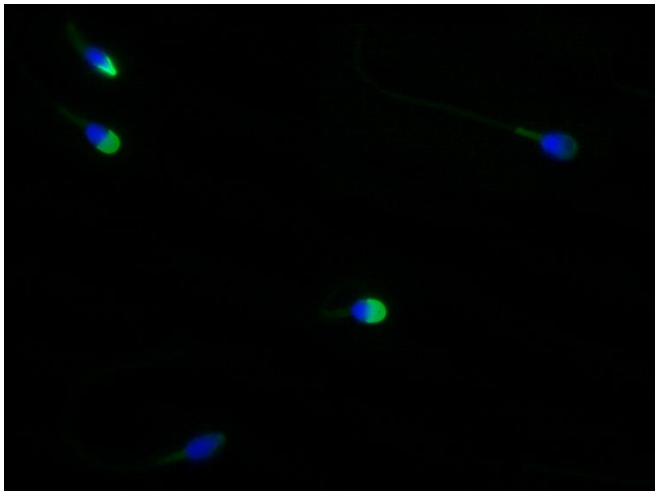

Figure 13 - Acrosomal integrity evaluated by PSA-FITC. Spermatozoa presenting homogeneous green fluorescence on the anterior region of the head possess intact acrosomes whereas acrosome-reacted spermatozoa only displayed the blue DAPI counterstain. 1000X magnification.

Additionally, and according with other functional parameters evaluated so far, the presence of a metabolizable substrate is essential for the maintenance of acrossomal integrity following 2 days of incubation, as evidenced by the results obtained with L-glucose (Figure 14).

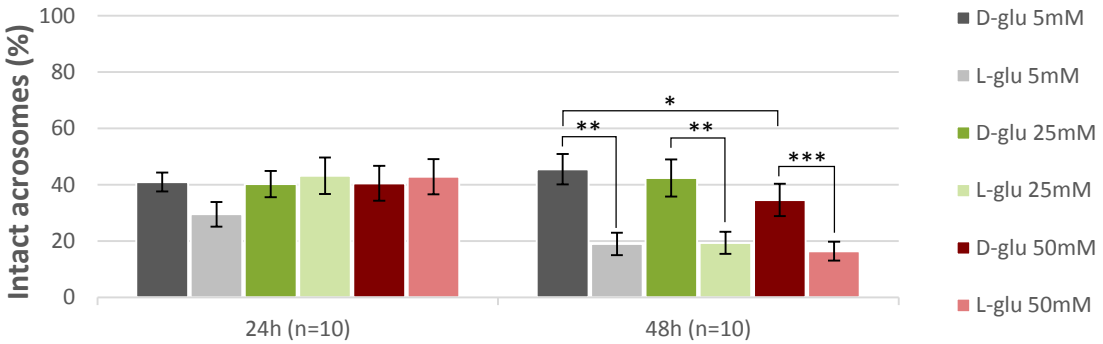

Figure 14 - Influence of different glucose levels on acrossomal integrity. Results represent the percentage of spermatozoa with intact acrosomes. Samples were incubated in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu). Results are presented as mean ±SEM and the number of experiments specified. *p≤0.05, **p≤0.01 and ***p≤0.001 were considered significant.

3.1.3. Spermatozoa mitochondrial function

Mitochondria plays a central role in sperm function since its correct activity has been extensively correlated with several sperm quality parameters including motility and viability, ultimately influencing sperm fertilizing ability (Marchetti *et al*., 2002; Gallon *et al*., 2006; Sousa *et al*., 2011; Amaral *et al*., 2013b). Moreover, mitochondrial impairment is also an important event in DM justifying the focusing of the subsequent analysis on sperm mitochondrial function.

3.1.3.1. Mitochondrial superoxide production

As mitochondria might mediate cell dysfunction via ROS production, mitochondrial superoxide generation was determined by flow cytometry using a mitochondrial-specific fluorescent probe, MitoSox-Red.

No differences were observed in samples incubated in the different described conditions. However, a significant increase of superoxide production was observed, at both day 1 and day 2, on L-glucose incubated spermatozoa (Figure 15). This increase in superoxide production parallels the observed decrease in motility at day 1 and on other parameters at day 2.

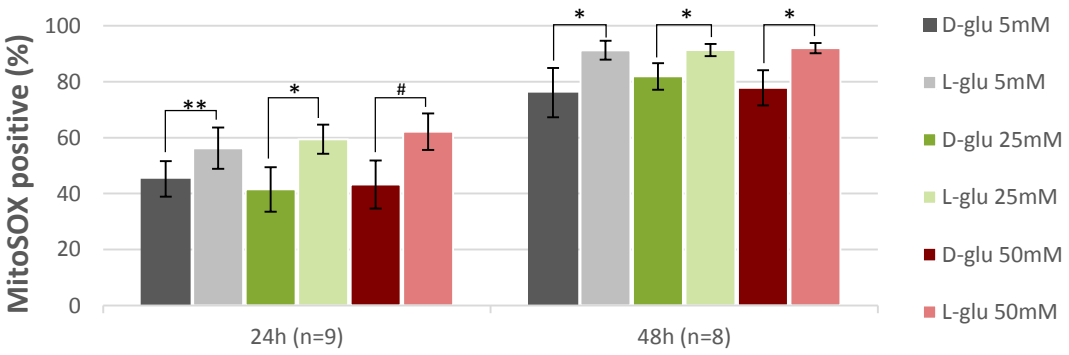

Figure 15 - Influence of high glucose levels on sperm mitochondrial superoxide production. Samples were incubated in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu). Flow-cytometry assay was performed as described in the materials and methods section. Results indicate the percentage of cells MitoSOX-Red positive. Data is presented as mean ± SEM and the number of experiments specified. *p≤0.05 and **p≤0.01 were considered significant; # represents a statistical trend.

3.1.3.2. Mitochondrial Membrane potential

To further determine the possible contribution of mitochondria on sperm impairment, MMP was evaluated by flow cytometry using JC-1, a MMP-dependent fluorescent probe.

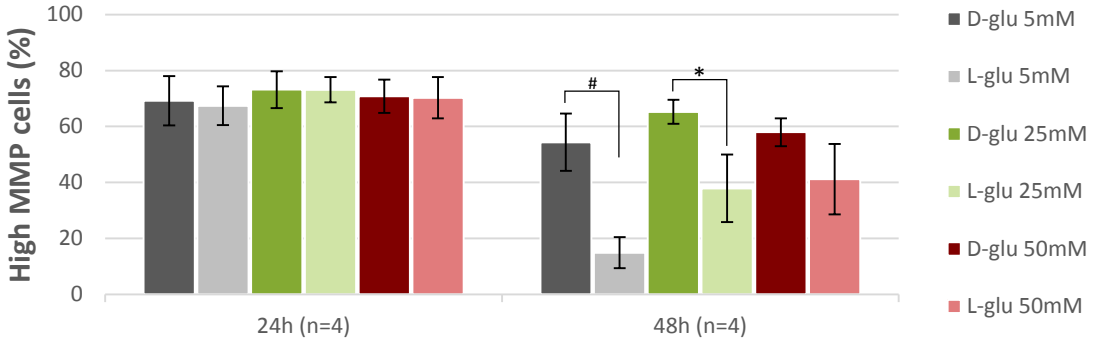

Figure 16 - Influence of high glucose (glu) levels on sperm mitochondrial membrane potential (MMP). Samples were incubated in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu). Flow-cytometry assay was performed as described in the materials and methods section. Results indicate the percentage of cells with high MMP. Data is presented as mean ±SEM and the number of experiments specified. *$p \leq 0.05$ was considered significant and # represents a statistical trend.

No significant glucose-induced effects on MMP were found at any time of incubation (Figure 16). Once again, substrate availability was shown to be important for mitochondrial activity, as proved by the decrease of MMP in spermatozoa incubated in L-glucose media after 2 days.

Globally, these results indicate that hyperglycemia does not seem to affect a variety of functional sperm parameters - motility, viability MMP and mitochondrial superoxide production. Merely acrosomal integrity and capacitation status were influenced by supra-physiological levels of glucose and at a particular time of incubation (day 1 and 2, respectively). Furthermore, it was proved that substrate availability is essential for sperm function as observed by the collectively worse results obtained in all of the assessed parameters when incubated with PBS-based media with non-metabolizable L-glucose. Of note, no osmotic effects were observed in any of the experiments conducted.

3.2. *In vitro* spermatogenesis evaluation under high glucose conditions

As already referred, the spermatogenic process has also been described to be altered by DM. However it remains to be determined at which levels these alterations occur.

When assessing the effects of DM on reproductive function *in vivo*, there are several factors that are difficult to control, such as altered hormonal regulation, inflammation as well as other associated physiological modifications. The *in vitro* organ culture system allows us to dissect the role of a single factor in the spermatogenesis progression without the confounding effects of other variables present in the *in vivo* model of diabetes.

We therefore performed organ culture experiments according to the previously described interphase gas-liquid method (Sato *et al*., 2011) and the testicular tissue development was weekly followed by optical microscopy. During this period it was possible to monitor the organs mainly in terms of tubular volume and necrotic central area (Figure 17-a).

3.2.1. Morphometric analysis

The influence of glucose on spermatogenesis progression was evaluated through a histomorphometric analysis of testicular fragment sections, specifically germ and somatic cell numbers and both tubular and luminal areas (µm) were determined.

The performed analysis revealed that, comparatively to control (5mM), high glucose levels seem to increase the Sertoli cell number, although only with statistical significance at the highest D-glucose concentration (50mM). Additionally, the number of Sertoli cells present in tubules of testis fragments cultured in L-glucose complemented media was identical to the control. Except for the 50mM concentration, there was no evident difference in the number of Sertoli cells, between fragments cultured in D-glucose *versus* L-glucose media (Figure 19).

In what concerns germ cells, the quantity of both spermatogonia and spermatocytes remain unaltered regardless the D-glucose concentration used. However, although, the number of spermatogonia was not related with glucose concentration available, the number of spermatocytes present in fragments cultured in

media containing 50mM of D-glucose was statistically superior to the number present in 50mM L-glucose media incubated fragments (Figure 19).

Additionally in high D-glucose circumstances (25 and 50mM) there is a considerable reduction in luminal area of the evaluated tubules when compared to D-glucose at 5mM (Figure 20).

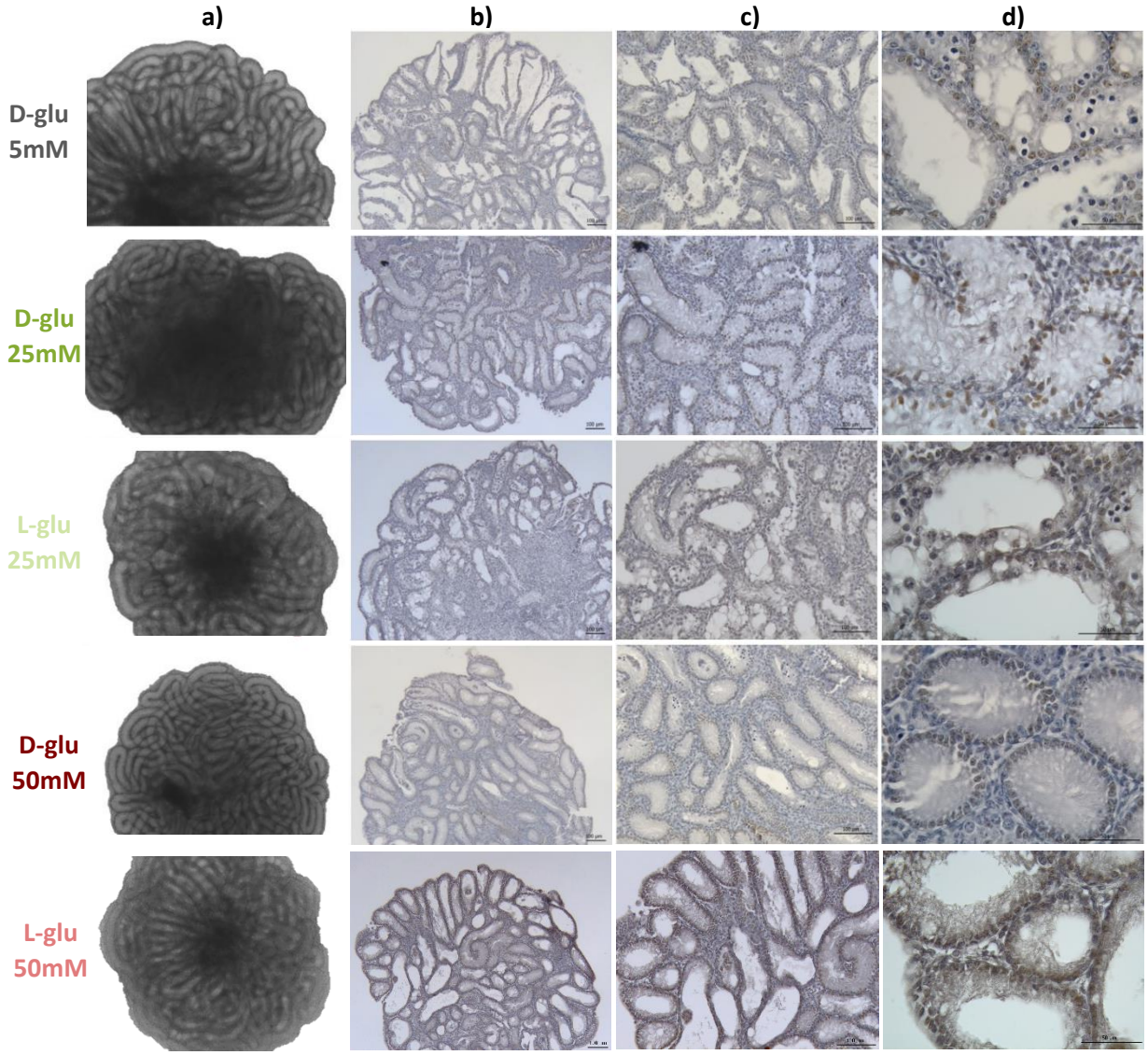

Figure 17 - Testis fragments after 3 weeks in culture in the different experimental conditions. Osmotic controls were incubated in medium with L-glucose (L-glu) **a)** Microscopic view. Magnification: 50X. **b-d)** Histological sections. SOX-9 (brown); hematoxylin (purple). Magnification: 100X, 200X and 630X, respectively.

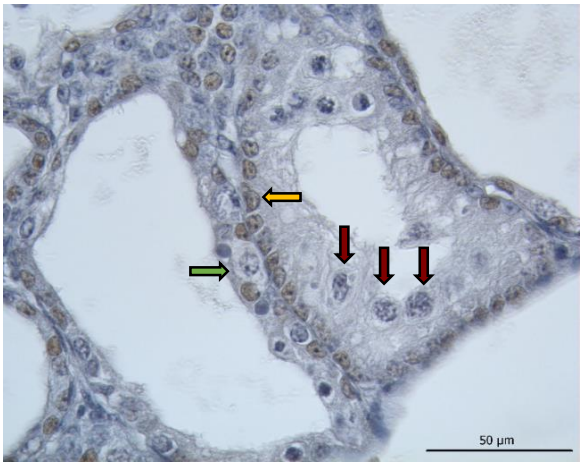

Figure 18 - Microscopic view (630X magnification) of the testicular cell types present in a testis fragment histological section. SOX-9 (brown); hematoxylin (purple). Distinct colored arrows indicate different cell types: green- spermatogonial cell; red- spermatocyte; yellow- Sertoli cell.

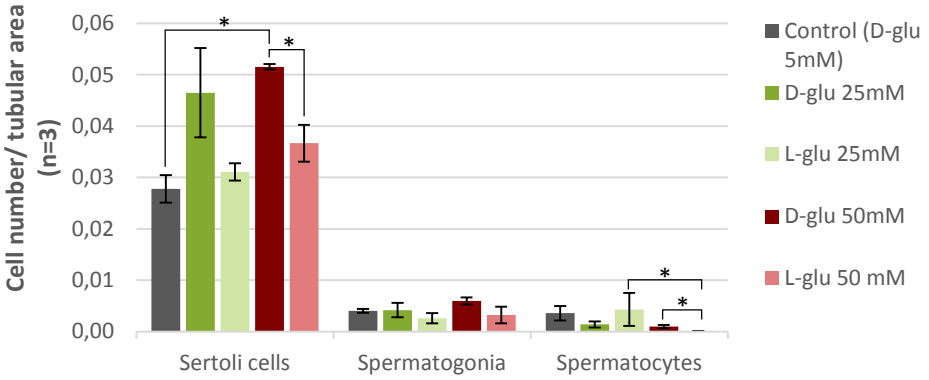

Figure 19 - Influence of different glucose (glu) levels on testicular cell number in testis fragments after 3 weeks in culture. Osmotic controls were incubated in medium with L-glucose (L-glu). Morphometric analysis was performed using Image J software. Results are presented as the mean± SEM ratio of cells according to tubular area. *$p \leq 0.05$ was considered significant.

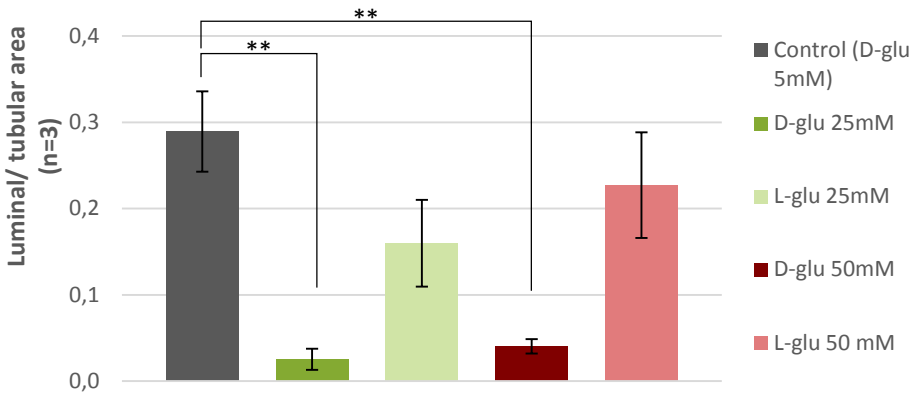

Figure 20 - Influence of different glucose (glu) levels on testis fragments luminal area, after 3 weeks in culture. Osmotic controls were incubated in medium with L-glucose (L-glu). Morphometric analysis was performed using Image J software. Results are presented as the mean± SEM ratio between luminal and tubular area. **$p \leq 0.01$ was considered significant.

Chapter 4

Discussion

4. Discussion

4.1. Effects of glucose in spermatozoa functionality

Although with some contradictions, overall the literature suggests that DM might alter sperm function and semen quality, even though the mechanisms involved are however poorly understood. In fact, current knowledge mostly depended on assessment of conventional sperm parameters and the differences found in the distinct studies point out the limitations of routine semen analyses in the determination of fertility status (Jequier, 2005). It should also be essential to evaluate the impact of DM according to the type and duration of the disease, degree of glycemic and metabolic control, type of treatment applied and, importantly, the presence of additional complications (la Vignera *et al*., 2012).

Given the extension and multifactorial nature of diabetes-induced physiological changes, there are many mechanisms that might be responsible for the associated reproductive dysfunction. In the present study we took advantage of a formerly described *in vitro* spermatozoa culture system (Amaral *et al*., 2011), closely mimicking *in vivo* physiological conditions, in order to strictly address the isolated and direct effect of high glucose levels in human spermatozoa without the *in vivo* confounding factors entailed in the disease.

The used *in vitro* system was previously proven, by a range of studies with a variety of objectives, to be a good tool to evaluate what conditions might lead to spermatozoa functional impairment. For instance, *in vitro* incubations of human spermatozoa allowed to test a wide range of substances varying from putative contraceptives and spermicide agents (Baptista *et al*., 2013), verify the influence of drugs as Sildenafil citrate (Sousa *et al*., 2014) and even the effect of environmental endocrine disruptors (Tavares *et al*., 2013) on spermatozoa function. Therefore, with the purpose of clarifying how high glucose levels affect spermatozoa functionality, a 2-day long incubation in a PBS-based medium with different concentrations of D-glucose were performed.

Although conventional procedures for spermatozoa quality evaluation involves the assessment of motility and viability (WHO, 2010) neither of these parameters were

found to be compromised by high glucose levels (25 or 50 mM) at any day of incubation (Figure 10).

Given the absence of glucose-induced alterations on basic sperm parameters (motility and viability), we then hypothesized that possibly the glucose effects relied on subtle aspects that are also important for sperm quality and function that might not be mirrored in terms of motility or viability. We therefore evaluated additional parameters, with great importance for spermatozoa functionality, but often ignored both in *in vivo* and *in vitro* studies.

As already referred, to become functionally competent, spermatozoa must undergo capacitation, a series of physiological and functional alterations occurring within the female tract conferring sperm fertilizing ability (Ecroyd *et al*., 2003). Even though glucose has been reported to be beneficial for human sperm capacitation (Mahadevan *et al*., 1997; Williams & Ford, 2001) (as discussed below), no knowledge about the effects of high glucose levels on capacitation exists. Here, the influence of different glucose levels on the capacitation status was determined by immunocytochemical identification of phosphotyrosines. We observed no effects of high glucose concentration on sperm capacitation, when compared to control suggesting that high glucose levels are not interfering with this process (Figure 12).

Subsequent to capacitation, the occurrence of acrosomal reaction is indispensable for fertilization. Since it is an event without which sperm penetration through the *zona pellucida* and oocyte fertilization cannot occur *in vivo*, acrossomal integrity is considered an important marker of sperm function (Ramalho-Santos *et al*., 2007). Although glucose levels did not affect the percentage of spermatozoa displaying intact acrosomes at day 1, the supra-physiological concentration of D-glucose (50mM) significantly reduced acrosomal integrity at day 2 (Figure 14), an effect not justified by alteration in sperm viability.

Overall, the observed effect of high glucose levels (50mM) on acrosomal integrity after 2 days, with no noticeable changes on capacitation, leads to suggest that the former process is more prone to alterations which can possible interfere with the fertilization process. However we should highlight that this glucose levels are not physiologically relevant.

Considering the impact of mitochondrial impairment on both diabetes (Ceriello, 2003) and reproductive function (Ramalho-Santos *et al.* 2009; Amaral *et al.* 2013b), it was pertinent to investigate the possible effect of glucose in sperm mitochondria, particularly on ROS production and membrane potential, one of the most important factors to the oxidative phosphorylation process, reflecting therefore mitochondrial functionality (Ramalho-Santos *et al*., 2009). To our knowledge, this is the first study addressing the effect of different glucose levels on human sperm mitochondria.

Our results show that neither the generation of mitochondrial superoxide (Figure 15) nor MMP (Figure 16) were significantly affected by the described conditions. The absence of alterations in sperm mitochondrial parameters might suggest that the alterations previously reported in diabetic sperm function are mitochondria-independent, in accordance to what has been reported on rat sperm and testicular mitochondria (Amaral *et al*., 2009b) or alternatively, and more likely, that other factors besides hyperglycemia might be involved. Suresh and colleges recently described an increased proportion of epididymal sperm with low MMP in diabetic-induced rats (Suresh *et al*., 2013). The obtained contrasting results may arise from the fact that (1) an *in vivo* model has been used, (2) the fact that mitochondria from human sperm greatly differ from their mouse sperm counterparts and also (3) the different technics used in general considered to be less powerful and more subjective (epi-fluorescent microscopy).

Globally, the obtained and described results in the present study indicate that high glucose concentrations *in vitro*, do not seem to directly affect a variety of spermatozoa functional parameters including motility, viability, MMP and mitochondrial superoxide production. Solely acrosomal integrity was influenced, although by supra-physiological levels of glucose (50mM).

Therefore, the effect of DM on human sperm reported in other studies thus may arise from alterations at the spermatogenesis level and/or from factors other than glucose (e.g. oxidative stress) or, alternatively, from a combined effect of glucose and other factors. In that vein, the influence of glucose at a spermatogenesis level was also evaluated as will be further discussed.

4.2. Effects of substrate availability in spermatozoa functionality

During the *in vitro* experiments involving human spermatozoa, osmotic controls were performed using a PBS-based medium with a non-metabolizable glucose (L-glucose). L-glucose-incubated spermatozoa presented a significantly inferior motility and viability than spermatozoa incubated with metabolizable glucose (D-glucose) (Figure 10). Since at day 1 motility was affected, without interfering with cell viability, it is possible to conjecture that this effect is due to a lack of substrate and not to cell death-related processes. Nonetheless, at day 2 we cannot exclude that differences in sperm motility may be due to a decrease in viability.

It is known that mammalian sperm require metabolic energy for a variety of functions, most notably to support motility (Williams & Ford, 2001; Ferramosca & Zara, 2014). Results regarding reduced motility in L-glucose incubated spermatozoa indicates that spermatozoa require glucose to support optimal motility, even when media is supplied with lactate and pyruvate that support mitochondrial respiration. In fact, it is known that human spermatozoa obtain a significant proportion of their energy from glycolysis (Williams & Ford, 2001). Nevertheless, the origin of ATP for sperm motility is still intensely discussed, since human sperm can remain motile in media lacking a glycolysable sugar (Suter *et al*., 1979; Ford & Harrison, 1981; Williams & Ford, 2001).

Our data emphasize the importance of glucose for energy production and motility in human sperm and it would be interesting to further determine whether the effect of glucose on motility can be explained by their effect on ATP levels. Additionally, our results showed that glucose availability seems also to be important for sperm capacitation (Figure 12) and acrosomal integrity (Figure 14). This was particularly noticeable at the second day of incubation, when both the percentage of capacitated spermatozoa and percentage of spermatozoa with intact acrosome was significantly lower than that obtained when cultured in D-glucose containing media. Through the evaluation of viability it is possible to exclude the hypothesis that this differences are due to sperm cell death at day 1, although the same is not true for day 2. In accordance, a number of studies have already demonstrated that human spermatozoa capacitation substantially rely on glucose, suggesting a key role in sustaining hyperactivation (Williams & Ford, 2001), tyrosine phosphorylation (Travis *et al*., 2001) and ensuring fertilization *in vitro* (Mahadevan *et al*., 1997; Rogers & Perreault, 1990). Although it is

still unclear whether the beneficial effects of glucose derive from the provision of extra metabolic energy through glycolysis or from the generation of other metabolic products**:** glucose may be necessary to produce NADPH, to allow a putative NADPH oxidase to generate superoxide to promote protein tyrosine phosphorylation (Aitken *et al*., 1997; de Lamirande *et al*., 1997; Williams & Ford, 2001)**.**

Regarding acrossomal integrity, the higher acrosomal loss observed in L-glucose incubated spermatozoa may be explained by an early-triggered acrosome reaction. Moreover, as result of the incubation in media with L-glucose, spermatozoa produced higher amounts of mitochondrial superoxide (Figure 15). The increased levels of superoxide may result from the lower viability and motility previously describe or *vice versa*, i.e. the higher oxidative stress might further contribute towards declining sperm viability and motility due to damage on cellular components such as membranes and macromolecules (Tremellen, 2008). To investigate whether the increase in superoxide generation was associated with mitochondrial dysfunction, we assessed MMP and found that spermatozoa incubated without D-glucose had affected mitochondrial function only after 2 days, as observable by the decrease in the number of cells presenting mitochondria with a high MMP (Figure 16). Interestingly, the observed increased production of ROS occurred before any alteration in MMP became apparent, an effect that has been previously described in human spermatozoa (Koppers *et al*., 2008; Aitken *et al*., 2012; Amaral *et al*., 2013b; Sousa *et al*., 2014).

Given the importance of mitochondria for spermatozoa function, the worst functionality of L-glucose-incubated spermatozoa might be explained by mitochondrial impairment, as might also be related with the observed significant decrease in capacitation achievement. In accordance, several studies demonstrated that concomitant with capacitation there is an abruptly increase in sperm mitochondrial activity both in mice (Boell, 1985; Fraser & Lane, 1987) and humans (Hicks *et al*., 1972; Stendardi *et al*., 2011). Therefore if mitochondrial activity is altered it will be expectable that capacitation might be affected in some extent as observed in our study. However we cannot exclude that other factors might be involved

Overall, glucose availability is essential for *in vitro* long-term maintenance of spermatozoa function as observed by the collective worst results obtained herein for all the assessed parameters. This is in accordance with the beneficial enhancing effects of

exogenous glucose in human sperm maintenance previously described (Amaral *et al*., 2011).

4.3. Diabetes and testicular histological alterations

4.3.1. *In vitro* spermatogenesis evaluation under high glucose conditions

Compared to clinical studies involving diabetic patients, the association between DM and decreased male fertility is considerably clearer in the various animal models employed to study this condition. Animal models are important research tools, providing insights almost impossible to duplicate and control in human populations (McIntosh & Pederson, 1999).

Most studies using rodent models of DM strongly suggest that, as in humans, this pathology significantly impairs male fertility, as revealed by marked reduction in fecundity (Scarano *et al*., 2006; Shrilatha & Muralidhara, 2007; Kim & Moley, 2008; Mulholland, 2011) and sperm quality, particularly diminishing sperm concentration, motility (Hassan *et al*., 1993; Amaral *et al*., 2006; Scarano *et al*., 2006; Kim & Moley, 2008; Navarro-Casado *et al*., 2010; Mulholland, 2011) and morphology (Navarro-Casado *et al*., 2010).

Additionally, other *in vivo* studies revealed that the spermatogenic process may also be affected suggesting that diabetic reproductive dysfunction might reflect alterations at this level, although the mechanisms of altered spermatogenesis in animal models (and diabetic patients) are poorly understood and frequently contradictory. While there are studies showing testicular and epididymis structural, morphological and histological changes, as reduction in organ size and lower diameters of the tubules and lumen (Cameron *et al*., 1985; Seethalakshmi *et al*., 1987; Hassan *et al*., 1993; Baccetti *et al*., 2002; Soudomani *et al*., 2005; Scarano *et al*., 2006; Amaral *et al*., 2009b) and spermatogenic impairment in diabetic animals (Kuhn-Velten *et al.*, 1984; Soudomani *et al*., 2005; Wankeu-Nya *et al*., 2013), others failed to detect any changes at these level (Navarro-Casado *et al*., 2010). The conflicting data may result from the fact that when *in vivo* assessing the effects of DM on spermatogenesis there are several factors that are difficult to control, such as altered hormonal regulation, inflammation, oxidative stress and other alterations associated with the disease that might contribute to a general dysfunction.

With the aim to determine the isolated effect of hyperglycemia in testicular cells and spermatogenesis progression, organ culture experiments were performed. Morphometric analysis of testicular fragment sections revealed that, after 3 weeks in culture, high glucose conditions (25 and 50mM) seem to increase Sertoli cell number, reaching statistical significance at the highest D-glucose concentration (50mM) (Figure 19). Additionally in these circumstances a considerable reduction in the luminal area was observed (Figure 20).

As already referred Sertoli cells are odd cells with a panoply of functions that are essential for spermatogenesis. In addition to nourishing and guiding germ cell development, Sertoli cells are also responsible for the production and secretion of tubular fluid essential for sperm transport and, due to its peculiar constitution, for maintenance of germ cells. The observed alteration in the luminal area in high glucose concentration suggests therefore an altered function at the Sertoli cell level.

Surprisingly, an increase in the number of Sertoli cells when testis fragments are subject to high glucose conditions was observed. We believe that this might be associated with a higher proliferation rate of this cells, an event that might be related to Sertoli cell maturational state as described *in vivo*. In fact is already known that to become fully competent Sertoli cells most undergo a radical change, around puberty, in their morphology and function, sifting from an immature, high proliferative state to a mature, non-proliferative state (McLaren *et al*., 1993; Sharp *et al*., 2003). A failure in Sertoli cell functional maturation, have inevitable consequences in terms of the ability to support the survival and development of the various germ cells (Boujrad *et al*., 1995; Sharp *et al*., 2003). Additionally it is also known that immature cells are not capable of the same functions as mature ones, such as observable in infertile men due to Sertoli cell-only syndrome (lack of germ cells in the seminiferous epithelium), in which Sertoli cells present the morphology characteristic of immature cells (Sharp *et al*., 2003).

Our findings are consistent and might be explained by what occurs *in vivo*. As only immature Sertoli cells proliferate we assume that the increased number of Sertoli cells present in testis fragments cultured in high glucose conditions are a result of higher proliferation, indicative of immaturity. Therefore, these cells may present an altered functionality. As Sertoli cells assume a hub role in the regulation, maintenance and subsequent success of spermatogenesis (Riera *et al*., 2002; Weinbauer *et al*., 2010),

impaired functionality of Sertoli cells might have repercussions in terms of spermatogenesis progression (Holstein *et al*., 2003), although not seen in our studied system.

Hence, high glucose levels may affect proliferation rate and/or avoid maturation of Sertoli cells, ultimately affecting its functionality, as mirrored by a reduction in the tubular luminal area.

4.4. General considerations and future directions

Taking advantage of the previously described *in vitro* methods, the present work helped to elucidate the particular and isolated effect of high glucose at two distinct levels: i) the spermatozoa, focusing on its functionality and at ii) the spermatogenic progression.

The results obtained lead us to assume that high glucose levels *per se*, if they have any effect on male reproductive function, this will result from alterations at the spermatogenic level, particularly through a possible impairment of Sertoli cell function/ maturational state as mirrored by a reduction in the luminal area and higher proliferation. Alternatively, the previously reported defects in diabetic spermatozoa might result from other factors than glucose. It is known that there are many biochemical alterations (e.g. altered insulin levels, increased inflammatory factors and oxidative status) in diabetic patients, and if those alterations are reflected at the testicular level, along the reproductive tract or at seminal plasma, the influence of these factors on sperm function will be cause for concern. Particularly, using the described *in vitro* approach for sperm culture it would be important to test in the future, in an isolated, efficient and quick manner, the effect of other factors characteristically altered in diabetic patients. In fact, to address this question we are at the moment testing the effects of oxidative stress and inflammatory factors on sperm function. Additionally, considering the key role of Sertoli cells on spermatogenesis it would be interesting to perform additional studies in order to clarify what contributes to the changes reported in our work. This studies could be conducted either in Sertoli cells in culture (as we are doing at the moment in our laboratory) or using the *in vitro* organ culture method here presented in order to better mimic and understand consequent occurring changes at seminiferous epithelium. In fact, it is our aim to clarify how Sertoli cells are altered in a

diabetic environment, performing a complete characterization of their function evaluating aspects such as viability, bioenergetics and metabolism.

In conclusion, many studies regarding the effects DM have particularly centered their attention in spermatozoa functionality. However, as emphasized here, understanding the mechanisms underlying diabetes-induced alterations of spermatogenesis is essential for the development of a strategy to prevent or alleviate those effects (Wankeu-Nya *et al*., 2013).

Chapter 5

Concluding remarks

5. Concluding remarks

Given the extension and multifactorial nature of diabetes-induced physiological changes, it remains unclear what are the mechanisms that may contribute for the reproductive dysfunction described in male diabetic patients. During this project two different *in vitro* approaches were used to address the isolated effect of high glucose concentrations on i) sperm function and ii) spermatogenesis progression, thus avoiding the *in vivo* confounding effects of the disease.

Regarding sperm function, neither motility, viability nor sperm mitochondrial functionality were directly affected by hyperglycemic conditions (25 and 50mM D-glucose). Only acrosomal integrity was influenced, although by a supra-physiological concentration of glucose (50mM). Moreover it was corroborated that glucose availability is essential for maintenance of sperm function as observed by the significant decrease in motility, viability, percentage of capacitated sperm and with intact acrosome and also by increase in ROS production when media containing only non-metabolizable L-glucose was used.

In vitro mimicking the spermatogenic process, through organ culture experiments, allowed to verify that high glucose level seem to affect this process increasing Sertoli cell number while decreasing tubular luminal area, therefore suggesting an impairment of this somatic cell type with hub importance in spermatogenic control.

Although more studies should be performed to clarify some raised and remaining questions, the general obtained results leads us to assume that the reported diabetic male reproductive dysfunction result not via an direct impact of glucose on sperm cells, but either from alterations at the spermatogenic level or from other factors involved in the disease and that were not addressed in this study.

Chapter 6

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6. References

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