UNIVERSIDADE DA BEIRA INTERIOR

Ciências da saúde

Avaliação de Marcadores de Morte Celular e Parâmetros Reprodutivos em Modelos de Diabetes Mellitus

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Resumo

A diabetes mellitus (DM) representa uma das maiores ameaças à saúde global moderna e a sua incidência está a aumentar rapidamente em todo o mundo. Esta doença consiste numa desordem metabólica, caracterizada por hiperglicemia, resultante de uma secreção defeituosa de insulina, resistência à ação da insulina ou ambas. Existem dois tipos de DM, tipo 1 e tipo 2, ambas as condições relacionadas com a infertilidade masculina. A DM tipo 1 está associada com a privação de insulina e embora os efeitos globais *in vivo* na função reprodutiva sejam bem conhecidos, há uma falta de estudos relativamente ao controlo da insulina sobre as funções fisiológicas das células do sistema reprodutivo. Sobretudo, os estudos *in vivo* são frequentemente feitos depois de a doença estar completamente estabelecida, mas sabe-se que há um estado pré-diabético, caracterizado por resistência à insulina, que antecede o desenvolvimento da DM, especialmente da DM tipo 2. Com o nosso trabalho, pretendemos investigar mais profundamente a ligação entre a DM e a infertilidade masculina, através da análise de vários marcadores de morte celular e de parâmetros reprodutivos. Para isso, simulámos o estado de DM tipo 1 humano em células de Sertoli de rato e analisámos os níveis de expressão de mRNA e proteína de vários marcadores de morte celular envolvidos na via mitocondrial. Por outro lado, também desenvolvemos um modelo animal de pré-diabetes de modo a reproduzir esta condição patológica e avaliar alterações produzidas nos parâmetros reprodutivos, assim como nos níveis de expressão de mRNA e proteína de marcadores de morte celular envolvidos na via mitocondrial.

Os resultados obtidos levam-nos a sugerir que a insulina interfere com a interação entre proteínas pró- e anti-apoptóticas. Uma vez que esta interação pode decidir o destino celular e exercer um controlo rigoroso sobre a sinalização apoptótica, a insulina terá um papel chave na manutenção da espermatogénese. O modelo de rato utilizado compartilhava muitas das características clínicas e metabólicas do estado pré-diabético observado em humanos, tais como resistência à glucose e a progressão de normoglicemia/normoinsulinemia para hiperglicemia/hiperinsulinemia moderada devido à ingestão de alimentos. Este estado pré-diabético induziu alterações significativas na morfologia dos espermatozoides da cauda do epidídimo, mostrando que esses animais poderão desenvolver problemas de subfertilidade ou de fertilidade. Na sinalização apoptótica na cauda do epidídimo, os animais sujeitos à dieta de alta energia (HED) apresentaram níveis mais baixos de mRNA Bax e da proteína citocromo C, embora a avaliação quantitativa do *endpoint* apoptótico, a atividade da caspase-3, não tenha evidenciado quaisquer alterações entre a situação HED e controle. Isto sugere que o processo apoptótico pode ser controlado por outros mecanismos que não somente os proteicos pró-apoptóticos mitocondriais, como por exemplo os sistemas anti-apoptóticos celulares.

Os resultados obtidos com estes dois modelos experimentais levam-nos a concluir que os problemas de subfertilidade/infertilidade causados pela DM podem ser mediados pela insulina, que tem um efeito importante na regulação da interação entre proteínas pró e anti-apoptóticas e, por esse motivo, deverá ser dedicada uma atenção especial às disfunções ocorridas no estado pré-diabético, onde observámos alterações cruciais na morfologia de espermatozoides epididimais de ratos. Devido à crescente incidência da DM e às complicações associadas ao nível da infertilidade masculina é fundamental aprofundar o conhecimento nestes dois sistemas, de modo a isolar possíveis mecanismos envolvidos e a avaliar os efeitos globais, como uma estratégia para desenvolver possíveis abordagens terapêuticas.

Palavras-chave

Diabetes mellitus; Infertilidade masculina; Parâmetros reprodutivos; Marcadores de morte celular.

Resumo alargado

Nos mamíferos, os testículos são os elementos centrais do sistema reprodutor masculino e estão envolvidos na síntese de esteroides e na produção de espermatozoides. A espermatogénese é o processo de expansão e desenvolvimento das células germinativas que ocorre dentro dos túbulos seminíferos dos testículos determinando a fertilidade masculina. Uma espermatogénese normal envolve um equilíbrio entre a proliferação celular e a apoptose, porque, mesmo durante condições normais, um número de células germinativas morre por apoptose antes de atingir a maturidade. Depois de completarem a espermatogénese, os espermatozoides são células diferenciadas incapazes de nadar ou fertilizar um óvulo. Essa capacidade de fertilização será adquirida durante as diferentes fases que ocorrem até ao encontro com o gâmeta feminino, sendo uma delas a passagem através do epidídimo. Este consiste num túbulo longo e enrolado que cria um microambiente único que ajuda a transformar os espermatozoides imaturos e sem motilidade em células competentes e completamente férteis. Permite também, o armazenamento dos espermatozoides férteis num estado viável dentro da cauda do epidídimo até serem ejaculados.

As células de Sertoli (SCs) desempenham um papel importante no desenvolvimento funcional dos testículos e consequentemente na expressão do fenótipo masculino. Elas fornecem nutrientes e fatores regulatórios para a sustentação das células germinativas. Para além disso, têm uma grande capacidade para produzir lactato que é um elemento importante para as células germinativas devido ao seu efeito anti-apoptótico e ao seu papel como fonte de energia. Nas SCs, a glucose é metabolizada para lactato para depois este ser usado pelas células germinativas como o principal substrato para a produção de ATP. Por isso, a regulação do metabolismo da glucose nas SCs é crucial para uma espermatogénese normal e para a fertilidade. A insulina é uma hormona na regulação do metabolismo celular e a sua disfunção (deficiência ou resistência) tem atraído muita atenção, uma vez que se traduz em patologias como a diabetes mellitus (DM). A DM representa uma das maiores ameaças para a saúde global moderna e a sua incidência está a aumentar rapidamente em todo o mundo. Esta doença poder ser divida em dois tipos, tipo 1 e tipo 2, que podem ser descritos sumariamente como desordens metabólicas caracterizadas por um estado de hiperglicemia (baixos valores de glucose no sangue) resultando de uma secreção defeituosa de insulina, resistência à ação da insulina, ou ambas. A DM tipo 1 desenvolve-se geralmente em idade jovem e é caracterizada por uma destruição autoimune das células beta do pâncreas (produtoras de insulina) em indivíduos geneticamente susceptíveis, resultando na dependência de um tratamento com insulina exógena. A DM tipo 2 ocorre quando a produção de insulina pelas células beta não é suficiente para manter os níveis de glucose no sangue dentro de valores

fisiológicos normais, levando a exaustão funcional das células beta. A DM tipo 2 pode ser prevenida se for detetado precocemente o estado de pré-diabetes que usualmente antecede o aparecimento desta doença. A transição de um estado de pré-diabetes para DM tipo 2 ocorre quando a capacidade secretora das células beta não é capaz de compensar a resistência à insulina.

As alterações hormonais e metabólicas associadas com a DM tipo 1 e tipo 2 comprometem a fertilidade masculina. A influência prejudicial da DM e da obesidade na fertilidade tem recebido maior atenção uma vez que a sua prevalência e incidência têm aumentado em todo o mundo, enquanto a idade do primeiro diagnóstico para ambas as doenças tem diminuído continuamente. Devido a esta situação, cada vez mais, os problemas de fertilidade afetam indivíduos antes e/ou durante os seus anos reprodutivos. Um grande número de casos de infertilidade masculina está associado a uma baixa qualidade dos espermatozoides devido a parâmetros reprodutivos anormais, sendo, por isso, a análise do sémen o teste inicial para a avaliação do fator de infertilidade masculina.

Com este trabalho, pretendeu-se investigar a associação entre a DM e a infertilidade masculina através da análise de vários marcadores de morte celular envolvidos na via mitocondrial e parâmetros reprodutivos dos espermatozoides (motilidade, viabilidade, concentração e morfologia). Um dos objetivos deste trabalho foi reproduzir a doença humana de DM tipo 1 em SCs de rato cultivadas (*in vitro*) para analisar o seu efeito nas vias apoptóticas de morte celular. Para isso, foram isoladas SCs de ratos Wistar normais e foram sujeitas a uma situação de privação de insulina, de modo a simular a total falta de insulina característica da DM tipo 1. Por outro lado, pretendeu-se também desenvolver um modelo animal de pré-diabetes para analisar o feito deste estado patológico na função reprodutiva masculina. Para isso, administrou-se diariamente uma dieta de alta energia a ratos Wistar e analisaram-se os parâmetros reprodutivos e efeitos ao nível das vias apoptóticas no epidídimo desses animais. Para analisar eventuais alterações nas vias apoptóticas analisámos os níveis de expressão de mRNA e proteína (por RT-PCR e western blot, respetivamente) de alguns dos marcadores de apoptose envolvidos na via mitocondrial, tais como: p53, Bax, caspase-9, caspase-3 e citocromo C. Também foram feitos ensaios enzimáticos com a caspase-3 para estudar a cinética enzimática como medida aproximada dos níveis de apoptose.

Os resultados obtidos levam-nos a sugerir que a insulina interfere com a interação entre proteínas pró- e anti-apoptóticas. Uma vez que esta interação pode decidir o destino celular e exercer um controlo rigoroso sobre a sinalização apoptótica, a insulina terá um papel chave na manutenção da espermatogénese. O modelo de rato utilizado compartilhava muitas das características clínicas e metabólicas do estado pré-diabético observado em humanos, tais como resistência à glucose e a progressão de normoglicemia/normoinsulinemia para hiperglicemia/hiperinsulinemia moderada devido à ingestão de alimentos. Este estado pré-diabético induziu alterações significativas na morfologia dos espermatozoides da cauda do

epidídimo, mostrando que esses animais poderão desenvolver problemas de subfertilidade ou de fertilidade. Na sinalização apoptótica na cauda epidídimo, os animais sujeitos à dieta de alta energia (HED) apresentaram níveis mais baixos de mRNA Bax e da proteína citocromo C, embora a avaliação quantitativa do *endpoint* apoptótico, a atividade da caspase-3, não tenha evidenciado quaisquer alterações entre a situação HED e controle. Isto sugere que o processo apoptótico possa ser controlado por outros mecanismos que não somente os proteicos pró-apoptóticos mitocondriais, como por exemplo os sistemas anti-apoptóticos celulares.

Os resultados obtidos com estes dois modelos experimentais levam-nos a concluir que os problemas subfertilidade/infertilidade causados pela DM podem ser mediados pela insulina, que tem um efeito importante na regulação da interação entre proteínas pró e anti-apoptóticas e, por esse motivo, deverá ser dedicada uma atenção especial às disfunções ocorridas no estado pré-diabético, onde observámos alterações cruciais na morfologia dos espermatozoides epididimais de rato. Devido à crescente incidência da DM e às complicações associadas ao nível da infertilidade masculina é fundamental aprofundar o conhecimento nestes dois sistemas, de modo a isolar possíveis mecanismos envolvidos e a avaliar os efeitos globais, como uma estratégia para desenvolver possíveis abordagens terapêuticas.

Abstract

Diabetes mellitus (DM) represents one of the greatest threats to modern global health and its incidence is rapidly rising worldwide. It describes a metabolic disorder characterized by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action, or both. There are two types of DM, type-1 DM (T1DM) and type-2 DM (T2DM), both associated with male infertility. T1DM is associated with insulin deprivation and although the overall *in vivo* effects in the reproductive function is well known, there is a lack of studies concerning about the insulin control over the physiological functions of cells from the reproductive system. Importantly, the *in vivo* studies are often focused after the disease is fully establish, but it is known that a prediabetic state, which is characterized by insulin resistance, precedes the development of DM, especially T2DM. With our work, we aimed to further investigate the association between DM and male infertility by analyzing several apoptotic markers and reproductive parameters. To do so, we simulated type 1 DM in cultured rat Sertoli cells and analyzed the mRNA and protein expression levels of several cellular markers involved in the mitochondrial apoptotic pathway. We also developed an animal model of prediabetes to evaluate the effect of this pathological state in the reproductive parameters as well as in the mitochondrial apoptotic pathway.

Our results lead us to suggest that insulin interferes with the interaction between pro and anti-apoptotic proteins. As the interaction of these proteins decide the cell fate and exert a strict control over the apoptotic signaling, insulin has a key role in the maintenance of the spermatogenesis. Our rat model shared many of the clinical and metabolic characteristics of the prediabetic state observed in humans such as glucose resistance and progression from normoglycaemia/normoinsulinemia to moderate hyperglycaemia/hyperinsulinemia due to food intake. This prediabetic state induced important alterations in cauda epididymis spermatozoa morphology showing that these animals may develop subfertility or fertility problems. The apoptotic signalling in cauda epididymis spermatozoa of the high-energy (HED) fed animals presented lower Bax mRNA levels and lower cytC protein levels although the apoptotic endpoint, caspase-3 activity, was not altered. This suggests that the apoptotic process may be controlled by other mechanisms rather than the mitochondrial pro-apoptotic proteins, such as the anti-apoptotic cellular systems.

Those two experimental models led us to conclude that the subfertility/infertility problems caused by DM may be mediated by insulin, which has an important effect in the regulation of the interaction between pro and anti-apoptotic proteins and special attention must be taken in the prediabetic state where crucial alterations in rat spermatozoa occurred. Due to the rising incidence and associated complications of DM and male infertility it is

crucial to further investigate in these two systems to isolate possible mechanisms and evaluate the overall effects as a strategy to develop possible therapeutics.

Keywords

Diabetes mellitus; Male infertility; Reproductive parameters; Apoptotic markers.

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AIF – Apoptosis inducing factor

Apaf-1 – Apoptotic peptidase activating factor 1

Apaf-3 - Apoptotic peptidase activating factor 3

ArKO – Aromatase knockout

BEB – Blood-epididymis barrier

BTB – Blood-testis-barrier

CytC – Cytochrome C

DM – Diabetes mellitus

EDs – Efferent ductules

ERs – Estrogen receptors

Erα – Estrogen receptor α

ErαKO - Estrogen receptor α knockout

Erβ – Estrogen receptor β

FSH – Follicle-stimulating hormone

GnRH - Gonadotropin releasing hormone

HED – High energy diet

IFG – Impaired fasting glucose

IGT – Impaired glucose tolerance

IR – Insulin resistance

LH – Luteinizing Hormone

OS –Oxidative stress

PIGs – p53-induced genes

ROS – Reactive oxygen species

SCs – Sertoli cells

STF - Seminiferous tubular fluid

T1DM – Type 1 diabetes mellitus

T2DM - Type 2 diabetes mellitus

TJ – Tight junctions

WHO – World Health Organization

I. Introduction

1. The Mammalian Testis

Mammalian testes, the central elements of the male reproductive tract, are paired, whitish, ovoid organs, suspended outside the abdomen in the scrotum, which is an outpouching of the abdominal wall and is internally divided into two sacs, one for each testis (Setchell 1978). The two main functions of the testes are steroid synthesis and spermatozoa production (Jayle *et al.* 1962). Their structural compartmentalization determines the topographical division of its dual functions: spermatogenesis occurring in the seminiferous tubules (avascular compartment) and steroidogenesis taking place in the interstitium (vascularized area) (Schlatt *et al.* 1997). The interstitial area is encased by a tough fibrous membrane called tunica albuginea (Figure 1) (Saladin 2003). There is also an outer tissue layer, known as tunica vaginalis, a thin serous sac, derived from the peritoneum during the descent of the testes, which covers the anterior and lateral surfaces of the testes but not their posterior surfaces (Setchell 1978; Kent 2001). Fibrous inward septum, extending from the tunica albuginea, partition the testis in 250 to 300 wedge-shaped testicular lobules, each containing one to three loop-shaped seminiferous tubules with both ends opening to a region called mediastinum (Kent 2001; Rabbani *et al.* 2010). Each seminiferous tubule is bounded by a basement membrane and has a fluid-filled lumen containing spermatozoa (Vander *et al.* 2001). The contents of the seminiferous tubules are received in the interconnecting tubules of the mediastinum known as rete testis, which in turn is connected to the efferent ductules (EDs) (Rabbani *et al.* 2010). EDs are a major site for fluid homeostasis, as they reabsorb more than 95% of the luminal fluid released from the seminiferous epithelium. These small-coiled ductules transport sperm from the rete testis and play a major role in concentrating it prior to his maturation in the epididymis (Picciarelli-Lima *et al.* 2006; Hahn *et al.* 2009; Lee *et al.* 2009).

Seminiferous tubules, which represent about 80% of the testicular mass, are lined by layers of germ cells in various stages of development (spermatogonia, spermatocytes, spermatids, spermatozoa) and mainly supporting Sertoli cells (SCs) (Sharpe 1984; Colborn *et al.* 1993; Sikka and Wang 2008). Each seminiferous tubule, considered the functional unit of the testis, is surrounded by mesenchymal cells. Among these are the peritubular myoid cells whose contractile elements generate peristaltic waves along the tubules (Gaytan *et al.* 1994; Gaytan *et al.* 1994). In the other hand, interstitium consists of loose connective tissue, blood and lymphatic vessels and various cell types, including Leydig cells, fibroblasts, macrophages and leukocytes (Sharpe 1984; Colborn *et al.* 1993).

Leydig (or intersticial) cells are the predominant source of the male sex steroid hormone testosterone (Vander *et al.* 2001). These cells arise from interstitial mesenchymal tissue between the tubules during the eighth week of human embryonic development and

play vital roles in downstream masculinization events and in descent of the testes into the scrotum (Akingbemi 2005; Sikka and Wang 2008).

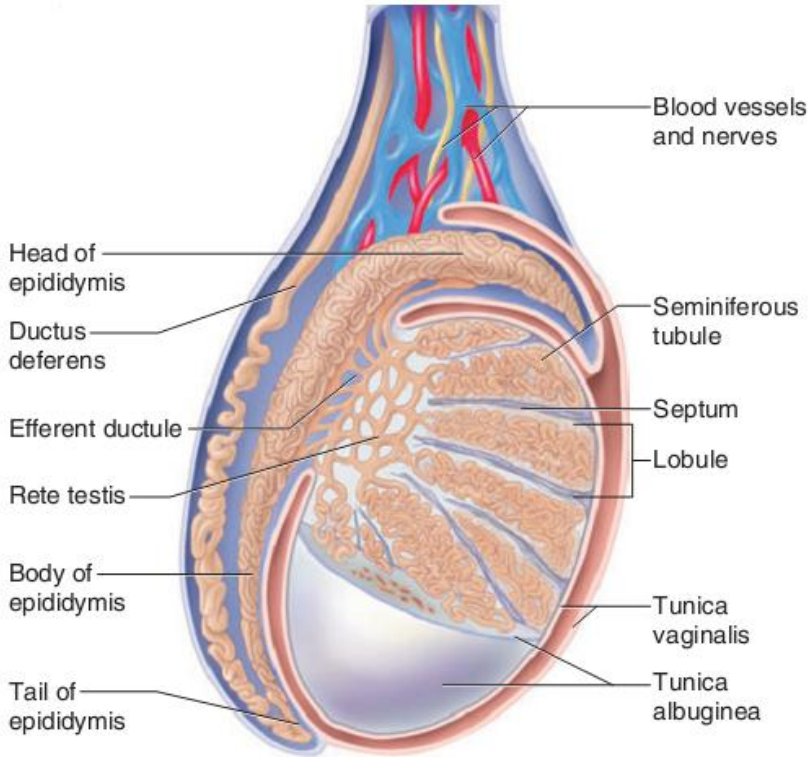

Figure 1 - Schematic representation of the mammalian testis and epididymis. The testis is encased by two tissue layers, from the inside to the outside, tunica albuginea and tunica vaginalis. Various septum extending from the tunica albuginea divides testis in lobules where seminiferous tubules are located. Seminiferous tubules converge to the rete testis that is connected to the efferent ductules (EDs). The head of the epididymis is linked to the testis by various EDs. Adapted from Saladin (2003).

1.1 Spermatogenesis

Spermatogenesis is a multi-step process of germ cell expansion and development which occurs within the seminiferous tubules of the testes determining male fertility (Walker 2010). This process has a duration of approximately 40–50 days in rodents (Sikka and Wang 2008) and 80 days in humans (Sikka and Wang 2008) and refers to the development of mature spermatozoa with half the number of chromosomes (haploid) from the most immature germ cell in the testis, spermatogonia (diploid) (Figure 2) (Han *et al*. 2009; Bettegowda and Wilkinson 2010). Spermatogenesis is composed of three phases, mitotic, meiotic and

spermiogenesis, and occurs during the 14 stages of the seminiferous epithelial cycle of spermatogenesis in rats, 12 stages in mice and 6 stages in men (Cheng *et al*. 2010). First, spermatogonia multiply by mitosis, producing two types of daughter cells called type A and type B spermatogonia (Saladin 2003). Type A cells remain outside the blood-testis-barrier (BTB) and continue to multiply from puberty until death. Thus men never exhaust their supply of gametes and normally remain fertile throughout old age. Type B spermatogonia migrate closer to the tubule lumen and differentiate into slightly larger cells called primary spermatocytes (Saladin 2003). In the second phase, primary spermatocytes undergo two divisions, meiosis I and meiosis II, to give rise to secondary spermatocytes and haploid round spermatids, respectively (Papaioannou and Nef 2010).

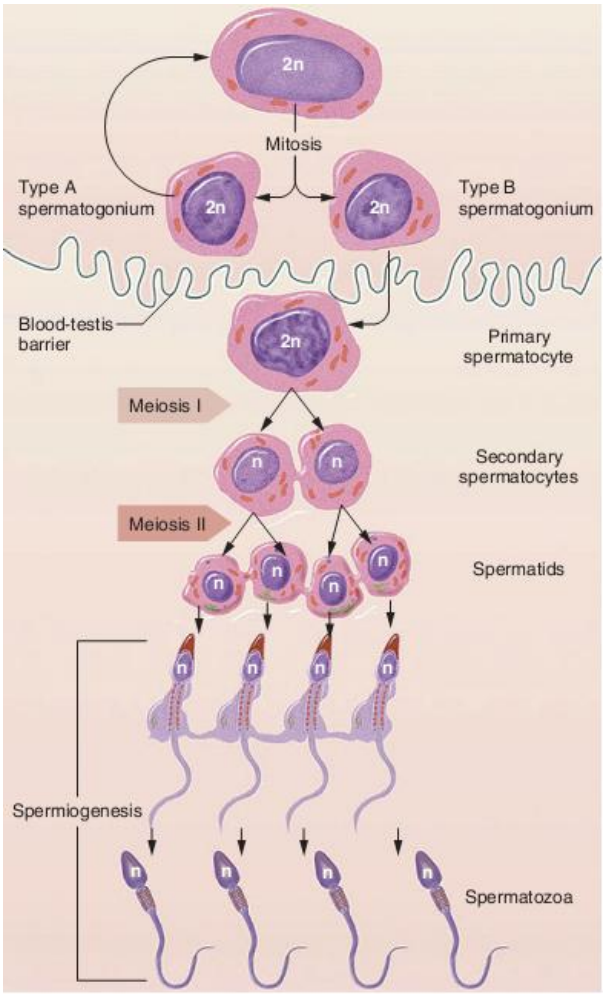

Figure 2 - The process of spermatogenesis. By mitosis spermatogonia produce type A and type B spermatogonium. Type B spermatogonium crosses the blood-testis-barrier (BTB) and differentiates into a primary spermatocyte. After the first meiotic division, secondary spermatocytes are formed, and then divide again resulting in haploid round spematids. The spermatids elongate and differentiate until spermatozoa are formed. Adapted from Saladin (2003).

Finally, takes place the spermiogenesis, which comprises the transformation of spermatids into elongated flagellar germ cells and culminates with the release of spermatozoa (spermiation) into the lumen of the seminiferous tubule (Papaioannou and Nef 2010; O'Donnell *et al*. 2011). During spermiogenesis, spermatids undergo a metamorphosis that does not involve cell division, but a number of morphological changes (acrosome formation, nuclear condensation, development of the flagellum, and cytoplasm reorganization) that eventually result in the generation of the spermatozoa (Papaioannou and Nef 2010).

After completing spermatogenesis and spermiation, spermatozoa are fully differentiated cells incapable of swimming or fertilize the egg. The fertilizing ability will be acquired in a temporally controlled manner during different stages towards the encounter with the female gamete: (1) along the transit through the epididymal duct, (2) at the encounter with the seminal plasma during the ejaculation, (3) during the transit through the female reproductive tract, (4) during the interaction with oviductal epithelial cells and (5) during the interactions with the different female gamete structures. Sperm changes occurring in the male reproductive tract have been defined as maturational changes where as those occurring in the female counter part are known as capacitation-associated changes (Caballero *et al*. 2010).

1.1.1 Hormonal Regulation of Spermatogenesis

The mammalian spermatogenesis regulation includes communication between the hypothalamus–hypophysis axis and the gonad itself (Aragon *et al*. 2005). The master control hormone is the gonadotropin releasing hormone (GnRH), a decapeptide produced by specialized neurons in the hypothalamus (Walker and Cheng 2005). Pulsatile GnRH reaches the anterior pituitary via the hypothalamo-pituitary portal vessels triggering the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that then act on the testes to regulate the spermatogenic potential (Figure 3) (Vander *et al*. 2001). The LH binds to receptors on the surface of leydig cells and stimulates testosterone production, a steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules only SCs possess receptors for testosterone and FSH (Walker and Cheng 2005). Thus, testosterone and FSH acts synergistically on SCs leading to the secretion of paracrine agents that are essential for spermatogenesis (Aragon *et al*. 2005).

Some negative feedbacks exerted by testicular hormones are also part of this regulation. The protein hormone inhibin secreted by the SCs can act on the anterior pituitary, decreasing the FSH levels, or on the hypothalamus decreasing GnRH levels. On the other

hand, testosterone inhibits mainly LH secretion. It can do so in two ways: acting on the hypothalamus to decrease the frequency of GnRH bursts, resulting in a decreased amount of GnRH reaching the pituitary and less secretion of the gonadotropins; or acting directly on the anterior pituitary leading to a decreased LH secretion in response to any given level of GnRH (Vander *et al.* 2001). Thus, testosterone is essential to maintain spermatogenesis at numerous levels. In the absence of testosterone or the androgen receptor, formation of the BTB is compromised, germ cells are unable to progress beyond meiosis, immature germ cells are prematurely displaced from SCs and mature spermatozoa cannot be released from SCs. The disruption of any of these testosterone-dependent steps results in the failure of spermatogenesis and infertility (Walker 2010).

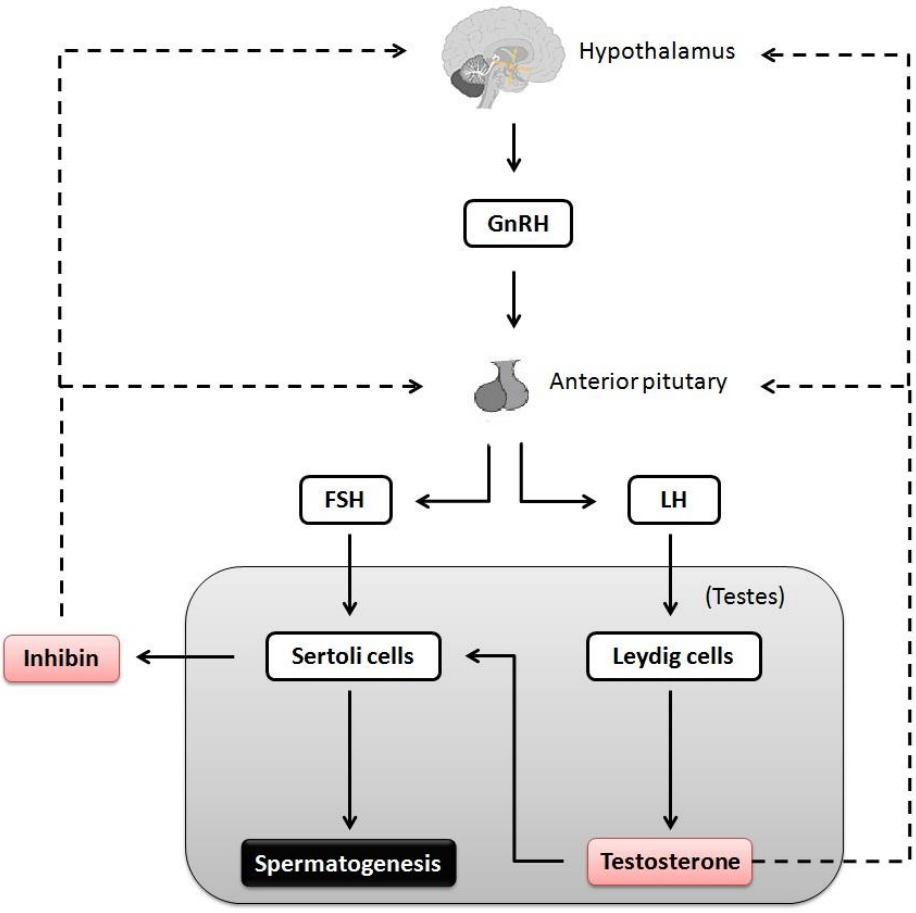

Figure 3 - Summary of hormonal control of male reproductive function. Hypothalamus synthesizes the gonadotropin releasing hormone (GnRH), which in turn stimulates the anterior pituitary to produce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH acts only on the Sertoli cells (SCs) leading to the stimulation of spermatogenesis, whereas LH acts only on the Leydig cells that produce testosterone. The secretion of FSH is inhibited mainly by Inhibin, a proteic hormone secreted by the SCs, and the secretion of LH is inhibited mainly by testosterone. Solid line – Stimulates; Dashed line – inhibits.

Apart from gonadotropins and androgens, spermatogenesis is a highly regulated developmental process occurring under a complex multifactorial control (Carreau and Hess 2010) and estrogens are now recognized as potential regulators in numerous species including humans (Carreau *et al.* 2010). The aromatase cytochrome P450, which is the last step in the steroidogenesis pathway leading to the formation of estrogens from androgens, is localized in the cellular endoplasmic reticulum of numerous tissues (Carreau and Hess 2010). In the male reproductive tract, aromatase and estrogen receptors (ERs) are mainly found in germ cells including spermatocytes, round spermatids, and elongated spermatids, as well as in leydig and SCs (Cheng *et al.* 2010). In order to exert a biological effect, estrogens should interact with ERs which in turn can modulate the transcription of target genes (genomic effect) and/or activate different signalization pathways located on the membrane (non genomic effect) (Carreau *et al.* 2012). Two main types of nuclear ERs have been described: estrogen receptor α (ERα) and estrogen receptor ß (ERß) (Carreau and Hess 2010). The infertility in mice lacking a functional ERα (ERαKO) was the first definitive demonstration that estrogen was required for male fertility (Lubahn *et al.* 1993; Korach 1994; Eddy *et al.* 1996; Hess *et al.* 1997). The ERαKO mice are infertile primarily due to a defect in EDs development and function (Hess *et al.* 1997; Lee *et al.* 2000). Mice lacking a functional aromatase gene (ArKO) are also infertile; however, this appears to be primarily due to a specific defect in germ cell development (Robertson *et al.* 1999).

Estrogen is clearly involved in the negative feedback effects of testosterone on the brain to control pituitary gonadotropin secretion, and hence an absence of, or inappropriate exposure to, estrogens leads to disturbances in the delicate balance of the hypothalamo-pituitary-testis axis in both mice and men. Once the development and spermatogenic potential of the testis is reliant upon this axis, such disturbances are likely to have a deleterious effect on spermatogenesis and fertility (O'Donnell *et al.* 2001). So, to ensure the well-functioning of spermatogenesis it is required a regulated balance between androgens and estrogens, with aromatase serving as a modulator (Shaha 2008; Carreau and Hess 2010).

1.2 Sertoli cells

Sertoli cells are highly polarized epithelial cells that extend upwards from the basement membrane of the seminiferous tubule to its open lumen (Mruk and Cheng 2011). They play a central role in the functional development of the testis and hence in the expression of the male phenotype (Mackay 2000). From Enrico Sertoli works, in 1865, came out the concept that SCs function as “nurse cells”, that is, they provide nutrients and regulatory factors for the sustenance of germ cells (Griswold 1995). SCs also facilitate germ

cell movement and mature germ cell release (Mruk and Cheng 2004), and secrete the seminiferous tubular fluid (STF) that helps the transport of spermatozoa into the epididymis (Waites and Gladwell 1982; Sikka and Wang 2008).

The SCs fate is established in the embryonic gonad at the time of sex determination (Hacker *et al.* 1995; Lovell-Badge and Hacker 1995) and is followed by a phase of rapid cell proliferation and differentiation. At the onset of puberty, SCs become terminally differentiated postmitotic cell population that support spermatogenesis (Chaudhary *et al.* 2005). Maturational process is marked by a reduced ability or the total loss of proliferation and irreversible changes in SCs morphology and physiology (Jegou 1992), heralding the switch from an immature, proliferative state to a mature, non-proliferative state. Morphologically, the nucleus enlarges and becomes tripartite and the nucleolus becomes more prominent (Sharpe *et al.* 2003). The changes associated with terminal differentiation of SCs include exit from the cell cycle and the formation of the BTB (Norton and Skinner 1992; Bremner *et al.* 1994; Law and Griswold 1994; Schlatt *et al.* 1996; Ketola *et al.* 2002; Holsberger *et al.* 2003).

Each SC has a fixed capacity for the number of germ cells that it can support (Orth *et al.* 1988), though this capacity varies between species. The Sertoli:germ cell ratio is approximately 1:50 in the adult rat testis (Weber *et al.* 1983; Wong and Russell 1983). The number of SCs will determine the number of germ cells that can be supported through spermatogenesis and hence the daily sperm production, a factor with obvious bearing on fertility (Orth *et al.* 1988; Sharpe *et al.* 2003).

The BTB is created by adjacent SCs that forms tight junctions (TJ) with each other such that nothing larger than 1000 daltons can pass from the outside to the inside of the tubule (Mruk and Cheng 2004; Cheng *et al.* 2010). At the beginning of meiosis, germ cells located outside of the barrier pass through the TJ. Once beyond the BTB, germ cells are dependent on SCs to supply nutrients and growth factors (Mruk and Cheng 2004). The BTB divides the seminiferous epithelium into the basal compartment (containing spermatogonia and spermatocytes) and adluminal (apical) compartment (containing different stages of meiotic spermatocytes, round spermatids, elongated spermatids, and spermatozoa) (Mruk and Cheng 2004; Su *et al.* 2011). In rats, the BTB forms at the age of 15–18 day's post-partum (Mruk and Cheng, 2008). This barrier consists in three components: an anatomical/physical barrier to restrict entry of molecules into the adluminal compartment, an immunological barrier, that limits the movement of immune cells and regulates the level of cytokines in the seminiferous epithelium and a physiological barrier, that are highly dynamic to encounter the needs of germ cells (Sikka and Wang 2008; Mital *et al.* 2011). Together, these components are essential to the function of BTB in testes and the special microenvironment created is responsible for proper development of the spermatogenesis.

Sertoli cells provide factors required to fuel germ cell metabolism, including lactate, which is a key element for germ cells due to its anti-apoptotic effect and its role as energy source (Erkkila *et al.* 2002; Michael K. Skinner 2005). In SCs, glucose is efficiently metabolized via cytosolic glysolysis to pyruvate, which is likely channeled via gap junctions to germ cells to maintain their survival, and lactate, the preferred energy source of germ cells (Robinson and Fritz 1981; Grootegoed *et al.* 1986). It is well known that spermatogonia may utilise glucose as the major energy substrate (Nakamura *et al.* 1984), but spermatocytes and spermatids suffer a rapid decline in their ATP content in glucose-supplemented media, thus, they require lactate/pyruvate for the maintenance of their ATP concentrations (Jutte *et al.* 1981; Mita and Hall 1982). In contrast, spermatozoa use glucose/fructose as the major source of energy (Mann 1964). So, it appears that, at each stage of spermatogenesis, there is a change in the substrate required for energy (Bajpai *et al.* 1998).

The metabolism of SCs is under the complex control of hormones, growth factors and even paracrine, autocrine and juxtracrine reactions occurring between the various cell types in the testis (Guma *et al.* 1997). The FSH and insulin regulate glucose metabolism in SCs, thus stimulating lactate production (Jutte *et al.* 1982; Jutte *et al.* 1983; Oonk *et al.* 1985; Oliveira *et al.* 2012). These stimulatory effects of FSH and insulin do not require protein synthesis, suggesting a modulation of enzyme activity and/or regulation of glucose transport (Oonk *et al.* 1985; Oonk and Grootegoed 1987). Insulin dysfunction (deficiency or resistance) has attracted much attention since it spreads the outcome results to diabetes mellitus (DM), hypertension, dyslipidaemia, among others (Reaven 1988). The SCs can adapt to conditions of glucose deprivation to ensure the adequate lactate concentration in the microenvironment where germ cell develop and even when glucose levels are low or in the complete absence of glucose, the SCs still produce lactate (Riera *et al.* 2009; Oliveira *et al.* 2012). The regulation of glucose metabolism in SCs is crucial for normal spermatogenesis and fertility (Boussouar and Benahmed 2004), but it is not completely understood (Rato *et al.* 2012).

1.3 Mammalian Spermatozoon

A mature sperm cell, or spermatozoon, comprises a head and a tail region (flagellum) (Figure 4) (Vander *et al.* 2001). The head contains the nucleus, acrosome, cytoskeletal structures and a small amount of cytoplasm. The nucleus fills most of the head and contains a haploid set of condensed, genetically inactive chromosomes (Saladin 2003). It is covered by the acrosomal cap, a membrane-enclosed cytoplasmic vesicle containing hydrolytic enzymes (Vander *et al.* 2001). The flagellum is divided successively into the midpiece (body), principal piece and end piece regions (Saladin 2003). It contains a central complex of microtubules forming the axoneme, which is responsible for spermatozoon motility. It is essentially a long,

specialized cilium surrounded in turn by outer dense fibers extending from the neck into the principal piece. The midpiece contains the mitochondrial sheath, a tightly wrapped helix of mitochondria surrounding the outer dense fibers and axoneme (Rabbani *et al.* 2010). The end piece consists of the axoneme only and is the narrowest part of the sperm (Saladin 2003).

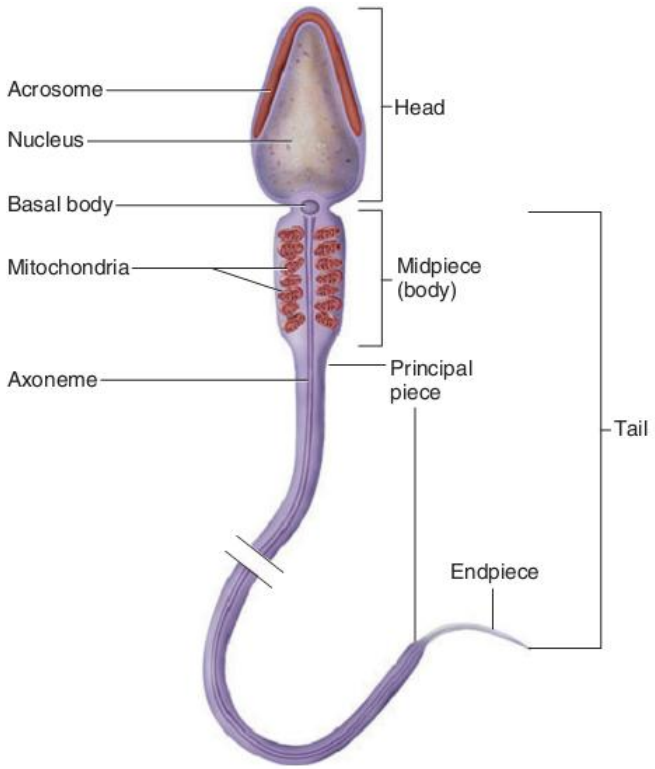

Figure 4 - General features of the mammalian spermatozoon. The mammalian spermatozoon has two parts: a pear-shaped head and a long tail (or flagellum). In the head, the acrosome is a lysosome in the form of a thin cap covering the apical half of the nucleus. The basal body of the tail flagellum is nestled in an indentation at the basal end of the nucleus. The regions of the flagellum are the middle piece which contains the mitochondrial sheath, the principal piece (major part of the tail) that consists of the axoneme surrounded by a sheath of fibers, and the end piece, which consists of the axoneme only and is the narrowest part of the spermatozoon. Adapted from Saladin (2003).

There are substantial species-specific differences in the size and shape of the head, in the length and relative amount of the different components of the flagellum (Rabbani *et al.* 2010). The rat sperm head is approximately 2.5 µm long and resembles a hook (Figure 5). It contains a dense nucleus and has a less dense tip referred to as the acrosome. The midpiece contains the centrioles and a spirally coiled sheath of mitochondrial material, while the tail contains a long axial filament that becomes vibratile for a brief period when the spermatozoon is mature (IRDG 2000).

The specialized structural features of mammalian spermatozoa reflect the unique functions of this cell type. The acrosome contains enzymes essential for penetrating the investments of the egg to achieve fertilization, while the flagellum contains the energy sources and machinery to generate the motility necessary for the sperm to reach the egg (Rabbani *et al*. 2010).

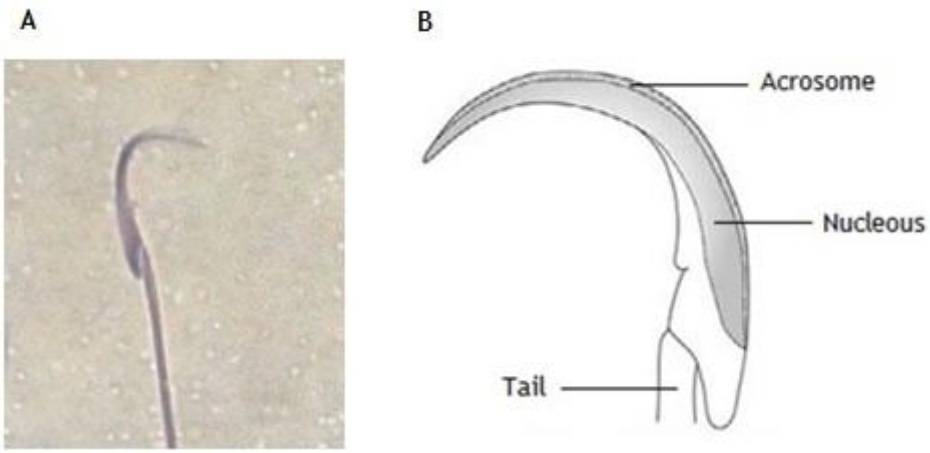

Figure 5- Lateral view of the falciform-shaped head of rat spermatozoa. A - Light microscopy image of a typical rat spermatozoon with Eosin/Negrosin staining (100× magnification). B – Schematic representation of the rat spermatozoa tail and head structures: acrosome and nucleous. Figure B adapted from Robaire *et al*. (2010).

2. The Epididymis

The epididymis is derived from the Wolffian duct and at birth consists mainly of mesenchymal tissue. The epididymis undergoes considerable remodeling including duct elongation and convolution, so that by puberty it has acquired its fully differentiated state (Carmen M. Rodriguez 2002). It consists of one convoluted and long tubule which, in humans and if uncoiled, measures about 5.5 m (Kent 2001). It links EDs to the vas deferens and is lined by a continuous layer of epithelial cells joined by TJ (Joseph *et al.* 2011). In humans, it can be morphologically divided into three principal regions (Figure 6): the caput and corpus regions, involved in the early and late sperm maturational events, respectively, and the distal cauda epididymis, which primarily serves as a storage site for functionally mature spermatozoa (Thimon *et al.* 2007; Dube *et al.* 2008; Cornwall 2009). The most proximal epididymal region, in some species such as the mouse, is also known as the initial segment (Figure 6) (Cornwall 2009). In the human, the initial segment is not as extensive, but a very tall epithelium is present (Yeung *et al.* 1991).

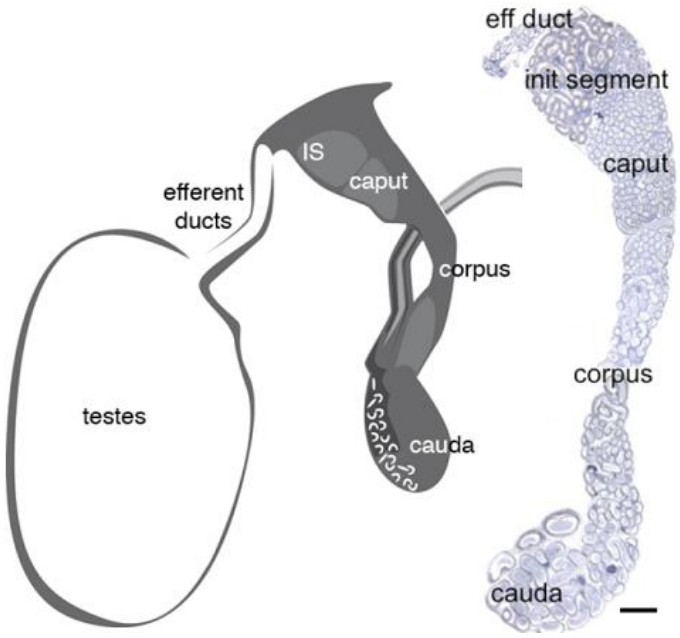

Figure 6 - Schematic and histological representation of the male reproductive tract and excurrent ducts. Schematic (left) showing relative orientation of the efferent ductules (EDs) and the proximal (IS and caput) and distal segments (corpus and cauda) of the epididymis. The cauda connects to the ejaculatory duct or vas deferens. Sagittal section (right) of the EDs and epididymis depicting its convoluted nature as well as its complex and changing epithelium. eff duct - efferent ductules; IS and init segment - initial segment; Bar = 1.5 m. Adapted from Joseph *et al.* (2011).

Histologically, the epididymis is a tube of smooth muscle lined by a pseudostratified epithelium. From proximal to distal, the muscular wall increases from a single circular layer to three layers. Proximally, the smooth muscle exhibits slow rhythmic contractility which gently moves spermatozoa towards the vas deferens, while distally, it is richly innervated by the sympathetic nervous system which produces intense contractions of the lower part of the epididymis during ejaculation (Kent 2001). The interstitial component that surrounds the duct contains blood vessels, connective tissue septa, lymphatic vessels, macrophages, and lymphocytes (Mital *et al*. 2011).

Each of the epididymal regions has a complex and different epithelium and can be further divided into intraregional segments or lobules of coiled tubule that are separated by septae (Turner *et al*. 2007; Joseph *et al*. 2011). Recently, microdissection of the mouse, rat and human epididymides has demonstrated that they are divided into 10, 19 and 7 intraregional segments (Figure 7), respectively and it has been shown that the connective tissue septae that separate the segments can establish borders for epididymal gene expression, protein presence and epithelial responses to lumicrine factors (Turner *et al*. 2007; Shum *et al*. 2011).

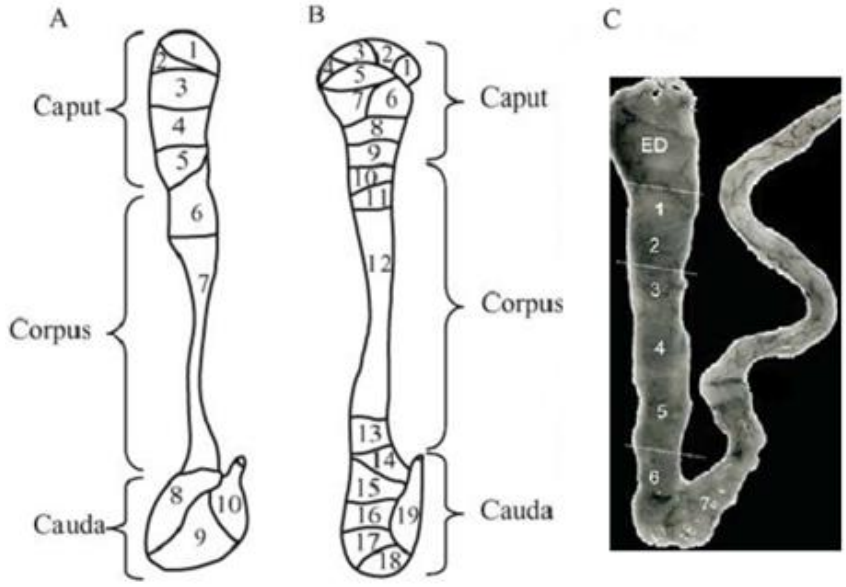

Figure 7 - Segmental structure of the epididymis. Typical schematic patterns of mouse (A) rat (B) and human epididymal segmentation (C). The mouse and rat epididymis are not drawn to scale. The average rat epididymis is approximately 2.5-times the length and has 40-times the total mass of the mouse epididymis (data not shown). Figure A and B adapted from Jelinsky *et al*. (2007), Figure C adapted from Dacheux *et al*. (2006).

Several different cell types can be identified in the epididymal epithelium (Figure 8): principal, narrow, apical, clear, basal cells and halo cells, surrounded by multiple layers of peritubular myoid cells (Joseph *et al.* 2011; Mital *et al.* 2011; Shum *et al.* 2011). Although the exact regional localization of the cell types differs between species (in part due to the inconsistency of identification of the different regions) in general, all the cell types appear to be present in all mammalian species (Rabbani *et al.* 2010). Each cell type contributes to the establishment and regulation of a unique luminal environment for the concentration, maturation, storage and viability of spermatozoa (Cornwall 2009; Shum *et al.* 2011).

The primary cell type throughout the tubule is the principal cell which constitutes approximately 80% of the epithelium and is responsible for the bulk of the proteins that are secreted to, and also endocyted from, the epididymal lumen (Cornwall 2009). The morphology of the principal cell reveals a prominent, branched, microvillus, absorptive border, but the cell changes dramatically from a tall columnar structure in the initial segment to low cuboidal cells in the cauda epididymis (Abe *et al.* 1983; Abou-Haila and Fain-Maurel 1984).

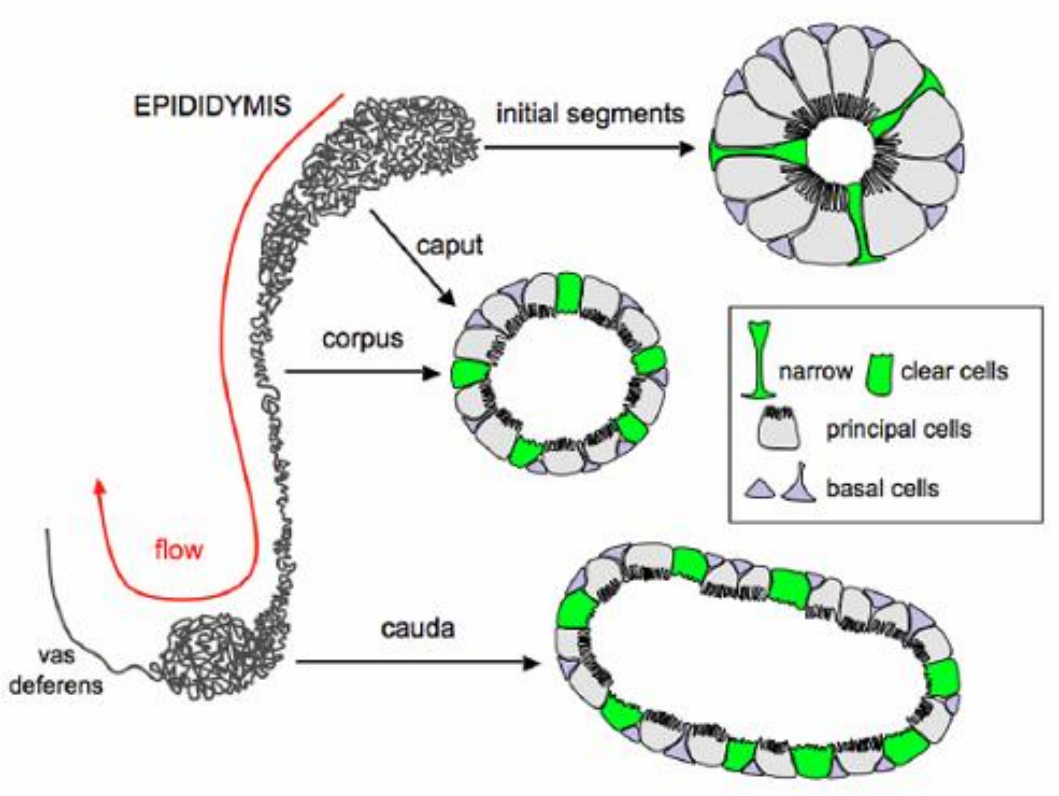

Figure 8 - Schematic view of the main epithelial cell types in the epididymis: narrow, clear, principal and basal cells. While principal and basal are present in all epididymal regions, narrow cells are located exclusively in the initial segment, and clear cells are present in the caput, corpus and cauda epididymis. Adapted from Shum *et al.* (2011).

Narrow cells are exclusively present in the initial segment (Shum *et al*. 2011). Their cytoplasm tapers between principal cells as it touches the basement membrane, but its apical cytoplasm may bulge slightly into the lumen with numerous vacuoles, endocytic vesicles, lysosomes and mitochondria, whereas apical cells have no such contacts (Hermo *et al*. 2000). Narrow cells are involved in endocytosis and secretion of protons (H^+) into the lumen (Hermo *et al*. 2000), while apical cells are also active in endocytosis and additionally contain many proteolytic enzymes (Adamali and Hermo 1996).

Clear cells are large endocytic cells interspersed between principal cells and are found in the caput, corpus and cauda epididymis. They are characterized by their lack of microvilli, as well as an apical region with numerous coated pits, vesicles, endosomes, multivesicular bodies, lysosomes, as well as lipid droplets (Abou-Haila and Fain-Maurel 1984; Hermo *et al*. 1988; Rabbani *et al*. 2010). These are thought to be the epididymal cells with the most endocytic activity and they are particularly active in the cauda epidydimis (Sun and Flickinger 1979; Hermo *et al*. 1988). These cells are also responsible for the uptake of a number of different proteins excreted by the epididymal epithelium in a region-specific manner and also participate in the regulation of luminal fluid acidification (Sun and Flickinger 1979; Flickinger *et al*. 1988; Hermo *et al*. 1988; Vierula *et al*. 1995).

Basal cells, as their name suggests, are situated on the basal side of the epithelium (Veri *et al*. 1993). They are present in all epididymal segments (L. Hermo 2002), and constitute 15%–20% of the total epithelium (B. Robaire 1988; Marengo and Amann 1990; Adamali and Hermo 1996). Basal cells do not access the luminal compartment and are in close association with the overlying principal cells, as indicated by the presence of basal cell cytoplasmic extensions between principal cells, and thus may regulate its functions (Veri *et al*. 1993; Seiler *et al*. 1999). Their morphology is typical to the structure of endocytotic cells and it is believed that they can endocytose factors derived from blood or principal cells and may help those in the regulation of electrolyte and water transportation (Veri *et al*. 1993; Hermo and Papp 1996). Some authors consider another type of epididymal cells, known as apical cells (Hermo *et al*. 2000; Cornwall 2009). They are "apical-reaching" basal cells (Shum *et al*. 2011) that endocytose luminal components (Cornwall 2009). While a few of them are present in the initial segment and caput, their numbers increase progressively in the corpus and reach a maximum in the distal corpus and proximal cauda regions (Shum *et al*. 2011).

Halo cells appear to be the primary immune cell in the epididymis (Cheung *et al*. 2005). They are easily recognized in several histological stainings by their narrow rim of clear cytoplasm, and are present in all epididymal segments (Rabbani *et al*. 2010). These cells are usually located at the base of the epithelium and contain variable numbers of dense core granules. In young adult animals, halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes (Flickinger *et al*. 1997; Serre and Robaire 1998; Serre and Robaire 1999).

2.1 Functions of the Mammalian Epididymis

The mammalian epididymis has two main functions. First, it creates a unique microenvironment within the lumen of the duct that helps transform immotile, immature testicular spermatozoa into fully fertile competent cells, and second, it stores fertile spermatozoa in a viable state within the cauda epididymis/vas deferens regions until they are ejaculated (Amann *et al.* 1993; Kirchhoff *et al.* 1998; Jones 2004).

From the rete testis to the end of the epididymis, spermatozoa are bathed in a continuously and progressively changing medium of fluid proteins and other chemical components (Guyonnet *et al.* 2011). Many luminal proteins may not play direct roles in sperm maturation but rather help to create the appropriate environment that is conducive for this process to occur (Cornwall *et al.* 2011). This can include proteins involved in the regulation of luminal pH, osmolality, regulation of oxidative stress (OS) and regulation of protein folding/misfolding. There are also mechanisms to remove secreted proteins from the lumen, presumably once their functions have been carried out or if misfolding occurs (JL Dacheux 2002). The formation of this luminal environment is the result of net secretory and absorptive processes of the epithelium, which continually changes along the duct (Hermo and Jacks 2002; Sullivan *et al.* 2007; Rabbani *et al.* 2010). It is a complex mixture of water, inorganic ions, small organic molecules and proteins, which accompanies the spermatozoa as they travel from the testis to the epididymis through the EDs, and is mostly composed of secretions by the SCs (Rato *et al.* 2010).

2.1.1 Epididymal Sperm Maturation

Epididymal sperm maturation, which is essential for the acquisition of progressive motility and the ability to fertilize (Rabbani *et al.* 2010), depends on the unique luminal environment created by the secretion and absorption of proteins and ions by the epithelium and the selective transport of molecules across the blood-epididymis barrier (BEB) (Hinton and Palladino 1995; Dacheux *et al.* 2003; Dacheux *et al.* 2006). This barrier comprises apical TJ between principal cells that create a seal between the cells and forces the selective transport of molecules across the epithelium (Dube *et al.* 2010).

The maturation process is androgen-dependent and conducts several biochemical and functional changes in spermatozoa. Epididymal originated proteins may change the membrane properties of the spermatozoa in several ways: they may bind to the sperm surface and/or

modify the structure or the arrangement of the existing membrane molecules (Vreeburg *et al.* 1992). Some of these proteins contribute to the stabilization of the sperm plasma membrane which prevents the occurrence of premature capacitation and are, therefore, known as "decapacitation factors", whereas others have been implicated in the acquisition of the sperm ability to bind and recognize the egg (Lefebvre *et al.* 2009; Cohen *et al.* 2011; Joseph *et al.* 2011).

2.1.2 Epididymal Sperm storage

Species presenting internal fertilization concentrate and store their maturing spermatozoa in the epididymis prior to ejaculation. This makes the spermatozoa amount in the ejaculate more controllable and less dependent on testicular production, once it can be maintained normally while spermatozoa are transported to the epididymis (Jones 1999). The normal transit time in mammals through the cauda epididymis is in the range of 3 to 10 days, but spermatozoa can be stored in this tissue for periods extending beyond 30 days (Rabbani *et al.* 2010). The survival of spermatozoa in the cauda epididymis depends on the species and incubation temperature (Jones 2004). In scrotal mammals, the combination of a unique luminal milieu and lower temperatures (30–32ºC) are thought to be major contributors to sperm survival. However, if spermatozoa are removed from the cauda and incubated at 32ºC *in vitro*, their fertility and viability is measured in hours rather than days (Jones 2004).

Spermatozoa need to be protected from damage caused by OS and microorganisms, and their motility needs to be suppressed until it is required to conserve energy and maintain structural integrity (Jones 1999). The BEB isolates the contents of the epididymal lumen from the rest of the body, which is important as certain proteins in spermatozoa are recognized as foreign objects by the immune system (Itoh *et al.* 2005). However, since immune cells are isolated from the epididymal lumen, it is required an alternative method to protect the spermatozoa against harmful microorganisms. With the high incidence of sexually transmitted infections and effect on fertility in humans, increased interest towards such factors has resulted (Hall *et al.* 2002). It has been described that genes expressed in the epididymis encoding cysteine-rich secreted proteins have antimicrobial properties and are involved in sperm maturation and defense against microorganisms (Hall *et al.* 2007; Diao *et al.* 2011; Zhao *et al.* 2011).

One of the most important factors contributing to poor quality semen has been reported to be OS (Tuncer *et al.* 2010). It is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly known as reactive oxygen species (ROS) (Sikka *et al.* 1995). Uncontrolled production of ROS that exceeds the

antioxidant capacity of the seminal plasma leads to OS which is harmful to spermatozoa (Desai *et al.* 2010). All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of OS (Agarwal *et al.* 2008).

The production of ROS by spermatozoa correlates with lipid peroxidation, DNA oxidation, poor sperm function and reduced fertility (Moustafa *et al.* 2004; Aitken and Koppers 2011). However, recent evidence suggests that redox activity is physiologically important in promoting normal sperm function (de Lamirande and Gagnon 1992; Aitken 1999; Aitken 2000). Small amounts of ROS are required for sperm capacitation, the final maturation steps associated with hyperactive motility and a physiological acrosome reaction (Griveau and Le Lannou 1997; Agarwal *et al.* 2004). Thus, it is essential to the proper functionality of the spermatozoa to maintain a delicate balance in ROS production and recycling. It has been estimated that OS is a contributor in 30–80% of cases of male infertility thereby making it an important area of research (Tremellen 2008).

2.2 Epididymal sperm abnormalities

Sperm abnormalities have long been associated with male infertility and sterility in most species studied. These abnormalities vary from morphological defects that are evident upon clinical examination, to those, which are more subtly defective (Chenoweth 2005). Morphology can be defined as the structure and form of organisms to include the anatomy, histology and cytology at any stage of its life history. It is important to compare this to morphometry, or the measurement of the forms of organisms. The latter, while relating to some aspects of viability is less relevant to the function of an organism as an entity (IRDG 2000). Abnormal sperm morphology is a reflection of negative stress factors working on the body without affecting the overall health of a specific male (Menkveld *et al.* 2011). Most of the published data are on sperm concentration, being the semen characteristic most commonly assessed and the one least subject to methodological bias. In contrast, there are fewer studies reporting data on sperm motility, viability or morphology. The assessment of these characteristics is more subjective by nature with an overall noticeable inter-technician and inter-laboratory variability (Neuwinger *et al.* 1990; Cooper *et al.* 1992; Matson 1995; Ombelet *et al.* 1998; Auger *et al.* 2000). Otherwise, the preparation, fixation and staining methods of the semen smears, have a severe influence on viability and sperm morphology evaluation results. In 1971, Eliasson made an important contribution to the classification system, which has been commonly used. He stated that for the complete morphological rating of human spermatozoa, sperm abnormalities should be classified in three main groups: those of the sperm head, the mid-piece and the tail(Eliasson 1971).

3. Diabetes mellitus and male infertility

Diabetes mellitus represents one of the greatest threats to modern global health and its incidence is rising rapidly. In the year 2000, the World Health Organization (WHO) reported that 177 million people were affected by diabetes worldwide and that by 2025 this figure is estimated to rise to an incidence of over 300 million individuals (Agbaje *et al*. 2007; Bener *et al*. 2009).

Diabetes mellitus describes a metabolic disorder characterized by hyperglycaemia (poor glucose in the blood) resulting from defective insulin secretion, resistance to insulin action, or both (Alberti 1998). Type 1 or insulin-dependent diabetes mellitus (T1DM), which generally develops at young age, is a multi-factorial immune-mediated disease characterized by the autoimmune destruction of insulin-producing pancreatic islet beta cells in genetically susceptible individuals, resulting in a dependency of exogenous insulin treatment (Burul-Bozkurt *et al*. 2010; Grieco *et al*. 2012). Type 2 or non-insulin-dependent diabetes mellitus (T2DM) is responsible for 90–95% of diabetes cases. In T2DM, liver, muscle and fat cells are resistant to insulin action. The compensatory attempt by the beta-cell to release more insulin is not sufficient to maintain blood glucose levels within a normal physiological range, finally leading to the functional exhaustion of the surviving beta-cells. Despite genetic predisposition, the risk of developing T2DM in humans increases with age, obesity, cardiovascular diseases and a lack of physical activity (Golay and Ybarra 2005; Carneiro *et al*. 2010).

Prospective studies of the natural history of T2DM have shown that the prediabetic state is characterized by impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), or both. The prediabetic state, which is associated with insulin resistance (IR) and that usually precedes T2DM, is also characterized by resistance to an insulin-mediated glucose disposal and a compensatory slight hyperinsulinemia. The transition from prediabetes to T2DM occurs when the secretory capacity of the pancreatic B cell is no longer able to compensate for the IR. However, frank hyperglycemia in patients with T2DM is not associated with absolute hypoinsulinemia and day-long circulating insulin concentrations in patients with T2DM are comparable in absolute terms to the values in non-diabetic patients (Reed *et al*. 2000). Pre-diabetes commonly associates with the metabolic syndrome, which in turn is closely associated with obesity (Ai *et al*. 2005). The progression from prediabetes to T2DM occurs over many years, strong evidence to support intervention to delay the progression from prediabetes to diabetes (Aroda and Ratner 2008).

Infertility according to the WHO is defined as the inability to conceive after 1 year of unprotected intercourse (Zegers-Hochschild *et al*. 2009) and affects about 13%–18% of couples (Dube *et al*. 2008). In about half of them, male factor is the sole cause or contributes to the infertility problem (Shukla *et al*. 2012). Male infertility can occur either as an isolated disease or as part of a complex syndrome. The main causes of infertility in men are diverse such as genetic, physiopathologic and anatomopathologic abnormalities, intense and prolonged physical exercise, aging, drugs and even excessive time of sexual abstinence (World Health Organization 2010). But in more than half of infertile men, the cause of their infertility is unknown (de Kretser 1997; Seshagiri 2001; Dohle *et al*. 2005). A large number of the male infertility cases are also associated to suboptimal sperm quality due to abnormal parameters - motility, morphology, concentration and DNA fragmentation (Iammarrone *et al*. 2003; du Plessis *et al*. 2011) and, thus, semen analysis represents the initial test for evaluating male-factor infertility.

Male fertility is compromised by the hormonal and metabolic changes associated with T1DM and T2DM (Bener *et al*. 2009; Mallidis *et al*. 2009), obesity (Pauli *et al*. 2008) and the metabolic syndrome, the latter sharing essential pathological features with the DM pathologies (Kasturi *et al*. 2008). The deleterious influence of DM and obesity on fertility is receiving increasing attention since their prevalence and incidence is escalating worldwide, while the age of first diagnosis for both diseases is in continuous decline (Lavizzo-Mourey 2007; Harjutsalo *et al*. 2008). Due to this situation, the fertility of a growing number of individuals is affected prior to and during their reproductive years (Delfino *et al*. 2007; Nguyen *et al*. 2007).

The prevalence of sexual dysfunction in diabetic male individuals has been reported to approach 50% of the cases (Kandeel *et al*. 2001). In particular, decreased sperm concentration and motility, abnormal sperm morphology and increased seminal plasma abnormalities were detected (Amaral *et al*. 2008). Furthermore, DM is associated with increased OS which damages sperm nuclear and mitochondrial DNA (Kort *et al*. 2006; Kasturi *et al*. 2008), retrograde ejaculation, premature ejaculation, decreased libido, delayed sexual maturation and impotence (Kandeel *et al*. 2001; La Vignera *et al*. 2011). Finally, spermatogenesis disruption and germ cell apoptosis in T1DM may relate to a local autoimmune damage, whereas IR, obesity and other related co-morbidities may impair sperm parameters and decrease testosterone serum levels in patients with T2DM (La Vignera *et al*. 2011). Low testosterone levels have also been found to predict IR and the future development of T2DM from a prediabetic state (Kapoor *et al*. 2007).

4. Apoptotic pathways in the testis

Apoptosis is a result of a complex network of signalling pathways, which allows the organism to tightly control cell numbers and tissue size, and to protect itself from rogue cells that threaten homeostasis (Hengartner 2000; Mallat and Tedgui 2000). It is an energy-dependent process originated from physical damage to the cell or through non-lethal stimuli that signal the cell to die (Goswami *et al*. 1999).

Cellular apoptosis appears to be a constant feature in the adult testis and during early development (Shaha *et al*. 2010). Normal spermatogenesis involves a balance between cell proliferation and cell death, because, even during regular conditions, a number of germ cells die by apoptosis before reaching maturity (Dunkel *et al*. 1997; Rodriguez *et al*. 1997; de Rooij 2001). In testis, 75% of all male germ cells produced are discarded through apoptosis, in order to equilibrate the number of germ cells with the supportive capacity of SCs (Hsueh *et al*. 1996; Sinha Hikim and Swerdloff 1999; Shaha *et al*. 2010).

There are two major apoptotic pathways: the extrinsic (or death-receptor) pathway and the intrinsic (or mitochondrial pathway), and molecules of one pathway can influence the other (Figure 9) (Igney and Krammer 2002). The process of apoptosis is important in the context of germ cells because the cells are actively undergoing both mitosis and meiosis and errors during the process may lead to the need for induction of cell death to eliminate cells with genetic defects (Shaha *et al*. 2010). The central component of both apoptotic pathways is a proteolytic system involving a family of proteases called caspases (Majors *et al*. 2007). Over a dozen caspases have been identified in humans and about two-thirds of these have been suggested to function in apoptosis. These enzymes participate in a cascade that is triggered in response to pro-apoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell (Thornberry and Lazebnik 1998).

In the extrinsic pathway, death receptors, such as CD95 (Apo-1/Fas), aggregate and form membrane-bound signalling complexes. This complexes recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity (Muzio *et al*. 1998). The activation of caspase-8 initiates a series of caspase-dependent events leading to cell death (Hengartner 2000).

The mitochondrial pathway can occur when cells activate an internal program of self-destruction in response to an internal signal, withdrawal of survival factors, changes in haemodynamic parameters or loss of contact (Haunstetter and Izumo 1998). Every cell contains all the components of the suicide machinery and is ready to engage in self-

destruction unless it is actively signalled not to do so (Mallat and Tedgui 2000). The diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family (Hengartner 2000). Bcl-2 family members are of particular importance to apoptosis regulation as they can influence the permeability of the outer mitochondrial membrane (Donovan and Cotter 2004; Schinzel *et al*. 2004; Sharpe *et al*. 2004). This family consists of both pro- (Bax, Bad, Bak, Bim) and anti-apoptotic (Bcl-2, Bcl-xL) members. Pro-apoptotic proteins normally reside in the cytosol (Wolter *et al*. 1997) but target the mitochondria during apoptotic signaling, causing the release of apoptotic signaling molecules, including cytochrome c (cytC) and apoptosis inducing factor (AIF) (Harris and Thompson 2000; Arden and Betenbaugh 2004). In contrast, the anti-apoptotic members of the Bcl-2 family maintain the mitochondria membrane potential and prevent the release of cytC during apoptotic insults (Kluck *et al*. 1997; Vander Heiden *et al*. 1997; Yang *et al*. 1997).

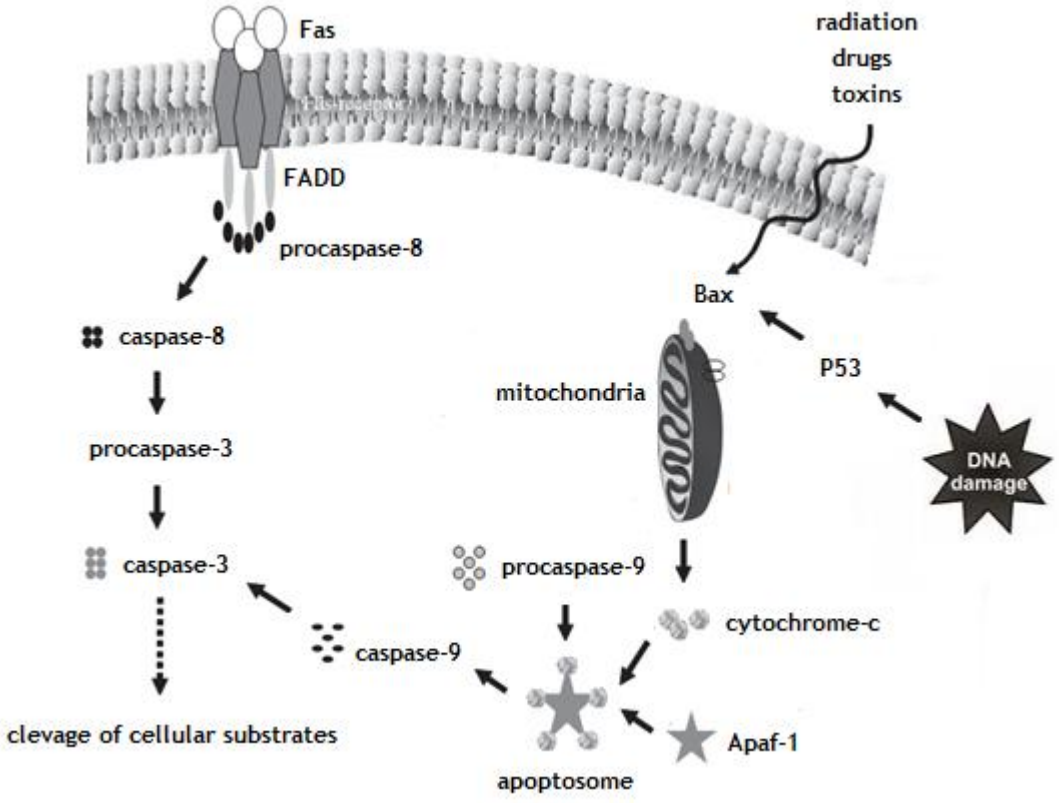

Figure 9 - The two main apoptotic pathways. The mitochondria-mediated cell death pathway involves a complex set of proteins that regulate the integrity of the mitochondrial membrane. Apoptotic insults activate Bax, causing the protein to translocate to the outer mitochondrial and promoting the release of cytC. Released cytC coupled with caspase-9 and Apaf-1 form the apoptosome that leads to caspase activation and apoptotic cell death. Binding of death ligands to the tumor necrosis family of death receptors triggers the activation of the extrinsic or death receptor pathway through direct activation of caspase-3 via caspase-8. Adapted from Shaha *et al*. (2010).

In the cytosol, cytC promotes the oligomerization of apoptotic peptidase activating factor 1 (Apaf-1), leading to recruitment and activation of caspase-9 in a large complex called the apoptosome (Li *et al*. 1997; Zou *et al*. 1997; Riedl and Salvesen 2007). Once activated, caspase-9 cleaves and activates the downstream effector caspase-3, -6, and -7. These effector caspases then directly cause the morphological and biochemical changes associated with apoptosis by specifically cleaving "death substrates" (Earnshaw *et al*. 1999). Grossly, apoptotic cells exhibit a variety of identifiable characteristics such as reduction in cell volume, membrane blebbing, chromatin condensation, and typically, fragmentation into membrane-enclosed vesicles (Earnshaw et al., 1999). Biochemical changes include the degradation of chromosomal DNA into high-molecular-weight oligonucleosomal fragments and cleavage of anti-apoptotic proteins such as Bcl-2 and Bcl-XL (Lee *et al*. 2003).

The tumor suppressor p53 is believed to be a guardian of genome integrity (Lane 1992). Upon stress conditions, such as DNA damage and hypoxia, increased levels of p53 cause cell cycle arrest in dividing cells or induce apoptosis (Figure 9) (Clarke 1990; Kuerbitz *et al*. 1992; Dulic *et al*. 1994; Graeber *et al*. 1996). High levels of testicular p53 protein are found in germ cells, especially in the primary spermatocytes (Schwartz *et al*. 1993; Sjoblom and Lahdetie 1996). Recent studies suggest that p53-induced apoptosis involves: (1) activation of redox-related genes also known as PIGs (p53-induced genes); (2) generation of ROS and (3) oxidative degradation of mitochondrial components permitting the release of apoptotis-inducing factors, including AIF, cytC, Apaf-1, Apaf-3 into the cytosol to activate the caspases (Sinha Hikim and Swerdloff 1999). Wild-type p53 is known to be a regulator of Bax, as a p53-binding site has been found in the Bax gene promoter. Thus, p53 is responsible for regulating of cell death through Bax imbalances (Miyashita and Reed 1995).

II. Aims of the present study

The rising incidence of DM all over the world and the fact that male infertility is affecting each time more young people and couples, has led us to develop this work. Therefore, with our work, we aimed to further disclose the association with DM and male infertility.

Diabetes mellitus type 1 is a hormonal condition classically associated with insulin deprivation. A first objective of this study was to examine the effect of insulin deprivation on rat SCs *in vitro*, analyzing its effect on various cell death markers. To do so, it was determined the mRNA expression and protein levels of some apoptotic markers involved in the mitochondrial apoptotic pathway (Bax, p53, caspase-3, caspase-9). It was also measured the enzymatic activity of caspase-3 as a rough measurement of the apoptosis levels of the SCs samples.

A second goal of this study was to develop an animal model of pre-diabetes state, using a high-energy diet (HED) to feed male Wistar rats, in order to evaluate its effects on the male reproductive function and on the expression of specific epididymal mitochondrial apoptotic pathway markers. For that, epididymal sperm parameters, such as sperm motility, viability, concentration and morphology were evaluated. Moreover, the mRNA expression and protein levels of some apoptotic markers such as Bax, p53, caspase-3, caspase-9 and cytC were determined in the cauda epididymis, as well as the enzymatic activity of caspase-3 as a rough measurement of the apoptosis levels of the cauda epididymis samples.

III. Materials and methods

1 – Chemicals

HBSS (Hanks balanced salt solution), DMEM:Ham's F12 (Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12), Collagenase, ExtrAvidin-Peroxidase Staining Kit, DAB (3,3-diaminobenzidine) hydrochloride, trypsin-EDTA, ITS (insulin-transferrin-sodium selenite) supplement, TRI Reagent and other drugs were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). FBS (fetal bovine serum) was obtained from Biochrom AG. Polyclonal antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). M-MLV RT (Moloney-murine-leukaemia virus reverse transcriptase), random hexamer primers, DNA ladder and primary antibody against vimentin were obtained from Invitrogen. dNTPs were obtained from GE Healthcare (Buckinghamshire, U.K.). 1×Buffer and Taq DNA Polymerase were obtained from Fermentas Life Sciences (Ontario, Canada). Caspase-3 substrate was purchased from Calbiochem® (Darmstadt, Germany). Differential Quick Stain Kit was purchased from Baxter Dale Diagnostics AG (Dubinger, Switzerland).

2 – Animals

The present study used 12 male Wistar rats 2-months old. They were obtained from our accredited animal colony (Health Sciences Research Center, University of Beira Interior) and maintained on *ad libitum* food and water in a constant temperature (20±2ºC) room on a 12 hour cycle of artificial lighting. Rats were divided randomly into normal control group and high-energy diet group (HED group) (6 in each group) and placed in individual cages. The control group animals were fed with a standard chow diet (4RF21 certificate, Mucedola, Italy) and the HED group received an additional high-energy emulsion, consisting of 20 g lard, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 5 g sucrose and 5 g saccharose, 20 mL Tween 80, 30 mL propylene glycol prepared in a final volume of 100 mL by adding distilled water and stored at 4ºC. In the first 5 days of treatment, were given progressively 1 to 5 mL of emulsion so that the animals could adapt. Thereafter, were administrated 5 mL daily until reach one month of treatment. The administration was performed by the oral route using a probe which directs the emulsion directly to the stomach. Water, food, weight and glucose blood levels were monitorized every 2 days in both experimental groups, during all the treatment. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the rules for the care and handling of laboratory animals (Directive 86/609/EEC).

3 - Glucose tolerance test

At 3 months of age, the male Wistar rats of both groups were submitted to a glucose tolerance test as described by (Nunes *et al*. 2007). Briefly, 14 hours before the test, food was taken from the cages. Immediately before the test, animals were weighed and an intraperitoneal injection with 6 mL glucose 30% (w/v)/kg of body weight was given to each animal. Glucose blood levels were immediately measured after the glucose administration, using a blood glucose meter. During the next two hours, glucose was measured every 30 minutes and animals remained without food until the end of the test.

4 – Epididymal sperm collection

Animals were anesthetized with a mixture of 90 mg/Kg of Ketamine and 10 mg/kg of Xylazine, and sacrificed by cervical dislocation. Epididymides were immediately removed and caudas were isolated. One cauda epididymidis of each animal was immediately stored at -80ºC for posterior RNAt and protein extraction. The other was placed in prewarmed (37ºC) 3 mL of HBSS (Hanks Balanced Salt Solution Ca2+/Mg2+ free; Sigma Aldrich) containing 10000 U/mL of penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B (pH 7.4). Each epididymal cauda was minced with a scalpel blade, to allow sperm to disperse into the medium, and the suspension was then incubated for 5 minutes at 37ºC. Subsequently, sperm suspensions were used to analyze sperm motility, concentration, viability and morphology and a sample from each animal was transferred to a criovessel and stored at -80ºC.

4.1 – Sperm Motility

Motility was evaluated by placing a drop of 100 µL of the sperm suspension in a prewarmed (37º) slide and covered with a cover slip. Then under a light microscope (x100 magnification), the percentage of motile sperm was assessed in 10 random fields, and the average was used as the final motility.

4.2 – Sperm Concentration

The concentration of epididymal sperm in the samples was determined using a dilution of 1:50 in HBSS to fill the two grids of a Neubauer counting chamber. The number of sperm cells was then counted under an optical microscope (x400 magnification).

4.3 – Sperm Viability

To assess sperm viability a nigrosin-eosin stain was used because it is effective, simple and, in addition of allowing sperm to be readily visualized, permits one to assess sperm membrane integrity. A total of 5 µL of sperm suspension was mixed with 10 µL of 0.5% eosin/negrosin stain and placed on a pre-warmed glass microscope slide. A total of 333 sperm were counted in random fields under a light microscope, with oil immersion (x1000 magnification), to determine the percentage of viable sperm. Live sperm remained white (Figure 10A) while dead sperm stained pink (Figure 10B), since the integrity of their plasma membrane had been compromised causing an increase in membrane permeability, which led to uptake of the dye.

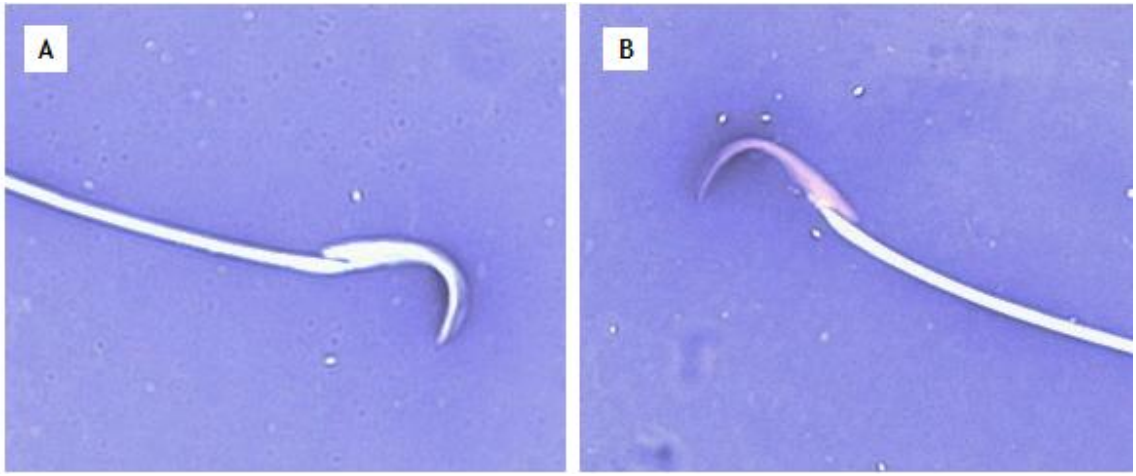

Figure 10 - Light microscopy image of a typical rat spermatozoon. Viable rat spermatozoon (A) and non-viable rat spermatozoon (B) stained with Eosin/Negrosin (1000× magnification).

4.4 – Sperm Morphology

To evaluate sperm morphology a Differential Quick Stain Kit was utilized. The Diff-Quick kit is composed of 3 sequential solutions containing (1) fixative, (2) eosin (dye) and (3) a thiazin-like stain (dye). A total of 10 µL of sperm suspension was placed on a pre-warmed glass microscope slide and air dried at 37ºC. The dried glass microscope slide was then stained using the supplier's protocol. A total of 333 sperm were evaluated, in random fields, under a light microscope, with oil immersion (x1000 magnification). To be classified as normal a sperm cell must have a hook-shaped head and no defects of head, neck or tail (Figure 11A). Otherwise, sperm were considered abnormal. Defects were subdivided into: defects of the head - decapitated head (Figure 11B), flattened head (Figure 11C), pin-head (Figure 11D);

midpiece defects - bent neck (Figure 11E); or tail defects (Figure 11F). When there was more than one abnormality in a sperm cell, only the most important was considered, which is considered the head defect.

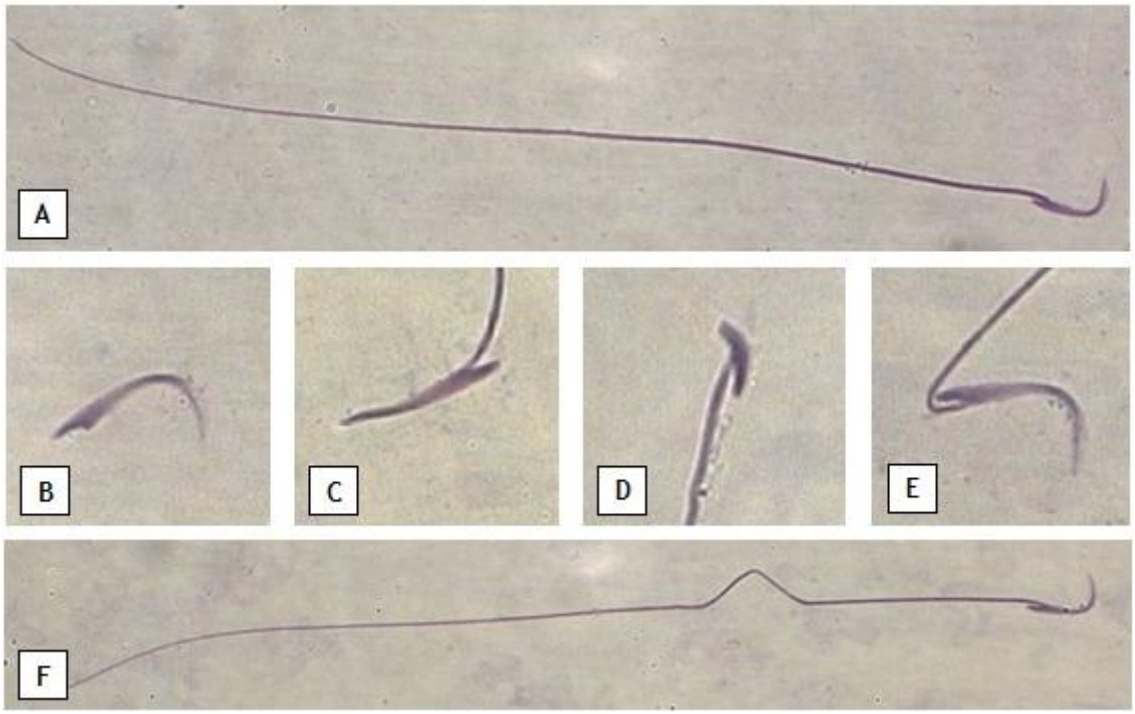

Figure 11 - Microscopic images of a normal rat spermatozoon and various morphological defects. A – Normal sperm. B – Decapitated head. C – Flatenned-head. D – Pin-head. E – Bent neck. F – Tail defect. (1000x magnification).

5 - Sertoli cells isolation procedure

After the animals were sacrificed, the testis were immediately excised in aseptic conditions and washed two times in a 50 mL conical tube in 30 mL of ice cold HBSS. After adherent epididymis and vas deferens removal, testis were decapsulated in HBSS, and the loosen tissue was washed three times in the same solution. SCs were isolated by a modification of a method described previously by (Oliveira *et al.* 2009). Briefly, tissue from decapsulated testes was placed in petri plates containing a collagenase solution (20 mL, 0.5 mg/mL in 1X HBSS, pH 7.4, 34ºC, 10-15 min, shaking 80 oscillation/min) to disperse the seminiferous tubules. It is important to not fragment the seminiferous tubules during the collagenase incubation, because tubule fragmentation results in poor yield and purity. The dispersed tubules were gently forced through a large-pore Pasteur pipette to "unravel" the tubules and further release the interstitial tissue/cells. The supernatant which contains

interstitial cells was discarded and tubules were washed three times in HBSS. Then, they were incubated in a trypsin solution (20 mL, 0.5 mg/mL in 1X HBSS, pH 7.4 at 37ºC for 5-10 min, with gentle shaking). The SCs suspension, collected (by centrifugation 300xg for 3 minutes), was washed three times in HBSS and resuspended in SCs medium which consisted of a 1:1 mixture of DMEM: F12 Ham, supplemented with 15 mM HEPES, 50 U/ml penicillin and 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 µg/mL gentamicin and 10% heat inactivated fetal bovine serum (FBS). This cellular suspension was then forced through a 20G needle, in order to disaggregate large Sertoli clusters. For cell culture, the concentration of clusters on the cellular suspension obtained from the procedure described above was adjusted to 5000 clusters/ml plated and incubated at 37ºC in an atmosphere of 5% CO_2, 95% O_2. The day of plating was considered day 0 of culture. The cultures were left undisturbed until day 2.

5.1 Immucytochemestry

Sertoli cell culture purity was revealed by the immunoperoxidase technique. Briefly, cells were grown on petri culture plates, incubated overnight at 4ºC with primary polyclonal antibody and processed by the labelled streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit giving a brown coloration to the SCs after reaction with 3,3'-diaminobenzidine (DAB) hydrochloride. Medium was removed, the cells were rinsing three times in phosphate-buffered saline (PBS; 4ºC). Cells were fixed with methanol (-20ºC) for 5 minutes and rinsing three times in PBS (4ºC). Endogenous peroxidase activity was blocked by incubating the sections with 0.1% H_2O_2 for 10 minutes. Cells were rinsing three times in PBS (4ºC). Then, cells were permeabilized with 0.2% Tripton X-100 in PBS and rinsing three times in PBS to remove the excess of detergent. Nonspecific antibody binding was minimized by incubation with blocking solution (PBS containing 1% bovine serum albumin (BSA)) for 1 hour at room temperature. Cells were rinsing three times in PBS and then incubated at 4ºC overnight, with primary antibody against vimentin (rat anti-Vimentin) diluted in PBS, pH 7.4, and 1% BSA with 0.1% sodium azide (NaN_3). Therefore, cells were washed three times in PBS (4ºC) and then incubated with the secondary antibody (in 1:500 dilution solution of PBS, 1% BSA with 0.1% NaN_3) for 90 minutes at room temperature. Cells were rinsing three times in PBS. Antibody binding was detected using ExtrAvidin-Peroxidase staining kit and 3.3'-diaminobenzidine tetrahydrochloride according to the manufacturer's instructions. Cells were incubated with 150 µL ExtrAvidin-Peroxidase (diluted in PBS, 1% BSA) for 30 minutes at room temperature. After three washes in PBS cells were incubated with 150 µL of DAB solution, until brown colour was easily detected. Cells were rinsed three times in PBS. The cell nuclei were then stained with Mayers haematoxylin solution. Negative-control staining was performed by substituting the primary antibody by PBS.

5.2 - Hormonal treatments

SCs were allowed to grow until reach 90-95% of confluence, and after fully washed the medium was replaced by serum-free medium (DMEM: F12 1:1, pH 7.4). SCs cultures were divided into two groups: in the control group (ITS Group) the SCs medium was supplemented with Insulin-Transferrin-Sodium Selenite (10 mg/mL – 5.5 mg/mL - 5 μg/mL, respectively) while the insulin-deprived group (TS Group) was supplemented with Transferrin-Sodium Selenite (5.5 mg/mL – 5 μg/mL, respectively). After 96 hours of treatment, cells were detached from the plates using a Trypsin-EDTA solution and collected for RNA and protein extraction. SCs number was determined with a Neubauer chamber. Trypan blue exclusion assays were used to determine the viability of the SCs as described by (Mullaney *et al*. 1994).

6 – RT-PCR

Total RNA (RNAt) isolated from the cultured SCs and from the epididymal caudas was extracted using TRIzol reagent according to the manufacturer's instructions, respectively ressuspended in 10-30μL or 500-600 μL of RNase-free water and stored ate -80ºC. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, Germany). RNAt (1 μg) was denatured for 5 min at 75ºC, then cooled on ice before adding the enzyme. The reverse transcriptase reaction was performed in a final volume of 20 μl containing: 200 U of M-MLV RT, 250 ng of random hexamer primers and 0.5 mM of each dNTP. The reaction was carried out at 37ºC for 60 min followed by 70ºC for 15 min. The resulting cDNA was used to amplify caspase-9, caspase-3, p53 and Bax cDNA fragments with exon-exon spanning primer sets. Each PCR reaction contained 1 μl of cDNA, 200 nM of each 5' and 3' oligonucleotide primers, 0.5 U of Taq polymerase, 2.5 μl of taq polymerase 10x buffer, 1.5 mM of $MgCl_2$, 1 μl of dNTPs 10 mM and distilled water up to 25 μl. Primer sequences, optimal annealing temperature, the number of cycles required for exponential amplification phase of fragments and fragment sizes are indicated in Table 1. Caspase-9, caspase-3, p53 and Bax mRNA levels were normalized with 18S gene expression as internal control. Liver mRNA was used as positive control and cDNA-free sample was used as negative control. At the end of the experiments, samples were run in 1% agarose gel electrophoresis, during 30 min at 120V, to confirm further the absence of nonspecific amplification. The size of the expected products was compared to a DNA ladder (100-bp ladder). Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods (Picado *et al*. 1999). For each condition, the obtained band density was divided by the respective 18S band density and the results were expressed as fold induction/reduction versus the control group.

Table 1 - Oligonucleotides and cycling conditions for PCR amplification of Bax, p53, caspase-3, caspase-9 and 18S.

Gene	Primer sequences	AT	C	Size (bp)
Bax	Forward – CGC GTG GTT GCC CTC TTC TAC TTT Reverse – CAA GCA GCC GCT CAC GGA GGA	59ºC	35	124
P53	Forward – CTG CCC ACC ACA GCG ACA GG Reverse – AGG AGC CAG GCC GTC ACC AT	59ºC	35	471
Caspase-3	Forward – AGG CCT GCC GAG GTA CAG AGC Reverse – CCG TGG CCA CCT TCC GCT TA	61ºC	35	255
Caspase-9	Forward – TGC AGG GTA CGC CTT GTG CG Reverse – CCT GAT CCC GCC GAG ACC CA	61ºC	35	130
18s	Forward - AAG ACG AAC CAG AGC GAA AG Reverse - GGC GGG TCA TGG GAA TAA	56ºC	25	159

Abbreviations: AT - annealing temperature; C - Number of cycles during exponential phase of amplification.

7 - Western Blot

Total proteins were isolated from the epididymal caudas and from the isolated SCs using RIPAS buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, supplemented with 1% protease inhibitor cocktail, aprotinin and 100 mM sodium orthovanadate). Protein concentration was determined by BioRad (Hemel Hempstead, UK) Bradford micro-assay according to the manufacturer's instructions. Protein samples (50 μg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue, pH=6.8) in a final volume of 20 μL, sonicated for 10 minutes at 4ºC and denatured for 10 minutes at 55ºC. Proteins were fractionated in 12% poliacrylamide gels and electrophoresis (SDS-page) was carried out for 90 min at 30 mA per gel. The separated proteins were then transferred during 75 min at 0.75 A, to previously activated polyvinylidene difluoride membranes and then blocked for 90 min in a 5% non-fat milk solution at room temperature. Afterwards, the membranes were incubated overnight at 4ºC with rabbit anti-Bax (1:4000, Cell Signaling, 2772, Massachusetts, USA) or rabbit anti-caspase-9 IgG (1:1000, Cell signalling techonology, 9507, Massachusetts, USA), or mouse anti-cytochrome C (1:1000, 556433, BD pharmigen). Mouse anti-α-tubulin (1:5000, Sigma Aldrich, St. Louis, MO, USA) was used as the protein loading control for SCs samples. The immunoreactive proteins were detected separately and visualized with goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany) or goat anti-mouse IgG-AP (1:5000, Santa Cruz

Biotechnology Heidelberg, Germany). Membranes were reacted with ECF™ (GE, Healthcare), a fluorescent substrate for alkaline phosphatase-based detection, and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods (Picado *et al*. 1999). The band density attained was divided by the corresponding α-tubulin band intensities and expressed in fold variation (induction/reduction) versus the control group.

8 – Enzymatic assays

The activity of caspase-3 was spectrophotometrically assessed by determining the cleavage of the respective colorimetric substrate as previously described by (Alves *et al*. 2011). Briefly, proteins (25 µg) were incubated with the assay buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37º C. The caspase-3-like activity was determined by detection of the chromophore p-nitroanilide, measured at 405 nm in a spectrophotometer. The method was calibrated with known concentrations of p-nitroanilide. The attained activities were expressed in percentage versus the control group.

9 - Statistical analysis

The statistical significance of mRNA and protein expression, caspase-3 activity and reproductive parameters among the experimental groups was assessed by one-way ANOVA, followed by Dunn post-test. All experimental data are shown as mean ± SEM (n=5 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

IV. Results

A – Apoptosis signaling pathways in insulin-deprived rat Sertoli cells

1. Rat Sertoli cells culture

Sertoli cells, isolated from male Wistar rats (3 months old), purity and viability were assessed as described by Rato *et al.* (2012). Whole preparations were examined in a phase contrast microscope (Olympus CKX41Cell) and showed that contaminants were below 5% after 96 h in culture. Purity was confirmed by the immunoperoxidase technique using a polyclonal antibody against vimentin, a SC marker (data not shown). SCs viability was not altered in the insulin-free SCs cultures as evaluated by trypan blue exclusion (data not shown). Viability averaged 85–90 %, always with values higher than 85 % (data not shown).

2. Insulin deprivation decreases p53 mRNA levels in *in vitro* cultured rat Sertoli cells

In response to a myriad of stress signals, the p53 protein can be activated and depending on the tissue and the extent of the cellular damage, triggers adequate cellular response including apoptosis (Vousden and Lane 2007). To analyse the possible effect of insulin-deprivation on mRNA expression levels of p53 we performed a semi-quantitative RT-PCR. The mRNA expression levels of p53 in TS group were significantly lower when compared with the control group. P53 mRNA levels significantly decreased to 0.82±0.06 fold (Figure 12) after the 96 hours of treatment.

The apoptotic event can be enhanced by the activation of several pro-apoptotic proteins, such as Bax, in a process mediated by p53 (Majors *et al.* 2007). In order to further disclose the effects of insulin deprivation and the possible role of p53 pathway, we investigated the Bax mRNA levels. When compared with the control group, Bax mRNA levels in the insulin-free SCs culture were not significantly altered.

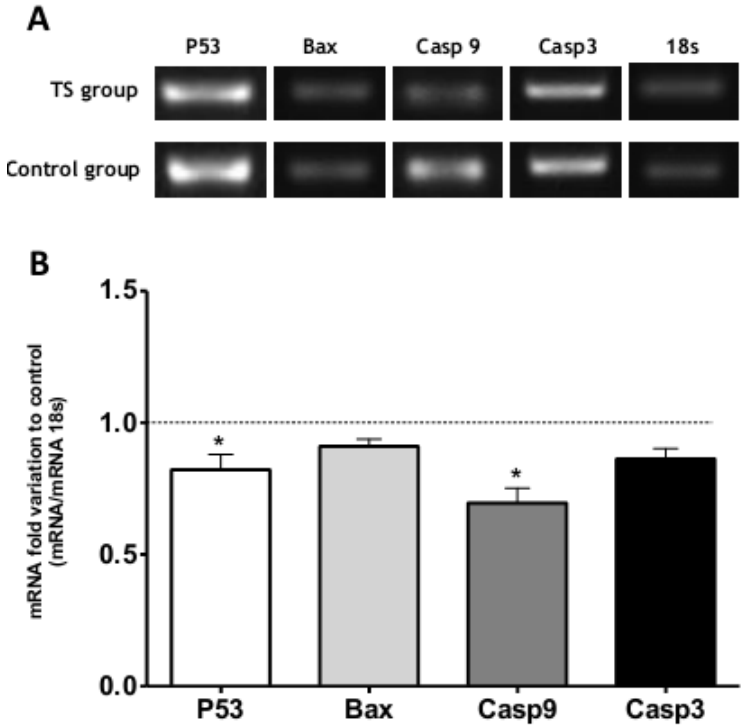

Figure 12 - Effect of insulin deprivation on p53, Bax, caspase-9 and caspase-3 mRNA levels in rat Sertoli cells. Panel A shows representative agarose gel electrophoresis. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA levels found in insulin deprived rat SCs when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

3. Insulin deprived rat Sertoli cells exhibit decreased caspase-9 mRNA levels

Caspase-9 is a cysteine aspartyl protease associated with the intrinsic or mitochondrial pathway of apoptosis (Li *et al.* 1997; Budihardjo *et al.* 1999). Once activated, caspase-9 cleaves and activates the effectors caspase-3 and -7, which target key regulatory and structural proteins for proteolysis to effect cell death (Taylor *et al.* 2008). We investigated the possibility of an alteration on the mRNA levels of caspase-9 and caspase-3. After performing the semi-quantitative RT-PCR, we observed a significantly decrease of caspase-9 mRNA levels in insulin-free treated cells to 0.70±0.06 fold (Figure 12B) when compared with SCs cultured in control conditions. However, caspase-3 mRNA levels did not show a significant alteration.

4. Insulin deprivation does not alter protein levels of Bax and caspase-9 in *in vitro* cultured rat Sertoli cells

Following the analysis of the mRNA levels of apoptotic markers, we also analysed the effect of insulin deprivation on protein levels of important pro-apoptotic markers such as Bax and cleaved caspase-9. The protein levels of these two pro-apoptotic markers were not significantly different in insulin-free treated cells when compared with the control group (Figure 13). However, both Bax and cleaved caspase-9 protein levels presented a non-significant increase to 1.28±0.11 and caspase-9 to 1.27±0.27 fold variation relatively to control cells, respectively.

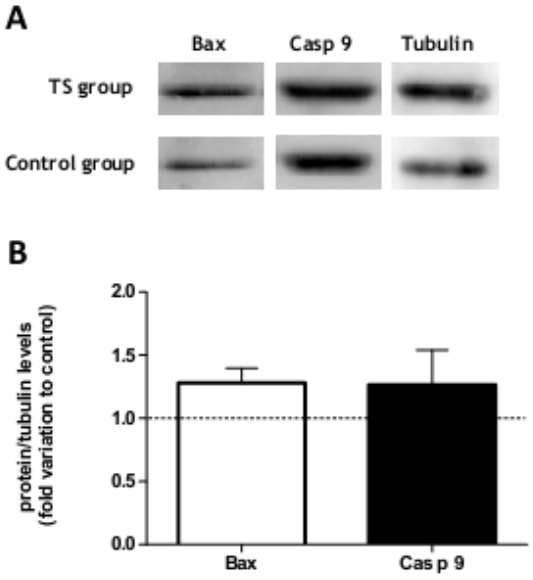

Figure 13 - Effect of insulin deprivation on Bax and caspase-9 protein levels in rat Sertoli cells. Panel A shows representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of protein levels found in insulin deprived rat SCs when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

5. Insulin deprivation does not alter caspase-3 activity in rat Sertoli cells

We also evaluated the caspase-3 activity in *in vitro* cultured rat SCs after the 96h of insulin deprivation, as an endpoint marker of apoptosis. We concluded that there were no significant differences between the both experimental groups (Figure 14).

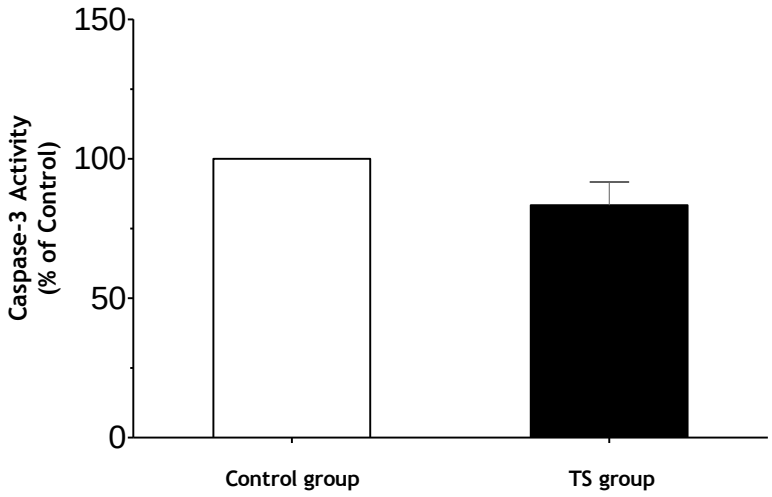

Figure 14 - Effect of insulin deprivation on caspase-3 activity in rat Sertoli cells. Figure shows pooled data of independent experiments, indicating the fold variation of caspase-3 activity levels found in insulin deprived rat SCs when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

B – Apoptotic signaling pathways in whole cauda epididymis

1. High-energy diet animal model

During all the treatment with the HED, animal weight and glucose blood levels were monitored every 6 days, in both experimental groups (Table 2). During the 30 days treatment, HED rats presented normal glycaemia values (as compared with the control group animals), although the animals weight was significantly reduced in HED rats. Ai et al. (2005) described similar results using this model and further described that HED rats had 170% more abdominal fat than control group animals.

Table 2 - Average values of the animals weight and glucose blood levels measured in the high-energy diet group and control group during all the treatment (n=6).

	Control group				HED group			
Days	**Glycaemia (mg/dL)**	**SEM**	**Weight (g)**	**SEM**	**Glycaemia (mg/dL)**	**SEM**	**Weight (g)**	**SEM**
0	102.33	4.81	262.17	7.33	114.83	4.77	296.67	8.70
6	94.33	7.54	285.83	9.02	109.67	9.46	299.83	9.05
12	101.00	3.36	299.83	9.69	102.67	4.56	295.50	11.30
18	102.67	3.77	314.83	10.29	113.00*	2.63	286.50*	7.55
24	101.17	3.66	327.00	10.24	115.67*	3.29	279.50*	7.56
30	102.00	6.43	335.67	8.69	109.17	5.25	274.50*	8.91

Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

2. Glucose tolerance test

The glucose tolerance test was performed to determine how quickly the given dose of glucose was cleared from the animals blood. As shown in figure 15, at the end of the test, the blood glucose levels were 165.0±14.1 mg/dL in the HED group and 118.2±4.3 mg/dL in the

control group. So, the blood glucose levels two hours after the glucose administration are significantly higher in the HED group when compared with the control group. Noteworthy, the glucose tolerance test show that the animals from the HED group present high glucose intolerance. The animals from HED group had a significantly different glucose metabolization during the test, presenting a distinct pattern that usually is associated with a prediabetic state (Aroda and Ratner 2008).

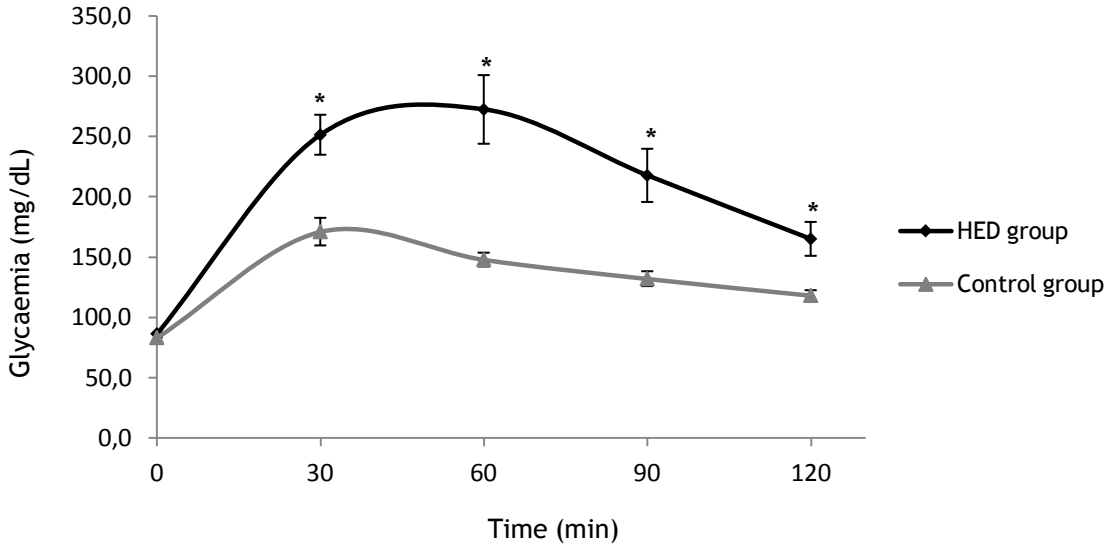

Figure 15 - Glucose levels of the high-energy diet (HED) group and the control group measured during the glucose tolerance test. Figure shows pooled data of independent experiments, indicating the blood glucose levels variation measured in HED rats when compared with the control group animals. Results are expressed as mean ± SEM (n=6 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

3. Motility is increased in cauda epididymis sperm of the high-energy diet fed animals

The spermatozoa flagellum contains the energy sources and machinery to generate the motility necessary for the sperm to reach the egg. So, motility is critical at the time of fertilization and it's assessment contributes to measure the semen quality (Rabbani *et al.* 2010). According to the results described in Table 3, sperm motility was significantly higher in the HED group animals when compared to the control group.

Sperm viability and concentration were also evaluated to assess semen quality, but none of these parameters showed significant differences in the HED group animals when compared with the control group animals.

Table 3 - Sperm motility, viability and concentration values of both experimental groups (n=6).

Sperm parameter	HED group	Control group
Motility	68 ± 2 % *	58 ± 5 %
Viability	24 ± 3 %	33 ± 2 %
Concentration	$2.8 \times 10^7 \pm 0.7 \times 10^7$ Cél/mL	$2.8 \times 10^7 \pm 0.3 \times 10^7$ Cél/mL

Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

4. Morphologically normal sperm decreased in epididymal sperm of the high-energy diet fed animals

Sperm morphology is another parameter evaluated in semen analysis. The shape of the head, midpiece and tail is usually used to examine the sperm morphology. The abnormal sperm morphology is a reflection of negative stress factors working on the body (Menkveld *et al.* 2011) and is a crucial parameter to evaluate the fertility potential of the individuals. In this work, the average percentage of normal epididymal spermatozoa decreased significantly in the HED group animals when compared with the control group (Table 4). Contrarily, all the other specific abnormalities analyzed (decapitated head, flattened head, pin-head, bent neck and tail defects) were not significantly different between both groups.

Table 4 - Average percentage of the morphology alteration evaluated in epididymal sperm of the high-energy diet group and control group animals (n=6).

Sperm Morphology	HED group (%)	Control group (%)
Normal sperm	45.9 ± 2.3*	61.0 ± 0.6
Decapitated head	7.5 ± 1.6	5.7 ± 1.3
Flatenned-head	1.0 ± 0.1	1.2 ± 0.5
Pin-head	0.10 ± 0.10	0.15 ± 0.10
Bent neck	5.8 ± 1.2	5.7 ± 1.0
Tail defects	38.9 ± 1.3	27.1 ± 0.5

Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

5. High-energy diet decreases Bax mRNA expression levels in cauda epididymis

Bax is a pro-apoptotic protein that facilitate caspases activation by promoting the release of cytC from the mitochondria (Lee *et al*. 2003). To analyse the possible effect of the HED on mRNA expression levels of Bax, we performed a semi-quantitative RT-PCR. The mRNA expression levels of Bax in the HED group were significantly lower, 0.82±0.04 fold, when compared with the control group (Figure 16B).

Following the significant decrease of Bax mRNA levels, we analyzed the mRNA expression levels of some pro-apoptotic proteins such as p53, caspase-9 and caspase-3. We concluded that none of these apoptotic markers showed significant alterations in the cauda epididymis of the HED group when compared with the control group animals.

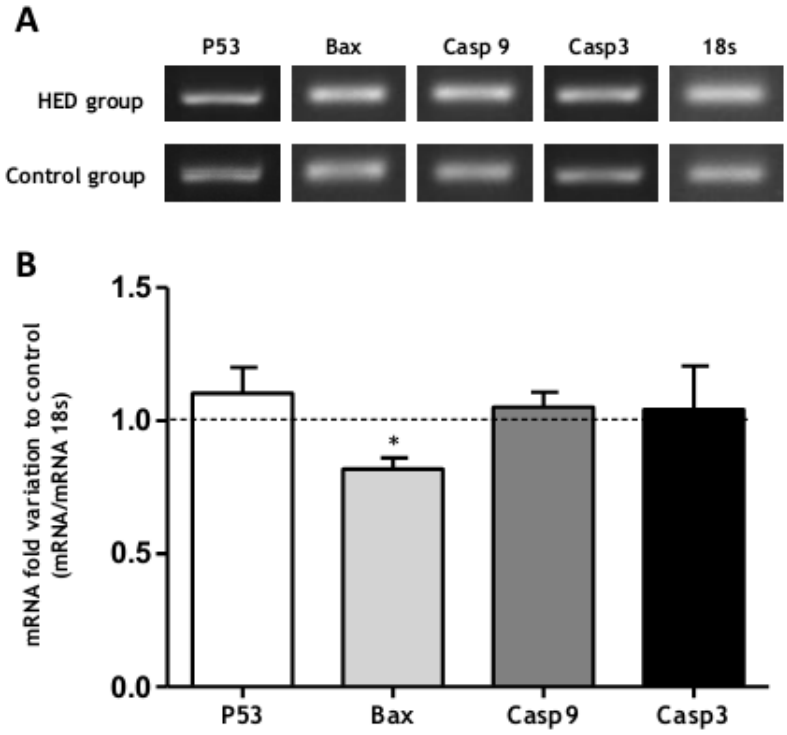

Figure 16 - Effect of high-energy diet (HED) on p53, Bax, caspase-9 and caspase-3 mRNA levels in rat cauda epididymis. Panel A shows representative agarose gel electrophoresis. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA levels found in cauda epididymis of HED rats when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control

6. High-energy diet decreases cytochrome c protein levels in cauda epididymis

After assessing the variation of mRNA levels in HED group, we also evaluated the effect on the protein levels of one relevant protein involved in apoptotic signalling - cytC. Cytochrome C initiates caspase activation when released from mitochondria during apoptosis (Liu *et al.* 1996). The protein expression levels of cytC in the HED group presented a significant difference of 0.77±0.05 fold relatively to the control group (Figure 17).

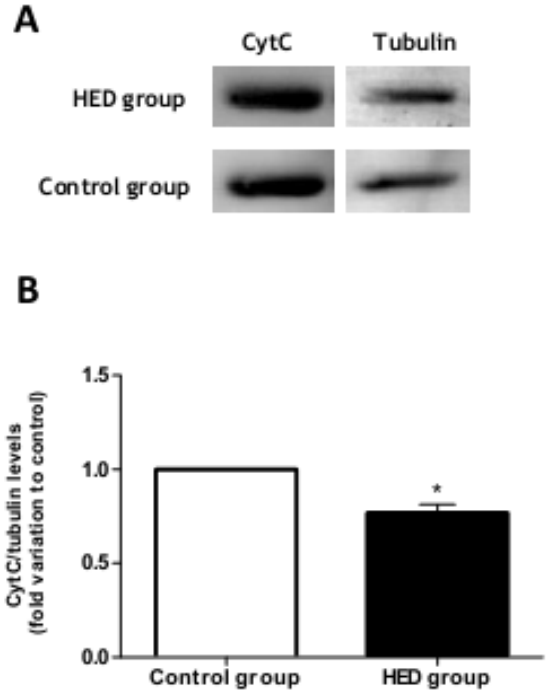

Figure 17 - Effect of high-energy diet (HED) on cytochrome C protein levels in rat cauda epididymis. Panel A shows representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of protein levels found in cauda epididymis of HED rats when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

7. High-energy diet does not alter caspase-3 activity in cauda epididymis

Caspase-3 activation plays a key role in the initiation of cellular events during the apoptotic process. Its activation requires the proteolytic cleavage of its inactive precursor enzyme, pro-caspase-3, and this is executed by activated caspase-9 (Zou *et al.* 2003). In our experiments, we determined the caspase-3 activity by measuring the cleavage of caspase-3 specific substrate and the release of a chromophore (p-nitroanilide). Using cauda epididymis homogenates of the rats from HED group, we performed a quantitative detection of caspase-3 activity that was not significantly altered when compared with the control group (Figure 18).

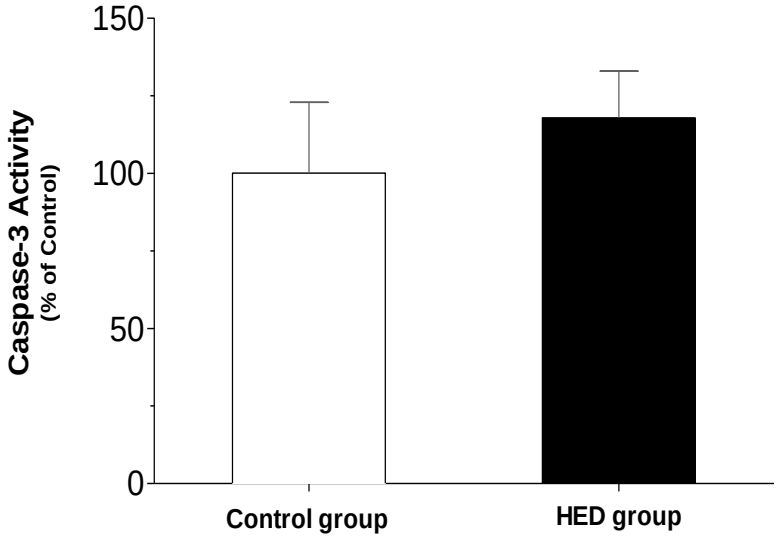

Figure 18 – Effect of high-energy diet (HED) on caspase-3 activity in rat cauda epididymis. Panel shows pooled data of independent experiments, indicating the fold variation of caspase-3 activity measured in HED rat cauda epididymis when compared with the control condition. Results are expressed as mean ± SEM (n=5 for each condition). Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

V. Discussion

Diabetes mellitus stands for a group of metabolic diseases marked by high blood glucose levels. This disease is classically associated with insulin deprivation or insulin resistance in cells, which is often described as a disorder of carbohydrate, lipid and protein metabolism. Diabetes mellitus has a significant impact on the fertility of male individuals, both directly and indirectly (Glenn *et al.* 2003). This disease affects male reproductive function at multiple levels such as on the endocrine control of spermatogenesis, spermatogenesis itself, or by impairing penile erection and ejaculation (Sexton and Jarow 1997). It is well-recognized that DM is a cause of male sexual dysfunction, which in itself may contribute to subfertility or even infertility. Thus, male infertility/subfertility problems may become more widespread as DM rates rise. Therefore, the enlightenment of the key regulatory mechanisms by which sperm production is affected in this disease is critical in order to highlight new therapeutic approaches.

A – Apoptosis signaling pathways in insulin-deprived rat Sertoli cells

In type 1 diabetes mellitus there is an absolute lack of insulin and testicular biopsies of diabetic patients also revealed numerous abnormalities in SCs (Cameron *et al.* 1985). Nevertheless, the mechanisms underlying these malfunctions remain largely unknown. Recently, Oliveira et al. (2012) described that insulin-deprived human SCs presented altered glucose consumption, lactate secretion and altered expression of metabolism-associated genes involved in lactate production export and glucose uptake. It has been also described that alterations of cell metabolism, particularly in glucose metabolism, are closely related with the apoptotic process (Majors *et al.* 2007). Decreases in ATP levels or increases in OS due to reduced glycolysis can lead to increased apoptosis (Moley and Mueckler 2000). Mitochondria seem to be the connection between apoptotic and metabolic signaling. Also, they are the source of several pro-apoptotic proteins. Hence, following previously reported results on the effect of insulin deprivation in human SCs metabolism, we hypothesized that the lack of insulin could actively modulate the regulation of these mitochondria related pro-apoptotic proteins and mechanisms. Although observations in SCs primary cultures may not exactly represent an *in vivo* situation, and must be analyzed under that scope, the results presented here are the first report of the effect of insulin deprivation on the apoptotic signaling markers of rat SCs. They are also a further step to identify key mechanisms by which this hormone can regulates SCs function, which can have a direct influence over spermatogenesis and thus male fertility. Noteworthy, there are few papers versed on the mechanisms behind the infertility that is known to occur in type 1 diabetic individuals. There is also a lack of literature concerning the insulin control of spermatogenesis. Thus, the experiments presented herein are a first step to elucidate the role of insulin in the apoptotic control of SCs.

The mitochondrial apoptotic pathway can be triggered in response to extracellular cues and internal insults such as DNA damage (Hengartner 2000). In this pathway, the tumor suppressor protein p53 is a key intervenient. This protein is also critical for cell growth and development and plays an important role in the intrinsic response to genome instability, including cell cycle arrest (Kubbutat *et al.* 1997; Haupt *et al.* 2003). When p53 is activated it can stimulate the expression of pro-apoptotic factors, such as Bax (Chen *et al.* 2007). We determined the effect of insulin deprivation on the expression of some apoptotic markers related to the mitochondrial pathway in rat SCs and observed a significant down-regulation of p53 mRNA levels, although no difference was observed on the Bax mRNA levels. Activation of the pro-apoptotic factor Bax leads to the mitochondria permeabilization, which causes the release of several apoptotic factors from the intermembrane space. This can trigger the caspase-dependent death pathway, through the sequential activation of caspase-9 and the effector caspase-3 (Schmitz *et al.* 2000), or the caspase-independent death effectors, which translocate to the nucleus and contribute to chromatin condensation and chromatinolysis (Vahsen *et al.* 2004). Although no difference was observed in Bax mRNA levels of insulin-deprived cells, we observed a significant down-regulation of caspase-9 mRNA levels. Following the apoptotic cascade, we assessed the cleaved caspase-9 protein levels and no differences were observed in SCs from insulin-deprived or control cells. Furthermore, no variation was observed in the mRNA levels or activity of caspase-3 in insulin-deprived SCs when compared with the control group.

These results seem to suggest that the mitochondrial apoptotic pathway is not perturbed in insulin-deprived SCs, as caspase-3 activity is not altered, which is usually known as an endpoint for apoptosis. The utilization of caspase-3 activity is widely used as a hallmark for apoptosis, as it allows a qualitative and a quantitative assessment of the apoptotic process (Köhler *et al.* 2002). Nonetheless, the absence of alteration on caspase-3 activity only suggests that the apoptotic process has not reached the non-returning point and, thus, can be counterbalanced by the action of the anti-apoptotic proteins such as Bcl-2 and Bcl-XL (Einat *et al.* 2005). The activation of anti-apoptotic proteins is often described as a critical point in the control of the apoptotic process. These anti-apoptotic proteins, belonging to Bcl-2 family proteins, function as antagonists of the pore forming in the mitochondrial membrane interrupting the apoptotic process (Epand *et al.* 2002). Importantly, the manipulation of this Bcl-2 system is suggested to be of extreme importance in pathological conditions related to apoptotic pathways dysfunction thus, it is expected that this system may also be important in SCs control of apoptosis. In fact, we observed important cues appointing to crucial alterations induced by insulin deprivation in the apoptotic process of SCs. The p53 levels were significantly reduced in insulin deprived cells although, as said, no differences were seen in the apoptotic endpoint. This could be due to either: (1) insufficient decrease on p53 levels to alter the homeostasis of the apoptotic process or (2) an increase in the expression of anti-apoptotic proteins, such as those from the Bcl-2 family. As previously discussed, the

activation of the caspase cascade is regulated by the balance between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family (Shaha *et al*. 2010) and although the evaluation of the levels of Bax showed no alteration, the expression of no other member of this family was evaluated. Thus, remains to be elucidated if the alterations of p53 levels are not counteracted by the increase of anti-apoptotic proteins. Supporting this suggestion that the anti-apoptotic system, such as the one controlled by Bcl-2, regulate the mitochondrial apoptotic signaling induced by insulin deprivation, is the fact that the protein levels of cleaved caspase-9 were not altered although the mRNA levels of this caspase were significantly decreased when compared to the control group. It is well known that the evaluation of the protein levels is more adequate to assess alterations in the protein global functioning and, as the activation of the caspase cascade requires the cleavage of pro-caspase-9, the most reliable measure to determine the effect of insulin deprivation was to evaluate the protein levels of the cleaved caspase-9. So, the obtained results suggest that if the mitochondrial apoptotic pathway is altered in insulin deprivation conditions, then the upstream pro-apoptotic events are counteracted by the anti-apoptotic proteins.

As referred, the role of insulin in the apoptotic pathways of SCs has not been investigated so far. Nevertheless in other cellular systems, such as neurons (Tanaka *et al*. 1995) insulin deprivation has proved to induce apoptosis. Noteworthy, high levels of insulin are also associated with stimulation of the apoptotic pathways in beta cells (Guillen *et al*. 2008) thus evidencing the importance of this hormone in the regulation of the apoptotic process. Sertoli cells are not an exception and our results point towards a possible role for insulin in the regulation of the interaction between pro- and anti-apoptotic proteins. As the apoptotic events controlled by SCs are crucial for the normal development of spermatogenesis, it is crucial to evolve towards new experiments in order to fully disclose the pathways where insulin exerts its control over apoptosis.

B – Apoptosis signaling pathways in whole cauda epididymis

Prediabetes is the leading risk factor for T2DM and consists of IFG and/or IGT (Edelstein *et al*. 1997). Insulin resistance occurs in the majority of patients with T2DM and in a large number of subjects with IGT (Aroda and Ratner 2008; Borai *et al*. 2011). T2DM has many complications and has high treatment costs associated, thus is crucial to further investigate the mechanisms behind its pathology and the associated effects.

For these reasons, we chose to use an animal model that exhibited some of the features characteristic of the prediabetic state, in order to evaluate and disclose the effects of these state on markers of the male reproductive function. Among these markers are the

motility, the morphology, the viability and the sperm concentration, which are the basic parameters in standard estimation of spermatozoa quality. Because of the paucity of studies and inconsistencies regarding the theoretical expected impact of prediabetes on semen quality, this disease is seldom looked for in the infertile patient.

After completing spermatogenesis, spermatozoa are fully differentiated cells incapable of swimming or fertilize the egg. Indeed, besides the spermatozoa quantity, the appropriate sperm motility and morphology is necessary for fertilization of the oocyte. In fact, the spermatozoa with abnormal morphology are not capable to fertilize the oocyte (Marzec-Wroblewska *et al.* 2012). Our results demonstrated that motility was significantly increased in the HED group, when compared with the control group, while viability and sperm concentration did not show significant differences. This increase in sperm motility is somewhat an intriguing result that can be due to a higher availability of glucose, related to the prediabetic state. It has been described that cauda epididymis spermatozoa are dependent on glucose as an energy source (Hoppe 1976; Williams and Ford 2001). Furthermore, it has been demonstrated that spermatozoa are capable of secreting its own insulin, which may provide an autocrine regulation of glucose metabolism according to their energetic needs independent of systemic insulin (Aquila *et al.* 2005). For that reason, an increase in the availability of glucose could promote an increase in sperm motility, as observed, due to an increase in ATP synthesis. Nevertheless, it is also well known that in more advanced stages of DM, sperm parameters (including sperm motility) are seriously compromised (Bener *et al.* 2009; La Vignera *et al.* 2011). One can hypothesize that, in those cases, due to the prolonged uprise on glucose availability, and consequent increase in sperm metabolism, spermatozoa tends to accumulate cellular damages derived from the resultant increased OS levels. Increased production of free radicals and deficiency of antioxidant systems has been described in T2DM individuals and is thought to be one of the main causes of the male infertility (Rabbani *et al.* 2010; La Vignera *et al.* 2011). Moreover, the increase in sperm abnormalities has also been associated with higher OS levels (Pasqualotto *et al.* 2000; Rabbani *et al.* 2010). So, the observed decrease in the number of morphologically normal spermatozoa in animals treated with the HED could be resultant from the augmentation in the OS, due to an increase on spermatozoa glucose metabolism. As the percentage of normal spermatozoa is decreased, this is a first cue suggesting that fertility is most likely compromised in the rats fed with a HED, even if spermatozoa present a higher motility.

Nevertheless, as the conventional semen analysis is now recognized to be of limited value in the determination of fertility status (Jequier 2005), we certainly need to further investigate the relation between prediabetes, sperm dysfunction and fertility potential of the individual. In order to further clarify some of the possible mechanisms by which the prediabetic state induced by the HED could compromise the spermatozoa maturation of treated rats, we evaluated the mRNA and protein levels of some apoptotic markers in rat cauda epididymis. The maturation (fertilizing ability) of spermatozoa is acquired in a

temporally controlled manner during different stages towards the encounter with the female gamete, being one of them the passage through the epididymis. It creates a unique microenvironment within the lumen of the duct that firstly helps the transformation of immotile, immature testicular spermatozoa into fully fertile competent cells, and secondly stores fertile spermatozoa in a viable state within the cauda epididymis until they are ejaculated. It is well known that apoptosis homeostasis is crucial in all physiological cellular processes and thus we hypothesized that the deregulation of cauda epididymis spermatozoa morphology could be associated with an increase of the apoptotic levels in this microenvironment. To test our hypothesis, we evaluated specific apoptotic markers from the mitochondrial apoptotic pathway in order to determine the effect of the prediabetic state in the development of mature spermatozoa, for it is well known that mitochondria are the major cellular sources of ROS (Andreyev *et al*. 2005).

In our work, we determined the mRNA levels of p53, a protein that is activated in response to a myriad of stress signals, including oxidative damage to DNA, and can trigger apoptosis (Vousden and Lane 2007), namely through the up-regulation of the pro-apoptotic factor Bax (Chen *et al*. 2007). We found that cauda epididymis from rats of the HED group showed no alterations in p53 mRNA expression levels. Nevertheless Bax mRNA levels in HED group showed a significant decrease, when compared with the control group. Furthermore, the mRNA expression of caspase-9 and the effector caspase-3, that are key players of the caspase-dependent death pathway (Schmitz *et al*. 2000), was not altered, clearly suggesting that the caspase dependent apoptotic signaling is not altered by the HED. This circumstance is further supported by the fact that caspase-3 activity also remained unaltered in the cauda epididymis of those animals. As said, caspase-3 activity was used as an endpoint marker for apoptosis because activation of effector caspases in cells is a key point of the apoptotic process (Lee *et al*. 2003).

The prediabetic state has been associated with increased apoptosis, particularly in pancreatic β-cells (Shimabukuro *et al*. 1998) but also in the testes, which presented an abnormal stage distribution of apoptosis in the seminiferous epithelium that resulted in an imbalance of spermatogenesis (Sainio-Pollannen *et al*. 1997). Nevertheless, in our experiments, no alteration on the apoptotic levels of cauda epididymis was observed. This could be due to several factors: (1) a particular resistance of this tissue to the imbalance caused by the prediabetic state; (2) an uneven sensibility of the different cells present in the cauda epidydimis (such as epithelial cells or spermatozoa) to the prediabetic derived apoptotic process or (3) triggering of alternative apoptotic pathways that are not dependent on caspase-3 activation. This suggestion is supported by the evaluation of cytC protein levels that significantly decreased in the HED group relatively to the control group. Once Bax is activated it binds to extracellular mitochondrial membrane promoting the formation of channels from where apoptotic signaling molecules such as cytC are released by mitochondria. Thus, the observed decrease in cytC levels is in agreement with the decrease

in Bax mRNA levels. Nevertheless one cannot disregard that we quantified the whole cytC present in the cauda epididymis and although this approach is a clear indicative for the apoptotic process, it is more accurate to measure the cytosolic and the intramitochondrial cytC fractions, in order to establish a ratio where is possible to infer about the release of this protein.

Taken together our results show that our rat model shares many of the clinical and metabolic characteristics of the prediabetic state observed in humans. The animals presented glucose resistance and progress from normoglycaemia/normoinsulinemia to moderate hyperglycaemia/hyperinsulinemia due to food intake. This prediabetic state induced by food intake was responsible for important alterations in cauda epididymis spermatozoa morphology, which clearly indicate that these animals may develop subfertility or fertility problems at some point. Noteworthy, these HED fed animals presented lower Bax mRNA levels and lower cytC protein levels although the apoptotic endpoint, caspase-3 activity, was not altered. This led us to suggest that the apoptotic signalling may be controlled by other mechanisms rather than the mitochondrial pro-apoptotic proteins, such as the anti-apoptotic cellular systems. Further works will be needed to clarify the possible apoptotic signalling responsible for the morphological alterations in the cauda epididymis spermatozoa.

VI. Conclusions

Approximately 15% of couples in reproductive age are subfertile or infertile and the increasing incidence of DM is coupled with falling fertility. In fact, the fertility of DM individuals has been somewhat under looked in the last few years especially due to the fact that, until recently, this endocrine disease has been associated with aged men with natural lower fertility status. This vision is being changed and according to a 2003 report of the International Diabetes Federation, there is a high rate of new-onset diabetes cases among adolescents worldwide and the numbers are expected to rise due to the new diet habits installed. Thus, it urges the study of the possible mechanisms related to DM and subfertility or infertility. In order to clarify some of those mechanisms, our first approach consisted in simulating an *in vitro* condition of T1DM by total absence of insulin. We chose to study SCs function as these cells are responsible for the establishment of the immunological and nutritional microenvironment where the germ cells develop. That approach lead us to suggest that insulin has an important role in the regulation of the interaction between pro and anti-apoptotic proteins. As the interaction of these proteins decides the cell fate and the overall apoptotic signaling, insulin proved to play a key role in the maintenance of the spermatogenic process. Although further studies are needed to fully clarify the mechanisms behind this action, insulin deprivation is in some extend deleterious to the apoptotic process, suggesting that apoptotic signaling can be modulated in individuals with T1DM due to the lack of insulin.

In the last few years, there are growing concerns about the possible severe consequences of the so called “prediabetic state”. The fertility potential of individuals under that condition was never fully evaluated. Thus, our first goal was to characterize an *in vivo* rodent model that could mimic that condition. The HED fed rats developed severe glucose intolerance and progressed from normoglycaemia/normoinsulinemia to moderate hyperglycaemia/hyperinsulinemia due to food intake, mimicking the characteristics of the prediabetic state as noted by the WHO. The cauda epididymis spermatozoa of these HED fed animals clearly presented abnormal alterations, which lead us to conclude that at some point they may develop subfertility or infertility. Our results does not clearly show a correlation between the alterations found in the cauda epididymis spermatozoa morphology and the pro-apoptotic proteins studies. Further studies concerning the anti-apoptotic proteins involved in these apoptotic signaling pathways will be needed to disclose the interaction between the cauda epididymis apoptosis in the prediabetic state and subfertility or infertility in these animals.

As DM grows to epidemic proportions, the identification and characterization of the *in vitro* and *in vivo* mechanisms by which the disease exerts its negative effects, is a crucial step forward to the development of a possible therapeutic.

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