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**New signalling pathways in reproductive tissues
associated with mitochondrial dysfunction
induced by metabolic diseases**

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Resumo

A diabetes mellitus é uma doença metabólica cuja incidência está a aumentar na população mundial. Classicamente pode ser dividida em dois tipos: tipo 1 que se caracteriza por um estado de insulino dependência, e o tipo 2 em que se verifica uma resistência à ação da hormona. Fatores externos associados ao estilo de vida, como os maus hábitos alimentares, em particular o consumo frequente de dietas altamente calóricas, em combinação com outros fatores como o sedentarismo, são as principais causas para o incremento de patologias como a diabetes mellitus tipo 2 (DMT2). A desregulação metabólica associada à DMT2 leva ao aparecimento de outras comorbidades, nomeadamente uma diminuição da fertilidade masculina. O eixo hipotálamo-hipófise-testículo, conhecido como eixo reprodutivo, é sensível às alterações metabólicas induzidas pela DMT2. Estudos recentes têm mostrado que alterações endócrinas e metabólicas associadas à DMT2 afetam a fisiologia dos órgãos reprodutivos, nomeadamente a bioenergética mitocondrial pelo que a manutenção da função mitocondrial nos órgãos reprodutivos é essencial. Assim, pretendemos estudar qual o impacto da DMT2 nas vias moleculares subjacentes à regulação da função mitocondrial testicular e epididimal. Para isso usou-se o modelo animal de DMT2 onde se avaliou a expressão de proteínas-chave envolvidas tanto na regulação da biogénese mitocondrial, como na ativação do sistema de defesa antioxidante. Também avaliamos os efeitos da DMT2 no número de cópias de DNA mitocondrial (mtDNA) e na expressão dos níveis dos complexos da cadeia respiratória mitocondrial em ambos os tecidos. Avaliamos ainda os danos induzidos pelo *stress* oxidativo, como a carbonilação e nitração de proteínas, assim como a peroxidação lipídica. Os resultados demonstraram que a DMT2 diminuiu a expressão da sirtuína 1 (SIRT1) e sirtuína 3 (SIRT3) no tecido testicular. Verificou-se uma diminuição na expressão dos complexos III e V da cadeia respiratória mitocondrial. Não se verificaram alterações no conteúdo do mtDNA testicular e nas atividades das enzimas antioxidantes. No entanto, estes resultados foram acompanhados por um aumento da peroxidação lipídica e nitração de proteínas. Ao nível epididimal, observou-se uma diminuição na expressão do regulador-chave da biogénese mitocondrial, o coativador 1 α do receptor γ ativado pelo proliferador de peroxissoma, assim como a expressão das SIRT1 e SIRT3. Embora não se tenham verificado alterações significativas no conteúdo do mtDNA, a DMT2 induziu uma diminuição significativa da expressão dos complexos II, III e V. Também se observou uma diminuição significativa na atividade das enzimas envolvidas no sistema de defesas antioxidantes, que culminou com o aumento da nitração de proteínas. Os resultados obtidos sugerem-nos que a DMT2 induz uma diminuição da expressão de reguladores-chave da biogénese mitocondrial dos órgãos reprodutivos, comprometendo assim as vias moleculares envolvidas na regulação da função mitocondrial e, consequentemente, na manutenção do sistema de defesas antioxidantes. Desta forma, torna-se essencial aprofundar os conhecimentos na bioenergética mitocondrial para que se possam desenvolver novas abordagens terapêuticas, de modo a atenuar o

aumento da infertilidade masculina, principalmente nos países mais desenvolvidos onde a elevada prevalência das doenças metabólicas é considerado um problema de saúde pública.

Palavras-chave:

Diabetes mellitus; Fertilidade masculina; Testículos; Epidídimo; Biogénese mitocondrial; Stress oxidativo

Resumo Alargado

Os testículos são os elementos centrais do sistema reprodutor masculino. As suas principais funções consistem na produção das células germinativas, processo designado de espermatogénese, bem como na síntese das hormonas esteroides. Nos testículos estão presentes diferentes tipos de células entre elas as células de Sertoli e as células de Leydig. As células de Sertoli desempenham um papel fundamental no desenvolvimento das células germinativas, uma vez que representam o suporte físico e nutricional para o desenvolvimento da espermatogénese. Após serem libertados nos túbulos seminíferos os espermatozoides são células altamente diferenciadas, mas incapazes de fertilizar. É no epidídimo que vão sofrer diversas modificações que vão permitir que esses espermatozoides se tornem maduros, adquirindo mobilidade e capacidade de fertilizar. Este ducto permite também o armazenamento dos espermatozoides férteis num estado viável, dentro da cauda do epidídimo, até serem ejaculados. Dado que tanto o tecido epididimal como o testicular requerem níveis adequados de energia é imprescindível assegurar o metabolismo celular para a manutenção da capacidade reprodutiva do indivíduo.

A diabetes mellitus (DM) é considerada como uma das doenças crónicas mais prevalentes no mundo, que causa sérias perturbações no metabolismo celular. Dado o aumento crescente de número de casos que se tem verificado de ano para ano, a DM é considerada como uma ameaça à saúde humana a nível global. Esta patologia é considerada como um distúrbio metabólico que pode ser dividido em dois tipos principais, tipo 1 e tipo 2. Estes dois tipos podem ser descritos sumariamente como desordens metabólicas caracterizadas por um estado de hiperglicemia (valores elevados 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 das células beta pancreáticas pelo sistema imune, 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 (72-99 mg/dl), levando a exaustão funcional das células beta. Ao longo dos anos tem-se vindo a verificar que a doença começa a ser detetada cada vez mais cedo em crianças e adolescentes.

Devido ao estado hiperglicémico, a DM está associada a uma exacerbada produção de espécies reativas de oxigénio (EROS), sobretudo na mitocôndria, onde ocorre a grande maioria dos processos metabólicos. Esta superprodução destes agentes oxidantes leva a danos celulares a nível do DNA (principalmente do DNA mitocondrial), dos lípidos e das proteínas, sendo um potencial alvo as células germinativas que se encontram desguarnecidas de defesas antioxidantes e dependentes do microambiente criado pelo tecido testicular e epididimal. Numa forma de tentar perceber quais os processos que levam à criação de defesas antioxidantes de forma a neutralizar as EROs, têm-se vindo a elucidar possíveis vias moleculares que ativam as defesas antioxidantes. Em várias delas podemos encontrar

proteínas chave na regulação da homeostase mitocondrial, como é o caso da sirtuína 1 (SIRT1), sirtuína (SIRT3) e o coativador 1 α do receptor γ activado pelo proliferador de peroxissoma (PGC-1 α). O PGC-1 α é considerado como o maior regulador da biogénese mitocondrial, sendo ativado em resposta a diversos estímulos ambientais, tais como a exposição ao calor ou em períodos de fome prolongada. Esta proteína tem a capacidade de ativar a biogénese mitocondrial através da ativação de diversos fatores de transcrição envolvidos no crescimento e divisão mitocondrial, e induz a expressão de proteínas que ativam enzimas antioxidantes, como é o caso da SIRT3. A SIRT3 pertence à família das desacetilases NAD⁺-dependentes, sendo também uma proteína chave como reguladora do metabolismo. SIRT3 exerce a sua função removendo grupos acetilo de proteínas envolvidas em repostas celulares ao *stress* oxidativo, como é o caso PGC-1 α . Além disso, a SIRT3 tem a capacidade de induzir a expressão de enzimas antioxidantes, como é o caso da superóxido dismutase e da catalase.

Assim o objetivo do trabalho foi avaliar os efeitos da DM tipo 2 na expressão das proteínas SIRT1, PGC-1 α e SIRT3 e as implicações em termos oxidativos. Para isso utilizou-se o modelo animal da DM tipo 2, ratos Gotokakizaki (modelo de ratos não obesos que desenvolvem espontaneamente a DM tipo 2). Os animais foram divididos em dois grupos, o primeiro grupo constituído por sete ratos saudáveis e o segundo constituído por sete animais com DM tipo 2. Após oito meses, os animais foram sacrificados e procedeu-se à recolha do tecido testicular e epididimal e avaliou-se a expressão de proteínas-chave envolvidas na regulação da biogénese mitocondrial e a atividade das enzimas envolvidas nas defesas antioxidantes, em particular a glutathione peroxidase, glutathione redutase, superóxido dismutase e catalase, bem como os danos causados pelo *stress* oxidativo nas proteínas e nos lípidos. Para além disso, avaliamos, também o número de cópias mitocondriais e a expressão dos complexos mitocondriais em ambos os tecidos.

Os resultados demonstraram que em ambos os tecidos o eixo molecular SIRT1/PGC-1 α / SIRT3 encontra-se desregulado, uma vez que as expressões destas proteínas se encontram diminuídas. Consequentemente, essa desregulação leva a uma diminuição na atividade das enzimas do sistema antioxidante no tecido epididimal, contrariamente ao que acontece no tecido testicular, onde as atividades das defesas antioxidantes permaneceram inalteradas. Apesar destas observações, verificou-se em ambos os tecidos um aumento da nitratação das proteínas e da peroxidação dos lípidos. Para além disso verificamos uma diminuição nos complexos III e V no tecido testicular e uma diminuição mais acentuada nos complexos II, III e V no tecido epididimal.

Podemos assim concluir que a DM tipo 2 causou uma desregulação das vias moleculares responsáveis pela manutenção da biogénese mitocondrial, como é o caso do eixo molecular SIRT1/PGC-1 α /SIRT3. Por conseguinte o sistema antioxidante também é afetado, promovendo danos oxidativos. No entanto, devem ser feitos mais estudos para elucidar estes mecanismos moleculares tendo em vista o desenvolvimento de novas abordagens terapêuticas de modo a diminuir a incidência da infertilidade masculina.

Abstract

Diabetes mellitus is a metabolic disease and its incidence is reaching epidemic proportions. Classically it can be divided into two types: type 1 diabetes mellitus, characterized by an insulin-dependent state, and type 2 diabetes mellitus (T2DM), where there is a resistance to the action of the hormone. External factors associated with lifestyle, such as eating behaviors, in particular, the excessive consumption of high caloric diets in combination with other factors, such as sedentary lifestyle, are the main causes for the increased incidence of T2DM. The metabolic deregulation associated with T2DM leads to the emergence of other comorbidities, notably a deregulation of male fertility. The hypothalamus-pituitary-testicle axis, also known as a reproductive axis, is sensitive to the metabolic changes induced by T2DM. Recent studies have shown that endocrine and metabolic alterations associated with T2DM affect the physiology of reproductive organs, mainly their mitochondrial bioenergetics. Maintaining mitochondrial function in reproductive organs is imperative for the maintenance of the reproductive capacity of the individual. Thus, we aimed to study the impact of T2DM on the molecular pathways underlying the control of the testicular and epididymal mitochondrial function. For this, we used an animal model of T2DM, in which we evaluated the expression of key proteins involved in the regulation of mitochondrial biogenesis and in mitochondrial function. We measured the activity of the enzymes of the antioxidant defense system. We also evaluated the effects of T2DM on the number of mitochondrial DNA copies and on the expression of the levels of the mitochondrial respiratory chain complexes in both tissues. Finally, we evaluated the parameters of oxidative stress (OS), such as carbonylation and proteins nitration, as well as lipid peroxidation.

Our results showed that T2DM decreased the expression of sirtuin 1 (SIRT1) and sirtuin 3 (SIRT3) in the testicular tissue. There was also a decrease in the expression of complexes III and V of the mitochondrial respiratory chain, but the content of mitochondrial DNA (mtDNA) remained unchanged in the testicular tissue. There were no alterations on the activities of the antioxidant enzymes, however, these results were accompanied by an increase in lipid peroxidation and nitrate of proteins. In the epididymal tissue, a decrease was observed on the expression of the key regulator of mitochondrial biogenesis, the peroxisome proliferator activated receptor γ co-activator 1 α (PGC-1 α), as well as on the SIRT1 and SIRT3 expression levels. Although there were no changes in the mtDNA content, DMT2 has induced a significant decrease in the expression of complexes II, III and V in the epididymis. There were also decreases in the activities of the enzymes involved in the system of antioxidant defenses, which were accompanied by an increase of protein nitration. The results suggested that T2DM disrupted the expression of key regulators of the mitochondrial biogenesis of the reproductive organs, thereby compromising the molecular pathways involved in the regulation of the mitochondrial function and, consequently in the maintenance of the antioxidant defense system. In this way, it is essential to deepen the knowledge in mitochondrial bioenergetics in order to develop possible therapeutic approaches to attenuate the increased decline of male

fertility, especially in developed countries where the prevalence of metabolic diseases is a major public health concern

Keywords: Diabetes mellitus; Male fertility; Testes; Epididymis; Mitochondrial biogenesis; oxidative parameters.

List of Abbreviations

3-NT - 3-nitrotyrosine
4-HNE - 4-hydroxy-2-nonenals
B2MG - nuclear-encoded beta-2-microglobulin gene
AMPK - 5' adenosine monophosphate-activated protein kinase
ATP - adenosine triphosphate
BMP4 - bone morphogenic protein 4
BSA - bovine serum albumin
BTB - blood-testis barrier
CAT - catalase
CREB - cyclic AMP response-element-binding protein
DHAP - fructose-1-phosphate aldolase yielding dihydroxyacetone phosphate
DM - diabetes mellitus
DNA - deoxyribonucleic acid
DNP - 2,4-dinitrophenyl hydrazone
ETC - electron transport chain
FOXOs- forkhead box transcription factors
FOXO3a- forkhead box O3
FSH - follicle-stimulating hormone
GCs - germ cells
GnRH - gonadotropin releasing hormone
GPx - glutathione peroxidase
GK rats- Goto-Kakizaki rats
GR - glutathione reductase
GSSG- oxidized glutathione
GTT-glucose tolerance test
HOMA-IR- Homeostasis assessment model-insulin resistance
HPT-axis - hypothalamus-pituitary-testicles axis
LCs - Leydig cells
LDH - lactate dehydrogenase
LH - luteinizing hormone
mtDNA - mitochondrial deoxyribonucleic acid
mtND1- mitochondrial ND1 gene
NAD⁺ - nicotinamide adenine dinucleotide
NRF1 - nuclear respiratory factor 1
NRF2- nuclear respiratory factor 2
O₂ - oxygen
OAA - oxaloacetate
OS - oxidative stress

PBS - phosphate buffer saline
PGC-1 α - peroxisome proliferator activated receptor γ co-activator 1 α
PPARs - peroxisome proliferator activated receptors
PUFAs - polyunsaturated fatty acids
PVDF - transferred to a polyvinylidenedifluoride
qPCR - quantitative polymerase chain reaction
ROS - reactive oxygen species
RNS - reactive nitrogen species
SCs - Sertoli cells
SCF- stem cell factor
SIRT1 - sirtuin 1
SIRT3 - sirtuin 3
SOD - superoxide dismutase
T - testosterone
T1DM - type 1 DM
T2DM - type 2 DM
TFAM - mitochondrial transcription factor A
TBS-T - Tris buffer solution with 0.05% Tween 20

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I. Introduction

1. Testicular Anatomy

The reproductive organs have an important role to ensure the survival of the species, once it is in the reproductive system that is created the fundamental units of life, the gametes. The sexual reproduction allows the transmission of the genetic code over generations through the variability induced by a combination of genes that ensures the evolution of the species.

The reproductive system is the only body system that is not fully functional at the time of birth, it requires the actions of sex hormones released at puberty to become fully functional. In addition, there are clearly differences between the male and female reproductive system which does not occur in the other body systems (Alves and Oliveira 2017).

Testes are the central element of the male reproductive tract, are paired, whitish, ovoid-shaped organs, suspended outside the abdomen located within the scrotum, which serves as a protective envelope and keeps the testicular temperature approximately 35°C (Alves and Oliveira 2017). The testicular capsule is composed by two layers of membranes: tunica vaginalis, tunica albuginea. The tunica vaginalis is the outer membrane that covers the surface of each testes, except where the testes attaches with epididymis and spermatic cord. The tunica albuginea is a tough fibrous membrane. Extensions of this membrane, in to the testes as fibrous septa result in the formation of approximately 250 pyramidal lobules each of which contains the seminiferous tubules (STs) (Alves and Oliveira 2017).

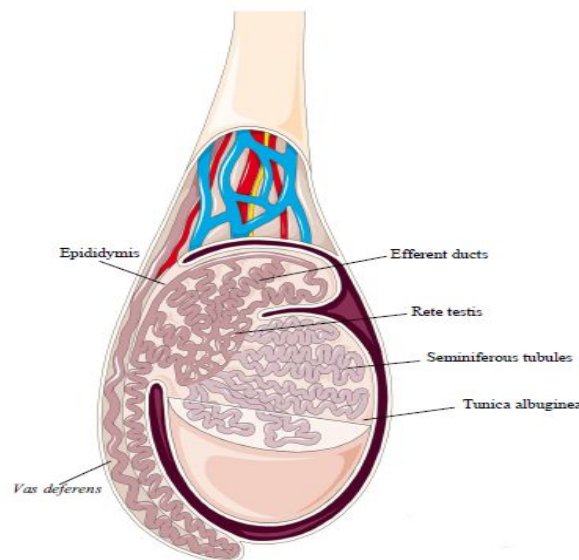


Figure 1: Representation of the human testis. The testicular capsule is composed by tunica albuginea which results in the formation of pyramidal lobules containing the seminiferous tubules culminating in the rete testis which transports the spermatozoa from the seminiferous tubules into the efferent ducts. Adapted from (Alves and Oliveira 2017).

Testes present a bi-functional and a compartmental organization which is highly conserved throughout evolution (Schlatt et al. 1997). Testes show an interstitial space where it can be found the somatic Leydig cells (LCs), blood and lymphatic vessels and cells of the immune system, whereas the seminiferous tubules are composed by peritubular myoid cells (PMCs), Sertoli cells (SCs) and germ cells (Schlatt et al. 1997). These organs present two main functions: the production of sex steroids hormones, mainly testosterone, and the formation of germ cells. The process of production of sperm designated, spermatogenesis, occurs in seminiferous tubules and the steroidogenesis in LCs (Schlatt et al. 1997).

STs are the functional unities of the testes which represents about 80% of the testicular mass (Rato et al. 2010). SCs are specialized polarized epithelial cells that extend from the base of the STs to its lumen. SCs are the first somatic cells to differentiate in the testes and are pivotal in the testes development. These cells contribute to the formation of the BTB (blood-testis barrier) which protects the germ cells from the immune system. The establishment of BTB provides a specific intratubular environment that is dependent on the function of SCs. This creates the adequate condition for the development of spermatogenesis (T. Dias et al. 2016). This is a complex and coordinated process, that takes place in the STs (Komsky-Elbaz et al. 2018). It consists of cell division and differentiation of spermatogonia which culminates in the production of the mature male gametes.

1.1. Sertoli cells

The main function of these cells is nursing germ cells since, behind the physical support, they also provide nutritional support to germ cell line (Rato et al. 2012). SCs produce and secrete, several proteins, and factors that are essential for the germ cell's development (Mruk and Cheng 2004). For instance, SCs produce transferrin, vitamin transporters, lactate, acetate, extracellular matrix components, glial cell-derived neurotrophic factor, fibroblast growth factor 2, growth regulatory factors (stem cell factor, transforming growth factors alpha and beta, bone morphogenic protein 4, and stem cell factor which initiate the differentiation of spermatogonia stem cells, and interleukins (Islam et al. 2017; Walker and Cheng 2005). On the other hand, these cells have an important role in order to maintain the balance in the number of germ cells, using phagocytosis to clean degenerating GCs or residual body from spermatids. This is one of the most critical function, because a considerable fraction of GCs is discarded during spermatogenesis, and the presence of these dead cells into the STs lumen could lead to the release of noxious contents that negatively impact sperm production. SCs can interact with other SCs but also with germ cells, this cell-to-cell interactions are crucial for the regulation of spermatogenesis (Mruk and Cheng 2004). This contact is possible through adherent, tight and gap junctions (Islam et al. 2017). The communication between germ cells and SCs is due to paracrine factors or ligand/receptor-mediated interactions (Rato et al. 2010). Besides that, studies have evidenced that SCs are capable to maintain a unique antiviral defense system through productions of some interferons (IFNs), ILs and Cytokines

(Dias et al. 2016). Metabolically SCs exert their action, producing various substrates, preferentially using glucose through the process glycolysis (Alves et al. 2013). The glucose enters in the cells mainly through GLUT 1 and GLUT 3 and is metabolized by glycolysis in pyruvate which can be converted into alanine, lactate or acetyl-CoA in mitochondria (Mruk and Cheng 2004; Rato et al. 2013). However, the preferred route of glucose is the production of lactate which is then transported to the germ cells mediated by active membrane monocarboxylate transporters (MCTs) via MCT4 (Dias et al. 2016). Lactate stimulates the synthesis of ribonucleic acid and proteins in developing germ cells and also helps to promote the activation of alternative metabolic pathways, such as gluconeogenesis and the pentose-phosphate pathway. Besides that, SCs also produce acetate, which is converted in acetyl-CoA in the cytoplasm or in the mitochondrial matrix by acetyl-CoA synthase. This metabolite is involved in the synthesis of cholesterol and other lipids and it has been proposed that acetate is required to the maintenance of the high rate of synthesis of the lipids and for the intense remodeling of the germ cells membranes during their development. Although, lipids are also used by SC as a source of energy by β -oxidation of fatty acids pathway which is the preferential way to obtain energy. Furthermore, SCs are also capable to convert the branched-chain amino acids in the corresponding branched-chain acids to produce ATP (Alves et al. 2017).

1.2. Leydig cells

Leydig (or interstitial) cells arise from interstitial mesenchymal tissue between the tubules during the eighth week of human embryonic development. These cells are located in clusters in the interstitium between blood vessels and STs and the presence of peritubular myoid cells at the basement membrane of the STs prevent direct physical contact between SCs (Xu et al. 2007). LCs play vital roles in downstream masculinization events and in descent of the testes into the scrotum (Akingbemi, 2005) and, also, act as a support to spermatogenesis, producing the hormone testosterone, under the regulation of pituitary luteinizing hormone (LH), to keep SCs and germ cells functionally and to regulate germ cells maturation (Wong and Cheng, 2009). The secretion of testosterone is around 3-10 mg/day, accounting for more than 95% of total circulating T in post-pubertal men. The hormone acts on SCs stimulating the release of several chemical messengers that function as paracrine agents to stimulate the proliferation and differentiation of the germ cells (Neto et al. 2016; Sharpe et al. 2003).

1.3. Spermatogenesis

Spermatogenesis is a complex and coordinated process, that occurs in the seminiferous epithelium of the STs (Komsky-Elbaz et al. 2018). It consists of cell division and differentiation of spermatogonia which culminates in the production of the mature male

gamete. It can be divided in several steps: the proliferation of spermatogonia; spermatogonial differentiation into spermatocytes; meiotic division of spermatocytes producing spermatids; maturation of round spermatids; and the release of highly specialized mature spermatozoa into the testicular tubule lumen (Bell et al. 2014). Firstly, the spermatogonia ($2n$) suffers mitosis, followed by a cellular transformation from type B spermatogonia into spermatocytes, that suffers meiosis giving rise to the spermatids ($1n$), that differentiate into spermatozoa (Wong and Cheng, 2009). Once formed these cells are released into the lumen and then proceed their journey through the epididymis where several molecular events will occur in order to become capable to fertilize (Bell et al. 2014). Even when spermatozoa reach the female reproductive tract it must ensure efficient energy production to successfully achieve acrosome reaction and capacitation, otherwise, fertilization will not occur. Thus, spermatozoa are able to maintain their energy levels through two manners: through glycolysis and oxidative phosphorylation (OXPHOS). Due to the highly specialized structure of spermatozoa, which allows the compartmentalization of energy production, the ATP generated through OXPHOS occurs in mitochondria, while the synthesis of ATP through glycolysis takes place in the head and the fibrous sheath, due to the absence of respiratory enzymes. Several glycolytic enzymes, such as hexokinase, PFK, LDH and glyceraldehyde-3-phosphate dehydrogenase are present in these segments of spermatozoa.

In fact, both processes may occur independently or in combination depending on the surrounding environment. The preferred substrates of spermatozoa are glucose and fructose. These hexoses are taken up from the seminal plasma fluid through several glucose transporters (GLUTs) present along the plasma membrane (Alves et al. 2013). Then, glucose is metabolized by germ cells originating two molecules of ATP, pyruvate, and nicotinamide adenine nucleotide (NADH). Pyruvate is further metabolized in lactate as an energetic substrate. Furthermore, fructose is internalized and phosphorylated by fructokinase to fructose-1-phosphate, which undergoes hydrolysis by fructose-1-phosphate aldolase yielding dihydroxyacetone phosphate (DHAP) and glyceraldehyde. DHAP is converted to glyceraldehyde-3-phosphate by triosephosphate isomerase or glyceraldehyde kinase. Thus, the intermediaries for the glycolytic pathway are obtained and can finally be converted to pyruvate and lactate for energy production (Dias et al. 2014).

However, in conditions when the availability of glycolytic substrates is scarce, the energy production is ensured by the oxidative phosphorylation in mitochondria. It is known that the most efficient energy process occurs under aerobic conditions and in these conditions, pyruvate is converted in acetyl-CoA by pyruvate dehydrogenase, which then enters the Krebs cycle to combine with oxaloacetate (OAA). OAA is then further oxidized to reduce NAD^+ and the complex II prosthetic group flavin adenine dinucleotide generating carbon dioxide. The oxidation of these substrates is coupled with the phosphorylation of adenosine diphosphate via the mitochondrial electron transport chain, with the subsequent production of ATP. These are the two main metabolic pathways in sperm metabolism, however, the spermatogenesis

process is a high energy process with a high consumption of oxygen (O_2), which consequently leads to an exacerbated production of ROS (reactive oxygen species). This uncontrolled and excessive ROS production, accompanied with a deficient capture of this agents, leads to an oxidative environment. The spermatozoa are the cells more prone to this species, so they are completely dependent (Alves et al. 2017).

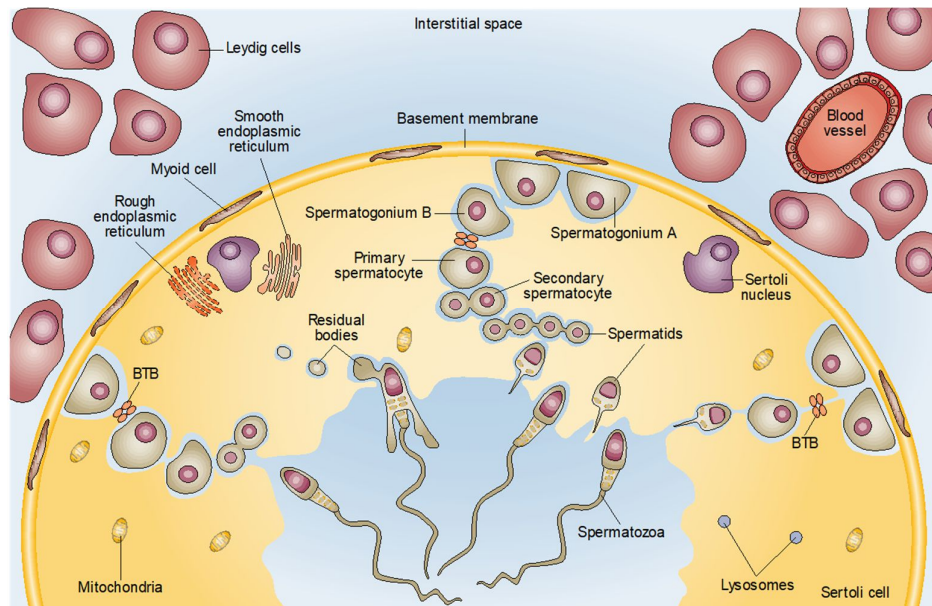


Figure 2: Representation of spermatogenesis. The seminiferous epithelium is composed of Sertoli cells and developing germ cells at different stages. Leydig cells and blood vessels are located in the interstitium. Spermatogenesis is the cellular division and transformation that produces male haploid germ cells from diploid spermatogonial stem cells. Adapted from (Rato et al. 2012).

1.4. Hormonal control of spermatogenesis

The regulation of spermatogenesis is complex and includes the communication between the hypothalamus-hypophysis axis and the gonads. It begins with the release of the gonadotropin-releasing hormone (GnRH) a decapeptide produced by specialized neurons in the hypothalamus that reaches the anterior pituitary via the hypothalamo-pituitary vessels and triggers the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Walker and Cheng 2005). Each of these hormones acts in different sites in the testes but work in concert to control spermatogenesis. FSH acts on the SCs activating the secretion of paracrine agents that are essential for spermatogenesis (Ramaswamy and Weinbauer 2014). LH acts on the LCs stimulating the secretion of the hormone testosterone, that acts as a paracrine agent, moving from the interstitial spaces into the seminiferous tubules (Ramaswamy and Weinbauer 2014). This hormone is essential for the development of the

spermatogenesis and plays an important role in masculinization and the maintenance of male secondary sexual characteristics.

However, some negative feedbacks exerted by testicular hormones are important in order to regulate the process of spermatogenesis. The inhibin secreted mainly by SCs can also suppress the release of FSH. Inhibin acts on the anterior pituitary decreasing the biosynthesis and secretion of FSH through a controlled mechanism that not affects the releasing of LH.

In the other hand, the testosterone produced by LC inhibits mainly LH secretion, through two different mechanisms: testosterone can act in the hypothalamic GnRH release, resulting in a decrease of FSH and LH hormones; or can acts directly into anterior pituitary leading to a decrease in the secretion of LH (Alves and Oliveira 2017). Thus, testosterone have an important role in the maintenance of males infertility once it plays a central role in the development of males reproductive tract and genitalia organs and also is required for the initiation and maintenance of spermatogenesis (Alves and Oliveira 2017). In the absence of testosterone, germ cells cannot undergo beyond meiosis, the formation of BTB is compromised and it prevents the release of mature spermatozoa. In sum, any disruption in this process can compromises the spermatogenesis process and infertility (Walker, 2010).

2. The Epididymis

The epididymis is characterized as a long-convoluted organ of the male reproductive tract. After leaving the STs, spermatozoa follow through the rete testis and efferent ducts, reaching the epididymis (Žaja et al., 2016). The epididymis of mammals, such as mouse and rat, is structurally organized into four main regions: initial segment, the caput (head), corpus (body) and cauda (tail) (Figure 3A), while the human epididymis is poorly differentiated since no initial segment can be distinguished (Cheng et al. 2017). Each section of epididymis has distinct functions: the caput and the corpus are responsible for early and late sperm maturation, respectively, and the cauda storage of functionally spermatozoa (Cheng et al. 2017; Dias et al. 2016). Each region of the epididymis is organized into lobules separated by connective tissue septa, which serves as a support for the organ, but also as the separation between lobules which allows the expression of several genes and proteins within individual lobules and may play a role in creating specialized micro-environments. Each of the epididymal regions has a complex and different epithelium and can be further divided into 10 and 19 intraregional segments, that are separated by septae (Figure 3B) (Sullivan and Miesusset, 2016).

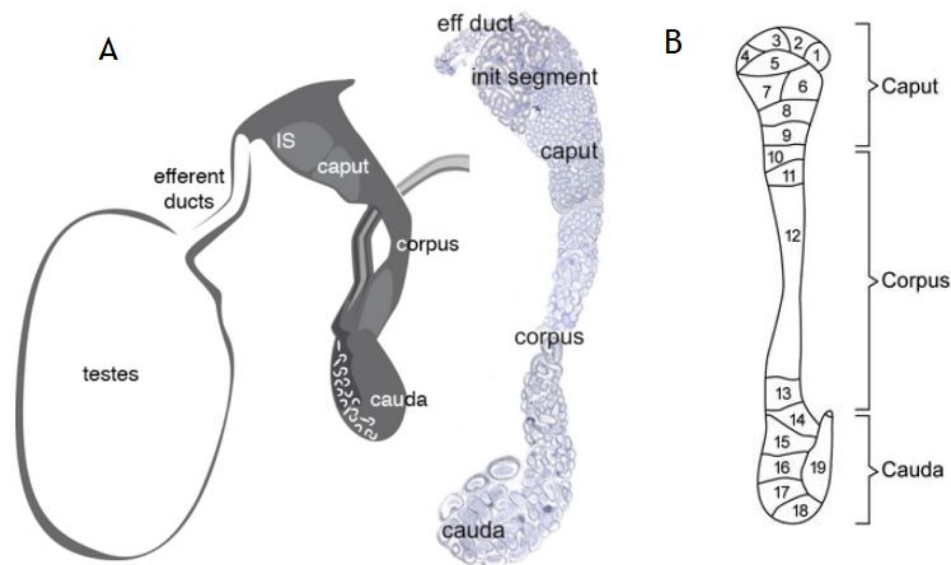


Figure 3: Schematic representation of the Epididymis (A) and typical patterns of rat epididymal segmentation (B). The epididymis can be divided into different parts: the initial segment, the caput and the corpus which are responsible for the early and late maturation of the germ cells respectively; and the cauda which stores the spermatozoa Adapted from (A) Joseph et al. (2011) and (B) Jelinsky et al. (2007)

The epididymal epithelium is composed of several types of cells: principal, apical, basal, narrow cells and halo cells, surrounded by multiple layers of peritubular myoid cells (Figure 4). 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 principal cells are the main cell type in epididymis corresponding a 65-80% of the epithelium. Morphologically, these cells present a columnar structure in the initial region which is converted into low cuboidal cells in the cauda of the epididymis. However, each segment presents differences in the structure and function, as the appearance and organization of the secretory apparatus, such as endoplasmic reticulum, secretory granules and Golgi apparatus, and endocytic apparatus (vesicular bodies, endosomes and lysosomes) (Abou-Haila & Fain-Maurel, 1984). These cells play important functions in transport, synthesize a large number of proteins that are secreted to epididymal lumen and are directly involved in the control of luminal protein concentrations (Robaire and Viger, 1995).

Apical cells have many mitochondria in the apical cytoplasm by few microvilli at the luminal border and by a spherical nucleus that is located in the upper half of the cell cytoplasm. These cells are present in the epithelium of the initial segment and in the epithelium of the intermediate segment and they do not contact with the basement membrane (Sun and Flickinger, 1980). Apical cells differ from principal cells and narrow cells in terms of protein expression. In terms of functions, little is known, but it is suggested that these cells may have an important role in the regulation of pH in the lumen and a possible role in the maintenance of spermatozoa in the quiescent state, through the production of enzymes of the carbonic anhydrase family (Hermo et al. 2005).

Narrow (pencil) cells can be characterized as elongated cells that are present within the epithelium of the initial region and intermediated zone of the epididymis (Adamali and Hermo 1996; Sun and Flickinger 1980). A characteristic of these cells is the fact that present numerous apically located cup-shaped vesicles that are involved in endocytosis and in the secretion of H^+ ions into the lumen (Hermo et al. 2000). These cells display an important role in process of endocytosis as well in the intracellular transport between the epididymal lumen and the epithelial cells (Hermo et al. 2000). One of the most important features of narrow cells is the important role in protecting spermatozoa against the electrolytic imbalance and for the degradation of specific proteins and carbohydrates within their lysosomes (Adamali and Hermo, 1996).

Clear cells are localized in the caput, corpus and cauda regions of the epididymis, these cells are characterized for being large endocytic cells with an apical region containing numerous coated pits, vesicles, endosomes, multivesicular bodies, lysosomes and lipid droplets (Hermo et al. 1988). Indeed, the endocytic activity by clear cells is more pronounced in the cauda of the epididymis. Furthermore, these cells are responsible for removing the contents of cytoplasmic droplets, that were created when spermatozoa are released and contain Golgi saccular components which are related to modifications of the structure of sperm membrane.

These cells are also in charge of the clearance of proteins from the epididymal lumen and participate in the regulation of luminal fluid acidification (Cornwall 2009; Hermo et al. 1988). Basal cells are triangular and flat cells which adhere to the basement membrane and do not have direct access to the lumen of the duct (Veri et al. 1993). These cells have cytoplasmic extensions which suggest a close association with principal cells, and thus may regulate its functions. Indeed, their plasma membrane is constituted by coated pits which may be associated with the receptor-mediated endocytosis of factors from the blood or principal cells. Besides that, it also being demonstrated that basal cells regulate principal cell electrolyte transport by releasing paracrine factors (Cornwall 2009).

Halo cells are easily recognized for being small cells with a narrow rim of clear cytoplasm and are present in all epididymal segments localized in the base of the epithelium. These cells belong to the immune system and are characterized for containing a variable number of dense core granules. In young adult animals, halo cells can be described as helper T lymphocytes, cytotoxic T lymphocytes, and monocytes. With age, in some specific regions, it was verified an increase of these immune cells but also some eosinophils and B lymphocytes, occasionally (Robaire and Viger 1995).

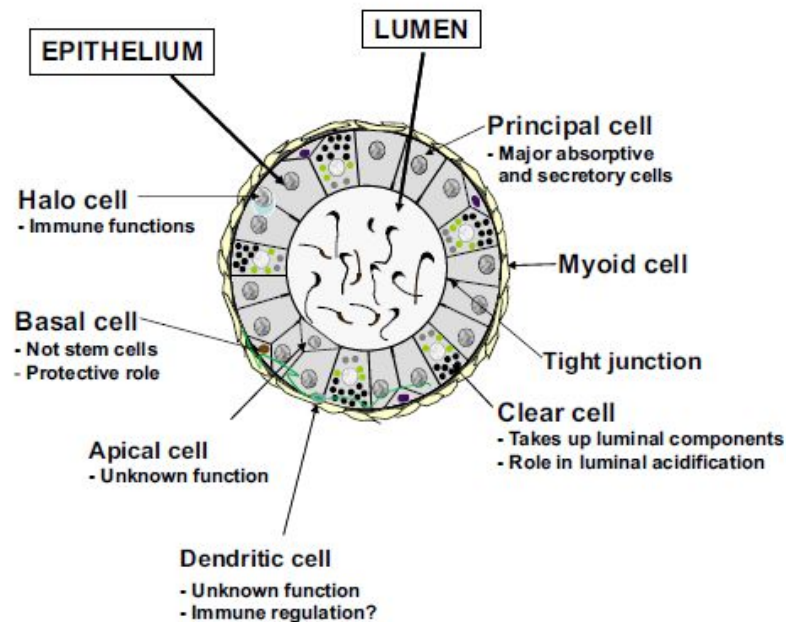


Figure 4: Different cells types and organization of the epididymis. The three epididymal compartments, as well as the relative position and distribution of each of the main cell types, are illustrated. The major function(s) associated with each cell type is also identified. Adapted from (Cornwall 2009).

2.1. Functions of the epididymis

The epididymis duct is a canal, which has the main functions to transport, mature spermatozoa in order to transform immature testicular sperm in competent cells to undergo fertilization, and their storage in a viable state in the cauda epididymis until they are ejaculated (Robaire and Viger, 1995). During the maturational process, spermatozoa include many changes in sperm physiological properties, such as the acquisition of forwarding motility, the ability to recognize and bind to the zona pellucida, and the capacity to fuse with the plasma membrane of an oocyte (Sullivan and Mieusset, 2016). Indeed, the epididymal lumen environment is extremely complex and it shows continuous and progressive changes in its composition from the caput to cauda regions.

Finally, the mature spermatozoa are stored for several days until ejaculation, in a quiescent state in the cauda epididymis. Thus, during the spermatozoa journey along the epididymal tract, it must ensure a controlled microenvironment in order to maintain the sperm viability. Mostly, protect the spermatozoa against the OS since they are highly vulnerable to ROS damages.

2.1.1. Epididymal sperm maturation

In the final stage of spermatogenesis, the spermatozoa are not completely mature, they leave the seminiferous tubules still immatures, without mobility and unable to fertilize the oocyte. Usually, sperm maturation takes place in the caput and corpus region of epididymis, where complex biochemical and physiological changes occur, to become movable and capable to fertilize (Cheng et al. 2017; Hu et al. 2017). In the epididymis lumen there are many components such as specific inorganic ions, small organic molecules, and proteins, that change continuously from the proximal to the distal epididymis to provide a specific environment for sperm maturation (Hu et al. 2017). The process of maturation it is accompanied by biochemical changes as formation of disulphide bonds within the sperm tail and nucleus, oxidation of sperm membrane sulfhydryl groups, increased capacity of glycolysis, modification of adenylate cyclase activity, and alteration in content in the lipid and protein composition of the membrane of spermatozoa (El-taieb et al. 2009). This includes the addition of new proteins, the removal or translocation of some specific proteins, or the modification of the structure of the proteins (Jankovičová et al. 2017). To help the whole process it secretes a specific fluid substance and it is essential for the transport, concentration, storage of spermatozoa and protection (Kamani et al. 2017).

Additionally, epididymis provides the adequate conditions to protect spermatozoa from oxidative injuries through antioxidant scavengers present in the luminal fluid that can be divided into enzymatic and non-enzymatic scavengers molecules (Hu et al. 2017). The non-

enzymatic antioxidants include taurine, glutathione, thioredoxin, and several studies also identified ascorbate as an important non-enzymatic scavenger antioxidant in caput epididymis. The antioxidant enzymes present in the epididymis are SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase) and the idolamina dioxygenase.

2.1.2. Epididymal sperm storage

When spermatozoa enter in the cauda of the epididymis they already get mature. This compartment of the epididymis not only stores the spermatozoa but also contribute to protect spermatozoa against from oxidative damage and to keep the quiescence state through ionic and non-ionic changes (Ghosh et al. 2017).

It takes approximately 10 days until the spermatozoa reach out the cauda of epididymis where they will be stored before ejaculation (Robaire and Hermo 1988). The survival of spermatozoa in the cauda epididymis depends on the species and incubation temperature. In scrotal mammals, the combination of a unique luminal milieu and lower temperatures (30-32°C) are thought to be major contributors to sperm survival. However, if spermatozoa are removed from the cauda and incubated at 32°C in vitro, their fertility and viability are measured in hours rather than days (Jones, 2014).

During this journey, spermatozoa are at risk due to the extreme susceptibility to oxidative damages because of membrane structure that is mainly composed of high quantities of PUFA. Interestingly, it is this membrane structure that allows the intrinsic fusogenic properties that will need to engage the membrane fusion events associated with fertilization (Vernet et al. 2004). However, the presence of high concentrations of PUFA in the membrane became spermatozoa a susceptible target to attack from ROS. Indeed, ROS are important in processes of signal transduction related with several physiological processes in sperm cells such as hyperactivation, capacitation, acrosome reaction, zona pellucida binding and oocyte penetration (Vernet et al. 2004). However, the uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress (OS) which is harmful to spermatozoa.

All cellular components including lipids, proteins, nucleic acids are potential targets of ROS (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). 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).

3. Diabetes mellitus and male fertility

Diabetes mellitus (DM) is one of the diseases of greatest global concern, with a progressive increase in the incidence of the disease over the years. At the beginning of the millennium, the World Health Organization (WHO) reported that 177 million people were affected by DM and around the year of 2030, it is estimated that more the 500 million people may suffer from DM (Agbaje et al. 2007). Many factors have contributed to the DM epidemics, such as the lifestyle of modern societies based on the erroneous eating habits, the lack of physical activity that all together predispose individuals to the development of DM.

DM is a metabolic disorder involving the derangement in carbohydrate, lipids and proteins metabolism. It can be divided into two types: type I and type II. Type I diabetes mellitus (T1DM) or insulin dependents diabetes mellitus which results in the destruction of the insulin-producing pancreatic beta cells by the autoimmune system, which generally develops at a young age but it may affect people of any age (Agbaje et al. 2007). The individuals with T1DM produce low quantities or no insulin, due to this condition is necessary exogeneous insulin to control their blood glucose levels (Canivell and Gomis 2014).

Type 2 diabetes mellitus (T2DM) or non-insulin dependent is the more common form of diabetes that results from defects in action or secretion of insulin. This type of diabetes can be described as the inability of cells to properly respond to insulin in a first stage, compensated by an increase in insulin levels to keep the normoglycemia, but in individuals predisposed to DM type II this mechanism is compromised resulting in a state of hyperglycemia (Asmat et al. 2016; Golay and Ybarra 2005). This is a progressive process because of the limited capacity of pancreatic cells to augment the secretion of insulin to counterbalance insulin resistance, maintaining glucose tolerance at normal levels. Normally the individuals in this condition do not need exogenous insulin action to survive (Golay and Ybarra 2005).

It has been observed an increase of T2DM in children and adolescents, which represents a threat to global health. The sustained hyperglycaemia can cause long term complications which include neuropathies, ophthalmopathies, kidney impairments, cardiovascular diseases and sexual dysfunctions that threaten the quality of life (Kyathanahalli et al. 2014; Aguirre-Arias et al. 2017). Indeed, the prevalence of DM has been increased in young people below reproductive age, moreover, data from animal models strongly suggest that DM impairs male fertility at multiple levels, such as endocrine control of spermatogenesis, spermatogenesis itself, or by impairing penile erection and ejaculation.

Male fertility is defined has the inability of males to produce or deliver fully functioning sperm. According to a medical point of view a couple is considered infertile to conceive if the pregnancy does not occur after one or two years of unprotected intercourse. It is estimated that over 10-15% of couples worldwide are infertile (Hosen et al. 2015). The causes behind

infertility, in some cases, are difficult to define, however, a large number of infertility cases are associated with the sperm abnormalities, such as morphology, motility, concentration and DNA fragmentation (Tunc et al. 2009).

Indeed, it was reported about 90% of chronic diabetic patients suffer from sexual dysfunctions including decreases in sexual libido, potency, erectile dysfunction and ejaculation difficulties (Al-Roujeaie et al. 2017). As well-known DM causes a hormonal and metabolic disruption which cause deleterious effects to male reproductive health. Several reporters had linked diabetes has a cause of infertility, the increase in glycaemic levels can lead to an oxidative environment which becomes toxic to the cells, causing several damages in DNA, proteins, and lipids (Rato et al. 2013). This is consistent with several studies in rats and humans that verified several deleterious effects of diabetes in male fertility. A study performed in type 2 diabetic men showed a decrease in semen parameters and an increased lipid peroxidation, so the authors concluded that OS may have detrimental effects on male fertility potential (Singh et al. 2014). Others have verified a significant reproductive dysfunction that resulted from a decrease in the reproductive organ weights and in sperm counts (Scarano et al. 2006). Also, a study reported in diabetic rats a deficient sperm quality, with a decreased in sperm concentration and motility and fertilization capacity as well as subsequent embryo development, they conclude that these abnormalities may be caused by alterations in steroidogenesis as a consequence of diabetes (Kim and Moley, 2008).

3.1. Diabetes mellitus and oxidative stress

In the testicular and epididymal tissue, spermatozoa possess metabolic processes to ensures the demands of their metabolism. One of the organelles that contribute the energetic of the germ cells are mitochondria which are known to produce significant amounts of ROS. It is due to hyperglycaemic state that increases the pyruvate production with a consequent production of the electron transfer donors, NADH and FADH, that subsequently enhances the electron flux through the mitochondrial electron transport chain. Consequently, there is an increase in the ATP/ADP ratio which leads to a hyperpolarization of the mitochondrial membrane potential. This creates a proton gradient, has a result of the high electrochemical potential difference, which leads to partial inhibition of the electron transport in complex III, resulting in the accumulation of electrons to coenzyme Q. Consequently, this leads to premature pass of electrons to O₂ originating the superoxide anion (Ahmad et al. 2017). It is thought that is this enhanced reduction of coenzyme Q and the generation of ROS that is the main cause for mitochondrial dysfunction, which is critical in diabetes-related metabolic disorders and tissue histopathology (Rolo and Palmeira 2006).

It is important to refer that in adequate concentrations this species plays an important role in some cellular signaling process such as the proliferation, differentiation, apoptosis and

growth regulation (Dobrakowski et al. 2017). For the germ cells, in normal concentrations, ROS are implicated in signal transduction mechanisms including the rate of hyperactivation, capacitation, the ability of the sperm to undergo acrosome reaction, spermatozoa-oocyte fusion and other processes implicated in fertility (Dobrakowski et al. 2017; Ferramosca et al. 2012). The problem is when there is an overproduction of this species and the rate of the removal, by the antioxidant defenses, is not sufficient to neutralize them, which makes them toxic to germ cells.

Sperm cells are vulnerable cells since the cytoplasm of germ cells contains a low concentration of scavenging enzymes and due to the chemistry of the cellular membrane which is mainly composed by large quantities of polyunsaturated fatty acids (PUFA). The main constituent of the lipid membrane is omega-3 polyunsaturated fatty acids which confer the fluidity of the plasma membrane and contribute to sperm structure formation, acrosome reaction, and sperm-oocyte fusion (Nichi et al. 2007; Tang et al. 2014). This species attacks the double bonds in molecules, which weaken the carbon-hydrogen bond on the adjacent hydrogen atom, making it susceptible to cleavage (Nichi et al. 2007).

Another important factor that can be responsible for the infertility is the fragmentation of DNA of the sperm nucleus, which is also a target for ROS (Naji et al. 2016). This species causes nucleotide modifications, such as, the 8-hydroxy-2'-deoxyguanosine, the most oxidized base, when DNA it is damage in infertile men (Hosen et al. 2015). Furthermore, in spermatogenesis occurs the packaging of the sperm DNA, when nuclear histones are replaced by protamines. This process requires transient breaks in sperm DNA through topoisomerase II that breaks DNA strains and replace the histones by protamines. However, if the enzyme topoisomerase has a deficient activity may lead to defective repair and residual DNA fragmentation (Tunc et al. 2009). In fact, a study showed that the levels of DNA damages are more pronounced in the sperms of males with diabetes compared to those without diabetes (Agbaje et al. 2007).

Thus, the damages caused by OS affected the sperm quality and the fertilizing ability (Said et al. 2005). In fact, Rato and collaborators (Rato et al. 2014b) found that diabetes alters testicular metabolism which it is linked with deficient spermatozoa, a decrease in concentration of sperm, the abnormal morphology, and reduced motility. Another study by Kamani and collaborators found that in diabetic rats occurs a decrease of epithelium height, density of epithelium, an increase of fibromuscular layer thickness and lower density of interstitial cells because of the overproduction of ROS by the disease (Kamani et al. 2017).

4. Mitochondria an overview

Mitochondria are pivotal organelles present in almost all eukaryotes, being responsible for multiple important metabolic events, such as the citric acid cycle (also known as Krebs cycle), β -oxidation of fatty acids, and OXPHOS. Besides that, it also plays an important role as a mediator in the induction and execution of apoptosis, to determinate the life and death of the cells. These organelles are classically called as the “powerhouse” of the cell, due to their role in the production of ATP, mitochondria are the principal local where ROS was produced, specifically during OXPHOS.

Each mammalian cell contains several hundreds of mitochondria, with different sizes, shapes, and abundance depending on the cell type. The number of mitochondria in a cell is determined by the processes of biogenesis and division of the organelles in order to maintain functional all the process underlying ATP production, to ensures an adequate supplying of energy to cells. Furthermore, mitochondria may also change under different energy demand and different physiological or environmental conditions (Lee and Wei 2005). This organelle is central in the human physiology so dysfunctional mitochondria may give rise to several pathologies has been implicated in a large range of diseases, such as Alzheimer’s disease. According to the endosymbiotic theory, mitochondria are descendants of the ancient bacterial having their own genome and the capacity of auto replication. The mitochondrial DNA is constituted by a double strand molecule containing 37 genes encoding for 13 proteins, 2rRNAs, and 22 tRNAs. The 13 proteins are the essential subunits of oxidative phosphorylation system, the complexes I, III, IV, and V. The other mitochondrial products are 22 tRNAs (transfer RNAs) and 2 rRNAs (ribosomal RNAs) important for the translocation of the respiratory subunit mRNAs within mitochondrial matrix (Benkhalifa et al. 2014). However, some mitochondrial proteins are encoded by the nucleus, in the cytoplasm of the cell and imported to the mitochondria (Wenz 2013) These proteins are transported by molecular chaperones, unfolded, and imported into mitochondria via the translocase of the outer membrane complex. After transfer across the outer membrane, certain precursors are directed through the import machinery of the inner membrane complex into the mitochondrial matrix in a membrane potential-dependent manner (Ventura-Clapier et al. 2008).

Indeed, the oxidative phosphorylation pathway in mitochondria is the major source of ATP production in eukaryotic cells. During this process, the electrons flow from the reduced substrates, NADH and FADH, through the electron chain composed of respiratory H^+ pumps (complexes I-IV) until being delivered to O_2 . The complex I and II received the electrons from NADH and FADH, respectively, and delivers those electrons to O_2 , at the complex IV. This electron flow is coupled to proton ejection, forming an electrochemical gradient that is used to produce ATP at complex V (ATP synthase). Although, during the electron flow the, can electrons form complex I and III can directly react to O_2 or other electron acceptors and origin

ROS. In fact, mitochondria are the major source of ROS production. Furthermore, it is estimated that during the oxidative phosphorylation 1-2% of the electrons are converted in the superoxide anion and hydrogen peroxide, through the incomplete reduction of O₂ (Santos et al. 2001).

Furthermore, mitochondria play an important role in the apoptosis process. This organelle has the important function to trigger cellular death in order to make a rapid decision (if necessary) to initiate programmed cell death (Lee and Wei 2005).

4.1. Mitochondrial Biogenesis

Mitochondrial biogenesis can be characterized as the growth and the division of pre-existing mitochondria which is campaigned by several variations in mitochondrial size, number, and mass. This is a well-coordinated process involving several proteins that regulates all the process. One of the most important is PGC-1 α (peroxisome proliferator activated receptor γ co-activator 1 α) that belongs to the family of transcriptional co-activators, being the most studied member. This protein is expressed in tissues with high energetic demands like the brain, liver, cardiac tissue and skeletal muscle. It is not only an important regulator of mitochondrial biogenesis program but is also involved in several stress programs and might thereby integrate mitochondrial biogenesis into cellular stress adaption.

PGC-1 α can be activated via PPAR (peroxisome proliferator activated receptors), AMPK (5' adenosine monophosphate-activated protein kinase) or SIRT1(sirtuin1) pathway (Hofer et al. 2014) and it interacts with several transcription factors including nuclear hormone receptors, nuclear respiratory factors, and specific transcription factors to regulate glucose metabolism and response to environmental stimuli such as cold exposure and prolonged starvation (Fu et al. 2016). Thus, PGC-1 α stimulate the mitochondrial biogenesis activating nuclear transcription factors leading to the transcription of nuclear respiratory factors 1 and 2 (NRF1 and NRF2). which up-regulates the transcription of several nuclear-encoded respiratory genes and induced the expression of mitochondrial transcription factor A (TFAM). Therefore, TFAM activates the mitochondrial transcription factors B1 and B2, essential components of the mtDNA (mitochondrial deoxyribonucleic acid) transcriptional machinery inducing the transcription and replication of mitochondrial genome. This is support by studies done in muscle cells that demonstrate the ability of PGC-1 α to bind and co-activate the transcription factor NRF1 e NRF2.

In addition, PGC-1 α interacts to with other transcription factors such as hormones, glucocorticoids, estrogen, an estrogen-related receptor α , and peroxisome proliferator-activated receptor α to enhance the transcription of mitochondrial fatty acid and β -oxidation (Fu et al. 2016). In cases of energy deprivation PGC-1 α has activated through NAD⁺ dependent deacetylase, SIRT1 which deacetylates multiple residues in PGC-1 α promoting mitochondrial fatty acid oxidation in response to low levels of glucose (Zachary Gerhart-Hines).

Furthermore, it has been reported that PGC-1 α in muscle cells can increase the expression of ROS scavenging enzymes, preventing mitochondrial OS, like GPx1 and SOD2 (Kong et al. 2010; St-Pierre et al. 2003). Besides that, PGC-1 α regulates the expression of genes involved in gluconeogenesis, as is the case of the protein phosphoenolpyruvate carboxyl kinase and glucose-6-phosphate (Kong et al. 2010; Wenz 2013). SIRT1 belongs to a conserved family of proteins, the Sirtuins (SIRT1-7) which are class III NAD⁺ dependent histone deacetylase (Kolthur-Seetharam et al., 2009) distinct from class I and II histone deacetylases. SIRTs also possess ADP-ribosyltransferase activity (Kong et al. 2010). This group of proteins is activated in response to different factors, such as an increase in NAD⁺ levels associated with metabolic and redox stresses, including several cellular processes like energy turnover, glucose metabolism, DNA repair and autophagy (Rogacka et al. 2016).

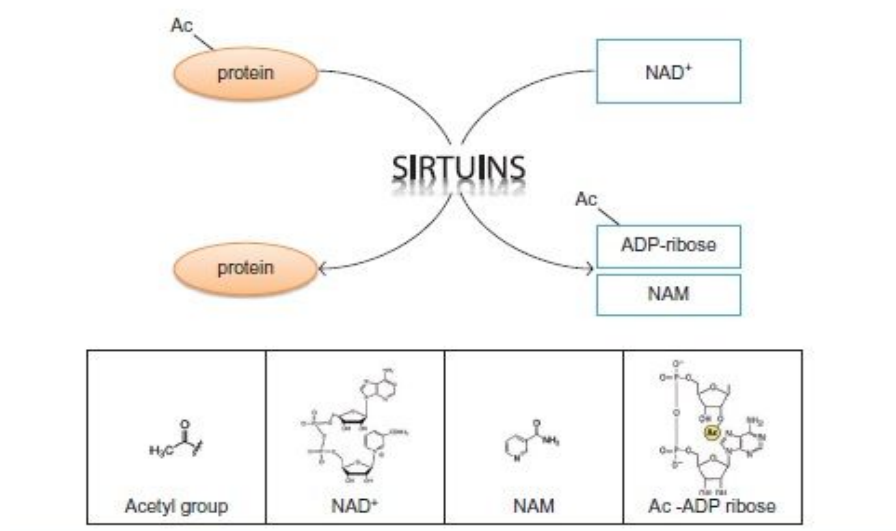


Figure 5: The reaction catalyzed by Sirtuins. Sirtuins deacetylates, i.e., removes the acetyl groups from their substrates in the lysine residue, in a reaction that consumes NAD⁺ (nicotinamide adenine dinucleotide) releasing NAM (nicotinamide), Acetyl-ADP (adenosine diphosphate) ribose and the deacetylated protein. Adapted from (Tatone et al. 2018).

SIRT1 is the most conserved mammalian NAD⁺-dependent protein deacetylase, occurring in various metabolic tissues and has been recognized as a key metabolic regulator. In the presence of NAD⁺, this protein catalyzes deacetylation of a variety of non-histone targets, with O-acetyl-ADP-ribose and nicotinamide as side products. NAD⁺ is an essential coenzyme found in all living cells, and its level regulates SIRT1 activity. The involvement of NAD⁺ in deacetylation associates the SIRT1 activity with metabolism (Rogacka et al. 2016).

In situations of caloric restrictions more, carbons are oxidized producing NAD⁺ from NADH what shifts the activations of sirtuins activity (Wang 2014). Indeed, when the ratio NAD⁺/NADH is high or the levels of ATP/AMP are reduced the AMPK becomes active (Cantó et al. 2009; Rogacka et al. 2016). AMPK is an important metabolic energy sensor and a master

regulator of metabolic homeostasis being closely linked with insulin resistance and glucose homeostasis (Rogacka et al. 2016; Tartarin et al. 2012). It is being suggested that AMPK activates SIRT1, both proteins act in concert with the major regulator of mitochondrial biogenesis, PGC-1 α , that suffers post-translational modifications, such as acetylation exerting its action on the respective target genes (Amat et al., 2009). In addition, when the redox status of the cells is altered SIRT1 activated several redox-related targets including p53, FOXOs (forkhead box transcription factors) (Cerutti et al. 2014; Nemoto et al. 2005) and the nuclear factor NF-Kappa B (Autiero et al. 2009). P53 is a transcriptional factor that activated genes involved in antioxidant defenses, like SOD2 and GPx1, as well, the FOXO3a (forkhead box O3) protein which induces antioxidants responses through regulation of SOD2 and catalase.

SIRT3 is another deacetylase, localized in mitochondria and it is synthesized as an inactive protein after being transported into the matrix where is activated through proteolytic processing at the N-terminus (Kong et al. 2010; Liu et al. 2017).

The main function of this protein is deacetylate enzymes involved in cellular metabolism, genomic integrity, tumor suppression, responsive to OS and mitochondrial integrity (Tseng et al. 2013). The first substrate for SIRT3 is Acetyl-CoA synthetase 2 (AceCS2), a mitochondrial enzyme that converts acetate into acetyl-CoA. In this case, the SIRT3 deacetylates AceCS2 at lysine 642 activating the AceCS2, which leads to an increase in acetyl-CoA production and in turn enters in the tricarboxylic acid cycle (Kong et al. 2010). Another target of SIRT3 is acyl-CoA dehydrogenase, an enzyme involved in the β -oxidation of long chain fatty acids. The deacetylation of this enzyme leads to its activation and consequently the catabolism of the lipids (Hirschey et al. 2010).

Additional physiologic SIRT3 substrates are glutamate dehydrogenase and a component of complex I, the subunit denominated NDUFA9, both are acetylated by SIRT3 at lysine 370 (Kong et al. 2010). It is thought that this subunit has an important role in stabilization of the enzyme complex and may be involved in regulation of its activity as well the preventing against ROS. Besides complex I, SIRT3 interacts directly with complex II, linking to this complex deacetylating it and regulates its enzyme activity. However, the mechanism of activation of complex III remains unknown (Bause and Haigis 2013).

In situations of disruption of the redox cellular environment, SIRT3 is able of activated several detoxifying enzymes including, SOD2 through deacetylation, directly, in lysine 122 (Liu et al., 2017). Besides that, SIRT3 can activate FOXO3a, a transcriptional activator involved in OS triggered cell response, which upregulates the expression of PGC-1 α and TFAM supporting the mitochondrial bioenergetic function (Tseng et al. 2013).

Indeed, several studies suggested that SIRT3 contribute to mitochondrial biogenesis through activation of PGC-1 α in hepatocytes due to phosphorylation of AMPK, which induce PGC-1 α gene expression. Kong and collaborators proposed another mechanism of activation of PGC-1 α

by SIRT3 is through coactivation of CREB (cyclic AMP response-element-binding protein) phosphorylation forming a positive-feedback loop (Kong et al. 2010).

In the other hand, SIRT3 can be also activated by PGC-1 α mediated by ERR α promoting an antioxidant environment. Summarizing, they propose that PGC-1 α /SIRT3 acts cooperatively in which PGC-1 α induces the expression of SIRT-3 and in return SIRT3 stimulates PGC-1 α gene expression. This synergy between PGC-1 α /SIRT3 it is required to keep the mitochondrial homeostasis (Kong et al. 2010; Rato et al. 2014).

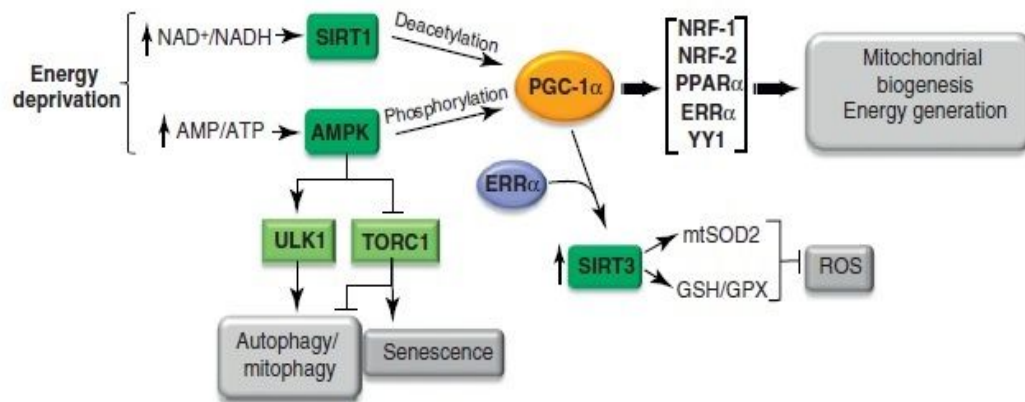


Figure 6: Integration of energy-generating and antioxidant pathways. PGC-1 α is activated via phosphorylation by AMPK or by deacetylation via SIRT1 in response to nutrient deprivation. Induction or activation of the coactivator can enhance mitochondrial biogenesis and oxidative function through the coactivation of multiple transcription factors, involved in respiratory gene expression. PGC-1 α activation may also promote an antioxidant environment by coactivating ERR α to induce SIRT3, a mitochondrial sirtuin that has been implicated in ROS detoxification. Adapted from (Scarpulla et al. 2012).

4.2. The role of mitochondria bioenergetics in male fertility

The role of mitochondria in male reproduction is extremely important, where these organelles play several roles in sperm maturation, capacitation, and motility. Furthermore, mitochondria also seem to be required to ensure the quality of the sperm, being a requirement for the success of the fertilization (Ferramosca et al. 2012).

In fact, the mitochondria of germ cells are structurally and functionally different from mitochondria of somatic cells. The main reason for these differences is due to the fact that spermatozoa, which are terminal cells, with the particular function of fertilizing the oocyte in the female genital tract. Thus, sperm has the ability to undergo metabolic adaptations according to the availability of substrates. Indeed, the spermatocytes, which are closer to luminal regions of STs and so away from blood vessels, the main energy pathway is glycolysis, while spermatogonia located on the basal membrane, and so close to the O₂ supply, prioritize

OXPHOS (Varum et al. 2009). Another important factor that it is important to ensure the functional spermatogenesis and development of normal mature sperm is apoptosis. This is a selective process that keeps the homeostasis in the organism. For the germ cells, this process occurs to counteract the excess of clonal expansion during spermatogenesis. Indeed, many studies emphasized that germ cells with alterations in mitochondrial structure and function in asthenozoospermic subjects suggested an important involvement of these organelles in energy production for sperm motility. It is very important ensures the motility of the spermatozoa, any disruption in mitochondria efficiency can compromise the metabolism/production of the substrates and consequently the production of ATP, which can lead to a decrease in spermatozoa motility and an increased in the ROS leading to oxidative cell injury (Darr et al. 2016).

Several factors can disrupt the mitochondrial viability as T2DM, that causes an overproduction ROS and consequently a disruption in the normal flux of the electrons in the electron transport chain of mitochondria, leading to an impairment of mitochondrial bioenergetics which may compromise the viability of the cells (Ahmad et al. 2017). Since, germ cells, due to the biochemical nature of the membranes are very prone to the attack of the ROS. Thus, it is important that these agents remain in the right concentrations in our cells in order to not become toxic to germ cells. Given that, it is noteworthy the role of several proteins as regulators of mitochondrial function as SIRT1, PGC-1 α , and SIRT3.

It has been demonstrated that SIRT1 induces GnRH acting as a modulator of the reproductive axis, which play an important role in the production of the hormones LH and FSH, extremely important for the development and maturation of the testes (Di Sante et al. 2014). Since SIRT1 is highly expressed in the gonads (Di Sante et al. 2014). Several studies have enhanced the role of SIRT1 in male fertility. For instance, a study done by Coussens and collaborators shows that if SIRT1 is deficient in mice displays a number of abnormalities. The authors verified that both mice with SIRT1-/- and SIRT1+/- shows a reduced number of germ cells, as well a small and abnormal seminiferous tubule, several abnormalities in the morphology of sperm and elevated DNA damages in germ cells (Coussens et al. 2008). Furthermore, Bell and collaborators reported that mice lacking SIRT1 in male germ cells show a decreased in sperm count, deficient morphology and an increase in DNA damages (Bell et al. 2014), which it agrees with the previous study. Indeed, this sperm defects observed in SIRT1-/- mice could be a consequence of OS. Since SIRT1 cooperates with the transcriptional co-activator peroxisome proliferator-activator receptor- α coactivator (PGC-1 α), and consequently to SIRT3 to trigger antioxidant defenses system.

It has been proposed by Kong and collaborators that PGC-1 α can be activated by SIRT3 mediating CREB phosphorylation (Kong et al. 2010). Furthermore, PGC-1 α coactivates ERR α which induces the expression of SIRT3 and consequently the antioxidant defences (SOD2, CAT). Additional, Rato and collaborators studied the interaction between these proteins in testes of prediabetes rats, and they observed that both proteins are reduced in the

prediabetic group, suggesting that the reduced expression of PGC-1 α influence the expression of SIRT3, leading to a testicular decrease in SIRT3 protein content (Rato et al. 2014). Consequently, an increase in levels of carbonyl content in testes and a decrease in testicular mtDNA causing by the oxidative environment due to the disruption of PGC-1 α /SIRT3 axis (Rato et al. 2014). Both reported the synergy between SIRT3 and PGC-1 α which is consistent, to the fact that both proteins stimulated the ROS-detoxifying system and the deregulation of both leads to an increase of ROS. Since fertility is very sensitive to redox perturbations related to aging and metabolic dysfunctions are essential to ensure the adequate oxidative environment to keep the viability of the cells. Thus, the mediators of OS, such as SIRT1, SIRT3, and PGC-1 α has being emerged as promising proteins in the responses of cellular stress.

II. Aims of the study

T2DM incidence has reached epidemic proportions coinciding, with an increase of the associated reproductive health problems. The disruption of testicular signaling pathways induced by T2DM has been on the basis of male infertility associated with this disease. Alterations induced by T2DM affect not only molecular pathways governing the metabolism of reproductive organs but also leads to mitochondrial dysfunction, compromising male fertility. The maintenance of normal mitochondrial function throughout male reproductive tract is essential to ensures the viability of the sperm and consequently fertility.

The general aim of this work was to disclose the effects of T2DM in the signaling pathways that govern the mitochondrial function in the testes and epididymis.

To achieve this, we evaluated the expression of protein levels of key regulators of the mitochondrial function, specifically the peroxisome proliferator-activated receptor α coactivator 1 α , sirtuin 1 and sirtuin 3. We also evaluated the protein levels of the complexes of the electron transport chain (CI-CV), as well as the mitochondrial DNA copy number. Then, we determined the impact of T2DM on the activities of enzymes of the antioxidant system, namely GPx, glutathione reductase (GRx), SOD, and CAT. Additionally, we also evaluated the OS damages, such as lipid peroxidation, protein nitration, and carbonylation, both in the testicular and in the epididymal tissues.

III. Materials and methods

1. Chemicals

Anti-3-Nitrotyrosine antibody (9691S) , and anti-SIRT3 (D22A3) were purchased from Cell Signalling, (Danvers, MA, USA); rabbit-anti 4-Hydroxynonenal (ab5605) and total OXPHOS (ab110413), was purchased to Abcam (Cambridge, United Kingdom); anti-DNP antibody (D9656) and anti- α -tubulin (T9026) was purchased from sigma Aldrich (St Louis, USA); rabbit anti-SIRT1 (sc-15404 and) and rabbit anti-PGC-1 α (sc-13067) were purchased to Santa Cruz (Biotechnology, USA); B-actin (MA515739) was purchased from Thermo Fischer (Waltham, EUA). The secondary antibodies goat anti-rabbit IgG-HRP (sc-2004), mouse anti-goat IgG-HRP (sc-2354) and goat anti-mouse IgG-HRP (sc- 2005) were purchased from Santa Cruz, Biotechnology, USA. Western Bright ECL substrate was purchased from Advansta (Menlo Park, CA, USA). Genomic DNA Kit Tissue GK03.0100, (Grip) was purchased from GRISP, Lda (Porto, Portugal), iTaqTM Universal SYBR Green Supermix, Rat Insulin Enzyme Immunoassay kit was purchased from SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Animal model and experimental design

The present study used 14 male rats: 7 middle-aged (8 months old) Goto-Kakizaki (GK) rats (a non-obese model that spontaneously develop T2DM early in life (Candeias et al., 2018) and 7 age-matched control rats. The animals were obtained from Charles River (Barcelona, Spain) and Taconic (Ejby, Denmark), respectively, and maintained in the animal colony of Animal Research Centre of the University of Coimbra, under controlled light (12 h day/night cycle) and humidity (45-65%) with standard hard pellets chow and sterile water *ad libitum*. Both groups received a sterile saline infusion. Signs of distress were carefully monitored and glucose tolerance tests (GTT) was used as a selection index. All animal experiments were used upon ethical approval by the Animal Welfare Committee of the Centre for Neuroscience and Cell Biology and Faculty of Medicine, University of Coimbra. 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 European directives for the care and handling of laboratory animals (Directive 2010/63/EU). In accordance with the Portuguese law (Ordinance no. 1005/92 of 23 October), the research team requested a permission to perform this animal experimentation study to the Portuguese “Direcção Geral de Veterinária” (Portuguese Veterinarian and Food Department).

3. Glucose tolerance test

At 8 months of age, rats of the control group and T2DM GK rats were submitted to a glucose tolerance test as described by (Assis et al. 2009; Bowe et al. 2014) with some alterations. Briefly, approximately 16 hours before the test, food was taken from the cages and animals were kept in fasting. The blood glucose levels were determined before intra-peritoneal injection of 2 mg D-glucose/g body weight (basal glycemia) and after 15, 30, 60, and 120 min. At the end of the test, cages were supplied with wet food. Results were expressed as milligrams glucose per decilitre blood and as area under the curve (AUC).

4. Insulin levels

Before the intra-peritoneal injection of D-glucose (0 min) blood from the caudal vein of fasted rats was collected and centrifuged at 572.g in a Sigma 2-16 PK centrifuge, for 10 min at 4 °C. The resulting plasma was used to determine fasting insulin levels through the Rat Insulin Enzyme Immunoassay kit (Montigny le Bretonneux, France), according to the manufacturer's instructions. Absorbance was read at 405 nm in a SpectraMax Plus 384 multiplate reader, when maximum binding (B0) wells ranged from 0.2 to 0.8 arbitrary units. Results were expressed nanograms per millilitre for plasma insulin levels. Homeostasis assessment model-insulin resistance (HOMA-IR) index was calculated using the formula: $\text{HOMA-IR} = (\text{fasting insulin } [\mu\text{U/mL}] \times \text{fasting glucose } [\text{mmol/L}]) / 22.5$.

5. Total protein extraction

The testicular and epididymal tissue were homogenized in an appropriate volume of lysis buffer (with freshly added 20mM of sodium fluoride, 100mM sodium orthovanadate and 1% of protease inhibitor cocktail) and allowed to stand 20 minutes on ice. The homogenates were centrifuged at 14000.g in Hettich Mikro 200R centrifuge for 20 minutes at 4°C. After centrifugation, the pellet was discarded. The total protein concentration was quantified using the Bradford Protein Assay Kit II from Bio-Rad (Hercules, USA) according to the manufacturer's instructions and the absorbances were measured by xMark Microplate Spectrophotometer from Bio-Rad (Hercules, USA). Protein concentration was determined using different bovine serum albumin (BSA) concentrations as standards for calibration. Optical densities of samples were determined at 595 nm. All sample volumes were adjusted with lysis buffer to have the same final protein concentrations.

6. Analysis of nitration and lipid peroxidation

To evaluate the protein nitration and lipid peroxidation the slot blot was used. Each sample of testicular and epididymal protein extract was diluted to a concentration of 0.05µg/µl using phosphate buffer saline (PBS) and transferred to a polyvinylidenedifluoride (PVDF) membrane.

Membranes were previously activated for 1 minute in methanol, 5 minutes in sterile H₂O and 15 minutes in PBS. The technique was performed using a Hybrid-Slot manifold system (Biometra, Göttingen, Germany). The membranes were then blocked for 60 min with 5% non-fat milk Tris buffer solution with 0.05% Tween 20 (TBS-T) containing 5% skimmed dried milk. Afterwards membranes were incubated overnight with rabbit anti-3-Nitrotyrosine antibody (1:5000; 9691S) and goat anti 4-Hydroxynonenal (1:5000; AB5605). Samples were visualized using a secondary antibody goat anti-rabbit IgG-HRP (1:10000; sc-2004) and mouse anti-goat IgG-HRP (1:10000; sc-2354), respectively. Membranes were then reacted with WesternBright™ ECL (Advansta, Menlo Park, USA) and visualized on the Chemidoc MP Imaging System from Bio-Rad (Hercules, USA). Densities from each band were obtained with Image Lab Software 5.1 from Bio-Rad (Hercules, USA).

7. Analysis of carbonyl groups

Protein carbonyl groups were evaluated by slot blot. Samples were derivatized using 2,4-dinitrophenylhydrazine described previously by Levine and collaborators (Levine et al. 1990). Briefly, 5µg of lyophilized testicular and epididymal tissue homogenized in PBS was mixed with the same volume of sodium dodecyl sulfate (12%). The samples were then mixed with two volumes of 2,4-dinitrophenylhydrazine 20mM diluted in trifluoroacetic acid 10% and incubated in the dark for 15-20 min at the room temperature. Afterwards, 1.5 volumes of 2M Tris with 18% of β-Mercaptoethanol were added to the samples to stop the reaction. Samples were then diluted to a concentration of 0.05µg/µl using PBS. PVDF membranes activated for 1 minute in methanol, 5 minutes in sterile H₂O and 15 minutes in PBS. Slot blot was performed using a Hybrid-Slot manifold system (Biometra, Göttingen, Germany). The membranes were then blocked for 60 min with 5% non-fat milk TBS-T containing 5% skimmed dried milk. Then blocked membranes were incubated overnight with rabbit anti-DNP antibody (1:5000; D9656, Sigma Aldrich). Membranes were incubated using a secondary antibody goat anti-rabbit IgG-HRP (1:10000; sc-2004, Santa Cruz, Biotechnology, USA). Membranes were then reacted with WesternBright™ ECL (Advansta, Menlo Park, USA) and visualized on the Chemidoc MP Imaging System from Bio-Rad (Hercules, USA). Densities from each band were obtained with Image Lab Software 5.1 from Bio-Rad (Hercules, USA).

8. Western Blot

Total protein extracted from testicular and epididymal (50 µg) was mixed with the supplemented lysis buffer plus loading buffer (50% Glycerol (v/v), 20% Tris-HCl (v/v), 10% Sodium dodecyl sulfate (SDS) (w/v), 1.25% β -mercaptoethanol (v/v) and 0.05% bromophenol blue (v/v), pH=6.8). Samples were separated in 11% polyacrylamide gel (SDS-page). After electrophoresis, proteins were transferred to previously activated PVDF membranes. Then, membranes were blocked at room temperature with 5% non-fat milk TBS-T containing 5% skimmed dried milk. The membranes were then incubated overnight at 4°C with rabbit polyclonal primary antibody against PGC-1 α (1:1000, sc-13067 rabbit), SIRT3 (1:1000; D22A3), and rabbit anti-SIRT1 (1:1000, sc-15404), total OXPHOS (1:1000; ab110413), β -actin (1:100000; MA515739) and anti- α -tubulin (1:10000; T9026). After washing in TBS, the membranes were incubated with a secondary antibody goat anti-rabbit IgG-HRP (1:10000; sc-2004) and goat anti-mouse IgG-HRP (1:10000; sc-2005), respectively. Membranes were reacted with WesternBright™ ECL (Advansta, Menlo Park, USA) and visualized with the Chemidoc MP Imaging System from Bio-Rad (Hercules, USA). Densities from each band were obtained with Image Lab Software 5.1 from Bio-Rad (Hercules, USA). The band density attained was divided by the corresponding α -tubulin or β -actin band intensity and expressed in fold-variation versus the control group.

Table 1: List of primary and secondary antibodies used in western blot technique.

Antibody	Host specie	Molecular weight (kDa)	Diluition	Reference
PGC-1 α	Rabbit	100	1:1000	sc-13067
SIRT1	Rabbit	75	1:1000	sc-15404
SIRT3	Rabbit	27	1:1000	D22A3
Total OXPHOS	Mouse	20,30,40, 48 and 53	1:1000	ab110413
α -tubulin	Mouse	50	1:10000	T9026
β -actin	Mouse	48	1:10000	MA515739
Rabbit	Goat	-	1:10000	sc-2004
Mouse	Goat	-	1:10000	sc-2005

9. Mitochondrial DNA relative copy number

Total DNA was extracted from tissues epididymal and testicular using the Genomic DNA Kit Tissue (GK03.0100, Grisp), according to the manufacturer's instructions. mitochondrial DNA (mtDNA) relative copy number of the experimental groups was determined by quantitative polymerase chain reaction (qPCR) analysis. Relative quantification of mtDNA levels was determined by the ratio between the mitochondrial ND1 (mtND1) gene and the single-copy, nuclear-encoded beta-2-microglobulin (B2MG) gene. qPCR reactions were carried out in CFX 96 Connect Real-time system (Bio-Rad, Richmond, CA, USA), and the efficiency of the reactions was determined for the selected primers using serial dilutions of DNA samples. The specificity of the amplicons was determined by melting curve analysis. qPCR amplifications used 1 µl of diluted cDNA in a 20 µl reaction containing: 1X iTaq™ Universal SYBR Green Supermix, 200 nM of forward and reverse primers for each gene and sterile nuclease-free H₂O. Amplification conditions comprised 3 minutes of denaturation at 95 °C, followed by 30 cycles at: 95 °C for 10 seconds, a specific annealing temperature for 30 seconds and 72 °C for 15 seconds. Each reaction was run in triplicate to calculate relative mtDNA copy number. Ct values of all samples were within the linear range. Ct value differences were used to quantify mtDNA copy number relative to the B2MG gene with the following equation: Relative copy number = $2^{\Delta Ct}$, where ΔCt is $Ct_{B2MG} - Ct_{ND1}$.

Table 2: Genes, oligonucleotide sequence and respective conditions for PCR amplification.

Gene	Sequence (5'-3')	Amplification	AT (°C)
ND1	Sense: GAGCCCTACGAGCCGTTGCC	271	58
	Antisense: GCGAATGGTCCTGCGGCGTA		
B2MG	Sense: GCGTGGGAGGAGCATCAGGG	264	58
	Antisense: CTCATCACCACCCCGGGGACT		

10. Glutathione peroxidase activity

The quantification of Glutathione Peroxidase (GPx) activity was performed we use an indirect determination assay, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalysed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing GR and NADPH. The oxidation of NADPH to NADP⁺ is indicative of GPx activity. To perform this assay, we diluted, 50 µg of the protein from epididymal tissue in glutathione peroxidase assay buffer (50 mM of Tris-HCl, pH 8.0 containing 0.5 mM EDTA). Then, we mixed 5 mM of NADPH, 42 mM of reduced glutathione and 10 U/ml of glutathione reductase with the assay buffer. The reaction was started by the addition of 30 mM butyl hydroperoxide solution. Glutathione peroxidase activity was measured following the decrease of absorbance at 340 nm using a spectrophotometer (UltrospecR 3000, Pharmacia Biotech, Cambridge, England) at 25°C. The activity of the enzyme was determined using the molar extinction coefficient of 6.22 mM⁻¹.cm⁻¹ and expressed as Units/mg.

11. Glutathione reductase activity

The assay of Glutathione Reductase activity was based on the reduction of GSSG and posterior reconversion to GSH by GR, using one molecule of NADPH. Briefly, 100 µg of epididymal protein homogenate were diluted in glutathione reductase assay buffer (100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA) and 1 mg/ml BSA. Then, this mixture was incubated for 10 min. at 25°C in a reaction buffer containing 2 mM oxidized glutathione solution, the glutathione reductase assay buffer and 3 mM of 5,5-dithiobis (2-nitrobenzoic-acid) (DNTB). The reaction was started by the addition of the 2 mM NADPH. The assay was performed in 96 wells microplate and the enzymatic activity was measured following the increase of absorbance at 412 nm using a spectrophotometer (Bio-Rad xMARKTM, Microplate Spectrophotometer). The activity of glutathione reductase was calculated by the mean of the slopes, obtained at the 0 seconds and at 88 sec, using a molar extinction coefficient of 14.15 mM⁻¹.cm⁻¹ and was expressed as Units/mg.

12. Superoxide dismutase activity

The assay for SOD activity is based on the reaction in which SOD reduces the superoxide anion to hydrogen peroxide and oxygen (Oguntibeju et al. 2016). Briefly, a reaction cocktail of pH 7.8 (containing distilled H₂O; 216 mM phosphate buffer (pH 7.8); 10.7 mM ethylenediaminetetraacetic acid solution (EDTA); 1.1 mM Cytochrome C solution and 0.108 mM xanthine solution) was incubated for 5 min at 25°C. Then we diluted, separately, 50 µg of testicular and epididymal protein homogenate in 216 mM potassium phosphate buffer (pH 7.8) which was added to the reaction cocktail. The reaction was started with the addition of the xanthine oxidase enzyme solution. The assay was performed in 96 wells microplate and the

enzymatic activity was measured in a spectrophotometer (Bio-Rad xMARK™, Microplate Spectrophotometer) at 550 nm. The activity of the enzyme was calculated by the mean of slopes, obtained at the 0 seconds and at 88 seconds. Concentrations of substrates were 0.05 mmol/L for xanthine and 0.025 mmol/L for INT. SOD was calculated by the degree of inhibition and expressed as Units/mg.

13. Catalase activity

The catalase activity assay is based on the measurement of the hydrogen peroxide substrate produced by the action of CAT. Briefly, it was extracted, separately, 50 µg of testicular and epididymal protein homogenate which was diluted in assay buffer (50 mM potassium phosphate buffer, pH 7.0). The reaction was started by the addition of the samples, the assay buffer and the 10 mM of H₂O₂ solution. This mixture was left to react for 5 min and the reaction was stopped by adding 15 mM sodium azide solution and transferred to a new well. Then, the color reagent (150 mM potassium phosphate buffer, pH 7.0; 0.25 mM 4-aminoantipyrine; 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid) was added and the mixture stand at room temperature for 15 minutes. The assay was performed in 96 wells microplate and the enzymatic activity was measured at 520 nm in a spectrophotometer (Bio-Rad xMARK™, Microplate Spectrophotometer). The activity of CAT was expressed activity µmol/min/ml in which one unit (U) of CAT activity correspond to the amount of the enzyme that decomposes 1 µmol of H₂O₂ in O₂ and H₂O per min. at pH 7.0 at 25 °C.

Statistical Analysis

Statistical significance, between experimental groups was assessed by *t*-Student test. All experimental data are shown as mean ± SEM (N=7 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Outliers, whenever present, were excluded using ROUT method with a *q* value of 1%. Results were considered significant when *p*-value < 0.05.

IV. Results

1. General characteristics of the T2DM animal model

T2DM GK rats are a non-obese model and develops spontaneously T2DM (Goto and Kakizaki 1981) . These animals presented a significant decrease (by 11%) in body weight ($418.80 \pm 6.11\text{g}$) when compared to the control rats ($470.60 \pm 11.95\text{g}$) (Table 3). At the end of the treatment glycaemic values of the T2DM rats were significantly increased $342.50 \pm 42.08\text{mg/dL}$ when compared to control group showing only $90.63 \pm 6.70\text{mg/dL}$ (Table 3). Blood HbA1c levels were also significantly increased (by 85%) in the T2DM group ($8.29 \pm 0.19\%$) when compared to the control animals ($4.46 \pm 0.07\%$) (Table 3). These results denote a prolonged state of hyperglycaemia and an impaired glucose metabolism. In fact, the results attained for the glucose tolerance test show that blood glycaemia of T2DM group increased during the 120 min of the test (Figure 7A), indicating the development of glucose intolerance. As can be seen by the significantly increase (by 137%) of the AUC_{GTT} values in T2DM animals ($4744 \pm 1123 \text{ a.u.}$) when compared to animals from the control group ($20010 \pm 2829 \text{ a.u.}$) (Figure 7B). Then we evaluated the insulin status and we observed a significant increase in the fasting insulin of the T2DM group ($0.26 \pm 0.02 \text{ ng/mL}$), whereas control group only exhibited ($0.15 \pm 0.02 \text{ ng/mL}$) (Table 3). The higher levels of insulin were consistent with the increased HOMA-IR in the T2DM animals (3.58 ± 0.36), as compared to control animals showed (0.67 ± 0.07), thus corroborating that T2DM animals developed insulin resistance (Table 3). Furthermore, we also observed a significant decrease (by 25%) in the gonads' weight of the T2DM rats ($2.81 \pm 0.03 \text{ g}$), as compared to that of the rats from the control group ($3.77 \pm 0.12 \text{ g}$) (Table 3). Accompanied by a decreased in the gonadosomatic index in GK rats (0.66 ± 0.02) when compared to the control rats (0.80 ± 0.03) (Table 3). Concerning the epididymis, those of T2DM group presented a weight of ($1.08 \pm 0.02 \text{ g}$), whereas as those of the animals of the control had a weight of ($1.47 \pm 0.05 \text{ g}$), corresponding to a 27% decrease (Table 3). The epididymal fat weight also decreased in T2DM group ($3.99 \pm 0.22 \text{ g}$) when compared to control group ($7.87 \pm 0.71 \text{ g}$) (Table 3).

Table 3: Effect of T2DM on average weight, glycaemia, HbA1c, insulin and reproductive organs weight from the control group and T2DM group.

Parameters	Control Group	T2DM Group
Weight (g)	470.60 ± 11.95	418.80 ± 6.11 *
Glycaemia (mg/dL)	90.63 ± 6.71	342.50 ± 42.08 *
HbA1c (%)	4.46 ± 0.07	8.29 ± 0.19 *
Fasting plasma insulin levels		
(ng/mL)	0.15 ± 0.02	0.26 ± 0.02 *
HOMA-IR	0.67 ± 0.07	3.58 ± 0.36 *
Gonads weight (g)	3.77 ± 0.12	2.81 ± 0.03 *
GSI	0.80 ± 0.03	0.66 ± 0.02*
Epididymis weight (g)	1.47 ± 0.05	1.08 ± 0.02 *
Epididymal fat weight (g)	7.87 ± 0.71	3.99 ± 0.22 *

Legend: GSI: gonadosomatic index; HbA1c: glycosylated haemoglobin; HOMA-IR: Homeostasis assessment model-insulin resistance; T2DM: type 2 diabetes mellitus. Results are presented as mean ± SEM. Significantly different results ($p < 0.05$) are indicated: * relatively to control.

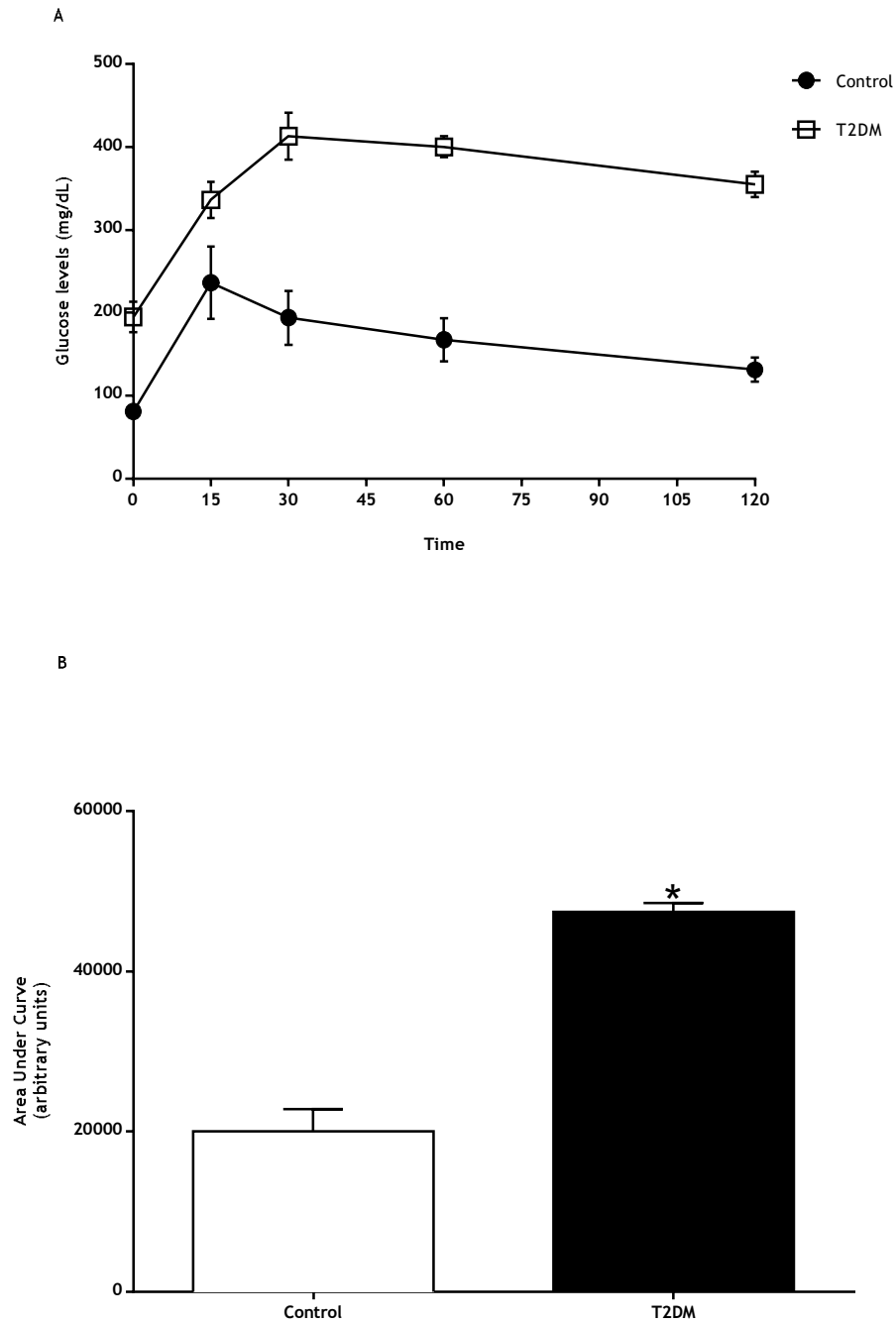


Figure 7: A) Blood glucose levels of control and T2DM groups measured during the intraperitoneal glucose tolerance test. B) AUC_{GTT} (area under curve of glucose tolerance test) values in T2DM rats and in the control group. Results are presented as mean \pm SEM. Significantly different results ($p < 0.05$) are indicated: * relatively to control.

2. T2DM decreased testicular levels of SIRT1 and SIRT3

SIRT1 is an important deacetylase that regulates the expression and the activity of PGC-1 α (Nemoto et al. 2005). The interaction between these two proteins is crucial to maintain a normal mitochondrial function, especially in tissues with high-energy demands, as is the case of testes. Then we evaluated testicular SIRT1 protein levels and we found a significant decrease (by 25%) in the T2DM group (0.75 ± 0.04 fold variation to control) when compared to the control group (1.00 ± 0.02 fold variation) (Figure 8).

PGC-1 α is the one of the key regulators of mitochondrial function and a decrease in its expression may compromise mitochondrial homeostasis. Our results showed a slight but not significant decreased expression of testicular PGC-1 α in the T2DM group (0.86 ± 0.04 fold variation to control) when compared to the control (1.00 ± 0.12 fold variation) (Figure 8). PGC-1 α is pivotal for the expression of SIRT3, so we further quantified the protein levels of this important mitochondrial deacetylase responsible for the modulation of mitochondrial metabolism and OS (Rato et al. 2014). We observed a decrease in the protein levels of SIRT3 (by 28%) in the T2DM rats (0.72 ± 0.11 fold variation to control) when compared to control (1.00 ± 0.16 fold variation) (Figure 8). Together these results led us to suppose that testicular mitochondrial function may be impaired, so we further assessed the mitochondrial integrity and the expression of mitochondrial respiratory complexes function.

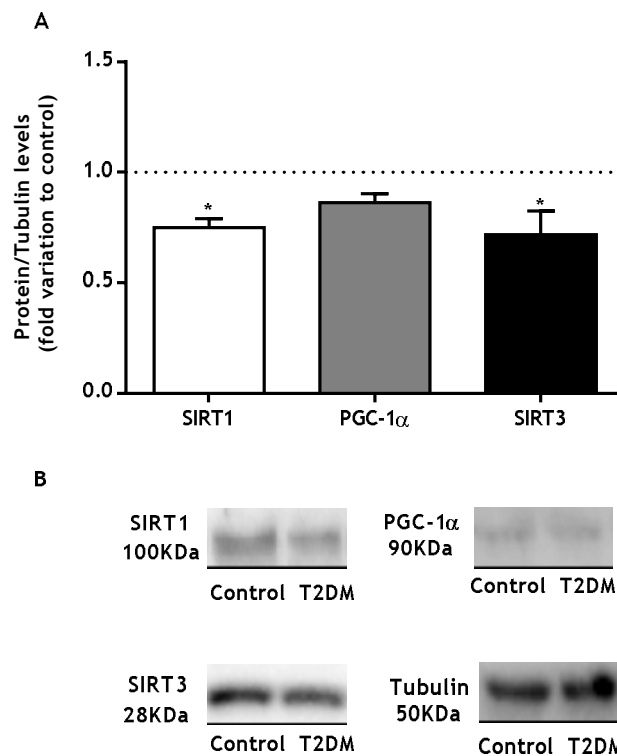


Figure 8: A) Effect of type 2 diabetes mellitus in testicular proteins levels of Sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor coactivator 1 α (PGC-1 α) and Sirtuin 3 (SIRT3). Panel B) Illustrative representation of Western Blot experiment. Results are presented as mean \pm SEM of three independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

3. T2DM did not alter testicular mtDNA copy number

mtDNA encodes for several of the multi-subunits that are part of the enzyme complexes of the respiratory chain. Therefore, changes in mitochondrial content may compromise the synthesis of the mitochondrial complexes and consequently the normal functioning of the electron transport chain. Our results showed a slight but not significant decrease in the mtDNA content of the T2DM group (942 ± 73 a.u.) when compared to the control (1173 ± 147 a.u.) (Figure 9). After that, we determined the expression of ETC complexes in order to evaluate the effects induced by T2DM.

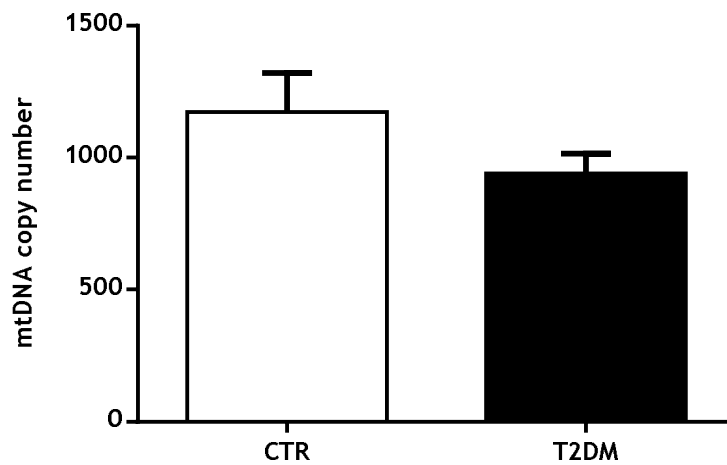


Figure 9: Relative testicular mtDNA copy number from the control and T2DM groups. Results are presented as mean \pm SEM of three independent experiments. Significantly different results ($p < 0.05$) are indicated: * relatively to control.

4. The expression of testicular mitochondrial respiratory complexes III and V was decreased by T2DM

The electron transport chain is composed by four multi-subunit complexes (complexes I-IV) interconnected between them, which create an electrochemical proton gradient across the inner mitochondrial membrane, resulting in ATP synthesis by the mitochondrial complex V, usually known as ATP synthase. We found a significant decrease (by 45%) in the expression of mitochondrial complex III in the testes of T2DM group (0.55 ± 0.14 fold variation to control) when compared to control rats (1.00 ± 0.15 fold variation) (Figure 10). Similarly, the expression of ATP synthase was significantly decreased by of 53% in the T2DM (0.47 ± 0.09 fold variation to control) when compared to control rats (1.00 ± 0.17 fold variation). The expression of testicular complexes I, II and IV was not altered (Figure 10).

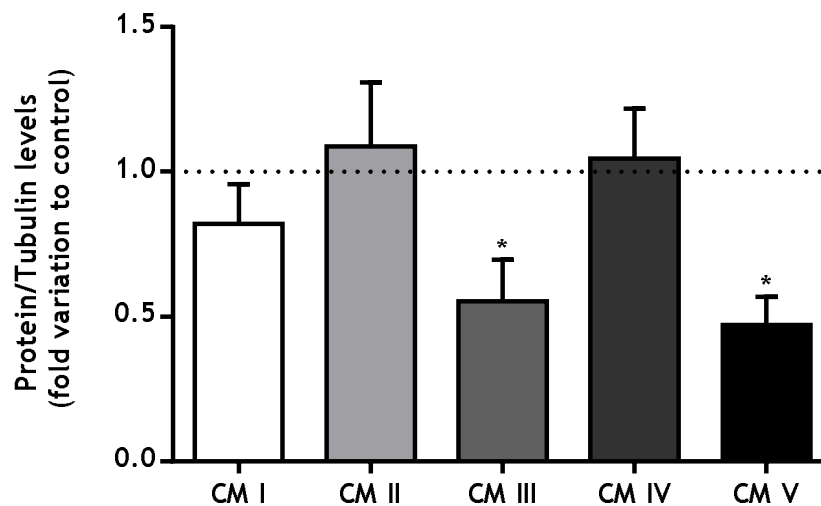


Figure 10: Expression of the testicular mitochondrial complexes I, II, III, IV, V from the control and T2DM groups. Results are presented as mean \pm SEM of three independent experiments. Significant differently results ($p < 0.05$) are indicated: * relatively to control.

5. T2DM did not alter the activity of the testicular antioxidant enzymes

We evaluated the activity of the following enzymes GR, GPx, SOD and CAT that belong to the antioxidant system in the reproductive tract. These enzymes exert their action, in order to counterbalance the production of ROS. GPx enzyme oxidized the GSH depending of the GR and cellular NADPH concentration (Maritim et al. 2003). This enzyme uses, GSH as a hydrogen donor for the reduction of non-specific substrates (hydrogen peroxide, lipid and non-lipid hydroperoxides. While SOD and CAT catalyses the conversion of superoxide anion to hydrogen peroxide and O₂ (Correia et al. 2008).

In our work the activity of these four enzymes did not show significant results (Figure 11).

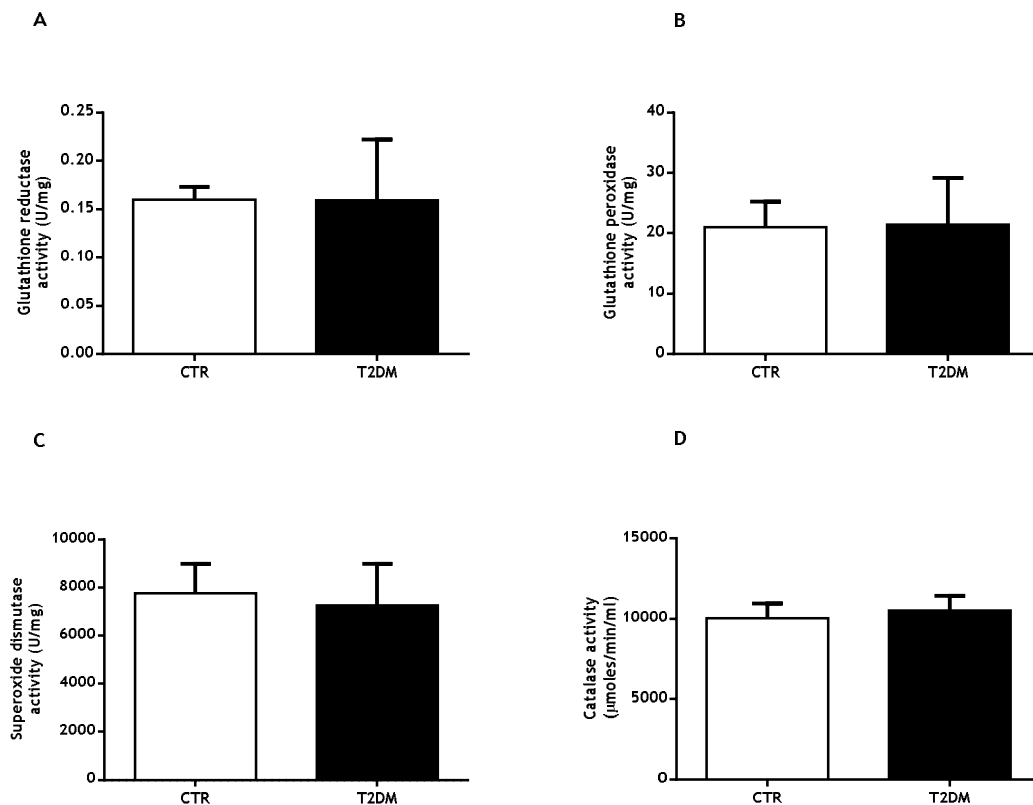


Figure 11: Activity of the testicular antioxidant enzymes Glutathione reductase, Glutathione peroxidase, Superoxide dismutase and Catalase from the control and T2DM groups. Results are presented as mean \pm SEM of three independent experiments. Significantly different results ($p < 0.05$) are indicated: * relatively to control.

6. T2DM increased testicular protein nitration and lipid peroxidation

T2DM favours the increase of the oxidative environment in the tissues due to the unbalanced production of ROS and an inefficient antioxidant system. This often leads to damages in the lipids and proteins. Testes are organs with high lipid content, which are the major targets of ROS. In this context we quantified testicular lipid peroxidation by measuring the relative levels of 4-HNE in this tissue. We found that in the T2DM group lipid peroxidation was significantly increased by 89% (1.89 ± 0.23 fold variation to control) in relation to the control group (1.00 ± 0.17) (Figure 12A). Proteins are also targeted by reactive nitrogen species (RNS) and thus may suffer oxidation. So, we evaluated the effects of T2DM in the protein carbonylation and nitration by measuring the levels of 2,4-dinitrophenyl hydrazone (DNP) and 3-nitrotyrosine (3-NT), respectively. The levels of 3-NT groups of proteins can be used indirectly to measure protein damage related to the oxidative state. Protein nitration was increased by 53% in the T2DM animals (1.53 ± 0.19 fold variation to control) when compared to control rats (1.00 ± 0.15) (Figure 12B).

Animals from the T2DM group exhibited a decrease in the testicular protein carbonyl content (1.09 ± 0.14 fold variation to control) when compared to control group (0.95 ± 0.11 fold variation) (Figure 12C).

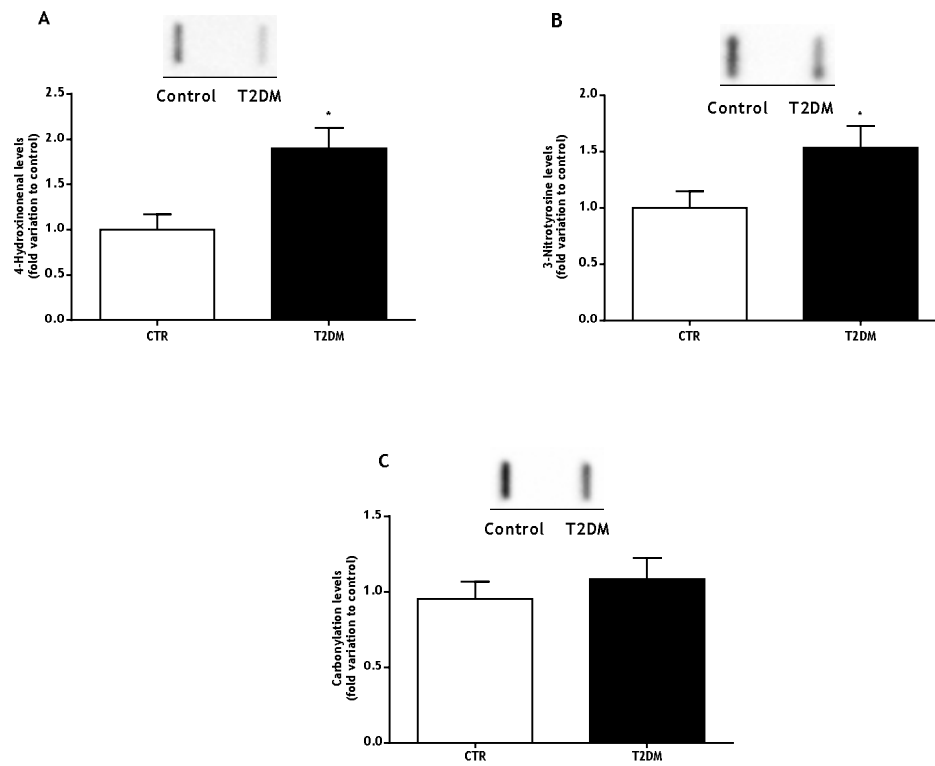


Figure 12: A) Testicular lipid peroxidation, B) 3-NT (3-nitrotyrosine) content and C) proteins carbonylation in control rats and T2DM rats. Results are presented as mean \pm SEM of seven independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

7. T2DM compromised the expression of key regulators of the mitochondrial biogenesis in the epididymis

We found a significant decrease (by 22%) in the expression of the SIRT1 in the epididymal tissue of the T2DM group animals (0.78 ± 0.12 fold variation to control), when compared with control group rats (1.00 ± 0.09 fold variation) (Figure 13). These results lead us to assess the protein levels of PGC-1 α in the epididymis and we observed that the rats T2DM group showed lower levels of PGC-1 α (0.68 ± 0.22 -fold variation to control) (by 32%) in relation those of the control group (1.00 ± 0.09 fold variation) (Figure 13). Then, we quantified the expression of SIRT3 and we observed T2DM induced a significant decrease in the expression of this deacetylase in the epididymal tissue. The rats of the T2DM group exhibited a decrease on the expression levels of SIRT3 (0.65 ± 0.11 - fold variation to control), corresponding to 35% reduction when compared to the animals of the control group (Figure 13).

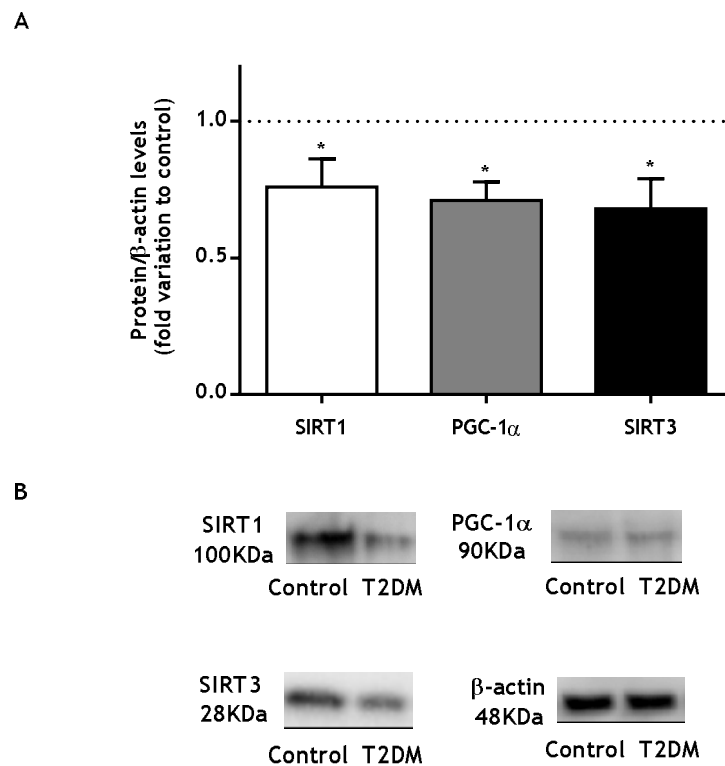


Figure 13: A) Effect of type 2 diabetes mellitus in epididymal proteins levels of Sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor coactivator 1 α (PGC-1 α) and Sirtuin 3 (SIRT3). Panel B) Illustrative representation of Western Blot experiment. Results are presented as mean \pm SEM of three independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

8. T2DM significantly decrease the activity of antioxidant enzymes in the epididymis

Our results showed that the activity of GR in the epididymal tissue was significantly increased by 30% from 0.14 ± 0.03 U/mg of protein in the animals of the control group to 0.20 ± 0.02 U/mg of protein in those of the T2DM group (Figure 14A).

Contrastingly, we measured the activity of GP which was also significantly decreased by 58%, with the animals of T2DM group exhibiting an epididymal activity of 4.93 ± 1.12 U/mg of protein and the control group an activity of 11.89 ± 0.74 U/mg of protein (Figure 14B).

We also, observed that the activity of SOD was significantly decreased by 87% in the epididymal tissue of rats from the T2DM group (4628 ± 545.90 U/mg of protein), compared with the animals of the control group (36535 ± 13554 U/mg of protein) (Figure 14C). Similarly, CAT activity was decreased by 25% in the T2DM group animals (1991 ± 165.30 U/mg of protein), with the epididymis of animals from the control group exhibiting an activity of 2668 ± 192.80 U/mg of protein (Figure 14D).

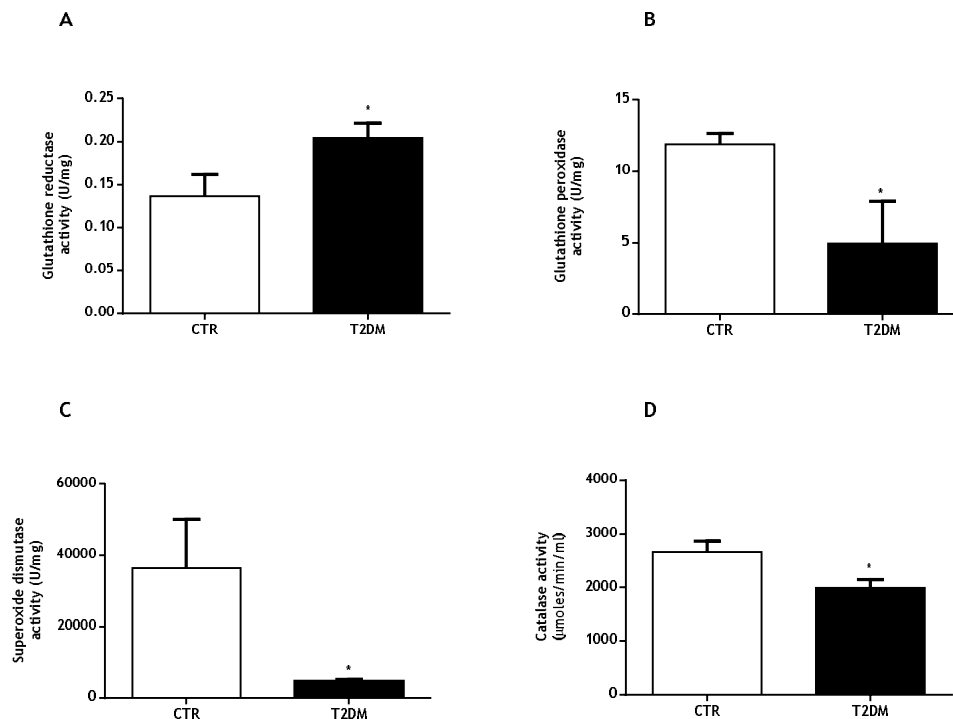


Figure 14: Activity of the antioxidant enzymes Glutathione reductase, Glutathione peroxidase, Superoxide dismutase and Catalase in the epididymal tissue from the control and T2DM groups. Results are presented as mean \pm SEM of three independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

9. T2DM did not alter epididymal mtDNA content

We evaluated whether T2DM induces alterations in the mtDNA copy number and we found no differences, where T2DM animals showed (2993 ± 278.10 a.u.) while control group showed (3632 ± 523.60 a.u.) (Figure 15).

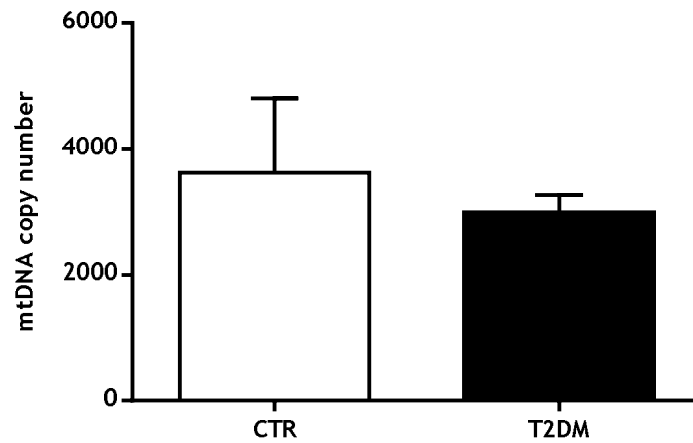


Figure 15: Relative mtDNA copy number in epididymal tissue from control and T2DM groups. Results are presented as mean \pm SEM of seven independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

10. Expression of the epididymal mitochondrial complexes II, III and V was altered under T2DM

T2DM significantly decreased the expression of the mitochondrial complex II, from (1.00 ± 0.12 fold variation) in the epididymis of rats from the control group to (0.76 ± 0.15 fold variation to control) in those of the animals from the T2DM group, corresponding to 24% of the decrease (Figure 16). Also, we observed a decreased expression of mitochondrial complex III (by 22%) with T2DM showing (0.78 ± 0.10 -fold variation to control) when compared to the control group (1.00 ± 0.12 fold variation). Complex V was decreased in the T2DM group by 23% in the T2DM group (0.77 ± 0.15 fold variation to control) when compared to control group (1.00 ± 0.12 fold variation). No differences were found concerning complex I and complex IV (Figure 16).

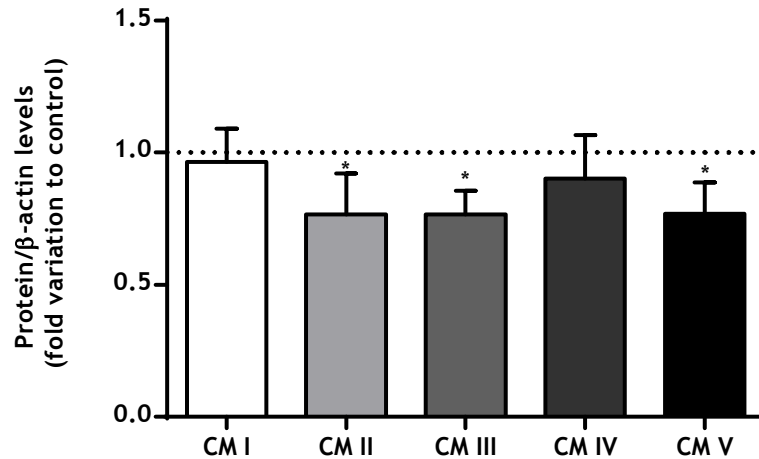


Figure 16: Expression of the mitochondrial complexes I, II, III, IV, V in the epididymal tissue from control and T2DM groups. Results are presented as mean \pm SEM of seven independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

11. T2DM increased protein nitration in the epididymal tissue

T2DM favours the increase of the oxidative environment in the tissues due to the unbalanced production of ROS and an inefficient antioxidant system. Proteins are targeted by ROS and RNS, suffering oxidation. So, we evaluated the impact of T2DM in epididymal protein carbonylation and nitration by measuring the levels of DNP and 3-NT, respectively. We, also, quantified epididymal lipid peroxidation by measuring the relative levels of 4-HNE and we observed a significant increase by 40% in T2DM rats (1.22 ± 0.10 -fold variation to control) in relation to those of the control group (0.87 ± 0.09) (Figure 17A). Protein nitration was significantly increased by 48% in the epididymis of T2DM animals (1.23 ± 0.11 -fold variation to control) when compared that of control rats (0.83 ± 0.10) (Figure 17B). The levels of epididymal carbonyl content in the T2DM group was (0.60 ± 0.02 -fold variation to control) when compared to control group (0.84 ± 0.04 fold variation) (Figure 17C).

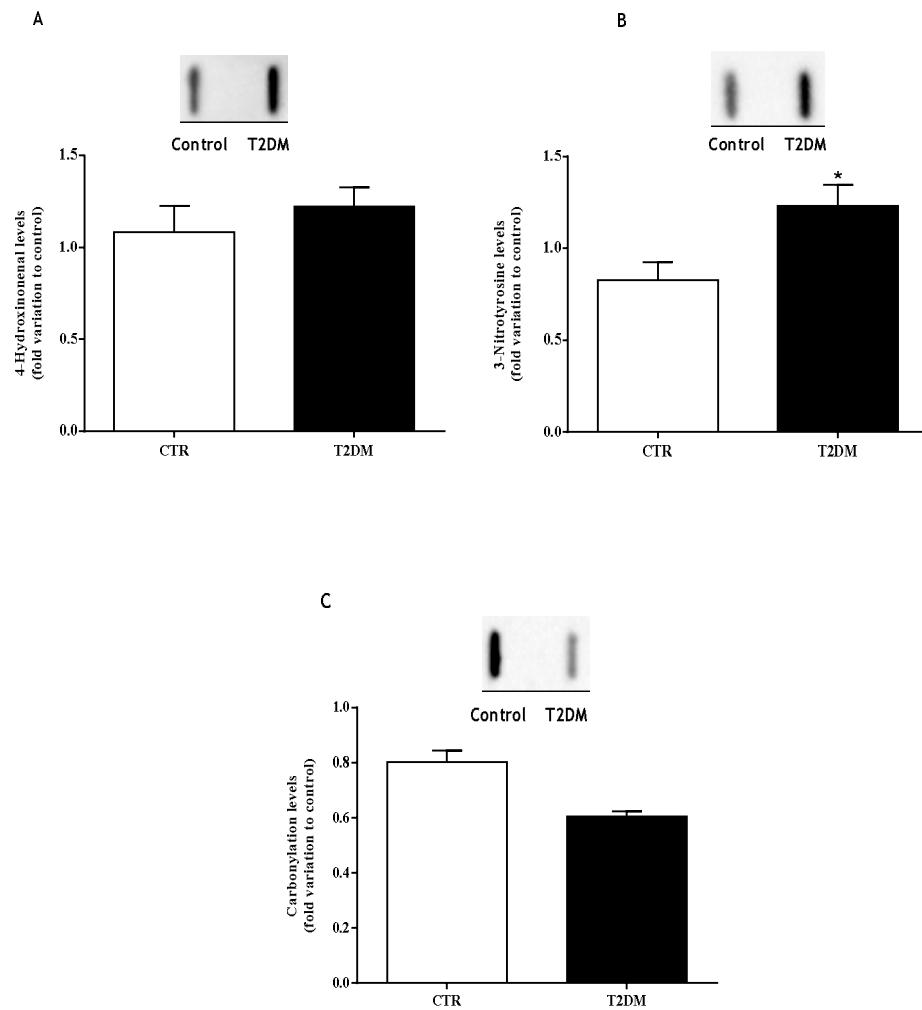


Figure17: Epididymal lipid peroxidation, 3-NT (3-nitrotyrosine) content and proteins carbonylation in control rats and T2DM rats. Results are presented as mean \pm SEM of seven independent experiments. Significantly differently results ($p < 0.05$) are indicated: * relatively to control.

V. Discussion

Diabetes is the one most prevalent metabolic disease and has been considered a public health concern. T2DM is the most prevalent type of diabetes in the world reaching over 95% of the individuals, and it is associated with a metabolic and endocrine impairment. Underlying this is the development of several pathologies. Indeed, the disruption of glucose metabolism and the resulting hyperglycemia induced by T2DM may be one of the causes for male infertility associated with T2DM. According to the latest data, the numbers revealed that there is more men suffering with diabetes than women, about 14 million more men than women (Federation, 2013). Besides that, the incidence of T2DM has increased among young males and today more than 50% of men with diabetes are subfertile/infertile (La Vignera et al. 2009). Several animal models have been used to study in detail the effects of T2DM in reproductive health, for ethical reasons or for inability to use human models.

In this study, we used a T2DM animal model which was developed in the earliest 80's by Yoshio Goto and Masaie Kakizaki and the animals are non-obese but spontaneously developed T2DM (Goto and Kakizaki, 1981). This model was developed by a selection of animals from a normal population, with a glucose tolerance test (GTT) slightly altered from the normal range. Crossing males and females, their descendants, have an impaired GTT. So, the repeat of this procedure over several generations would finally produce diabetic animals (Yoshio and Third, 1975). These animals develop several characteristics associated to T2DM as middle hyperglycaemia as 2 to 4 weeks after birth, and when the animals reach about 12 months exhibit a moderate but stable fasting hyperglycaemia without developing ketotic acidosis. At the end of treatment, the hyperglycaemia was higher in the animals from the T2DM group when compared to the control group. This was accompanied by glucose intolerance and increased levels of fasting insulin illustrating an insulin resistance state, which was corroborated by the significant increase of HOMA-IR.

T2DM animals presented a significant decrease in the gonadosomatic index as well in the reproductive accessory organs, indicating that the architecture of testes and epididymis was not maintained. This is consistent with several studies in diabetic models and in diabetic individuals which shows a decreased in semen parameters, deficient sperm quality, with a decreased in sperm concentration and motility and fertilization capacity as well as subsequent embryo development and an increased in lipid peroxidation (Kim and Moley 2008; Singh et al. 2014). Apart from morphologic alterations induced by T2DM, there are molecular changes that are on the basis of male infertility associated with T2DM.

T2DM is characterized as a hyperglycaemic state related with an increase in the electron transfer donors (NADH and FADH) and consequently the electron flux through the mitochondrial electron transport chain. This caused an increase in the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential. This high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complex III, resulting in an accumulation of electrons to coenzyme Q. In turn, this drives the partial reduction of O₂ to generate the free radical anion superoxide

(Nishikawa et al. 2000; Brownlee, 2001). This accelerates the reduction of coenzyme Q and generation of ROS that is one of the major causes of mitochondrial dysfunction. The increased glycolytic activity has been associated with an augmented oxidative environment that leads to several damages in the reproductive organ (Rato et al. 2015; Rato et al. 2014). Several studies have linked the subtle metabolic alterations induced by the prodromal stage of DM are sufficient to induce metabolic alterations and oxidative status in the testicular and epididymal tissues (Dias et al. 2016; Oliveira et al. 2015). Mitochondria are responsible for the maintenance of essential events for cellular viability playing an important role in the homeostasis of the redox environment. Thus, ensuring a normal mitochondrial function within reproductive tract is imperative, particularly under DM which induces large fluctuations of energy levels. DM promotes alterations in whole cellular metabolism, such as the unbalance of NAD^+/NADH ratio (Rato et al. 2015), and therefore in sirtuins action since, NAD^+ is an essential co-substrate that directly regulates sirtuins activity (Yoshida and Imai 2018). T2DM adversely affected testicular metabolic pathways, particularly glucose metabolism (Rato et al. 2015) and fatty acid oxidation probably through SIRT1/PGC-1 α mechanism, leading to the accumulation of metabolic intermediates that caused testicular oxidative damage and germ cell apoptosis (Jiang et al. 2015). Compelling evidence highlighted the relevance of SIRT1 in male fertility, because SIRT1 knockout animals are infertile (Kolthur-Seetharam et al. 2009; Seifert et al. 2012), showing decreased reproductive organs size and are not able to successfully mate (Kolthur-Seetharam et al. 2009; McBurney et al. 2003). Sperm from SIRT1 knockout mice has abnormalities in morphology and in molecular composition (Bell et al. 2014). SIRT1-deficient mice present an arrest of the spermatogenic process due to dysfunctional mitochondria (Seifert et al. 2012). Moreover, epididymal sperm from SIRT1 mutant animals were frequently immature and much less motile than those from wild-type individuals, suggesting that maturation within the epididymis was also defective.

In the testis and epididymis, we observed a significant decrease in SIRT1 protein levels. Indeed, SIRT1-deficient mice present an arrest of the spermatogenic process due to dysfunctional mitochondria (Seifert et al. 2012). Although there are no data reporting the effects of T2DM in epididymal SIRT1 levels, our results are consistent with other models showing the expression of SIRT1 is affected by DM (Kreutzenberg et al. 2010). SIRT1 interacts with mitochondrial proteins and both *in vitro* and *in vivo* studies indicate that SIRT1 is a major regulator of PGC-1 α (Rodgers et al. 2005; Scarpulla et al. 2012). Similarly, to SIRT1, the expression of PGC-1 α epididymal tissue was significantly decreased, although in the testis the decrease was not significant, this may illustrate a possible bioenergetic disruption in both tissues of these animals. SIRT1/PGC-1 α axis is a key regulatory point for the maintenance of mitochondrial function and bioenergetic capacity since together both proteins are involved in “mitochondrial renewal” (Lee et al. 2008). The decreased levels of PGC-1 α might be related with the significant decrease in the SIRT3 expression observed in testicular and epididymal tissue. As previously mentioned (Kong et al. 2010), PGC-1 α is pivotal for SIRT3 and we have

found that even in the prodromal stages of T2DM, the expression of PGC-1 α and SIRT3 is impaired (Rato et al. 2014).

Disruption of SIRT1/PGC-1 α /SIRT3 pathway has deleterious effects since the action of these proteins potentiates the expression and/or activity of several proteins and enzymes required for the proper functioning of mitochondria (Rato et al. 2014; Rodgers et al. 2005; Scarpulla et al. 2012). Then, we quantified the epididymal and testicular mtDNA copy number and we found a non-significant decreased for both tissues. The mtDNA encodes for the enzymatic mitochondrial complexes so we evaluated the expression of complexes I-V in the testicle and epididymal tissue. We found a significant decrease in the expression of testicular complexes III and V of the T2DM animals. These results were more pronounced in the epididymal tissue since there was a significant decrease in the expression of complexes II, III and V. Several studies have associated the impairment of the mitochondrial complexes, in different tissues under T2DM, as observed by Raza and collaborators who found reduced activities of mitochondrial complexes II/III and IV in pancreatic cells (Raza et al. 2016). Similar results were also reported by Xu and collaborators who found a decreased activity of mitochondrial complex III activity in the kidneys of diabetic rats (Xu et al. 2012). Also, Rato and collaborators observed a significant decreased in mitochondrial complex III, but in contrast, the authors observed a significant increase in complex I in the testicular tissue of prediabetic rats. They suggest that this increment may be a compensatory mechanism in order to maintain the normal electron flow to guarantee an adequate energy supply for all the metabolic testicular demands (Rato et al. 2014). This highlights the adverse impact of diabetes on the normal function of complexes activity. The complex II received the electrons from the FADH₂ molecule, once the expression of this protein is reduced may compromise the normal flux of the electrons (Jørgensen et al. 2012). The electrons flow sequentially through cytochrome c reductase (complex III), which funnels electrons from the coenzyme Q pool to cytochrome c (Rato et al. 2014).

The decrease in the expression of mitochondrial complexes III and V may be due to the decreased levels of PGC-1 α , since the expression of complex III and V encoded genes is PGC-1 α dependent (Andersson and Scarpulla 2001). Bearing in mind that complex III is the main source of ROS and complex V is responsible for ATP synthesis, the reduction of these two complexes will contribute to an inefficient ETC, leading to a possible production of O₂-free radicals and low ATP levels (Raza et al. 2016).

The testis and the epididymis play an important role in the maturational process of sperm and the microenvironment within this tubule must ensure sperm viability. The normal functionality of the sperm requires a delicate balance between ROS production and recycling. Indeed, small quantities of ROS are required for sperm function, such as motility, but also for capacitation and acrosome reaction that are pivotal for fertilization (Agarwal et al. 2008). However, uncontrolled production of ROS is detrimental for sperm since the presence of PUFAS in plasma membranes is a preferred target for ROS. Spermatozoa are highly susceptible

to oxidative injuries since they are practically devoid of ROS-scavenging enzymes and dependent on the existing antioxidant protection in the male reproductive tract, particularly in the epididymis (Sakkas and Alvarez 2010).

As aforementioned, SIRT1 is a major upstream activator of PGC-1 α which can itself activated redox-related targets as p53, FOXOs and the nuclear factor, each one plays different roles in response to changes in the redox status. p53 can activate genes involved in antioxidant defenses, like SOD2 and GPx1 and, also, FOXO which induces antioxidant responses via activation of SOD and catalase. Besides that, as aforementioned SIRT1 activates upstream PGC-1 α which acts in concert with SIRT3 to activate substrates implicated in ROS suppression as GPx1 glutathione peroxidase1, FOXO3a which stimulate the expression detoxifying factors like SOD2 and catalase (Tseng et al. 2013).

Thus, alterations in the molecular axis SIRT1/PGC-1 α /SIRT3 induced by the T2DM influence the activity of the antioxidant defenses. We evaluated the activity of several detoxifying enzymes involved in ROS suppression as GPx, GR, CAT, and SOD. SOD is one of the most important enzymes that converts the superoxide anion to hydrogen peroxide and O₂. GPx is a selenium-dependent enzyme that catalyze the reduction of H₂O₂ into two H₂O molecules using GSH as a hydrogen donor. After being oxidized GSH is transformed into GSSG, which is recycled by GR, using NADPH into the initial form, GSH (Maritim et al. 2003; Sakkas and Alvarez 2010) Catalase degrades hydrogen peroxide produced by peroxisomal oxidases to water and O₂ (Correia et al. 2008).

Interestingly, our results showed that the activity of the antioxidant enzymes remains unaltered in the testicular tissue. However, in the epididymis, our results show a significant decrease in the enzymatic activity of SOD and CAT in the T2DM rats when compared to control rats, this may be a consequence of T2DM. However, we observed a significant increase in the GR activity whereas GPx activity was decreased. Amaral *et al.* (2006) also observed similar results in testicular cells of T2DM rats. This can be explained as an attempt to regenerate the levels of GPx that might be oxidized by a GPx independent pathway (Amaral et al. 2006).

Indeed, the influence of T2DM in the enzymatic activity is a controversial issue, for instance, in the brain some studies reported that both GPx and GR activity is elevated (Correia et al. 2008), however in other studies GR is significantly increased in T2DM rats when the GPx and SOD remain unchanged (Santos et al. 2009). In cardiac tissue, it was reported that T2DM significantly decreased CAT, SOD, and GPx, while Rausher *et al.* (2000) reported that in heart CAT activity was significantly decreased and GPx and GR increased (Rauscher et al. 2000). Taking all this together, different tissues have different metabolic needs suggesting that the non-alterations in testicular enzymatic activity may be due to, different quantities in different cell types and cellular compartments of the antioxidant enzymes, and, also, the regulation of the antioxidant activity may be influenced by several factors as age, hormonal state, organ specificity, and amount of cofactors present.

Moreover, the decreased activity of epididymal antioxidant enzymes may also result in part from the disruption of the molecular axis SIRT1/PGC-1 α /SIRT3 resulting in the increase of ROS production. Studies evidenced the consequences of ROS through the evaluation of potential biomarkers of cellular OS in T2DM. In this work, we evaluated the levels of lipid peroxidation, protein nitration, and carbonylation. Lipid peroxidation causes the oxidative degradation of PUFAs in membranes of the cells, in other words, the overproduction of ROS oxidizes PUFAs in cellular membranes through free radical chain reactions and form lipid hydroperoxides as primary products (Yin et al. 2011), which may decompose and lead to the formation of reactive lipid electrophiles, specially 4-hydroxy-2-nonenals (4-HNE) (Yin et al. 2011). Taking into account the huge amount of PUFAs in sperm membranes we measured 4-HNE testicular and epididymal levels and it was observed a significant increase in lipid peroxidation, of both tissues in the T2DM group. This is consistent with what was observed by Chatterjee and collaborators (Chatterjee et al. 2013), who also found increased lipid peroxidation during maturation and storage in epididymis under diabetic conditions. Accordingly, high levels of lipid peroxidation were found in sperm with a reduced motility, as well as in sperm mitochondrial membrane (Kao et al. 2008).

Furthermore, several molecules are altered due to interactions with ROS including RNS in the microenvironment, and those changed in response to increased redox stress are considered biomarkers of OS (Teixeira et al. 2016). Proteins contain surface-exposed Tyr residues in their composition, due to the hydrophobic nature of the aminoacid allowed the process of nitration. The proteins suffer post-translational modification mainly caused by addition of NO of either free or protein-bound tyrosine, resulting in the formation of 3-NT. This resulting product is liable of being quantified, in our work we observed a significant increase in 3-NT content evidencing the susceptibility of epididymal and testicular tissue to ROS overproduction. A recent study reveals that nitro-oxidative stress in germ cells occurs in the midpiece and in the head of the sperm, and was correlated with sperm motility, which is linked with a disruption in mitochondrial ETC (Cassina et al. 2015). We also quantified the protein carbonylation which is a biomarker of protein oxidation. We can define protein carbonylation as the insert of reactive carbonyl groups, mainly in the form of aldehydes and ketones, into a protein structure. In order to measure the carbonylated content of proteins, it was use highly sensitive assays based on derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine, which leads to the formation of a stable 2,4-dinitrophenyl hydrazone product, liable to be measured (Colombo et al. 2016; Dalle-donne et al. 2003; Davies and Zhang 2017). Our results show that there are no significant alterations in protein carbonylation in the testes and in epididymal tissue.

Thus, both tissues are affected by the effects of diabetes that may culminate in a decrease of the fertility parameters. Indeed, the germ cells are very prone to the attack of the ROS due to the lipidic nature of their membrane. This disturbance in mitochondrial bioenergetic favors ROS production and impairing ATP synthesis leading to an oxidative environment in the

testes and epididymal of T2DM rats. This compromises the optimal microenvironment that both tissues create for the generation and maturation of the sperm, which leads to physiologic deficiencies in sperm and consequently to infertility.

VI. Conclusion

In the last few decades, the increase in the numbers of cases of DM has been uprising, being now considered a health problem. This increasing trend in the incidence of DM is accompanied by an increase in infertility cases. Both diseases are closely linked, with DM being one of the causes of this decline in fertility. In order to attenuate the implications of DM in fertility, numerous studies have been done in order to elucidate the mechanisms behind this disorder.

In our work we studied the effects of T2DM in the signalling pathways that govern the mitochondrial function in the testes and epididymis. We evaluated the molecular expression of the proteins involved in the molecular axis SIRT1/PGC-1 α / SIRT3 in the testes and epididymis, and we observed a decrease in their expression. As this metabolic pathway is involved in the activation of the antioxidant defence enzymes, we observed a decrease in the enzymatic activities of GPx, SOD and CAT in epididymis, contrarily to what happens to the testicular tissue in which the antioxidant defenses remain unaltered. We also observed a decrease in the expression of mitochondrial complexes III and V in testicular tissue and a in complexes II, III and V in epididymal tissue. Both tissues presented increased oxidative damages that are revealed by the increase of protein nitration and lipid peroxidation.

Thus, we can conclude that T2DM causes a disruption in the molecular axis SIRT1/PGC-1 α /SIRT3, which is more pronounced in epididymal tissue than in testicular tissue leading to an impairment in mitochondrial bioenergetics. This impairment could explain in part the relationship between the OS caused by DM. However, more studies should be performed to elucidate the molecular pathways underlying male's infertility associated to T2DM, in view of a potential therapeutic target to attenuate the incidence of infertility in men suffering from T2DM.

VII. References

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