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Efeito de Antidiabéticos em Células de Sertoli Humanas: Implicações Para a Fertilidade Masculina

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O conteúdo do presente trabalho é da exclusiva responsabilidade da autora:

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Resumo

A diabetes mellitus (DM) é uma doença metabólica que está a atingir proporções pandémicas, afetando um elevado número de homens numa idade muito jovem. É uma das principais causas de morbilidade e mortalidade em países desenvolvidos e em desenvolvimento. A diabetes mellitus tipo 2 (T2DM) é responsável pela grande maioria de casos de DM. Compreende indivíduos que apresentam resistência à insulina e uma relativa deficiência na secreção de insulina. Ao contrário da diabetes mellitus tipo 1 (T1DM), o principal fator para o desenvolvimento da doença parece ser o estilo de vida das sociedades modernas em vez de fatores genéticos.

O processo de produção de espermatozóides viáveis é altamente regulado, dependente de diversos fatores, e qualquer alteração pode resultar em problemas de fertilidade no homem. Entre outros aspectos, as células germinativas são dependentes da cooperação metabólica que estabelecem com as células de Sertoli (SCs), que mostrou ser essencial para a ocorrência da espermatogénese.

Dos diferentes antidiabéticos disponíveis no mercado, vários têm uma ação na homeodinâmica metabólica do indivíduo. Contudo, os seus mecanismos de ação são distintos e variam dependendo de diversos fatores (p. ex. tecido ou células alvo). Neste trabalho, avaliámos o efeito de dois antidiabéticos, a pioglitazona e a metformina, no metabolismo de células de Sertoli humanas (hSCs).

Os nossos resultados sugerem que, tal como a metformina, a pioglitazona é um antidiabético adequado para homens em idade reprodutiva. Para além disso, os resultados obtidos neste trabalho levam-nos a sugerir um possível papel protetor da pioglitazona na função reprodutiva masculina.

Palavras-chave

Pioglitazona; Metformina; Espermatogénese; Células de Sertoli; Infertilidade, Mitocôndria

Resumo Alargado

A diabetes mellitus (DM) é uma doença metabólica pandémica que afeta uma grande quantidade de homens numa idade muito jovem. Vários fatores contribuem para estas estatísticas. Nos próximos anos espera-se que o número de diabéticos aumente de forma alarmante. De notar, à medida que o número de homens que sofrem de doenças metabólicas aumenta, as taxas de fertilidade diminuem, o que ilustra uma associação que pode, ou não, ser direta. Na verdade, estes homens sofrem de uma variedade de problemas que podem conduzir a subfertilidade ou infertilidade. Além da conhecida disfunção hormonal induzida pela DM e os efeitos que promove no controlo endócrino da reprodução masculina, provas convincentes têm mostrado que o metabolismo testicular também é alterado pela DM.

O metabolismo testicular foi, durante muitos anos, um campo negligenciado da investigação. No entanto, o funcionamento metabólico adequado das células testiculares é crucial para a normal ocorrência da espermatogénese. No testículo ocorre um fenómeno metabólico muito específico designado como cooperação metabólica. As células germinativas em desenvolvimento dependem do lactato produzido pelas células de Sertoli (SCs), como fonte de energia. Neste processo, as SCs metabolizam a glicose para lactato. Este metabolito é posteriormente exportado para o fluido intratubular onde serve como combustível metabólico para as células germinativas em desenvolvimento. Esta cooperação metabólica é dependente de vários fatores e é sensível às flutuações de metabolitos e hormonas. Assim, não é de surpreender que a DM possa alterar esses processos comprometendo a saúde reprodutiva masculina. No entanto, o metabolismo dos espermatozóides também apresenta necessidades específicas que podem ser alteradas pela DM. Nos últimos anos, vários estudos têm sido realizados para desvendar os mecanismos pelos quais doenças metabólicas alteram o potencial reprodutivo dos homens. Por outro lado, novas terapias estão a ser estudadas para compensar os efeitos indesejáveis da desregulação hormonal e de glicose em pacientes diabéticos.

A pioglitazona é um potente agonista sintético dos recetores ativados por proliferadores de peroxissoma gama, sendo utilizada para tratar a diabetes mellitus tipo 2 (T2DM). A ativação destes recetores leva ao aumento da transcrição de genes relacionados com o metabolismo da glicose. Para além do tratamento da T2DM, a pioglitazona tem sido útil no tratamento de outras doenças, mas os seus efeitos na reprodução masculina permanecem desconhecidos. Este antidiabético é utilizado na prática clínica em monoterapia ou em terapia combinada, associado a fármacos como a metformina. A metformina é uma biguanida usada como primeira linha no tratamento da T2DM. Apesar de ser utilizada na prática clínica há mais de cinquenta anos, o seu mecanismo de ação específico ainda não é totalmente conhecido.

O objetivo deste trabalho foi investigar os efeitos da pioglitazona, isoladamente ou em combinação com a metformina, no metabolismo das SCs humanas (hSCs). Para isso, foram isoladas hSCs a partir de biópsias testiculares e foram cultivadas na ausência e na presença de concentrações crescentes de pioglitazona ou de pioglitazona e metformina. Para analisarmos eventuais alterações na glicólise, avaliámos os níveis de proteínas de alguns intervenientes nesse processo (por western blot) e a produção/consumo de metabolitos (através de ressonância magnética nuclear). Foi também avaliada a actividade de enzimas associadas à glicólise ou produção de lactato, assim como o estado funcional das mitocôndrias (potencial mitocondrial) das hSCs dos diferentes grupos experimentais.

Uma vez que a dose farmacológica de pioglitazona aumenta a produção de lactato pelas hSCs, sem causar dano às mesmas, e que diversos trabalhos têm demonstrado que o aumento de lactato tem um papel protetor na espermatogénese, os resultados por nós obtidos levam a sugerir que a pioglitazona é um antidiabético adequado para homens com T2DM em idade reprodutiva. A pioglitazona aparenta ter um papel positivo na cooperação metabólica testicular, o que sugere um papel protector no potencial reproductivo masculino de indivíduos com DM.

Devido à crescente incidência da DM e às complicações associadas na fertilidade masculina é fundamental aprofundar o conhecimento acerca das terapias utilizadas para o tratamento da T2DM, de modo a que estas não prejudiquem ainda mais a fertilidade destes pacientes. Por outro lado, tendo em consideração que o metabolismo testicular é essencial para a espermatogénese e estes antidiabéticos são moduladores metabólicos que têm demonstrado terem várias outras aplicações, os seus efeitos para a saúde reprodutiva masculina podem ser benéficos.

Abstract

Diabetes mellitus (DM) is a pandemic metabolic disease that affects an enormous amount of males at a very early age. It is a leading cause of morbidity and mortality, in both developed and in developing countries. Type 2 diabetes mellitus (T2DM) is responsible for the vast majority of DM cases. It comprises individuals who present insulin resistance and relative insulin deficiency. Unlike type 1 diabetes mellitus (T1DM), the main triggering factor of T2DM seems to be the new lifestyle of modern societies rather than genetic factors.

The process of production of viable spermatozoa is strictly regulated and dependent of several factors. Any alterations in this process may result in fertility problems in the male. The developing germ cells are dependent on the metabolic cooperation established with the Sertoli cells (SCs), being reliant on the lactate produced by these testicular somatic cells, as energy source.

Of the various antidiabetics available in the market, a number of them have a direct action in the individual metabolic homeodynamics. However, its mechanisms of action are different and vary dependent on several factors (e.g. tissue or target cells). In this work, we evaluated the effect of two antidiabetic drugs, pioglitazone and metformin, in human Sertoli cell metabolism (hSCs).

Our results suggest that, like metformin, pioglitazone may be a suitable antidiabetic drug for young men and those in reproductive age. These novel findings suggest a possible protective role of pioglitazone to male reproductive function.

Keywords

Pioglitazone; Metformin; Spermatogenesis; Sertoli cells; Infertility, Mitochondria

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List of Abbreviations

^{1}H-NMR	Proton nuclear magnetic ressonance
ACAC	Acetyl-Coenzyme A carboxylase
ACAD	Acyl-Coenzyme A dehydrogenase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BTB	Blood/testis barrier
CI	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8
CII	Succinate dehydrogenase complex, subunit B, iron sulfur
CIII	Ubiquinol-cytochrome c reductase core protein II
CIV	Mitochondrially encoded cytochrome c oxidase I
CoA	Coenzyme A
CV	ATP synthase alpha-subunit
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic acid
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GH	Growth hormone
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
HBSS	Hank's Balanced Salts Solution
hSC	Human Sertoli cell
IGF-1	Insulin-like growth factor I
IGT	Impaired glucose tolerance
ITS	Insulin–transferrin–sodium selenite
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
LDH	Lactate dehydrogenase
LDHA	Lactate dehydrogenase type A

LDHC4	Lactate dehydrogenase type C isoform 4
LH	Luteinizing hormone
MCT	Monocarboxylate transporter
Met	Metformin
M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTP	Mitochondrial trifunctional protein
NADH	Reduced nicotinamide adenine dinucleotide
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PDH	Pyruvate dehydrogenase
PFK1	Phosphofructokinase 1
PI3K	Phosphatidylinositol 3-kinase
Pio	Pioglitazone
PPAR*α*	Peroxisome proliferator-activated receptor *α*
PPARγ	Peroxisome proliferator-activated receptor γ
SC	Sertoli cell
SEM	Standard error of the mean
SGLT	Sodium dependent glucose transporter
SRB	Sulforhodamine B
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
T3	Tri-iodothyronine
T4	Thyroxine
TBS	Tris-buffered saline solution
TZD	Thiazolidinedione

I. Introduction

1. Testicular physiology

The establishment of male fertility is a complex process that requires specific and controlled interactions between different tissues and cells of the male reproductive tract and accessory glands. The male reproductive tract is formed of heterogeneous tissues, including the testis, efferent ducts, epididymis and vas deferens. The mammalian testis is a complex organ, coated for a capsule constituted by complex fibrous structure in various distinct tissue layers. The major component of the capsule, the tunica albuginea, is distinguished by the existence of fibroblasts interspersed in collagen fibers (Setchell, Maddocks et al. 1994), from which septations extend toward the testicular mediastinum, separating the human testis into 200–300 lobules (Niederberger 2011) (Figure 1). Tunica albuginea has several physiological functions in male reproduction, including preservation of the interstitial pressure within the testis, boost the transport of sperm out of the testis into the epididymis and control of the blood flow through the testis (Setchell, Maddocks et al. 1994).

Each testicular lobule encloses coiled seminiferous tubules (Figure 1). Within the testis two compartments are formed, the interstitial and tubular, that present different cell types and fluid flow (Weinbauer, Luetjens et al. 2010). The interstitial compartment is the space outside the seminiferous tubules. Here, the most important cells are the Leydig cells that actively produce and secrete testosterone, the main male sexual hormone (Haider 2004, Ge and Hardy 2007) (Figure 1). In humans, the interstitial compartment represents about 12–15% of the total testicular volume, 10–20% of which is composed of Leydig cells. Human testes have approximately 200×10^6 Leydig cells (Weinbauer, Luetjens et al. 2010). The proliferation rate of these cells in the adult testis is low and influenced by luteinizing hormone (LH) (see below). In addition to Leydig cells, the interstitial compartment contains macrophages, lymphocytes, mesenchymal fibroblasts, extracellular matrix, and small blood capillaries (Haider 2004). The tubular compartment represents about 60-80% of the total testicular volume. The seminiferous tubules are avascular and with no nerves penetrating through their walls (Setchell 1986). Overall, the human testis contains about 600 seminiferous tubules. The lobules filled by seminiferous tubules are separated by extensions of the tunica albuginea that open on both ends into the *rete testis* (Figure 1). Of note, all the processes involved in the production of male gametes (spermatogenesis), occur within the seminiferous tubules (Weinbauer, Luetjens et al. 2010). These tubules end at the *rete testis*, which is a network of tubules that empties into the efferent ductules. Spermatozoa are transported through the efferent ductules into the epididymis, and then enter the vas deferens, which through peristalsis propels them to the ejaculatory duct (Niederberger 2011).

The tubular compartment contains the germ cells and two different types of somatic cells, the Sertoli and peritubular cells (Niederberger 2011). The seminiferous tubules are sheltered by a lamina, which consists of a basal membrane, a layer of collagen and the peritubular cells (myofibroblastic cells). These cells are stratified and form concentric layers that are separated by collagen layers (Schell, Albrecht et al. 2010). The peritubular cells have an essential role in the transport of sperm (Romano, Tripiciano et al. 2005, Welsh, Saunders et al. 2009), and contribute to several testicular functions by secreting factors involved in cellular contractility (panactin, desmin, gelsolin, smooth muscle myosin and actin), components of the extracellular matrix (collagen, laminin, vimentin, fibronectin, growth factors, fibroblast protein) and adhesion molecules (Albrecht, Rämsch et al. 2006, Schell, Albrecht et al. 2008, Schell, Albrecht et al. 2010, Mayerhofer 2013, Flenkenthaler, Windschüttl et al. 2014).

The Sertoli cells (SCs) extend from the base to the apex of the epithelium in a direct interaction with the developing germ cells (Mruk and Cheng 2004) (Figure 1). About 35–40% of the volume of the germinal epithelium is comprised by these cells. The human testis of reproductive age contains about $800–1200\times10^6$ SCs (Zhengwei, Wreford et al. 1998). These somatic cells are commonly referred as “nurse cells”, because they are responsible for providing nutritional and energetic support to developing germ cells, and for creating an immunologically protected space for the development of germ cells (Rato, Alves et al. 2012). Adjacent SCs form tight junctions with each other in such a way that nothing larger than 1 kDa can pass from the outside to the inside of the tubule. The tight junctions formed between adjacent SCs create the Sertoli/blood–testis barrier (BTB) that physically divides the seminiferous epithelium into basal and apical compartments (Figure 1). This barrier regulates the movement of substances, such as nutrients and wastes, in and out of the seminiferous epithelium (Madara 1998, Alves, Rato et al. 2013). The cytoskeleton of SCs is responsible for the organization of the seminiferous epithelium and plays a fundamental role in facilitating germ cells movement. In addition to the tight junctions, other types of associations occur between the SCs to strengthen the BTB, such as adherens junctions (eg: ectoplasmic specialization, a testis-specific adherens junction type) and intermediate filament-based desmosome-like junctions (Pelletier 2001, Mruk and Cheng 2004, Rato, Alves et al. 2012). The relevance of these junctions goes far beyond the physical and nutritional support, as they must undergo extensive restructuring during germ cell migration on the course of spermatogenesis.

Several proteins, products and factors are known to be secreted by SCs, including: proteases, protease inhibitors, hormones, energy substrates, growth factors, paracrine factors, inhibin, transferrin, androgen-binding protein, plasminogen activator, glycoproteins, sulpho-proteins, myo-inositol and other extracellular matrix components (Fritz, Rommerts et al. 1976, Robinson and Fritz 1979, Elkington and Fritz 1980, Skinner and Griswold 1980, Marzowski, Sylvester et al. 1985, O'Brien, Gabel et al. 1993, Griswold 1998). Notably, the number of SCs is also an important determinant of testis size, and this number is directly related to the number of germ cells (Griswold 1998). Germ cells are dependent on SCs not only for structural support, but also for nutritional and energetic support. The SCs convert glucose to lactate that is known to be used as substrate and to influence the survival of germ cells. Although glucose is one of the most used substrates by SCs, they can also metabolize other substrates such as ketone bodies and fatty acids (Griswold 1998, Rato, Alves et al. 2012). These processes are vital for the production and export of lactate that can be conditioned by several factors. For instance, it was recently reported that insulin-deprived SCs altered the expression of metabolism-associated genes implicated in the export and production of lactate, as well as the consumption of glucose and secretion of lactate (Oliveira, Alves et al. 2012). The SCs also control the composition of the seminiferous tubular fluid and the physicochemical milieu where spermatogenesis occurs. The seminiferous tubular fluid serves as a mean of transport of sperm, and also helps to maintain a proper microenvironment required for a normal spermatogenesis (Rato, Socorro et al. 2010, Rato, Alves et al. 2011).

2. Spermatogenesis

Spermatogenesis is a complex biological process that produces spermatozoa in the seminiferous tubules of the mammalian testis. It involves an important balance between self-renewal and differentiation of spermatogonial stem cells to ensure an endless production and release of mature spermatozoa (Sharpe 1994). A fertile man produces more than 40 million spermatozoa per day, beginning at puberty and spanning his entire reproductive life (Cheng and Mruk 2013). The duration of the complete spermatogenic process varies according to species and in human it spans 74–76 days (Sharpe 1994). The SCs are responsible for the movement of germ cells from the base of the tubule toward the lumen and for the release of mature spermatozoa into the lumen. Moreover, as discussed above, the BTB, composed by SCs, divides the epithelium into two compartments: the basal compartment, in which spermatogonia, preleptotene, and leptotene spermatocytes exist; and the adluminal compartment, in which meiotic spermatocytes and spermatids in different stages reside (Hess and de Franca 2008, Cheng, Wong et al. 2010) (Figure 1). Spermatogenesis is controlled by several factors, (endocrine, paracrine and autocrine) and can be divided into four different phases that include: mitosis, meiosis, spermiogenesis and spermiation. In the early steps of spermatogenesis, diploid spermatogonia (2n) proliferate and generate two different populations of cells: one subpopulation of stem cells identical to their progenitors; other subpopulation, the majority, starts a differentiation process and differentiate into spermatozoa. In this process there is a subpopulation of spermatozoa and germ cells in different stages that undergo apoptosis (Dym 1994, Hofmann 2008, Sá, Neves et al. 2008, Sá, Miranda et al. 2013). Spermatogenesis is of great complexity and requires a time-specific period for completion depending of the species. For instance, it requires 6–9 weeks for completion in human. Spermatogonia are the most primitive diploid germ cells (2n) that divide by mitosis and reside on the basement membrane of seminiferous epithelium (Figure 1). In the mitotic phase, spermatogonia are self-renewed or undergo differentiation, with both cases involving several mitotic divisions. Little is known about the division of spermatogonia in humans, but three different types of spermatogonia have been identified in human: type A Dark, type A Pale, and type B (Rowley, Berlin et al. 1971, Dym 1994, Sá, Neves et al. 2008, Sá, Miranda et al. 2013). The main morphological features used to distinguishing between these four types of spermatogonia were the shape and staining characteristics of the nucleus, the position of the nucleolus, the structure of the mitochondrial cristae, the association of the endoplasmatic reticulum with mitochondria, the presence or absence of glycogen in the cytoplasm of the cells and the presence of the filamentous structures of cytoplasm are evaluated to distinguish (Rowley, Berlin et al. 1971). The Type A Dark spermatogonia are suggested to be the least morphologically differentiated and are considered the subpopulation of stem cells. Each spermatogonium is in touch with the basal membrane in the seminiferous tubule. Notably, this proximity progressively decreases with the degree of spermatogonia differentiation. The type A Dark spermatogonia are the flat cells lying parallel to the basal membrane, through the type A pale and type B (Rowley, Berlin et al. 1971, Hess and de Franca 2008). Type B are the most differentiated type of spermatogonia since this differentiation only occurs in two thirds of the type A pale spermatogonia. Type B spermatogonia enter meiosis and originate preleptotene spermatocytes, which in turn differentiate into leptotene, zygotene, pachytene and diplotene spermatocytes.

During these phases the chromosomes condense, form pairs of homologous chromosomes, synapses are completed and then substituted by crossing-over and homologous recombination. In diplotene stage, chromosomes are unsynapsed and the cell divides. The BTB separates these two states of spermatocytes, the preleptotene spermatocytes located in the basal compartment and the pachytene spermatocytes in adluminal compartment (Mruk and Cheng 2004, Cheng and Mruk 2013). Meiosis is characterized by the separation of chromosomes that occurs during the metaphase, anaphase and telophase of the first meiotic division, after which secondary spermatocytes are originated (Phillips, Gassei et al. 2010) (Figure 1). The prophase of the first meiosis lasts 1–3 weeks, whereas the other phases of the first meiosis and the entire second meiosis are completed within 1–2 days in man (Holstein 1994, Weinbauer, Luetjens et al. 2010). Secondary spermatocytes contain haploid chromosomes in duplicate. During the second meiotic division secondary spermatocytes originate four haploid spermatids (n) (Grootegoed, Siep et al. 2000, Weinbauer, Luetjens et al. 2010) (Figure 1). Spermiogenesis and spermiation are the last phases of spermatogenesis in which the spermatids undergo several alterations. During spermatid development, the nucleus elongates and condenses. The nucleus of spermatids contains compacted DNA following the replacement of nucleosomal histones by transition proteins and subsequently by protamines (Meistrich, Trostle-Weige et al. 1992). Finally, differentiated elongated spermatids and spermatozoa (Figure 1) are produced. For human, originally 12 spermatid maturation steps were described. These steps include nucleus condensation, the formation of a flagellum and the extrusion of a large part of cytoplasm (Weinbauer, Luetjens et al. 2010). The differentiation into the extremely specialized sperm cells is one of the most significant cell developmental processes that occur in biological systems. It involves phases of acrosome development, nuclear elongation and condensation, the formation of middle piece and tail, and the reduction of cytoplasmatic volume (Grootegoed, Siep et al. 2000). The SCs play an essential role in reducing the cytoplasmatic volume of the elongated spermatids. The adhesive contacts and ectoplasmic junctional specializations between SCs and spermatids are shattered and elongated spermatids are thereafter released into the lumen of seminiferous tubule occurs, in a process named spermiation (Griswold 1998, Grootegoed, Siep et al. 2000).

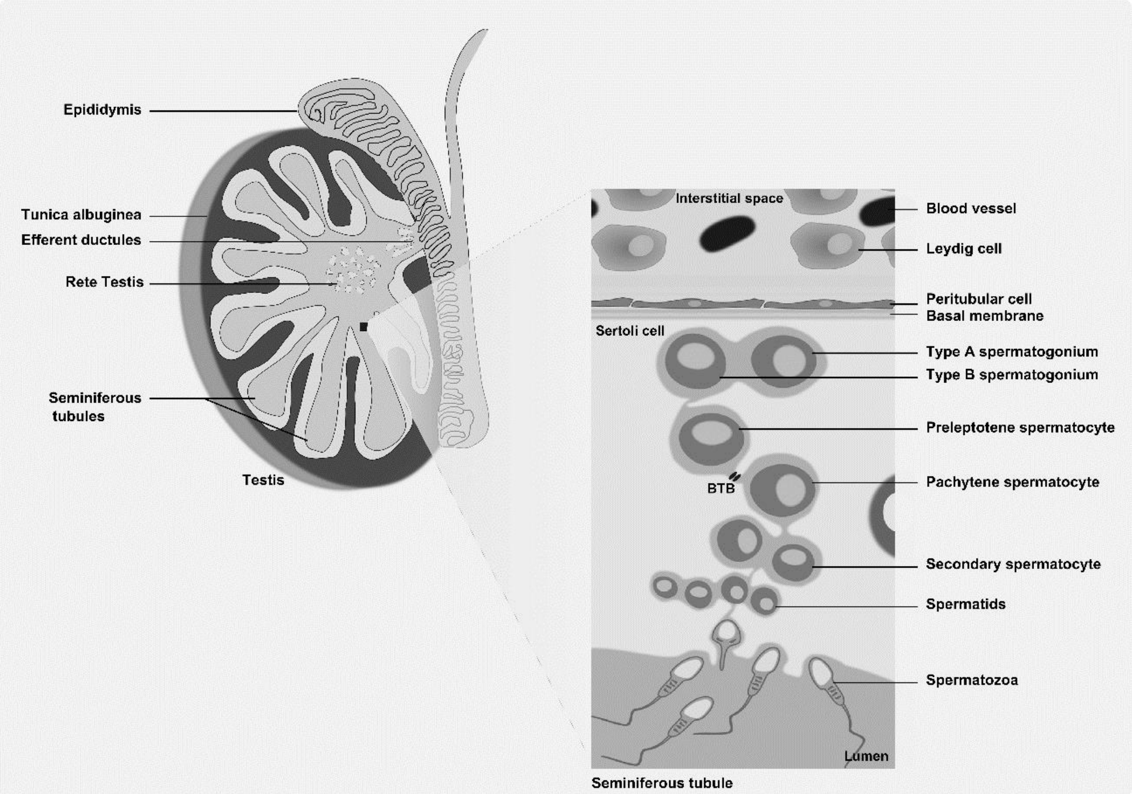

Figure 1: Schematic representation of the human testis and spermatogenesis. The testis is coated by tunica albuginea and divided in lobules. The seminiferous tubules, where spermatogenesis occurs, are coiled in the testis lobules. The Leydig cells and blood vessels are found in the interstitial space. The seminiferous epithelium is composed of Sertoli cells, which form the Sertoli/blood-testis barrier (BTB), and developing germ cells at different stages. The Sertoli cells adhere to the basal membrane where spermatogonia are also adherent. Type A Spermatogonia divide and develop into type B spermatogonia. The primary spermatocytes (preleptotene and pachytene) start meiosis and divide in secondary spermatocytes, which in turn divide into haploid spermatids that migrate towards the lumen where the fully formed spermatozoa are finally released.

2.1 Hormonal regulation of spermatogenesis

Spermatogenesis is an intricate process, controlled by several interacting mechanisms that differ according to the consecutive developmental stages: fetal, infantile, pubertal and adult. It is tightly regulated by the hypothalamus–pituitary–testis axis. Within this axis, neurons of the hypothalamus produce gonadotropin releasing hormone (GnRH). Regulation of gonadotropin secretion involves a complex interaction between stimulation by GnRH from the hypothalamus and feedback control by sex steroids and inhibin from the testes, besides autocrine/paracrine modulation by other factors within the pituitary (Walker and Cheng 2005, Niederberger 2011). Pulsatile GnRH signals stimulate gonadotropic cells in the anterior pituitary to release follicle-stimulating hormone (FSH) and LH that then act on the testis to regulate the spermatogenic potential (Walker and Cheng 2005, Rato, Alves et al. 2012, Alves, Rato et al. 2013). LH is of upmost importance for the stages of immature and mature adult Leydig cells, being the main inducer of these adult cells differentiation and the responsible for the maintenance of its high rates of proliferation (Habert, Lejeune et al. 2001). LH acts on Leydig cells through LH receptors present in the surface of these somatic cells of the testis and stimulates the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Testosterone is the main secreted product of the testis, with daily production rate being 5–7 mg in men (Niederberger 2011). Interestingly, testosterone plasma levels are strictly correlated with LH levels and it was described that pulsatile LH concentrations stimulate testosterone secretion in a dose-dependent manner (Walker and Cheng 2005, Cheng and Mruk 2013). SCs are the only testicular cells that express receptors for testosterone and FSH. FSH is a member of the glycoprotein hormone family which binds to its receptor in SCs and has typically been described to be involved in the beginning of pubertal spermatogenesis (Sharpe 1994). This hormone is crucial to stimulate spermatogenesis, as it maintains testicular size, seminiferous tubular diameter and a suitable number of sperm with normal motility (Walker and Cheng 2005, Niederberger 2011). FSH also regulates DNA synthesis in spermatogonia, spermatogonial proliferation and differentiation (Rossi, Albanesi et al. 1991, Dym 1994).

The hypothalamus–pituitary–testis axis is regulated by feedback mechanisms. The production of inhibin by SCs, as well as testosterone and 17β-estradiol by Leydig cells lead to a negative feedback that decreases the secretion of GnRH in the hypothalamus and of LH in the pituitary (Tilbrook and Clarke 2001). Sex steroids seem to play a slight role in the feedback control of FSH secretion while inhibin is the key factor implicated in the testicular regulation of FSH secretion. Notably, there is an inverse relationship between inhibin's circulating level and FSH ones (Niederberger 2011). In the last years, there has been an increasingly comprehension that sex steroid hormones are involved in several biological mechanisms, including the homeostasis of energy balance and energy metabolism in male reproductive activity (Wade, Schneider et al. 1996, Hill, Elmquist et al. 2008). The GnRH pulse is extremely susceptible to energetic deficits, environmental contaminants and intense exercise (Nindl, Kraemer et al. 2001, Saradha and Mathur 2006, Trumble, Brindle et al. 2010). For instance, Trumble and collaborators (2010) have described that fasting causes suppression of GnRH pulses, which consequently causes a decrease in the levels of LH and testosterone production levels by Leydig cells disturbing male reproductive function.

Androgens are considered the main sex male hormones, namely testosterone, while estrogens are usually referred as the sex female hormones. Nevertheless, androgens and estrogens are present in both sexes. Therefore, sexual distinctions are not qualitative differences, but result from quantitative divergence in hormone concentrations and differential expressions of steroid hormone receptors (Carreau and Hess 2010). In men, most serum estradiol is produced by peripheral aromatization of androgens secreted by Leydig cells. The cytochrome P450 enzyme aromatase is responsible for catalyzing this reaction and is primarily present in adipose tissue but also functions in skin and liver. In the testis, aromatase activity is primarily localized in Sertoli and Leydig cells (Inkster, Yue et al. 1995). It has been shown that estrogens have an essential role in regulating the hypothalamus-pituitary-testis axis and thus indirectly regulate LH and testosterone equilibrium through a feedback loop (O'Donnell, Robertson et al. 2001). Besides from gonadotrophins and steroid hormones, thyroid hormone has also been shown to play an important role in testicular physiology (Cooke, Holsberger et al. 2004, Mendis-Handagama and Ariyaratne 2005). Thyroid gland produces thyroxine (T4) and tri-iodothyronine (T3) (Mendis-Handagama and Ariyaratne 2005). It is known that T3 regulates the maturation and growth of the testis, in rats and other mammalian species, by inhibiting immature SCs proliferation and by stimulating their functional differentiation (Cooke, Hess et al. 1991, Hess, Cooke et al. 1993). Likewise, thyroid hormone has been shown to play a critical role in the onset of Leydig cell differentiation and stimulation of steroidogenesis in postnatal rat testis (Mendis-Handagama and Ariyaratne 2005). Though the mechanisms implicated in the regulatory actions of thyroid hormone in testicular cells are still undefined, the presence of thyroid hormone receptors in human and rat testis throughout development and in adulthood implies that T3 may act via the classical genomic pathway in testis (Benbrook and Pfahl 1987, Jannini, Olivieri et al. 1990).

Insulin-like growth factor I (IGF-1) is also biosynthesized in the testis by SCs (Itoh, Nanbu et al. 1994). Its receptors are identified in Leydig cells, peritubular cells, and spermatocytes (Niederberger 2011). It is known that IGF-1 stimulates the proliferation of Leydig cell precursors, spermatogenesis and spermatid maturation (Itoh, Nanbu et al. 1994). LH, FSH and testosterone may cooperate in spermatogenesis through stimulation of IGF-1 secretion by SCs (Itoh, Nanbu et al. 1994, Niederberger 2011). Growth hormone (GH) is not classically considered as a reproductive hormone, although it has important roles in reproductive function. It plays a role in steroidogenesis and spermatogenesis exerting an endocrine action either directly at gonadal sites or indirectly via IGF-1. GH influences Leydig cell steroidogenesis by regulating the secretion of IGF-1 and by increasing the expression of several genes that code for steroidogenic enzymes, including 3β-hydroxysteroid dehydrogenase, responsible for the conversion of pregnenalone to progesterone (Spiteri-Grech and Nieschlag 1992, Gomez, Weil et al. 1999, Hull and Harvey 2000, Niederberger 2011). Progesterone is another hormone very relevant for male reproduction. The maturation of progesterone to androstenedione is catalyzed by 17α-hydroxylase/ C17–20 lyase (CYP17), while further conversion of androstenedione to testosterone depends on 17β-hydroxysteroid dehydrogenase activity, in Leydig cell smooth endoplasmic reticulum (Niederberger 2011). Therefore, it is essential to unravel the complex hormonal network and signalling involved in the control of spermatogenesis to understand all the mechanisms relevant to male fertility, and unveil its flaws in cases of infertility.

3. Metabolic cooperation in testis

Metabolic cooperation is defined as the interchange of metabolism-associated molecules and metabolic substrates between cells (Hooper and Subak-Sharpe 1981). Although metabolic cooperation between neuronal and other cell types (Pellerin 2003) has been studied for decades, the study of these processes in the testis are relatively recent. In fact, latest advances provided compelling evidence that the metabolic cooperation between SCs and developing germ cells is crucial for the normal development of spermatogenesis (Rato, Alves et al. 2012). Moreover, it has been proposed that alterations in these processes result in serious consequences to male fertility (Alves, Martins et al. 2013). One of the major intervenient in the metabolic cooperation in the testis and to the occurrence of a normal spermatogenesis are SCs (Oliveira, Martins et al. 2015). As discussed, junctions between adjacent SCs form the BTB, which physically divides the seminiferous epithelium (Rato, Alves et al. 2011). Thus, the SCs are responsible for the selective passage of substances from blood plasma and testicular lymph to the *rete testis* fluid (Setchell 1980). Due to this function, these cells are responsible for providing nutritional support for the developing germ cells (Griswold 1995). Although this latter function of SCs has been underestimated for many years, it is now accepted that there is a close metabolic relationship between these cells and developing germ cells, a condition that is affected by many diseases and drugs (Alves, Dias et al. 2014). It has already been shown that there is a transference of carbohydrates, vitamins, lipids, amino acids and metal ions between SCs and germ cells (Mruk and Cheng 2004). Notably, SCs metabolism presents some distinctive characteristics. Cultured SCs convert a great percentage of glucose to lactate, the major energy substrate to germ cells (Robinson and Fritz 1981). This is a very interesting metabolic behaviour since SCs prefer the pathway of lactate production instead of Krebs cycle, using a less effective pathway concerning to adenosine triphosphate (ATP) production, in a clear evidence of a Warburg-like metabolic effect. Moreover, SCs present a high glycolytic flux, equally to what occurs in cancer cells

(Oliveira, Martins et al. 2015). This is a consequence of the metabolic cooperation needed between SCs and developing germ cells for the normal occurrence of spermatogenesis, illustrating the relevance for this process.

Glucose is essential for the occurrence of spermatogenesis. However, it is present in very low levels in the seminiferous tubules due to its rapid metabolism (Voglmayr, Waites et al. 1966, Robinson and Fritz 1979). Moreover, glucose is very hydrophilic and thus, it can pass through the lipid bilayer by simple diffusion in a very inefficient way. Therefore, there are two families of glucose transporters: the glucose transporters (GLUTs) and the sodium dependent glucose transporters (SGLTs) (Thorens 2001, Scheepers, Joost et al. 2004) that are responsible for the transport of this metabolite to cells. From these transporters, GLUTs are present in most tissues and are reported to play a key role in mediating passive glucose transport through membranes (Thorens 2001) (Figure 2). Different isoforms were already identified in SCs, particularly GLUT1 (Carosa, Radico et al. 2005, Galardo, Riera et al. 2008), GLUT2 (Kokk, Veräjänkorva et al. 2003), GLUT3 (Galardo, Riera et al. 2008) and GLUT8 (Carosa, Radico et al. 2005). However, the latter isoform is not reported to be responsible for glucose transport from the extracellular milieu since it has not been localized in the plasma membrane (Piroli, Grillo et al. 2002). Moreover, GLUT8 has been reported to be related with lysosomes and membranes of the endoplasmic reticulum (Schmidt, Joost et al. 2009). Once a molecule of glucose has passed from the blood into the cell, it is gradually metabolized in a controlled sequence of biochemical steps catalyzed by various enzymes. Firstly, it is oxidized into two molecules of pyruvate (Martins, Alves et al. 2013), through a process known as glycolysis (Figure 2). The first rate-limiting step of glycolysis, is the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (Chehtane and Khaled 2010). This reaction is catalyzed by phosphofructokinase (PFK) whose activity is also known to be linked with the energy status of the cell (Morgante, Tosti et al. 2011). The pyruvate produced during glycolysis can then follow three main pathways. One of those is the Krebs cycle where pyruvate is transported to the mitochondria and oxidized, as well as acetyl CoA, to produce NADH and $FADH_2$ for ATP synthesis in the respiratory chain (Freedman and Graff 1958, Oliveira, Martins et al. 2015). Another pathway is the conversion of pyruvate into alanine by alanine aminotransferase (ALT). The last pathway that pyruvate can follow is the conversion of this end product of glycolysis to lactate, by lactate dehydrogenase (LDH) (Rato, Alves et al. 2012) (Figure 2). For many years, lactate was reported as a waste product of glycolysis (Gladden 2004). However, this has been challenged and nowadays it is known that lactate is the main energy source for several cells, including the developing germ cells (Boussouar and Benahmed 2004).

In the last years, several authors have debated the role of the lactate produced by SCs in the survival and nutritional support of developing germ cells (Erkkilä, Aito et al. 2002, Boussouar and Benahmed 2004, Gladden 2004). Some studies have shown that lactate production is stimulated by FSH (Mita, Price et al. 1982), as well as epidermal growth factor (Mallea, Machado et al. 1986). It has been shown that both stimulate lactate production by enhancing the glycolytic metabolism of rat SCs, since they increase the glucose uptake into the cells (Hall and Mita 1984, Mallea, Machado et al. 1986). These mechanisms are thought to be highly regulated through a complex signalling network. The greater glucose uptake may possibly result from the interaction between FSH and phosphatidylinositol 3-kinase (PI3K). Deprivation of insulin has also been reported to alter glucose consumption and lactate production by SCs (Oliveira, Alves et al. 2012). Insulin deprivation altered the expression of several genes involved in the production and export of lactate, such as LDH but also induced a similar adaptation in the expression of GLUTs in conditions of glucose deprivation (Riera, Galardo et al. 2009, Oliveira, Alves et al. 2012). Moreover, 5α-dihydrotestosterone (100 nM) decreased lactate production in human SCs (Oliveira, Alves et al. 2011). This decrease was reported to be due to a decrease in mRNA levels of LDHA, since glucose consumption was increased. Thus, it seems that 5α-dihydrotestosterone redirects glucose metabolism to Krebs cycle (Oliveira, Alves et al. 2011).

After being produced in SCs through the action of LDH, lactate is exported to the intratubular fluid through monocarboxylate transporters (MCTs) (Figure 2). From the 14 members of this family, only MCT1 to MCT4 are thought to be involved in the transport of lactate (Boussouar and Benahmed 2004). However, not all are present in testicular cells. While MCT1 and MCT4 can be found in SCs, MCT2 can be found in elongated spermatids. Moreover, MCT1 was found in developing germ cells and is reported to play a major role in the import of lactate from the intratubular fluid. Lactate is converted by LDHC4 (Shi, Wang et al. 2005), the specific isoform of LDH in testis, into pyruvate. The latter is then converted by pyruvate dehydrogenase into acetyl-CoA, which enters the mitochondria to be used in the Krebs cycle. Besides lactate, acetate is also produced by SCs and may be used by developing germ cells for lipid synthesis and membrane remodelling (Alves, Socorro et al. 2012) (Figure 2). Acetate has been described as an intermediate metabolite and is the most common intermediate for the synthesis of fatty acids and cholesterol (Alves, Socorro et al. 2012). It results from the conversion of acetyl-CoA by acetyl-CoA hydrolase.

While developing germ cells rely on the lactate produced at high rates by SCs, the resulting mature sperm cells present specific metabolic needs, using external hexoses, such as glucose, as their main substrate (Frenkel, Peterson et al. 1973). Following the uptake, the hexose metabolism is crucial to preserve spermatozoa function and motility (Williams and Ford 2001). Thus, either oxidative phosphorylation and/or glycolysis can be used to provide the energetic needs to spermatogenic cells (Storey 2008).

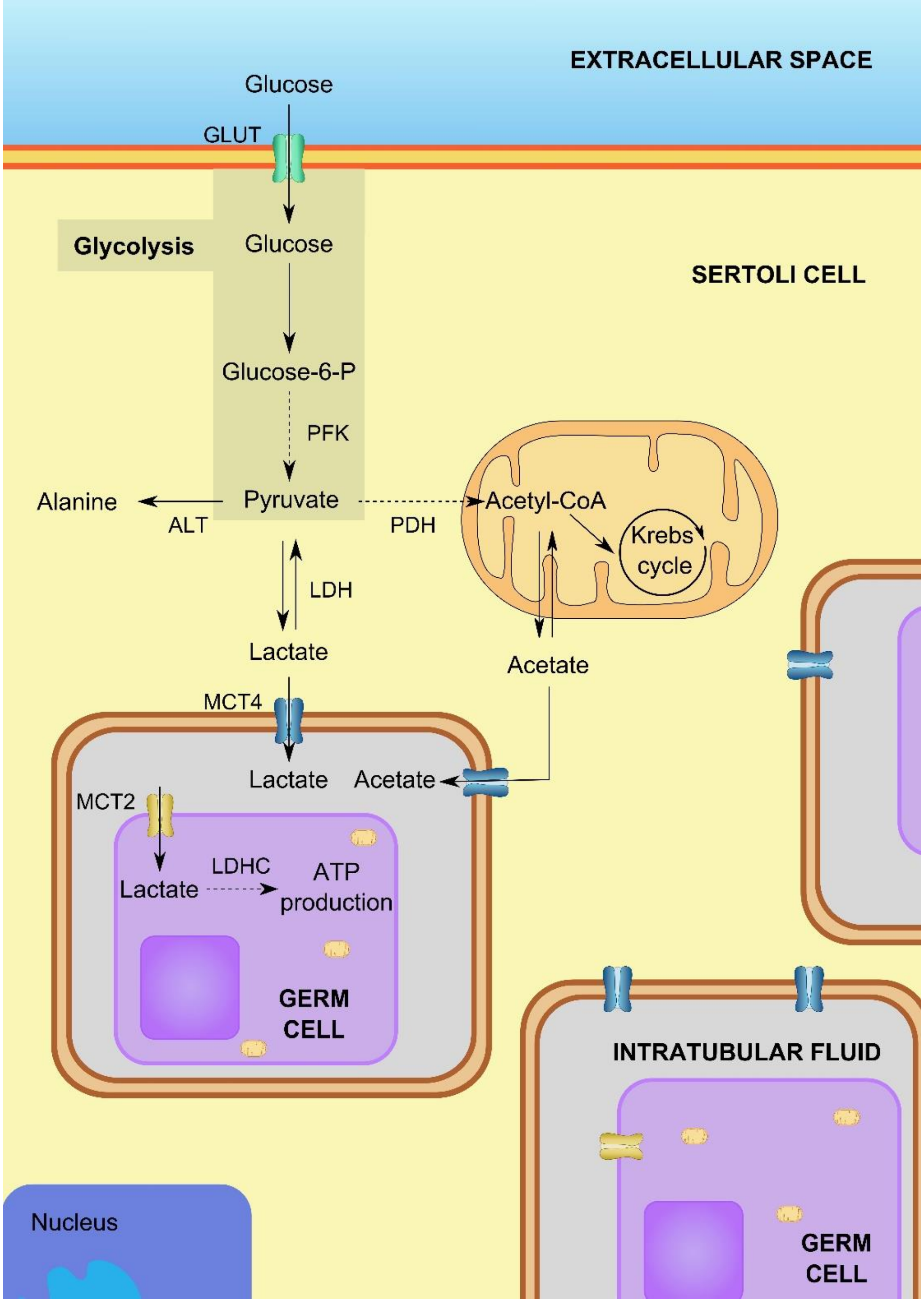

Figure 2: Representative diagram of the metabolic cooperation established between Sertoli cells and developing germ cells in the testis. The glucose from the extracellular space enters the Sertoli cells through glucose transporters (GLUT), and is converted into glucose-6-phosphate (Glucose-6-P). Phosphofructokinase (PFK) catalyzes the rate limiting step of glycolysis, which results in the formation of pyruvate. The pyruvate can then be: converted into alanine by alanine aminotransferase (ALT); converted into lactate by lactate dehydrogenase (LDH); or transported into the mitochondrial matrix to form acetyl-CoA. Pyruvate dehydrogenase (PDH) catalyzes the rate limiting step of the latter conversion of pyruvate. The lactate produced by Sertoli cells is exported to the intratubular fluid by monocarboxylate transporters (MCTs), mainly MCT4. The germ cells uptake the lactate produced by Sertoli cells through MCT2 that is used for ATP production by the isoform LDHC. Moreover, acetyl-CoA may be converted into acetate, which is then exported to the intratubular fluid and may be used by germ cells for membrane remodelling.

4. (Pre)-diabetes in brief

DM is one of the most widespread chronic diseases. It is a leading cause of morbidity and mortality, in both, developed and in developing countries. The World Health Organization estimates that 347 million of people have DM (Alberti and Zimmet 1998). According to a recent report, the number of diabetic patients may reach 592 million until the year 2035 (Guariguata, Whiting et al. 2014). These alarming statistics clearly illustrate that is urgent to study the impact of this pathology on cells, organs and whole body. DM is a chronic, metabolic disease of multiple aetiologies, characterized by hyperglycemia that can result from defects in insulin secretion, insulin action or both (American Diabetes Association 2014). Moreover, it causes impaired carbohydrate, fat, and protein metabolism (Hamden, Jaouadi et al. 2011). All body metabolism is affected and thus, DM can lead to a variety of co-morbidities, including cardiovascular diseases, retinopathy, obesity, sexual disorders, nephropathy and diabetic foot (Struijs, Baan et al. 2006, Meneses, Sousa et al. 2015). The most prevalent types of DM are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM, also known as insulin-dependent DM, is characterized by an autoimmune reaction of T lymphocytes on insulin-producing beta cells in genetically susceptible individuals (Killestein 2002, Lehuen, Diana et al. 2010). The destruction of pancreatic beta cells is progressive and results in a dramatic decrease or even elimination of insulin production. Consequently, these patients are dependent on exogenous insulin treatment to survive (Van Belle, Coppieters et al. 2011). The first symptoms of T1DM generally appear before the age of 30 for what it is known as juvenile-onset DM. Accompanying the trends of DM, T1DM has also been drastically increasing in recent years. Of note, it is expected that the prevalence of cases in individuals younger than 5 years will double between 2005 and 2020 (Patterson, Dahlquist et al. 2009).

T2DM is responsible for the vast majority of DM cases (American Diabetes Association 2014). It comprises individuals who present insulin resistance and relative insulin deficiency (Henriksen, Diamond-Stanic et al. 2011). Unlike T1DM, the main triggering factor for T2DM seems to be the new lifestyle of modern societies rather than genetic factors, although the latter can also play a crucial role in the establishment of the disease (Hu, Manson et al. 2001). Insulin, which has a preponderant role in DM, is the main hormone responsible for glucose homeostasis. In the early stages of the disease, the cells have decreased sensitivity to insulin, failing to respond to normal levels in circulation. This characteristic of T2DM is known as insulin resistance (Hu, Manson et al. 2001). Thus, pancreatic beta cells augment the rate of insulin secretion to compensate the resistance of the tissues to this hormone (Kahn 1998). However, with the progression of the disease, beta cells loose the capacity to release adequate amounts of insulin to compensate the insulin resistance, leading to the development of impaired glucose tolerance (IGT) (DeFronzo, Bonadonna et al. 1992). IGT is the main characteristic of pre-diabetes, which is an intermediate condition between normal glucose tolerance and T2DM (Tabák, Herder et al. 2012). In fact, this condition may be identified through an oral glucose tolerance test, once pre-diabetic individuals present glycemic variables that are higher than normal, but lower than T2DM thresholds (Tabák, Herder et al. 2012, Alves, Martins et al. 2013). Like T2DM, pre-diabetes may be triggered by sedentary lifestyle and inadequate diets, such as the ingestion of high caloric and saturated fat rich diets (Rato, Alves et al. 2013). Consequently, these patients present metabolic modifications in diverse organs, leading to an increased probability of developing several co-morbidities, like cardiovascular disease (American Diabetes Association 2014, Buysschaert, Medina et al. 2015). In fact, pre-diabetic patients present a higher risk of stroke (Lee, Saver et al. 2012) and a 2- to 3-fold increase in the risk of developing macroangiopathy (Huxley, Barzi et al. 2006). Moreover, the prevalence of chronic kidney disease is also high among pre-diabetic patients (Plantinga, Crews et al. 2010, Buysschaert, Medina et al. 2015). Despite the similarity between T2DM and pre-diabetes, the latter does not imply the progression to T2DM. Lifestyle and/or drug-based interventions can be done to revert the condition, illustrating the relevance of the early diagnosis of this prodromal stage of T2DM.

5. Modifications in testicular metabolic cooperation promoted by (pre)-diabetes

The metabolic cooperation between testicular cells is crucial for germ cells development and any alteration in those processes may have drastic consequences for male fertility potential (Alves, Dias et al. 2014, Reis, Moreira et al. 2015). However, the precise molecular mechanisms of testicular glucose metabolism in diabetic patients are far from being unveiled. However, a study performed during the 80s reported that rat SCs and peritubular cells were very sensitive to glucose concentrations. In fact, SCs produced more lactate when they were in contact with a higher glucose concentration (Hutson 1984).

There is increasing information about the precise molecular mechanisms of testicular glucose metabolism in diabetic patients through *in vitro* and morphological studies of human biopsies from those individuals. Studies using testicular biopsies from diabetic men have shown an extensive vacuolization and a high degree of degeneration in SCs (Cameron, Murray et al. 1985). In addition, and despite germ cells presented normal morphology, the seminiferous tubules were depleted and Leydig cells presented lipid droplets and variable number of vacuoles (Cameron, Murray et al. 1985). In fact, all these alterations have drastic consequences to testicular cells' metabolism, particularly to the metabolic cooperation discussed above. Moreover, a study described that DM caused a decrease in lactate production and an increase of endogenous oxygen uptake by testicular cells (Sharaf, Kheir El Din et al. 1978).

A recent study described that insulin depletion and hyperglycemia in streptozotocin-induced DM did not regulate the expression of GLUT8 in testes, once its expression was not modified in these conditions (Gómez, Ballester et al. 2009). However, another study explored the effect of insulin deprivation on several markers of apoptotic signalling in cultured rat SCs. The latter showed a significant decrease on mRNA levels of p53, Bax, caspase-9 and caspase-3 followed by a significant increase of Bax and a decrease of caspase-9 protein levels relatively to the control. These results provided clear evidence that insulin deprivation decreases caspase-dependent apoptotic signalling in cultured rat SCs, evidencing a possible mechanism by which the lack of insulin can affect spermatogenesis and fertility (Dias, Rato et al. 2013).

Although the exact mechanisms by which DM modulates glucose metabolism in testicular cells are not easy to follow *in vivo*, *in vitro* tests showed some interesting mechanisms. Some studies demonstrated that both rat and human SCs metabolism is influenced by sex steroid hormones, which are known for being altered in DM (Kanter, Aktas et al. 2012). Importantly, treatment with 5α-dihydrotestosterone resulted in decreased glucose consumption, leading to reduced lactate production and, consequently, less energy for developing germ cells (Oliveira, Alves et al. 2011). Besides, the decrease in lactate production may lead to increased apoptosis of developing germ cells once lactate as an important anti-apoptotic effect (Dias, Martins et al. 2013). 17β-estradiol and 5α-dihydrotestosterone also modified gene transcript levels of GLUTs, LDH and MCT4. These proteins are all related with the glycolytic pathway and lactate production or transport, thus these modifications will affect germ cells (Oliveira, Alves et al. 2011, Rato, Alves et al. 2012). Moreover, impaired levels of steroid sex hormones may lead to apoptosis and necrosis of SCs, since they regulate apoptotic signalling pathways (Royer, Lucas et al. 2012, Simoes, Alves et al. 2013). Nevertheless, when analysing the effects of DM in the glycolytic pathway, one cannot neglect the possible involvement of glycogen. Although some reports from the 80s report that SCs possess glycogen and glycogen phosphorylase activity, these reports were not consolidated (Leiderman and Mancini 1969, Slaughter and Means 1983). However, a recent study has shown that T2DM enhances testicular glycogen accumulation in rats, by modulating the availability of the precursors for its synthesis.(Rato, Alves et al. 2015) Thus, the influence of glycogen metabolism to SCs is most likely underestimated, mainly under abnormal physiological conditions since these cells possess the necessary machinery to metabolize glycogen. In fact, when glucose is not available, glycogen is a mobilizable fuel storage that can be readily metabolized (Villarroel-Espíndola, Maldonado et al. 2013). Thus, glycogen should deserve special attention in the future to comprehend its real relevance on these processes, particularly under pathological conditions.

Spermatogenesis and sperm maturation are also affected by pH establishment in the several luminal fluids. In fact, disturbances of acid-base homeostasis in the reproductive tract have been associated with male infertility/subfertility in mammals (Pastor-Soler, Pietrement et al. 2005, Bernardino, Jesus et al. 2013). Moreover, it is also known that pre-diabetes cause an alteration in bicarbonate homeodynamics in the lumen of the epididymis (Bernardino, Martins et al. 2013). This disturbance may affect the establishment of a proper environment for sperm storage and viability and, hence, male reproductive potential since the regulation of pH and ionic properties of the seminiferous tubular fluid is essential for spermatogenesis (Rato, Socorro et al. 2010, Martins, Bernardino et al. 2014).

6. Effects of diabetes mellitus in sperm physiology and metabolism

As previously discussed, the prevalence of DM has been dramatically increasing (Guariguata, Whiting et al. 2014). Interestingly, on the contrary, the fertility rate has declined in the recent decades. In fact, these two events appear to be interconnected for various reasons. One of the possible explanations is an increased frequency of diabetic men on reproductive age (Delfino, Imbrogno et al. 2007). As discussed in the previous subchapters, the metabolism of testicular cells is pivotal for spermatogenesis. Moreover, glucose and insulin are key players in the control of testicular cells metabolic cooperation. Thus, the deregulation promoted by DM in glucose and insulin might be a key factor to the decline in fertility rate observed in countries with high incidence of metabolic diseases, particularly DM and pre-diabetes.

Testicular function is primarily controlled by pituitary hormones: FSH and LH. While LH controls Leydig cell function, FSH regulates spermatogenesis (Schulz and Miura 2002). Thus, both pre-diabetes and DM may affect male reproductive function due to their effects on the endocrine control of spermatogenesis (Agbaje, Rogers et al. 2007). Several of the effects promoted by DM in testicular function have been attributed to the lack of insulin (Ballester, Munoz et al. 2004). In men with these diseases, the absence of the stimulatory effect of insulin and an insulin-dependent decrease in FSH causes a decrease in testosterone production by Leydig cells. As FSH acts on SCs and is crucial to stimulate spermatogenesis, the decrease in FSH also causes a decrease in the sperm output and fertility (Ballester, Munoz et al. 2004). Moreover, diabetic patients present an abnormal feedback of the hypothalamus pituitary axis by gonadal steroids, either due to inefficient steroid transport into effector cells or reduced pituitary sensitivity (Dong, Lazarus et al. 1991, Baccetti, la Marca et al. 2002, Dias, Alves et al. 2014). These modifications have a direct effect on germ cell development, namely during spermatogenesis, spermiogenesis and on sperm metabolism (Chiodini, Di Lembo et al. 2006) leading to abnormalities such as abnormal ultrastructure of ejaculated sperm (Baccetti, la Marca et al. 2002).

DM can cause other sexual disorders including erectile dysfunction (Dey and Shepherd 2002), retrograde ejaculation (Ellenberg and Weber 1966), impotence (Ellenberg 1971) or decreased libido (Nakanishi, Yamane et al. 2004). Some studies have reported anomalous sperm parameters and quality markers in diabetic men. However, the literature shows several contradictory results (Alves and Oliveira 2013). While the majority of the studies report one or more anomalies in sperm parameters of diabetic men, such as lower sperm counts (Ranganathan, Mahran et al. 2002), significant differences in sperm motility (Bartak 1978, Ali, Shaikh et al. 1993, Delfino, Imbrogno et al. 2007) and morphology (Bartak 1978, Delfino, Imbrogno et al. 2007, Rato, Alves et al. 2013), others did not find any significant differences (Padrón, Dambay et al. 1984). However, it is less controversial that diabetic patients present higher levels of glucose and fructose in sperm (Padrón, Dambay et al. 1984, Delfino, Imbrogno et al. 2007). This fact, along with sperm ineffective metabolic control, led to the establishment that impairment of sperm parameters in diabetic men may be related with hexose metabolism in these cells (Padrón, Dambay et al. 1984). In fact, diabetic patients are known to have a defective glucose transport due to a depletion of GLUTs (Handberg, Vaag et al. 1990). Moreover, it was found that DM is associated with increased sperm nuclear and mitochondrial (mtDNA) damage (Agbaje, Rogers et al. 2007), probably due to oxidative damage, which may impair male fertility and reproductive health. Interestingly, a study has shown that the ejaculate of diabetic men contains higher concentrations of spermatozoa with disrupted mitochondrial transmembrane potential, activated caspase-3, reactive oxygen species and fragmented DNA when compared with nondiabetic donors (Roessner, Paasch et al. 2012). Moreover, these results were more pronounced in men with T2DM (Roessner, Paasch et al. 2012). Despite some contradictory studies, it seems clear that diabetic patients have fertility problems. Thus, the discovery of the molecular mechanisms by which DM affects male fertility is essential.

7. Antidiabetic Drugs

In the last decades, the number of diabetic patients has alarmingly increased, particularly due to the increased rates of T2DM. Besides the health problems, this is associated with severe economic and sociologic problems. The treatment of diabetic individuals involves high costs and the amount of money spent every year in their treatment is increasing. There are several antidiabetic drugs in the market that can be used either in monotherapy or in combination. The mechanisms of action for each compound are different and may vary depending of several conditions, including the doses. The antidiabetic drugs aim to control glucose metabolism and, in a non-specialized approach, we can affirm that their gold objective is to lower blood glucose levels. Therefore, most of their mechanisms of action are intimately linked with glucose metabolism. Unfortunately, most of these compounds compensate loss of insulin sensitivity, of insulin action or of insulin secretion, as well as other mechanisms responsible for the disease, but are unable to avoid or treat some of the deleterious effects. In addition, this is a field of research in constant change with the development of new products and intense research.

Glycemic control in diabetic patients is sometimes quite difficult do attain. Therefore, it is urgent that the scientific community provides new and better options to improve the use of the current available drugs and to highlight the most suitable therapies. Of particular relevance is the study of the pathophysiological alterations that diabetic individuals suffer and how the available drugs may affect those processes. There are several options for the treatment of diabetic patients, with distinct modes of action. Notably, some of those compounds were developed or are in development to treat other diseases, particularly obesity, which is a major cause for the establishment of DM. Thus, the complexity of compounds available, their modes of action and their biological activities are hot topics of debate for multiple areas of the human health, particularly for cardiovascular, renal, neurologic and even cancer pathologies.

DM is a multifactorial disease, which indicates that a complex analysis is needed when searching for new targets to treat this disease or the mode of action of compounds with potential antidiabetic activity. Moreover, several of these compounds, if not all, have the ability to alter cellular metabolism in a way that may be of benefit in some organs, but cause damage in others. This is a tricky problem that hampers the development of new drugs and obliges to a constant monitoring of how the patients take each compound alone or how combined therapies may evolve.

7.1 Biguanides

Several glucose-lowering guanidine derivatives were introduced in the 1920s, due to the finding that *Galega officinalis*, a traditional herb historically used as treatment for DM, was rich in guanidine (Oubre, Carlson et al. 1997). These agents were almost forgotten as insulin became widely available and used (Bailey 1992). It was not until the 1950s that biguanides were re-investigated for the treatment of DM. In the late 1950s, three biguanides with antidiabetic action were reported: phenformin (McKendry, Kuwayti et al. 1959), buformin (Bailey 1992) and metformin (Met) (Gottlieb and Auld 1962). The use of phenformin and buformin has been discontinued in many countries due to a high incidence of lactic acidosis (Misbin 1977), leaving Met as the main biguanide used worldwide (Williams and Palmer 1975, Bailey 1992). Indeed, Met has been used to treat T2DM for over 50 years. It is usually described as the first line treatment to this disorder, along with diet and exercise (Stumvoll, Nurjhan et al. 1995). Met is an insulin-sensitizing drug that exerts its antihyperglycemic effects by blocking liver gluconeogenesis (Alves, Martins et al. 2014) through regulation of the gluconeogenic flux, rather than direct inhibition of gluconeogenic gene expression (Viollet and Foretz 2013). It also increases skeletal muscle uptake of glucose (Musi, Hirshman et al. 2002) and reduces the absorption of glucose in the intestinal mucosa (Ikeda, Iwata et al. 2000). Despite almost 60 years of research, the exact mechanisms of Met action remain to be unveil. Nevertheless, it is known that Met has no effect on stimulating insulin secretion in pancreatic β-cell (Bailey 1992). In recent years, studies provided evidence that Met acts as an inhibitor of complex I of the electron transport chain (Owen, Doran et al. 2000, Andrzejewski, Gravel et al. 2014), inducing activation of AMP-activated protein kinase (AMPK) sensitive signalling (Musi, Hirshman et al. 2002) (Figure 3). AMPK acts as a regulator of glucose and lipid metabolism, as well as cellular energy regulation, through phosphorylation of some key proteins (Coughlan, Valentine et al. 2014). The increase in its activity results in several biological alterations including: stimulation of glucose uptake in muscle; fatty acid oxidation in liver and muscle; inhibition of hepatic glucose production, cholesterol and triglyceride synthesis; and lipogenesis (Winder and Hardie 1999). Studies showed results consistent with a model where increased phosphorylation and activation of AMPK leads to the effects on glucose and lipid metabolism observed after treatment with Met (Foster 2012, Hadad, Hardie et al. 2014). It has been suggested that activation of AMPK leads to the phosphorylation and inactivation of acetyl-CoA carboxylase (ACAC), inhibiting the rate-limiting step of lipogenesis (Hadad, Hardie et al. 2014, Rebecca, Morgan et al. 2015). Reduced synthesis of malonyl-CoA, the ACAC product, is also predicted to relieve inhibition of carnitine palmitoyltransferase 1, resulting in increased fatty acid oxidation (Foster 2012, Abo Alrob and Lopaschuk 2014) (Figure 3). These effects may contribute to the Met's *in vivo* ability to lower triglycerides (Abo Alrob and Lopaschuk 2014). It was also demonstrated, using an AMPK inhibitor, that AMPK activation is required for inhibition of hepatocyte glucose production by Met (Zhou, Myers et al. 2001). Moreover, an association between increased glucose uptake and AMPK activation was observed in isolated skeletal muscles, and it was suggested that Met's effect in augmenting muscle insulin action *in vivo* may be attributed to AMPK as well (Zhou, Myers et al. 2001). Another study showed that AMPK activation is absolutely required for the glucose-lowering action of Met *in vivo* but with genetic evidence. The genetic deletion of liver kinase B1, one of the upstream activators of AMPK, did not impair AMPK activation in muscle, yet it eliminated the effect of Met on serum glucose levels. This led the authors to hypothesize that in mice, Met primarily decreases blood glucose concentrations by decreasing hepatic gluconeogenesis (Shaw, Lamia et al. 2005). Interestingly, it was recently reported that Met could be considered as a suitable antidiabetic drug for male patients in reproductive age with T2DM. The authors showed that besides not having deleterious effects on human Sertoli cells (hSCs), the cells that support spermatogenesis (Alves, Martins et al. 2014, Dias, Alves et al. 2015, Oliveira, Martins et al. 2015), Met presented a possible antioxidant activity. Moreover, Met stimulated lactate production by those testicular cells (Alves, Martins et al. 2014), which provides nutritional support (Dias, Alves et al. 2015) and has an anti-apoptotic effect in developing germ cells (Erkkilä, Aito et al. 2002). Another suggested effect for Met is that it increases plasma levels of glucagon-like peptide-1, an incretin hormone with antihyperglycemic properties, and induces islet incretin receptor gene expression, through a mechanism that is dependent on peroxisome proliferator-activated receptor *α* (PPAR*α*) (Maida, Lamont et al. 2011). Met has a main advantage over other biguanides, which is the very low probability to promote lactic acidosis (Inzucchi, Bergenstal et al. 2012). However, it has some disadvantages, such as adverse gastrointestinal effects (Inzucchi, Bergenstal et al. 2012). Although considered the first-line pharmacological agent for T2DM individuals, in many patients the administration of this drug is insufficient to reach glycemic control and a second agent is added to the treatment (Rendell 2004, Ahren 2008, Derosa and Maffioli 2012).

Besides T2DM, Met is considered a therapeutic option for other diseases related with insulin resistance, such as polycystic ovary syndrome (PCOS), an endocrinopathy characterized by hyperandrogenism, menstrual irregularity/anovulation, and polycystic-appearing ovaries with a polycystic appearance on ultrasound (Johnson 2014, Vitek, Alur et al. 2015). However, the specific molecular pathways where Met acts on the ovary remain elusive (Viollet, Guigas et al. 2012). As in the case of T2DM, it is thought that AMPK is involved in the mechanism of action of Met in PCOS. AMPK is present in all cell compartments of the ovary and its expression could be different depending on the maturation stage. *In vitro* studies with primary cells of theca interna from the rat showed that the addition of Met was associated with increased activity of AMPK and inhibition of proliferation induced by insulin stimulation (Will, Palaniappan et al. 2012).

In addition, there are several studies associating Met to reduced cancer development and progression (Viollet, Guigas et al. 2012). The different mechanisms associated to this action of Met are fundamentally focused on inhibition of growth stimuli and alterations of metabolic processes, which control cancer cell growth. This characteristic began to be observed in epidemiological studies, but was later confirmed by *in vitro* and *in vivo* studies. In fact, it has been demonstrated that Met has an antiproliferative action in various cell lines and animal models (Viollet and Foretz 2013). After its uptake into the cells, Met seems to cause a reduction in ATP via inhibition of the mitochondrial respiratory chain complex 1 leading to activation of AMPK (Shackelford, Abt et al. 2013). AMPK activation then disrupts the expression of genes involved in gluconeogenesis, protein synthesis, lipogenesis and possibly angiogenesis (Morales and Morris 2014). Moreover, treatment with Met may decrease serum levels of insulin and insulin-like growth factor, therefore reducing the stimulus for cell growth. In fact, a decrease in insulin and insulin-like growth factor, whose receptors are expressed on many cancer cells, induced by caloric restriction have been shown to reduce the incidence of cancer in *in vivo* animal models (Kalaany and Sabatini 2009). Numerous studies have revealed growth-inhibiting effects of Met in breast, endometrial, lung, liver, gastric, and medullary thyroid cancer cell lines (Rizos and Elisaf 2013). Moreover, studies of the effects of some chemotherapeutics (cisplatin, carboplatin and paclitaxel) on endometrial (Dong, Zhou et al. 2012) and ovarian (Erices, Bravo et al. 2013) cancer cell lines have suggested a role for Met as adjuvant therapy. On endometrial cancer cells, it has been described that Met acted by a downregulation of glyoxalase I, an enzyme that is related with glycometabolism. Moreover, glyoxalase I is overexpressed in endometrial cancer cells and its gene silencing enhances the sensitivity of endometrial cancer cells to chemotherapeutic drugs (Dong, Zhou et al. 2012).

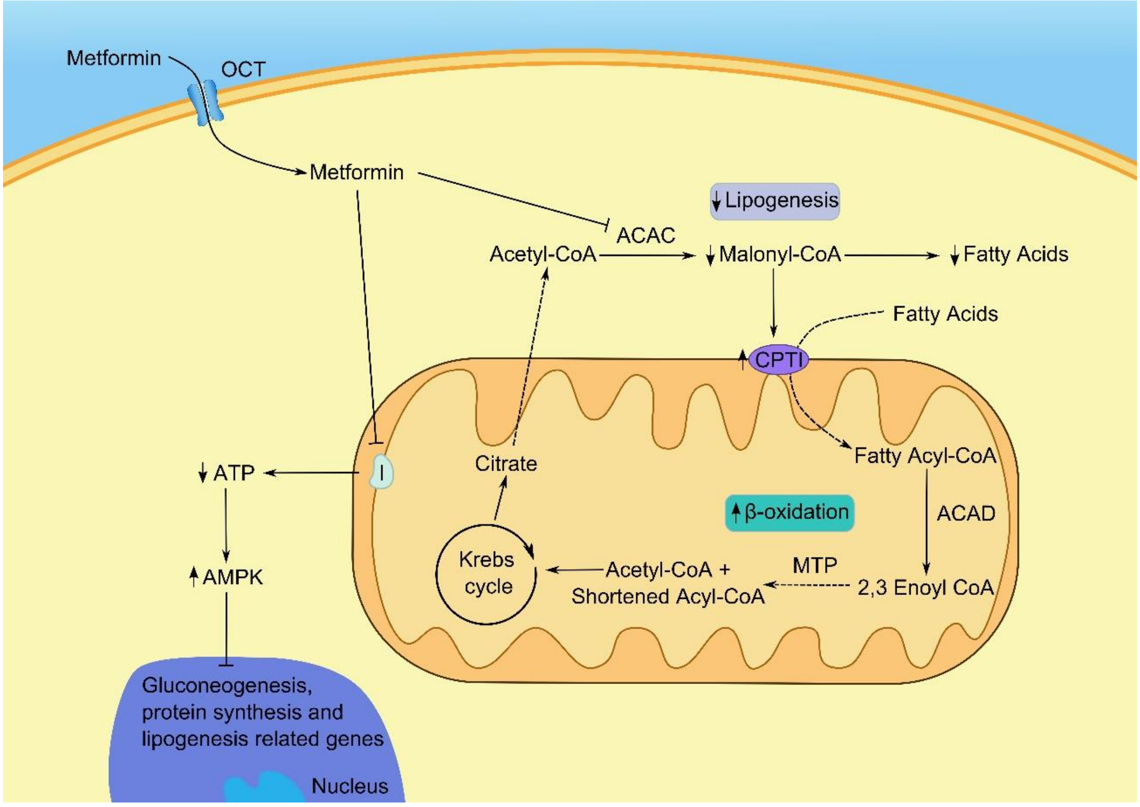

Figure 3: Possible mechanism of metformin action in cells. Metformin enters the cell through organic cation transporter (OCT) and may lead to the phosphorylation and inactivation of acetyl-CoA carboxylase (ACAC), which is responsible for the rate-limiting step of lipogenesis. The reduced synthesis of malonyl-CoA is also predicted to relieve inhibition of carnitine palmitoyltransferase 1 (CPTI), resulting in increased β-oxidation. Moreover, metformin seems to cause a reduction in ATP via inhibition of the mitochondrial respiratory chain complex 1 (I) leading to activation of adenosine monophosphate-activated protein kinase (AMPK). AMPK activation then disrupts the expression of genes involved in gluconeogenesis, protein synthesis and lipogenesis. ACAD – Acyl-CoA dehydrogenase; MTP – Mitochondrial trifunctional protein

7.2 Thiazolidinediones

Thiazolidinediones (TZDs), also termed glitazones, are a class of oral antidiabetic agents that were originally developed in the early 1980s as antioxidants (Yoshioka, Fujita et al. 1989). The blood glucose-lowering potential of this class of drugs was observed after the synthesis of ciglitazone, the first TZD. This effect was particularly pronounced in animals with genetic insulin resistance (Fujita, Sugiyama et al. 1983). TZD were considered insulin sensitizers (Tominaga, Igarashi et al. 1993), as biguanides. This was established after observing that glycemia improved in the absence of increasing insulin levels and that it did not affect insulin-deficient animals. However, due to liver toxicity, ciglitazone and englitazone were never subjected to clinical studies (Isley 2003). Troglitazone, the first marketed TZD, was introduced in Japan and USA in 1997, but was subsequently withdrawn due to hepatoxicity side effects (Gitlin, Julie et al. 1998). Today, two TZDs are available for clinical use: rosiglitazone and pioglitazone (Pio). These drugs are very similar in their effect on hyperglycemia, side effects and mechanism of action. Interestingly, both are also available in combination with other antidiabetic drugs such as Met or glimepiride (US Food and Drug Administration 2011). Importantly, an adverse effect of this class is fluid retention, making TZDs contraindicated in patients with heart failure, which is one of the leading causes of death among T2DM patients (Krentz and Bailey 2005). In addition, the therapeutic effect is only observed after 3 to 4 months of therapy, having a slow onset of action compared with the other available pharmacological treatments (Inzucchi, Bergenstal et al. 2012).

TZDs are potent synthetic activators of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) (Kung and Henry 2012). PPARγ is abundantly expressed in key target tissues for insulin action such as adipose tissue, but is also present in muscle, liver, endothelium and pancreatic β-cells (Ahmadian, Suh et al. 2013). It heterodimerizes with retinoid X receptor and binds to nuclear responsive elements, thus modulating the transcription of genes that play a role in the metabolism of glucose and lipids (Tontonoz and Spiegelman 2008). Notably, this stimulation promotes differentiation of pre-adipocytes, which enhance local effects of insulin. It is also discussed that signals resultant from the adipose tissue, e.g adiponectin or leptin, may mediate the improvement in skeletal glucose disposal induced by TZDs (Yamauchi, Kamon et al. 2001).

Pio, a potent PPARγ agonist (Suzuki, Arnold et al. 2010), increases insulin-stimulated glucose uptake in peripheral tissues as well as insulin sensitivity in hepatic and adipose tissue (Miyazaki, Mahankali et al. 2001). It also causes a minor activation of PPARα, which is related with anti-inflammatory effects, as well as with the decrease of plasma triglyceride levels (Qin, Liu et al. 2007). Pio has an oral bioavailability of approximately 83%, which is not modified by the presence of food on the gastrointestinal tract (Karim, Slater et al. 2007). Furthermore, it is rapidly absorbed and extensively metabolized by hydroxylation and oxidation in the liver, forming active and inactive metabolites (Lin, Ji et al. 2003, Jaakkola, Backman et al. 2006). Pio blood glucose-lowering effect evolves gradually over a period of weeks in a dose-dependent manner (Aronoff, Rosenblatt et al. 2000). Pio administration must be started at 15 mg once a day to a maximum daily dosage of 45 mg (US Food and Drug Administration 2011). Although pio is not reported to cause hepatoxicity, it is debated if it might elevate the risk of bladder cancer through an unknown mechanism (Azoulay, Yin et al. 2012). Some evidences suggest an effect associated with crystal formation and bladder irritation, rather than a pharmacologic effect through PPARγ (Suzuki, Arnold et al. 2010). However, there is some controversy since other studies reported that Pio should not be associated with an increased risk of bladder cancer (Wei, MacDonald et al. 2013). Notably, Pio is reported to possess multiple beneficial effects on lipid metabolism (Betteridge 2007), immune function as well as in endothelial function (Marx, Mach et al. 2000).

Pio, an agonist of PPARγ, increased the expression of glucose transporters GLUT1 and GLUT4 in 3T3-F442A preadipocyte cultures treated with Pio (1 μmol/L) and insulin (1 mg/L). This increase was mainly due to the stabilization of GLUT1 and GLUT4 transporter messenger RNA transcripts (Sandouk, Reda et al. 1993). The increase on GLUT4 expression was also observed in epididymal fat cells in an animal model of insulin-resistant T2DM, KKAy mice. This augment was registered after a treatment with Pio 20 mg/kg/day for 4 days. Besides, treatment with Pio partially corrected the reduction on mRNA encoding hexokinase II levels in epididymal fat and muscle in this animal model (Braithwaite, Palazuk et al. 1995). In addition, Pio also increases protein phosphatase-1, the enzyme that activates glycogen synthase activity, in hepatocytes from streptozotocin-induced diabetic Sprague Dawley rats treated with insulin 0.1 μM and Pio 5 μM (Pugazhenthi and Khandelwal 1998). An induction of adipose tissue secretion was also observed after treatment with 3 μM of Pio, particularly of the high molecular weight adiponectin (Bodles, Banga et al. 2006). Furthermore, Pio decreased angiotensin II-induced connective tissue growth factor expression and proliferation in atrial fibroblasts. This effect might be at least in part related with its inhibitory effect on transforming growth factor-β1 / Smad2/3, as well as on transforming growth factor-β1 / tumor necrosis factor receptor associated factor 6 / transforming growth factor-β-associated kinase 1 signaling pathway (Gu, Liu et al. 2013).

In concerning the female reproductive system, Pio is reported to counterbalance the inhibition of FSH-induced follicular development and steroidogenesis by tumor necrosis factor-α. These results suggest that, in patients with PCOS, Pio may directly act on ovarian functions through PPAR-γ activation (Hara, Takahashi et al. 2013). Pio can also suppress hepatic fetuin-A expression, a hepatokine that induces insulin resistance (Stefan, Fritsche et al. 2008), *in vitro* and *in vivo*. This seems to be a characteristic restricted to TZD derivatives compared to other antidiabetic drugs, such as Met (Ochi, Mori et al. 2014). TZDs were also found to have a renoprotective effect. This effect may be related to a decreased albuminuria and proteinuria (Sarafidis, Stafylas et al. 2010). Since functional PPARγ have been identified in renal glomerular and tubular segments (Nicholas, Kawano et al. 2001), TZDs can also protect against renal injury through these receptors at the kidney. The action of Pio in cell metabolism remains completely unknown and further studies are needed to unveil how these mechanisms alter cells and organs metabolic phenotype.

Rosiglitazone belongs to the TZDs class but has a different side chain from those of troglitazone and Pio (Lebovitz 2002). It reaches a 99% oral bioavailability and is extensively metabolised in the liver (Cox, Ryan et al. 2000). Thus, rosiglitazone is contraindicated for patients with liver disease. The major excretion routes of rosiglitazone are via the urine and faeces (Cox, Ryan et al. 2000). To achieve a significant antihyperglycemic efficacy, rosiglitazone should be given once or twice a day at a starting dosage of 4 mg/day, which may be increased, if required, to 8 mg/day, in combination with diet and exercise (Wagstaff and Goa 2002). Intriguingly, the risk of bladder cancer appears to be specifically associated with Pio, and not with rosiglitazone (Zhu, Shen et al. 2012). At a cardiovascular level, it has been suggested, based on a systematic review and meta-analysis of observational studies, that rosiglitazone presents a higher risk of complications (e.g. congestive heart failure and myocardial infarction) when comparing with Pio (Loke, Kwok et al. 2011).

II. Aim of Project

The sale of antidiabetics is rapidly increasing. On one hand, antidiabetic drugs directly interfere with glucose homeostasis and metabolism. Within the testis, glucose metabolism in the somatic SCs is pivotal for a normal spermatogenesis and, consequently, to male fertility. Thus, these drugs can alter the nutritional support of spermatogenesis. On the other hand, new therapeutic applications, even to female reproductive health have been suggested but their effects in male reproductive potential remains scarcely known.

Although both Pio and Met are well-known cellular metabolic modulators, their isolated and combined effect in hSCs metabolism remains largely unknown. Hence, we hypothesized that exposure to Pio alone or in combination with Met may alter SCs glycolytic profile, influencing the nutritional support of spermatogenesis.

III. Materials and Methods

1. Chemicals

D_2O (99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Cambridge, MA, USA). Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Insulin-transferrin–sodium selenite supplement (ITS supplement) and Pierce™ LDH Cytotoxicity Assay Kit and JC-1 dye were purchased from Life Technologies (Gaithersburg, MD, USA). Mammalian Protein Extraction Reagent (M-PER) and BCA Protein Assay Kit were obtained from Thermo Scientific (Whalthan, MA, USA). Dried milk was obtained from Regilait (Saint-Martin-Belle-Roche, France). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, USA). ECF™ substrate was purchased from GE Healthcare (Weßling, Germany) Pioglitazone hydrochloride was purchased from Abcam (Cambridge, MA, USA; ab120794). Metformin (1,1-dimethylbiguanide hydrochloride), Hank's Balanced Salts Solution (HBSS), Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Tetra Acetic acid (EDTA), soybean trypsin inhibitor, DNase, collagenase type I, Bovine Serum Albumin (BSA), trypsin-EDTA and other chemicals were all purchased from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise.

2. Patient selection, ethical issues and testicle tissue preparations

Testicular biopsies were performed at the Centre for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) according to Local, National and European Ethical Committees guidelines. The studies were all performed according to the Declaration of Helsinki. Testicular biopsies were obtained from patients under treatment for recovery of male gametes and were only used after informed written consent and after patient's treatment. hSCs were isolated from six testicular biopsies of men with conserved spermatogenesis, selected from patients undergoing fertility treatment due to previous vasectomy, psychological, vascular or neurologic anejaculation or traumatic section of the vas deferens.

3. Primary Culture of Human Sertoli cells

hSCs were isolated and cultured as previously described (Oliveira, Sousa et al. 2009). Each biopsy, once obtained, was transferred to sperm preparation medium (SPM; Medicult, Copenhagen, Denmark) containing penicillin and streptomycin until cell isolation.

Testicle biopsies were washed twice in cold HBSS (without Ca^{2+} or Mg^{2+}, containing 50 U/ml of penicillin and 50 mg/ml streptomycin sulfate (pH 7.4)) and minced in HBSS, shaken vigorously during 1 min to disperse tubules. The tissue was left to settle for 5 min on ice, and the supernatant was discarded. This procedure was repeated twice to mechanically remove red blood cells and free Leydig cells. The resulting pellet was digested in 5 mL of HBSS with collagenase type I and DNAse continuously shaken (100 r.p.m.) at 32°C during 25–35 min. The formed aggregate was removed, washed in HBSS and discarded. The washing HBSS was added to the cellular suspension resulting from the digestion.

The resulting suspension was washed twice and left to completely settle at 4°C. The resulting pellet was suspended in 5 mL of HBSS with 1 mg pancreatin and DNAse and digested at 32°C with continuous shaking (100 r.p.m.) during 15–25 min. The new aggregate formed was discarded and 0.1 mL of FBS was added to the cellular suspension, which was left to rest at 4°C for 5 min. The suspension was then centrifuged at 100 g during 5 min. The pellet was then gently suspended in 5 ml HBSS. This procedure was repeated twice and the resulting pellet was suspended in 5 mL of HBSS. This suspension was passed through a glass Pasteur pipette in order to loosen germ cells from the clusters, and then pelleted at 200 g for 5 min. This procedure was repeated twice. The resulting pellet was suspended in Sertoli culture medium (DMEM:Ham's F-12 1:1, containing 15 mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin sulfate, 0.5 mg/ml fungizone,50 µg/ml gentamicin and 10% heat inactivated FBS) and forced through a 20 G needle, in order to disaggregate large Sertoli clusters. Then, cells were plated on Cell+ culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 33°C, 5% CO_2 in air until used.

4. Experimental Groups

hSCs were allowed to grow until they reach 90–95% confluence, and then washed thoroughly. The medium was replaced by serum and phenol-red free media (DMEM:F12, 1:1, with ITS supplement: insulin 10 µg/mL; transferrin 0.55 µg/mL; sodium selenite 0.0067 µg/mL, pH 7.4). hSCs were either treated in the absence (control) or presence of Pio (1, 10 and 100 µM). The concentrations of Pio chosen were 1 µM, a pharmacological concentration of 10 µM (Gillies and Dunn 2000) and 100 µM. Finally, it was established a group of hSCs treated with Pio at 1.5 µM and Met at 10 µM, corresponding to the pharmacological concentration of the combined treatment with these two drugs (Solomon, Mishra et al. 1997). After a 24 h incubation period, cells were detached with a trypsin–EDTA solution and collected using standard methods. At the end of the treatment, the total number of cells was determined with a Neubauer chamber, the extracellular media was collected for ^{1}H-NMR analysis and the cells were collected for protein extraction and enzymatic assays.

5. Sulforhodamine B (SRB) cytotoxicity assay

The cytotoxicity of Pio or Pio plus Met was evaluated by the colorimetric SRB assay (Vichai and Kirtikara 2006). In brief, cells were cultured and treated with the concentrations of Pio or Pio+Met in study. After treatment, cells were washed in PBS and fixed in 1% acetic acid in methanol for 1 h at -20°C. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1h at 37°C. Then, cells were washed with 1% acetic acid solution to remove the unbound dye. Dye bound to cell proteins was extracted with 10 mM Tris solution (pH 10). The optical densities of the resulting media were determined at 540 nm. No cytotoxicity was observed for the concentrations used in this work (data not shown).

6. Protein extraction and quantification

Total protein was extracted from hSCs using the M-PER buffer (supplemented with 1% protease inhibitor cocktail and 100mM sodium orthovanadate). The cells were mixed for 10 minutes at 400 r.p.m. and the suspension was centrifuged at 14000.*g* for 20 minutes. The resulting pellet was discarded. The total protein concentration was determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Briefly, protein quantification of the samples was calculated by measurement of absorbance at 595 nm. Different bovine serum albumin concentrations were used as standard for calibration.

7. Western Blot

Western blot was performed as previously described (Oliveira, Sousa et al. 2009) to quantify the protein expression of GLUT1, GLUT2, GLUT3, PFK1, LDH, MCT4. Protein samples (50 µg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1% SDS, 2% ß-mercaptoethanol, 0.02% bromophenol blue, pH 6.8), denatured for 15 minutes at 55°C and sonicated for 10 minutes at 4°C. Proteins were fractionated in 12% polyacrylamide gels and electrophoresis was carried out for 75 min. The proteins were transferred from gels to previously activated polyvinylidene difluoride membranes in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) and then blocked for 90 min in a 5% non-fat milk solution at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies and the immune-reactive proteins were detected separately using the antibodies listed in Table 1. Membranes were reacted with ECF™ and read with the Bio-Rad GelDoc XR (Bio-Rad, Hemel Hempstead, UK). For the analysis of individual protein levels of oxidative phosphorylation (OXPHOS) complexes, 75 μg of proteins were mixed with sample buffer, and stirred for 15 minutes at 37°C. The protocol was followed as above, except that the proteins were fractionated in 15% polyacrylamide gels and membranes were blocked for 3 hours in a 5% non-fat milk solution at room temperature. The membranes were then incubated overnight at 4°C with MitoProfile® Total OXPHOS WB Antibody Cocktail and mouse anti-ß actin was used as a protein loading control (Table 1). In all cases, Quantity One Software (Bio-Rad, Hemel Hempstead, UK) was used to obtain band densities following standard procedures. The band density was divided by the respective ß-actin band density and then normalized with the control group value.

Table 1: List of the primary and secondary antibodies used in this study.

Antibody	*Host Specie*	*Molecular Weight (kDa)*	*Dilution*	*Vendor*	*Catalog #*
GLUT1	Rabbit	55	1:100	Santa Cruz Biotechnology	sc-7903
GLUT2	Rabbit	60-62	1:5000	Santa Cruz Biotechnology	sc-9117
GLUT3	Goat	48-70	1:200	Santa Cruz Biotechnology	sc-7582
PFK1	Rabbit	85	1:400	Santa Cruz Biotechnology	sc-67028
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology	sc-50329
LDH	Rabbit	37-38	1:10000	Abcam	ab52488
OXPHOS	Mouse	20, 30, 39, 47 and 53	1:500	MitoSciences	MS604
ß-Actin	Mouse	42	1:5000	Thermo Scientific	MA5-15739
Mouse	Goat	—	1:5000	Sigma-Aldrich	A3562
Rabbit	Goat	—	1:5000	Sigma-Aldrich	A3687
Goat	Rabbit	—	1:5000	Sigma-Aldrich	A4187

8. Lactate dehydrogenase activity assay

LDH activity was determined using a commercial assay kit following the manufacturers' instructions. In brief, 5 μg of proteins were homogenized in lysis buffer immediately before use. Similarly, a blank was prepared and boiled for 5 min at 90°C for protein denaturation. LDH assay substrate was added to all samples in a dark environment and left at 37°C for approximately 15 min. Then, a stop solution was used to end the enzymatic activity and absorbance at 490 nm was measured using a Bio-Rad model 680 microplate reader. LDH enzymatic activities were calculated as units per milligram of protein using the molar extinction factor (ϵ).

9. ^{1}H-NMR spectroscopy

^{1}H-NMR spectra of the extracellular media were obtained at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods (Alves, Neuhaus-Oliveira et al. 2013). Sodium fumarate (final concentration of 1 mM) was used as internal reference (singlet, 6.50 p.p.m.) to quantify the metabolites in solution (multiplet, p.p.m): lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (singlet, 1.9), pyruvate (singlet, 2.35) and H1-α glucose (doublet, 5.22). The relative areas of ^{1}H-NMR resonances were quantified offline using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis programme (Acorn NMR, Inc., Fremont, CA, USA).

10. Mitochondrial Membrane Potential

The mitochondrial membrane potential was measured using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). After treatment with Pio or Pio+Met, JC-1 was added to the media in a final concentration of 2 μM and the cells were incubated at 37°C for 30 min. The media with JC-1 were then replaced by media without JC-1. Fluorescence of each sample was read at excitation wavelength of 485 nm and 535 nm and emission wavelength of 530 nm and 590 nm using Cytation™ 3 (Biotek Instruments Inc., Winooski, VT). In healthy cells, a high concentration of JC-1 forms aggregates that yield red fluorescence at 590 nm. In unhealthy cells, JC-1 exists as a monomer at low concentration emitting green fluorescence at 530 nm. Results in fluorescence intensity were expressed as the ratio of 590- to 530-nm emission.

11. Statistical analysis

The statistical significance among the experimental groups was assessed by t-student tests. All experimental data are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). Possible outliers were removed using Grubbs’ method, alpha=0.2. $p<0.05$ was considered significant.

IV. Results

1. Pioglitazone 100 µM increases glucose consumption by human Sertoli cells

Both Pio and Met are antidiabetic drugs and thus, alter body glucose metabolism. We hypothesized that they should also modulate glucose metabolism by hSCs, which is essential for a successful spermatogenesis. Extracellular glucose uptake is the first key point. Our results showed a glucose consumption of 94.60 ± 26.72 pmol/cell by hSCs from the control group (Figure 4A). hSCs exposed to 1, 10 and 100 µM of Pio and 1.5 µM of Pio plus 10 µM of Met consumed 103.17 ± 24.04, 88.00 ± 22.35, 230.60 ± 52.95 and 59.00 ± 26.79 pmol/cell, respectively, with an increase in cells exposed to Pio 100 µM relative to that of cells from the control group (Figure 4A). Relatively to glucose transport, our results showed that exposure to Pio or Pio+Met did not significantly alter the protein levels of GLUT1 and GLUT2 in hSCs. However, GLUT3 protein levels were sensitive to the pharmacological and suprapharmacological concentrations of Pio (10 and 100 µM). In fact, GLUT3 protein levels were decreased in hSCs exposed to Pio 10 µM to 0.73 ± 0.04 – fold variation to control and those exposed to Pio 100 µM also decreased its levels to 0.75 ± 0.05 – fold variation to control (Figure 4C).

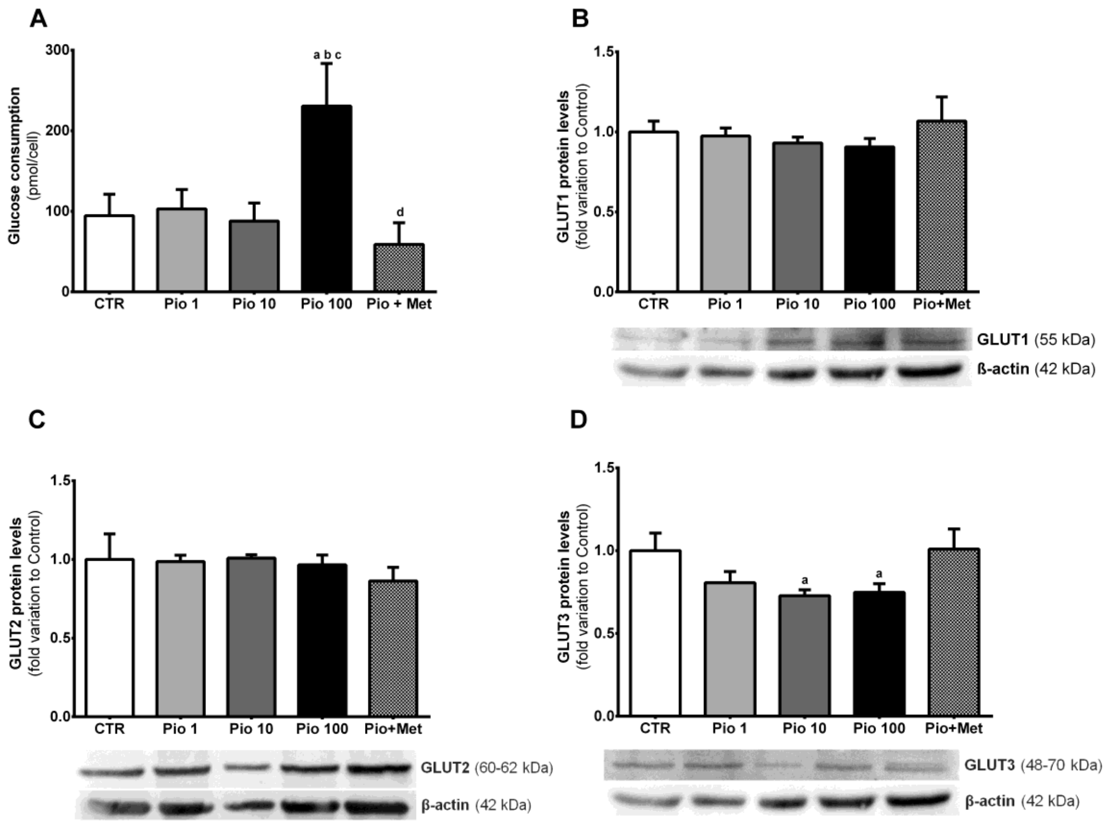

Figure 4: Effect of pioglitazone (1 µM, 10 µM, and 100 µM) and the combined action of pioglitazone 1.5 µM and metformin 10 µM (Pio+Met) in glucose uptake by human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating glucose consumption (panel A) and protein levels of glucose transporters and representative blots (panels B, C and D) found in hSCs cultured in the presence of pioglitazone or pioglitazone plus metformin when compared with the control condition. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a - relative to CTR; b - relative to Pio 1 µM; c - relative to Pio 10 µM; d - relative to Pio 100 µM.

2. Pyruvate metabolism is highly dependent of pioglitazone concentration

In hSCs, the vast majority of glucose taken from the extracellular media follows glycolysis. PFK1 catalyzes one main rate limiting step of this process. Thus, we have assessed PFK1 protein levels in hSCs cultured in our experimental conditions. Our data showed that exposure of hSCs to Pio alone or Pio+Met did not significantly alter PFK1 protein levels (Figure 5A). However, pyruvate variation was significantly increased in hSCs treated with Pio+Met, which indicates a production of this metabolite by 1.08 ± 0.5 pmol/cell) comparing to hSCs treated with Pio 100 µM (-0.62 ± 0.46 pmol/cell), which consumed pyruvate from the extracellular medium, similarly to the control group (-0.18 ± 0.25 pmol/cell) (Figure 5B). Similarly to hSCs treated with Pio+Met, hSCs treated with Pio 1 µM produced pyruvate (0.68 ± 0.37 pmol/cell) as well as hSCs treated with Pio 10 µM (0.54 ± 0.70 pmol/cell).

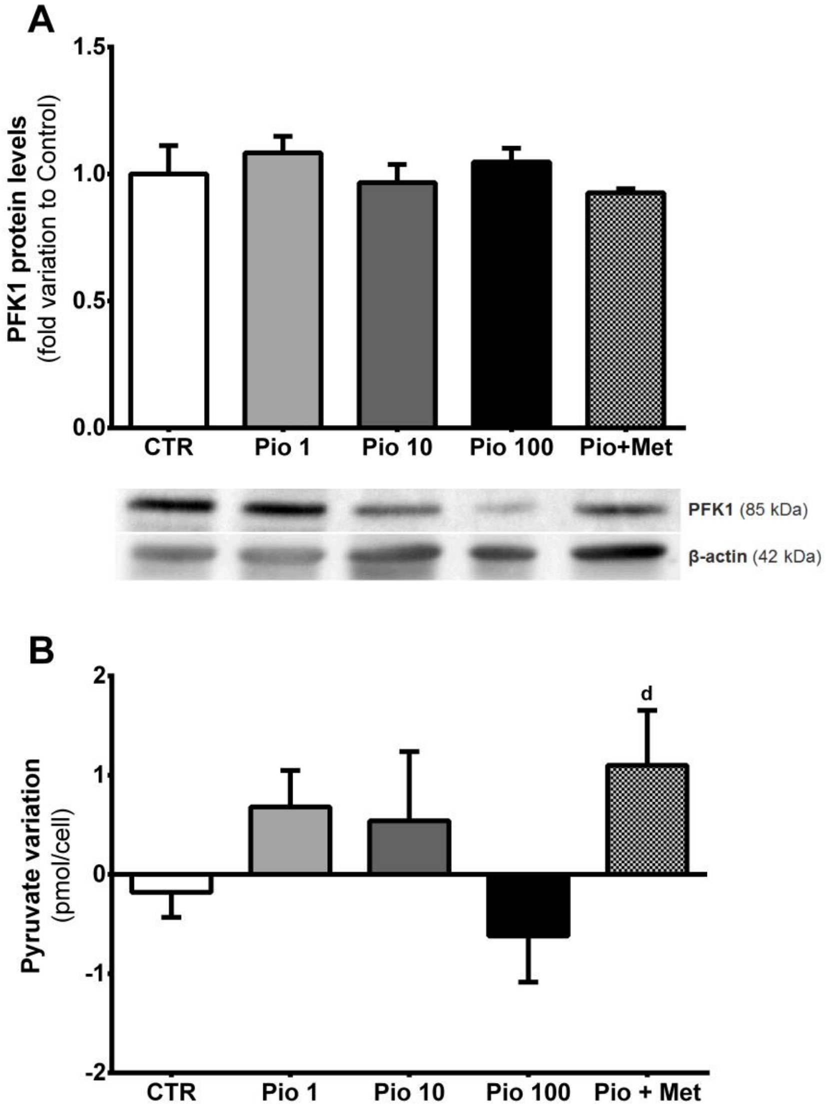

Figure 5: Effect of pioglitazone (1 µM, 10 µM, and 100 µM) and the combined action of pioglitazone 1.5 µM and metformin 10 µM (Pio+Met) in pyruvate metabolism in human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating phosphofructokinase-1 (PFK1) protein levels and representative blots (panel A) and pyruvate variation (panel B) found in hSCs cultured in the absence or presence of pioglitazone or pioglitazone plus metformin. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: d – relative to Pio 100 µM.

3. A pharmacological concentration of pioglitazone stimulates lactate production by human Sertoli cells

Pyruvate formed through glycolysis or attained from the extracellular media can be converted into lactate by LDH, as well as into alanine by ALT. Our data showed no significant differences in LDH protein levels (Figure 6A) but LDH activity was increased in hSCs exposed to Pio 100 µM to 52.3 ± 4.6 nmol/min/µg protein when comparing to hSCs treated with the pharmacological concentration of Pio (10 µM) which presented an LDH activity of 28.8 ± 7.1 nmol/min/mg protein (Figure 6B). Interestingly, hSCs treated with the pharmacological dose of Pio (10 µM) produced higher quantities of lactate (44.40 ± 2.80 pmol/cell) when comparing to hSCs of the control group (28.00 ± 4.60 pmol/cell) and to hSCs treated with the suprapharmacological concentration of Pio (100 µM) which produced 30.83 ± 3.07 pmol/cell (Figure 6C). Our data showed that hSCs treated with Pio 1 µM produced higher quantities of alanine (1.73 ± 0.29 pmol/cell) comparing to hSCs exposed to Pio 10 µM (1.00 ± 0.15 pmol/cell) and to hSCs treated with Pio 100 µM (0.5 ± 0.24 pmol/cell) (Figure 6D).

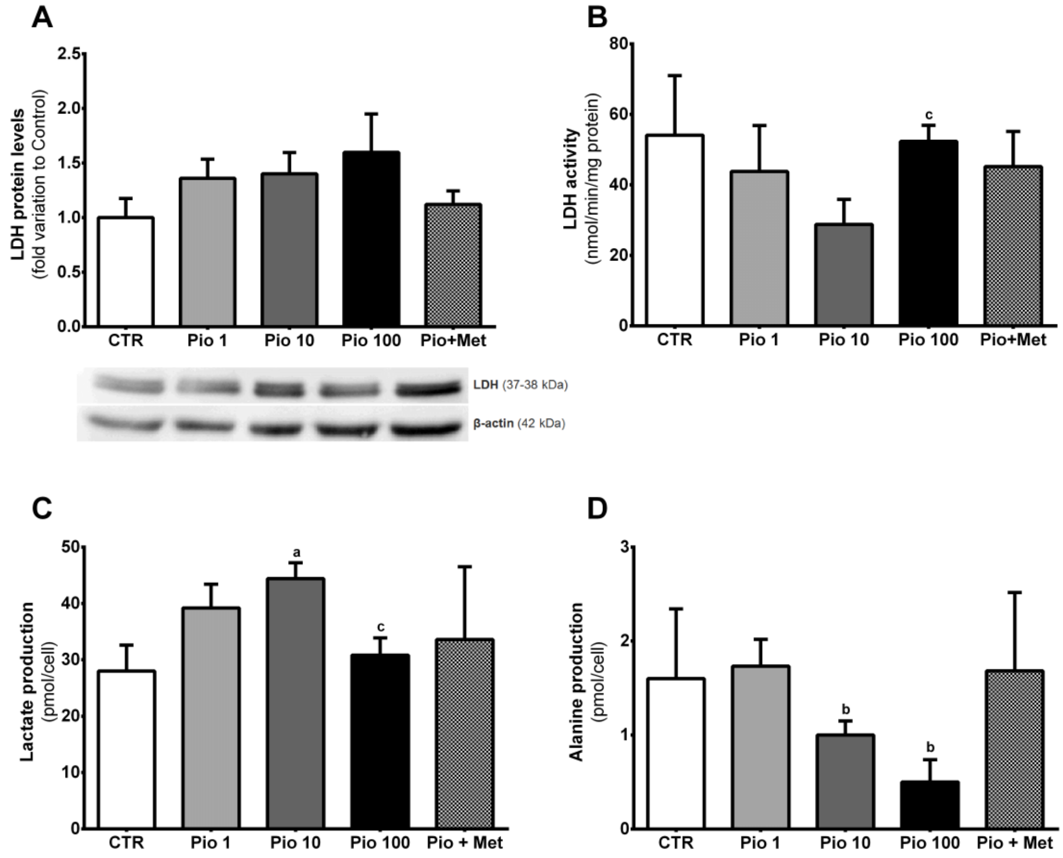

Figure 6: Effect of pioglitazone (1 µM, 10 µM, and 100 µM) and the combined action of pioglitazone 1.5 µM and metformin 10 µM (Pio+Met) in lactate and alanine metabolism by human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating lactate dehydrogenase (LDH) protein levels and representative blots (panel A), LDH activity (panel B), lactate and alanine production (panels C and D) by hSCs cultured in the absence or presence of pioglitazone or pioglitazone plus metformin. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to CTR; b – relative to Pio 1 µM; c – relative to Pio 10 µM.

4. A suprapharmacological concentration of pioglitazone decreases while the combined treatment with metformin increases mitochondrial membrane potential in human Sertoli cells

Analysis of OXPHOS complexes protein levels showed that Pio modulates its levels. The protein levels of complex I were decreased in cells treated with the pharmacological concentration of Pio 10 µM (0.94 ± 0.12), relative to cells treated with Pio 1 µM (1.30 ± 0.08) or Pio 100 µM (1.36 ± 0.14) (Figure 7A). Protein levels of complex II was increased in cells exposed to Pio 100 µM (1.52 ± 0.11 – fold variation to control) relative to cells from the control group. The protein levels of complex III in hSCs exposed to Pio 10 µM (0.84 ± 0.08) was decreased when compared to cells treated with Pio 1 µM (1.12 ± 0.11) or Pio 100 µM (1.17 ± 0.13) or Pio+Met (1.42 ± 0.19). Protein levels of complex IV were higher in hSCs exposed to the combination of Pio+Met (1.33 ± 0.20) comparing to cells exposed to the pharmacological concentration of Pio 10 µM (0.89 ± 0.11). Similarly, protein levels of complex V were increased in cells exposed to the combination of Pio+Met (1.81 ± 0.31 – fold variation to control) relative to the other groups (Figure 7A). Mitochondrial membrane potential was decreased in cells exposed to Pio 100 µM (0.97 ± 0.04) relative to hSCs from the control group (1.43 ± 0.15) and exposed to Pio 1 µM (1.41 ± 0.09), Pio 10 µM (1.28 ± 0.11) and to hSCs treated with Pio+Met (1.87 ± 0.41) (Figure 7C).

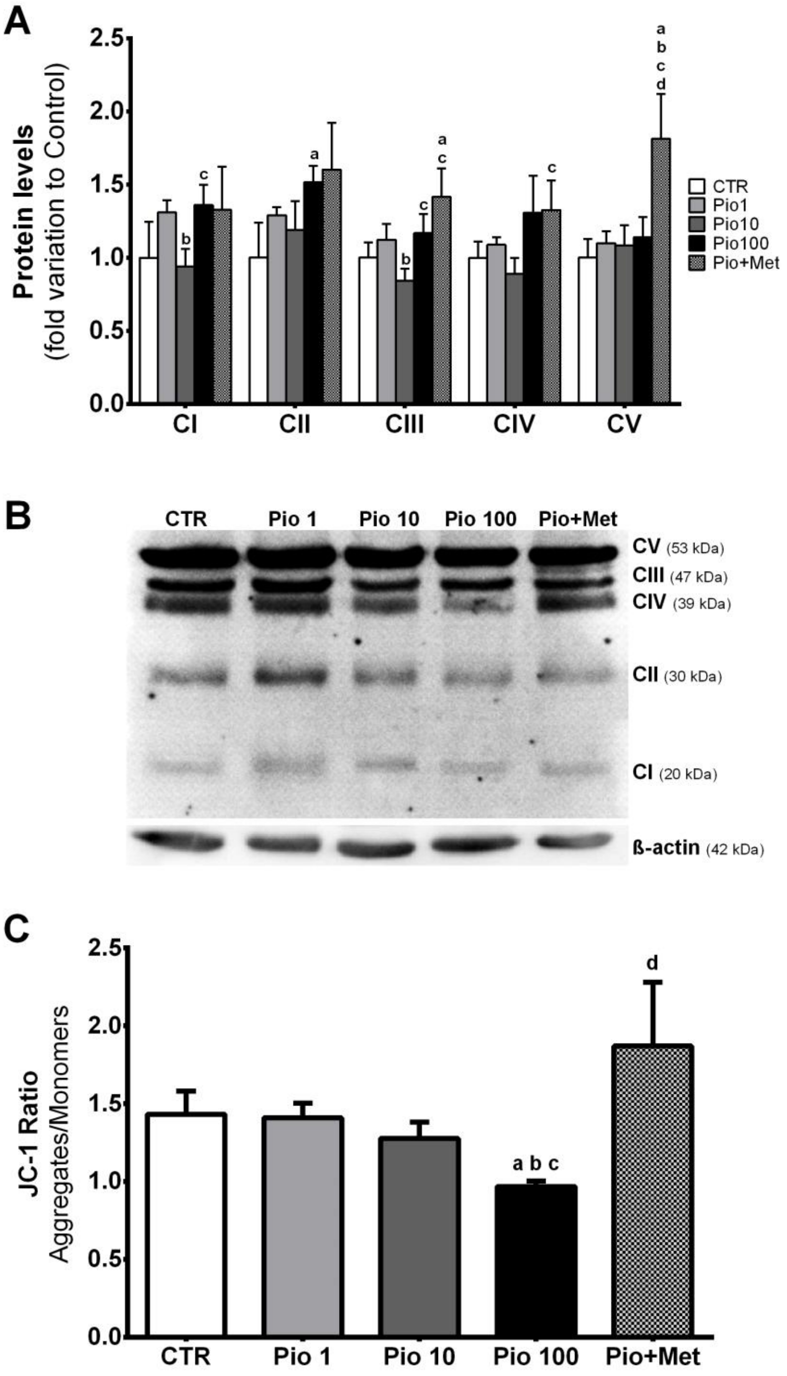

Figure 7: Effect of pioglitazone (1 µM, 10 µM, and 100 µM) and the combined action of pioglitazone 1.5 µM and metformin 10 µM (Pio+Met) in mitochondria of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating OXPHOS protein levels (panel A), its representative blots (panel B) and JC-1 ratio (panel C) found in human SCs cultured in the absence or presence of pioglitazone or pioglitazone plus metformin. Results are expressed as mean ± SEM (n = 6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to CTR; b – relative to Pio 1 µM; c – relative to Pio 10 µM; d – relative to Pio 100 µM.

5. The positive correlation between glucose consumption and lactate production detected in non-exposed human Sertoli cells is lost by exposure to pioglitazone alone or in combination with metformin

A correlation analysis between glycolysis-related steps showed that in non-treated cells, glucose consumption was positively correlated with lactate production (Table 2). Notably, only hSCs exposed to the pharmacological concentration of Pio presented significant correlations. MCT4 protein levels were found to be positively correlated with acetate production though it was negatively correlated with lactate production in those cells (Table 2). PFK1 protein levels were also positively associated with pyruvate variation and LDH protein levels were also positively associated with LDH activity (Table 2).

Table 2: Pearson correlation coefficients between glycolysis related-steps in hSCs cultured in control conditions as well as in those cultured with pioglitazone alone or in combination with metformin

Group	*Correlation*	*p*	*r*
CTR	Glucose consumption vs. Lactate production	0.0365	0.8397
Pio 10	MCT4 protein levels vs. Acetate production	0.0064	0.9337
Pio 10	MCT4 protein levels vs. Lactate production	0.0162	-0.8943
Pio 10	PFK1 protein levels vs. Pyruvate variation	0.0050	0.9416
Pio 10	LDH protein levels vs. LDH activity	0.0329	0.8479
Pio+Met	LDH protein levels vs. LDH activity	0.0025	0.9588

V. Discussion

DM is one of the most widespread chronic diseases and a leading cause of morbidity and mortality on both developed and in developing countries. T2DM, the most prevalent type of DM, is characterized by hyperglycemia that results from insulin resistance and variable degrees of inadequate insulin secretion (American Diabetes Association 2008). Although novel lifestyle of modern societies seem to be the major contributor for T2DM, genetic factors are also accountable (McCarthy 2010). The pancreatic β-cells inability to produce adequate amounts of insulin in postprandial glycemia is the main determinant of hyperglycemia progresses over time (Stumvoll, Tataranni et al. 2003). As a result, most patients with T2DM will progressively need more complex therapeutic protocols to control hyperglycemia. Indeed, the sales number of antidiabetics is increasing every year (Ashiya and Smith 2007). Met is an oral biguanide insulin-sensitizing drug recognized as the first-line treatment of patients with T2DM (Stumvoll, Nurjhan et al. 1995). Although Met is used in clinical practice for more than 50 years, its mechanisms of action remain under debate. Nevertheless, it is well known that Met is a metabolic modulator, which blocks liver gluconeogenesis, reduces the absorption of glucose in the intestinal mucosa and increases skeletal muscle uptake of glucose (Martineau 2012). Met can be used as single therapeutic or in combination with other antidiabetics, particularly Pio. Pio is an insulin sensitizing TZD also commonly used to treat T2DM. It is a potent synthetic activator of the PPARγ, which heterodimerizes with retinoid X receptor. This heterodimer binds to nuclear responsive elements, modulating the transcription of genes that play a role in the metabolism of glucose and lipids (Tontonoz and Spiegelman 2008). Interestingly, several effects not associated with the antihyperglycaemic functions have been reported to this drug, such as beneficial effects on lipid metabolism (Betteridge 2007) and immune and endothelial functions (Marx, Mach et al. 2000). Actually, there is some controversy relative to the relation of Pio and bladder cancer since some studies show a strong positive correlation (Azoulay, Yin et al. 2012), while others do not show any relation (Wei, MacDonald et al. 2013).

As opposed to DM prevalence, the fertility rates have been decreasing in the recent years. Several authors suggest that these two events may be related for various reasons. For instance, there is a growing number of men developing DM during reproductive age, particularly adolescents and young adults (La Vignera, Condorelli et al. 2012). In fact, the vast majority of T1DM cases are detected before the age of 30 (Patterson, Dahlquist et al. 2009) and there is an increasing number of children and adolescents with T2DM (Chen, Magliano et al. 2012). Nevertheless, the effects of DM in male reproductive health remain largely unknown. However, our previous study showed that Met could be considered as a suitable antidiabetic drug for male patients with T2DM in reproductive age. Indeed, the exposure of rat SCs to Met did not cause deleterious effects to these cells and increases lactate production, the main substrate for developing germ cells (Alves, Martins et al. 2014).

New beneficial effects have been recognised to Pio and Met. Although both are metabolic modulators and SC metabolism is essential for spermatogenesis, their isolated and combined effect in these cells metabolism remains scarcely known. Hence, we hypothesized that exposure to Pio alone or in combination with Met may alter SCs glycolytic profile, influencing the nutritional support of spermatogenesis. To test our hypothesis, we determined the effects of subpharmacological (1 μM), pharmacological (10 μM) and suprapharmacological (100 μM) concentrations of Pio and Pio in combination with Met (1.5 μM + 10 μM, respectively) on hSCs glycolytic and oxidative profile, as well as mitochondrial functionality.

As discussed, DM is a chronic disease and a leading cause for morbidity and mortality. There is an increasing number of children, adolescents and young adults with DM (Chen, Magliano et al. 2012) subjected to a long term use of antidiabetics. Glucose is essential for male reproductive function. Several reports discussed the impact of DM in male fertility and suggested that deregulated glucose metabolism is a key factor (Alves, Martins et al. 2013, Alves, Martins et al. 2013). Diabetic men face fertility problems with different degrees of severity depending of several factors. The sale of antidiabetics is rapidly increasing and their safety to male reproductive health remains unknown. Moreover, new clinical applications were reported for Pio and Met in cardiovascular system (van der Meer, Rijzewijk et al. 2009) and female reproductive system (Misso, Teede et al. 2012). As glucose metabolism of SCs exerts a tight control over spermatogenesis, we investigated the effects of Pio alone or Pio+Met on those mechanisms.

Pio is a synthetic insulin-sensitizing drug which acts as an agonist of PPARγ (Kung and Henry 2012), though PPARγ independent-mediated effects are also reported (Crosby, Svenson et al. 2005). It is an antidiabetic drug, approved for the treatment of T2DM by Food and Drug Administration and European Medicines Agency, known to alter cell metabolism and mitochondrial function. However, possible adverse side effects have been highlighted and remain under debate (Gillies and Dunn 2000, Kung and Henry 2012). Pio effects in male reproductive system remain unknown though it was recently reported that it improves erectile function of rats with bilateral cavernosal nerve injury (Aliperti, Lasker et al. 2014). Our data illustrates that Pio alone, and not Pio+Met, acts as a modulator of hSCs metabolic phenotype in a dose-dependent way. As expected, glucose consumption by non-exposed hSCs was positively correlated with lactate production. These cells are known for their particular metabolic features since they present a Warburg-like metabolism (for review (Oliveira, Martins et al. 2015)). SCs sustain a very high glycolytic flux even in the presence of adequate substrates and oxygen to maintain an active mitochondrial oxidative phosphorylation. Notably, treatment with Pio alone or in combination with Met disrupted this important correlation. Nevertheless, glucose consumption by hSCs was only stimulated by the suprapharmacological concentration of Pio. This was not followed by any change in GLUTs levels. Though exposure to Pio was reported to increase GLUTs mRNA expression in adipocytes (Tan 2000, Koenen, Tack et al. 2009), there are also studies reporting that it does not affect GLUTs protein levels in diabetic muscle tissue (Stuart, Howell et al. 2007). Our previous studies have shown that hSCs possess a high metabolic flexibility to guarantee adequate levels of lactate in the microenvironment where germ cells develop, particularly by modulating glucose transport, in response to unfavourable conditions (Oliveira, Alves et al. 2011). We propose that since Pio is an insulin-sensitizing drug, at suprapharmacological concentrations, it stimulates glucose uptake by GLUTs without altering their protein levels. Only the pharmacological concentration of Pio decreased GLUT3 protein levels. This decrease was accompanied by an increase in lactate production, which illustrates that a pharmacological concentration of Pio stimulates the glycolytic flux in hSCs. We have previously shown that GLUT3 protein levels are sensitive to these cells energetic needs (Alves, Martins et al. 2014). Thus, stimulation of glycolytic flux may induce a decrease in protein levels of this transporter to avoid an uncontrolled uptake of glucose, which would be deleterious for the cell. Interestingly, the same effect was recently reported after exposure to pharmacological concentration of Met in rat SCs (Alves, Martins et al. 2014).

Glucose enters the cell and usually follows the glycolytic pathway. There were no changes in PFK1 protein levels though interesting data were obtained concerning pyruvate variation, with a different metabolic behaviour for non-exposed cells and between cells exposed to different Pio concentrations (though they were not statistically different). LDH activity in hSCs was stimulated by the suprapharmacological concentration of Pio (100 µM) in comparison to that detected in hSCs exposed to the pharmacological concentration (10 µM). Notably, lactate production was decreased in hSCs exposed to 100 µM of Pio when compared with cells exposed to 10 µM of Pio. The pharmacological concentration of Pio maintains a high glycolytic flux without stimulating glucose uptake and LDH activity illustrating that it optimizes the synthesis and export of lactate. On the other hand, since the suprapharmacological concentration of Pio increases glucose uptake and LDH activity but decreases lactate production by hSCs when compared to Pio 10 µM it suggests that glucose metabolism is redirected to Krebs cycle or to synthesis of aminoacids, lipids or glycogen. Alanine production, a key feature of hSCs (Rocha, Martins et al. 2014) was decreased in hSCs exposed to both concentrations. The pool of alanine is linked with lactate pool to control the cellular redox state since the interconversion of pyruvate to lactate and/or alanine is directly related with re-oxidation of NADH to NAD$^+$ (O'Donnell, Kudej et al. 2004). Curiously, in rat SCs exposed to Met (Alves, Martins et al. 2014) it was proposed that SCs adjust alanine pools to compensate the unbalanced redox state that an increased production of lactate could induce. Thus, our results suggest that the pharmacological and suprapharmacological concentrations of Pio decrease alanine production to sustain redox balance and lactate production. Indeed, the lactate/alanine ratio was found to be maintained after exposure to any of Pio concentrations (data not shown). Concerning the glycolytic profile analysed, it is also important to highlight that several significant correlations were detected only in hSCs exposed to the pharmacological concentration of Pio. The protein levels of MCT4 in hSCs exposed to 10 µM of Pio were positively correlated with acetate production, while negatively correlated with lactate production. This correlation illustrates that MCT4 levels were pivotal for the control of lactate production when hSCs were exposed to the pharmacological concentration of Pio. Interestingly, exposure of hSCs to the pharmacological concentration of Met was also reported to decrease MCT4 protein levels, though increasing lactate production (Alves, Martins et al. 2014). This illustrates a novel mechanism by which insulin-sensitizing drugs modulate hSCs metabolism changing the nutritional support of spermatogenesis. Moreover, other positive correlations were detected in hSCs exposed to 10 µM of Pio, particularly between PFK1 and pyruvate variation, as well as between LDH levels and activity. Taken together, our results show that the pharmacological concentration of Pio stimulates the glycolytic flux and lactate production by establishing crucial positive correlations between protein levels/activity/production of key intervenient in those pathways.

TZDs are reported to control cells metabolism by altering the glycolytic flux and mitochondria functionality (Dello Russo, Gavrilyuk et al. 2003). TZDs can inhibit aerobic metabolism by changing mitochondrial complexes protein levels and/or activity (Brunmair, Staniek et al. 2004, Colca, McDonald et al. 2004). In skeletal muscle, TZDs elevate lactate production and were suggested to inhibit mitochondrial respiration (Brunmair, Gras et al. 2001, Dello Russo, Gavrilyuk et al. 2003). It was also previously identified an outer mitochondrial membrane Fe-S protein, known as MitoNEET, to which Pio was shown to bind this and stabilize its structure (Paddock, Wiley et al. 2007), illustrating a mechanism by which this TZD acts in mitochondria, causing less dysfunction. Our results suggest that exposure of hSCs to 10 µM of Pio did not alter mitochondrial complexes protein levels nor mitochondrial membrane potential, which is in agreement with previous works suggesting that Pio stabilizes mitochondrial structure. CI and CIII protein levels were lower in hSCs exposed to 10 µM of Pio than to the sub- and suprapharmacological concentrations of Pio, showing that OXPHOS protein levels are sensitive to Pio concentration. Notably, exposure of hSCs to 100 µM of Pio increased CII protein levels but decreased mitochondrial membrane potential. This may be a common effect of TZDs since troglitazone was also reported to cause mitochondrial membrane depolarization (Konrad, Rudich et al. 2005). Although Met is known for acting as an inhibitor of CI (Andrzejewski, Gravel et al. 2014), our data shows that the combined treatment of hSCs with Pio+Met not only did not alter CI protein levels but increased CIII and CV protein levels without changing mitochondrial membrane potential. Moreover, the combined treatment of Pio+Met did not altered the glycolytic profile of hSCs.

The emerging number of men in reproductive age with DM, together with an increasing incidence in children, adolescents and young adults highlight the need for studies assessing the safety of antidiabetic drugs on pivotal functions for male reproductive health. The nutritional support of spermatogenesis by SCs is highly dependent of glycolysis (Alves, Martins et al. 2014). The combined pharmacological treatment of Pio+Met did not alter the glycolytic profile of hSCs. Nevertheless, the pharmacological concentration of the peroxisome proliferator activated receptor-γ agonist Pio stimulates lactate production by hSCs, increasing the efficiency of the glycolytic flux and establishing crucial correlations among key intervenient of that pathway. Since lactate is the preferred substrate of developing germ cells and improves spermatogenesis *in vivo* (Courtens and Ploen 1999, Erkkilä, Aito et al. 2002), our results suggest that, like Met, Pio may be a suitable antidiabetic drug for young men and those in reproductive age. These novel findings suggest a possible protective role for this antidiabetic on male reproductive function.

VI. Conclusions

The prevalence of subfertility or infertility problems is alarmingly raising. Along with this increase, DM prevalence has also increased dramatically. One of the reasons for these increases is the alarming upraising of the number of men developing DM in reproductive age.

The vast majority of patients with T2DM will progressively need more complex therapeutic procedures to control hyperglycemia. Thus, it urges the study of the possible mechanisms of these antidiabetics to male fertility as they can have beneficial or deleterious effects that can lead to subfertility or infertility. In order to clarify some of those mechanisms, we studied SCs metabolism as these cells are responsible for the establishment of the nutritional microenvironment where the germ cells develop. Thus, our first approach consisted in evaluating the influence of Pio alone or in combination with Met in the glycolytic pathway of these cells. The resulting lactate from glycolysis is then used as metabolic fuel for developing germ cells and any alterations in the production of this metabolite leads to crucial modifications in the nutritional support of spermatogenesis and, consequently, in male fertility.

In summary, with this work we were able to assess the safety of Pio and Pio in combination with Met to the nutritional support of spermatogenesis and thus, male reproductive function of diabetic individuals. Using hSCs, we assessed that Pio alone, similarly to what was verified in a previous study for Met alone, is a suitable antidiabetic drug for diabetic males. In fact, the pharmacological concentration of Pio may have a protective role for male fertility, as it increases lactate production by hSCs and, this metabolite has been reported to improve spermatogenesis *in vivo*. Further studies will be needed to unveil more mechanisms by which Pio and Met can improve or protect male reproductive health.

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VIII. Annex I

List of publications resultant from the work developed during the M.Sc. in Biomedical Sciences

Meneses M. J., Sousa M., Alves M. G., Oliveira P. F. (2015). The Antidiabetic Drug Metformin and Male Reproductive Function: An Overview. *Int J Diabetol Vasc Dis Res*, 3(1e) 1-2.

Meneses M. J., Silva B. M., Sousa M., Sá R., Oliveira P. F., Alves M. G. (2015). Antidiabetic Drugs: mechanisms of action and potential outcomes on cellular metabolism. *Curr Pharm Des. (Accepted for publication – In Press)*

Meneses M. J., Bernardino R. L., Sousa M., Silva B. M., Sá R., Oliveira P. F., Alves M. G. (2015) Regulation of testicular glucose metabolism in (pre)diabetes and its implications to male reproductive health. In: Glucose Metabolism: Biochemistry, Regulation and Health Effects. Nova Science Publishers Inc., NY, USA. *(Accepted for publication – In Press)*

Meneses M. J., Bernardino R. L., Sá R., Silva J., Barros A., Sousa M., Silva B. M., Oliveira P. F., Alves M. G. (2015). Os antidiabéticos Pioglitazona e Metformina alteram a cooperação metabólica testicular. Revista CAPTAR. *(Accepted for publication – In Press)*

Meneses M. J., Bernardino R. L., Sá R., Silva J., Barros A., Sousa M., Silva B. M., Oliveira P. F., Alves M. G. (2015). The pharmacological concentration of pioglitazone increases the glycolytic efficiency of human Sertoli cells: implications for male fertility. *(Submitted)*

Dias T. R., Bernardino R. L., Meneses M. J., Sousa M., Sá R., Alves M. G., Silva B. M., Oliveira P. F. (2015). Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders. *(Submitted)*

List of oral communications resultant from the work developed during the M.Sc. in Biomedical Sciences

Meneses M. J., Bernardino R. L., Sá R., Silva J., Barros A., Sousa M., Silva B. M., Oliveira P. F., Alves M. G. (2015). The antidiabetic drugs Pioglitazone and Metformin alter the metabolic cooperation in the testis. IV Encontro Nacional Pós-Graduação em Ciências Biológicas, University of Aveiro, 30th March – 2nd April, Aveiro, Portugal.

Meneses M. J., Bernardino R. L., Sá R., Silva J., Barros A., Sousa M., Silva B. M., Oliveira P. F., Alves M. G. (2015). Pioglitazone alone alters the metabolic support of spermatogenesis but not in combination with metformin: *in vitro* evidence. III AEICBAS Biomedical Congress, Institute of Biomedical Sciences Abel Salazar, 20th – 22nd March 2015, Porto, Portugal. *Poster*