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SIRT1 and energy metabolism in rodent Sertoli cells

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DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração do presente trabalho académico e confirmo que não recorri à prática de plágio nem a qualquer forma de utilização indevida ou falsificação de informações ou resultados em nenhuma das etapas conducente à sua elaboração.

Mais declaro que conheço e que respeitei o Código de Conduta Ética da Universidade do Minho.

A SIRT1 E O METABOLISMO ENERGÉTICO DAS CÉLULAS DE SERTOLI DE RATINHO

RESUMO

Nos últimos anos, as sirtuinas têm sido apontadas como participantes ativos na coordenação metabólica em diversos órgãos e células, estando envolvidas na modulação de diversas vias metabólicas. De entre as desacetilases, a sirtuina 1 (SIRT1) tem sido das mais estudadas. Ainda assim, pouco se sabe acerca do seu papel na regulação do metabolismo testicular. A premissa deste trabalho é que a SIRT1 tem uma ação reguladora no metabolismo das células de Sertoli e consequentemente na espermatogénese. O objetivo deste trabalho foi estudar o papel da SIRT1 em células de Sertoli de ratinho (mSCs). Para isso expusemos as células a concentrações crescentes de um inibidor (EX-527) ou um ativador (YK-3-237) da SIRT1 e avaliámos a sua citotoxicidade e o seu impacto no perfil metabólico de uma linha de células de Sertoli de ratinho (TM4). O perfil citotóxico dos compostos foi estudado utilizando os ensaios de Brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio (MTT), libertação de lactato desidrogenase (LDH) e ensaio de sulforodamina B (SRB). Avaliámos a função mitocondrial, determinando os níveis de expressão dos complexos mitocondriais e o potencial mitocondrial (ensaio JC-1). O perfil metabólico das mSCs foi determinado por Ressonância Magnética Nuclear (^{1}H-NMR) e pela quantificação do glicogénio armazenado e reservas lipídicas. Foram também analisados os danos oxidativos (especificamente a peroxidação lipídica) e o perfil apoptótico, determinando a atividade da caspase-3 e a expressão de proteínas anti e pró-apoptóticas. A exposição das mSCs ao ativador ou inibidor da SIRT1 nas doses selecionadas não apresentou qualquer toxicidade para as células. No entanto, observou-se um aumento da atividade de caspase-3 quando as células foram expostas ao EX-527 (100 nM), sugerindo uma ativação da via apoptótica. Por outro lado, o ativador da SIRT1 (10000 nM) modulou a função mitocondrial, favorecendo a fosforilação oxidativa, sendo que as células nessas condições exibiram níveis mais elevados de peroxidação lipídica. Metabolicamente, quando a SIRT1 está ativada (YK-3-237, 10000 nM), há um aumento do consumo de glucose e piruvato, evidenciando um favorecimento do fluxo glicolítico, que se traduziu num aumento da produção de lactato (essencial para a espermatogénese). Adicionalmente, houve uma diminuição da acumulação lipídica, sugerindo a sua metabolização na mitocôndria. Em suma, a modulação da atividade da SIRT1 em mSCs leva a alterações do perfil metabólico dessas células. Em particular, a ativação da SIRT1 com YK-3-237 modula o desempenho metabólico das mSCs, favorecendo o metabolismo mitocondrial, sem afetar a viabilidade e proliferação celular, portanto, pode ser considerado como um ponto de controlo do metabolismo testicular.

Palavras-Chave: Células de Sertoli, EX-527, metabolismo testicular, SIRT1 e YK-3-237

SIRT1 AND ENERGY METABOLISM IN RODENT SERTOLI CELLS

ABSTRACT

In the last years, sirtuins have been identified as active participants in metabolic coordination of various organs and cells, being involved in the modulation of multiple metabolic pathways. Among all deacetylases, sirtuin 1 (SIRT1) has been the most studied. Still, little is known about its role in regulating testicular metabolism. We hypothesized that SIRT1 might have a regulatory action in Sertoli cells and consequently in spermatogenesis. The aim of this project was to study the role of SIRT1 in mouse Sertoli cells (mSCs). For that purpose, we exposed cells to increasing concentrations of an inhibitor (EX-527) or an activator of SIRT1 (YK-3-237) and we evaluated their cytotoxicity and the metabolic profile of TM4 cell line. The cytotoxic profile was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) release and sulfhorodamine B (SRB) assays. We evaluated mitochondrial function by determining the mitochondrial complexes expression levels and mitochondrial potential (JC-1 assay). The metabolic profile of mSCs was determined by Nuclear Magnetic Resonance (^{1}H-NMR) technique, and by the quantification of intracellular glycogen content and lipid accumulation. The oxidative damages in cells was also analyzed (specially lipid peroxidation) and the apoptotic profile was also assessed by determining caspase-3 activity and the expression of anti and pro- apoptotic proteins. The exposure of either SIRT1 activator or inhibitor at the selected doses presented no toxicity to mSCs. However, increased caspase-3 activity was observed when cells were exposed to EX-527 (100 nM), suggesting an activation of apoptotic pathway. On the other hand, SIRT1 activator (10000 nM) modulated mitochondrial function, favoring the functioning of the oxidative phosphorylation, and under these conditions, mSCs exhibited higher levels of lipid peroxidation. Metabolically, when SIRT1 is activated (YK-3-237, 10000 nM) there is an increase in glucose and pyruvate consumption, demonstrating that there is an enhancement in glycolytic flow, which translated into an increase in lactate production (essential for spermatogenesis). Additionally, there was a decrease in lipid accumulation, suggesting its metabolization in mitochondria. In sum, modulation of SIRT1 activity in mSCs leads to changes in metabolic profile. Particularly, the activation of SIRT1 with YK-3-237 modulates the metabolic performance, without affecting cell viability and proliferation and thus, SIRT1 can be considered as control point of testicular metabolism.

Keywords: EX-527, Sertoli cells, SIRT1, testicular metabolism and YK-3-237

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LIST OF ABBREVIATIONS

ALT – Alanine aminotransferase

AMPK - 5' AMP-activated protein kinase ATP- adenosine triphosphate

BSA – Bovine serum albumin

BTB - Blood-testis barrier

DMEM - Ham's F12- Dulbecos's modified Eagle Medium Ham's Nutrient Mixture F12

DMSO – Dimethyl sulfoxide

FBS - Fetal Bovine Serum

FOXO - Forkhead transcription factor

FSH - Follicle- stimulating hormone

GLUTs- Glucose transporters

GnRH - Gonadotropin releasing hormone

HPT - Hypothalamic-pituitary- testis

JC-1 - 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

LCs - Leydig cells

LDH - Lactate dehydrogenase

LH - Luteinizing hormone

MCT4 – Monocarboxylate transporter 4

MCT2 - Monocarboxylate transporter 2

mSCs - mouse Sertoli cells

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ - Nicotinamide adenine dinucleotide

NAM - Nicotinamide

NMR – Nuclear Magnetic Resonance

ORO - Oil Red O staining

OXPHOS - Oxidative phosphorylation

PBS - Phosphate Buffered Saline

PDH – Pyruvate dehydrogenase

PGC-1α - Peroxisome proliferator- activated receptor gama coactivator 1 α

PPAR - Peroxisome proliferator-activated receptor

RIPA – Radioimmunoprecipitation assay

ROS – Reactive Oxygen Species

RSV – Resveratrol

SCs - Sertoli cells

SIRT1 - Sirtuin 1

SIRT2 – Sirtuin 1

SIRT3 – Sirtuin 3

SIRT4 – Sirtuin 4

SIRT5 – Sirtuin 5

SIRT6 – Sirtuin 6

SIRT7 – Sirtuin 7

STAT3 - Signal transducer and activator of transcription 3

SRB - Sulforhodamine B

UCP 2 – Uncoupling protein 2

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Chapter I

INTRODUCTION

1. INTRODUCTION

1.1. Male Reproductive Tract – An Overview

The male reproductive tract provide the appropriate conditions for the development of germ cells, which can generate an entirely new organism, through the transmission of genetic information to the next generation, required for the survival of the species (1).

In order to maintain a normal male reproductive health, the coordination between the different type of cells, accessory glands and tissues is essential. In general, the mammalian testes are paired organs, covered by a layer, tunica vaginalis and an inner layer, tunica albuginea. Testis has lobules that include the seminiferous tubules, recognized as the functional units of the testis, which are immersed in loose connective tissue and some interstitial cells, namely Leydig cells (LCs) (2). The seminiferous tubules are compartmentalized by junctions between adjacent Sertoli cells (SCs), creating the blood-testis barrier (BTB), that physically divides the seminiferous epithelium into basal and adluminal compartments. The BTB is responsible for restricting or allowing the passage of substances that are present in the interstitial fluids into the tubular lumen (3). BTB represent an anatomical, immunological and physiological barrier, essential for the well-functioning of male reproductive tract. Blood and lymphatic vessels that are present in the interstitial space participate in the homeodynamics of hormones and nutrients in the testes (4), with the interstitial LCs being responsible for the synthesis of main male steroid hormone, testosterone (5). So, the testes are responsible for the synthesis of male sex hormones and the production of male gametes, spermatozoa. (6, 7). An impaired coordination between particularly SCs and LCs, interferes with the mature of spermatozoa (8, 9), and hence, has repercussions on male fertility.

1.2. Sertoli cells (SCs)

Sertoli cells were first described by Enrico Sertoli in the 19 th century (10) and the evidence of their fundamental involvement in the establishment of male fertility has been confirmed by several studies. Indeed, one SC can support up to 30 at 50 germ cells at different stages of development (11), although there is a variation on this number between species. Hence, the number of SCs is associated to the rate of spermatogenesis and therefore to the daily production of sperm per testis, that posteriorly will result on male fertility capacity (12-14). SCs are also responsible for the phagocytosis of residual bodies and degenerating germ cells (2, 15, 16). Moreover, SCs provide nutritional support that male germ cells need, through the production and release of specific metabolites to environment surrounding developing germ

cells. SCs must also produce different growth factors, signaling molecules, cytokines, bioactive peptides and several glycoproteins and peptides that form the molecular basis for Sertoli-germ cell interactions (17, 18).

Specialized junctions, like occludins, claudins and desmossomes, between adjacent SCs, are highly organized, located near the basement membrane, creating the BTB. Collagen, laminin and extracellular matrix components, also contributes to these specialized junctions. All those interactions contribute to the structural and physiological function of BTB (19).

BTB regulates the diffusion of water, electrolytes, nutrients and biomolecules (it specially prevents the movement of large molecules) from systemic circulation in to lumen of seminiferous tubules (20). It also limits the movement of immune cells and regulates the level of cytokines in the seminiferous epithelium (21, 22).

1.3. Sertoli cell metabolism and spermatogenesis

As already mentioned above, SCs are vital for the occurrence of a normal spermatogenesis. They are known as nurse cells, once they provide unique characteristics like the structural and nutritional support for the development of germ cells (23, 24). In fact, there is a metabolic dependence relationship between SCs and developing germ cells. SCs can secrete peptides, nutrients and metabolic intermediates (18, 25). A correlation between the levels of energetic substrates that SCs produce with the germ cells developed can be made, so they are essential for the development of spermatogenesis and hence for male fertility. (26).

Over the past years, multiple works pointed out that cultured SCs use preferably glycolysis and convert the majority of the resulting pyruvate to lactate, that is then exported from the cell. Indeed, Grootegoed et al., demonstrated that only 25% of the pyruvate produced from glucose go to Krebs cycle (27). Like cancer cells, it is known that SCs have a Warburg-like metabolism and prefer the fermentative rather than the oxidative metabolism of glucose (26). They exhibit a high glycolytic flux, however, is not accompanied with enhanced cellular proliferation, representing different aspect comparing to cancer cells.

As a starting point, specific carriers are needed to facilitate the diffusion of glucose across the membrane, since glucose is a hydrophilic and polar molecule and hence, it crosses the lipid bilayer very slowly by simple diffusion (Figure 1). There are specific membrane glucose transporters, named as sodium dependent glucose transporters (or solute carrier family 5 transporters - SLC5) and glucose transporters (GLUTs) (or solute carrier family 2 transporters – SLC2) (28, 29). Regarding GLUTs family, they are

divided in three sub-families: class I, which include GLUT1-4; class II (GLUT 5, GLUT7 and GLUT9 and GLUT11); and class III (GLUT6, GLUT8, GLUT10 and GLUT12). GLUTs are widely expressed in different tissues and cells. In testis, GLUT1 (30), GLUT2 (31), GLUT3 (32), GLUT5 (33) and GLUT8 have already been identified (34). Specially in SCs, the presence of GLUT1 to GLUT4 has also been confirmed (31, 35, 36). Curiously, Riera et al., reported that when glucose is removed from the culture medium, the expression of GLUT1 increase and GLUT3 expression decreases, demonstrating that glucose acts as signal to adequate an appropriate glycolytic flux in response to the glucose levels in the extracellular space (37).

Once inside the cell, glucose is metabolized by multiple enzymes, resulting in the production of pyruvate. The pyruvate can follow different ways: it can enter the Krebs cycle, it can be converted to alanine by the action of alanine aminotransferase, or it can be converted to lactate by the action of lactate dehydrogenase (LDH), with the simultaneous oxidation/reduction of NADH to NAD^+ (Figure 1). In fact, multiple authors, described that the majority of pyruvate produced by SCs is converted to lactate by LDH (26, 38, 39). Later, this lactate is exported via monocarboxylate transporters (MCTs). This family has 14 members and are expressed in different tissues and cells. Regarding male reproductive tract, MCT2 in found in elongated spermatids (40), MCT1 and MCT4 are present in SCs (41). In fact, MCT1 has a higher affinity for lactate than MCT4. MCT1 is most associated to the import of lactate from the extracellular space (42), unlike MCT4, which is primarily a lactate exporter, since it has lower affinity for lactate (41, 43). Thus, MCT4 seems to play an important role in SCs (39, 42), as it is expressed in cells with high glycolytic profile. In fact, the production of lactate is essential for the normal development of germ cells.

In adverse conditions, when glucose is decreased, SCs can adapt their metabolic behavior to guarantee an appropriate production and export of lactate into the adluminal compartment, they modulate GLUTs expression, trough different signaling pathways (37). Deprivation of insulin has also repercussions on lactate production (42), since it induces an adaptation in the expression of GLUTs. All of these evidences support the fact that SCs are essentials to ensure the metabolic needs of germ cells, in order to support properly the spermatogenesis.

Indeed, lactate has been reported to principal energetic substrate of germ cells and is essential to the maintenance of spermatogenesis *in vivo*. To support that, Trejo et al, demonstrated that pharmacological deprivation of lactate decreases the viability of male germ cells (44). Then, Courtens and Ploen, described that testicular perfusion of lactate was capable to suppress the loss of spermatocytes and spermatids in adult cryptorchid rat testicle (45). Furthermore, lactate was characterized to exert an anti-apoptotic effect on germ cells (46). All these data demonstrate that lactate is essential to spermatogenesis.

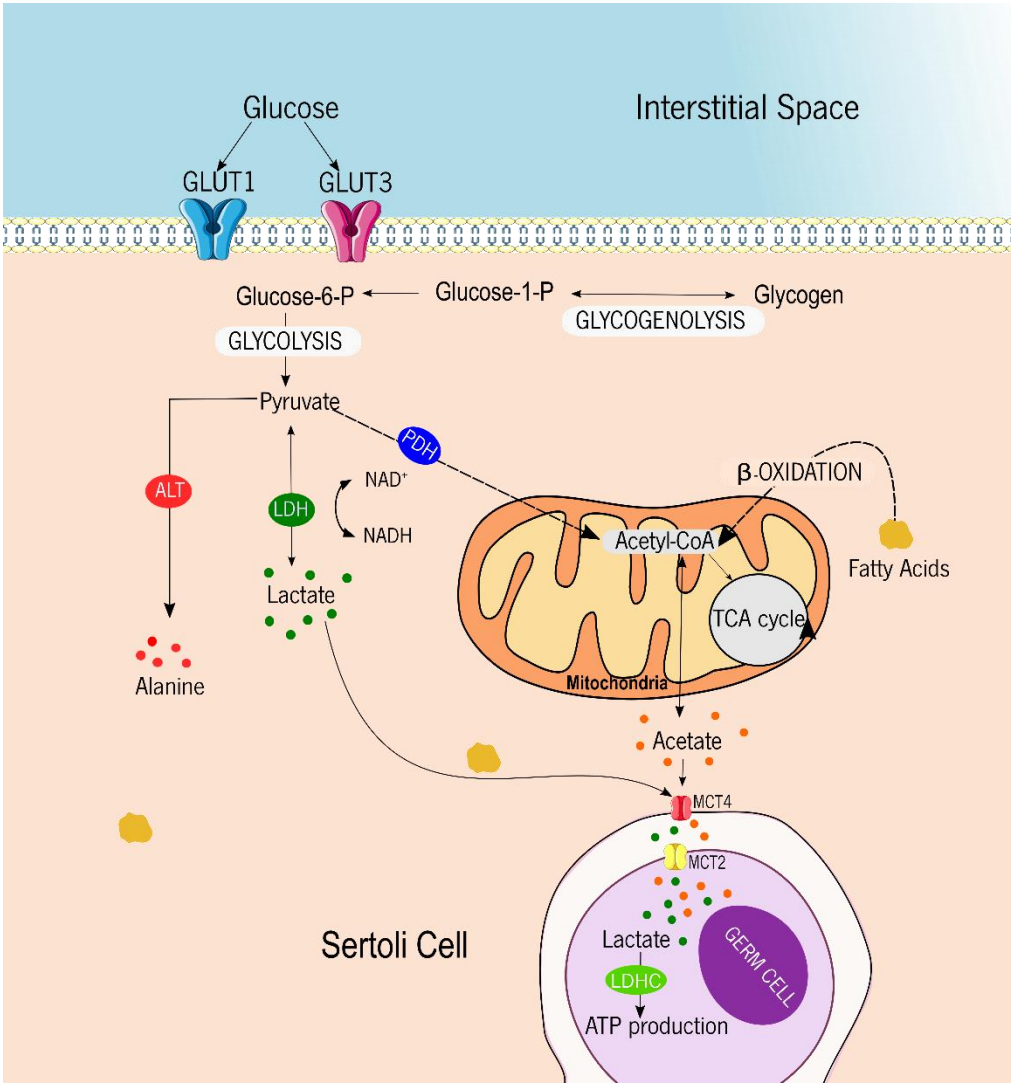

Figure 1. Representative diagram of the metabolic cooperation mechanisms established between Sertoli cells and developing germ cells. Glucose from interstitial space is taken preferentially through glucose transporters, GLUT1 and GLUT3, and is converted into glucose-6 phosphate (Glucose–6-P). Although, when glucose is not available glycogen can be used as an energy fuel, where it is converted into glucose-1-phosphate (Glucose-1-P) and then in glucose-6-P. Glucose is converted to pyruvate through multiples reactions that are compromised in glycolysis. The resulting pyruvate can follow three possible pathways. It can be converted in alanine by the action of alanine aminotransferase (ALT), it can be converted in lactate by the action of lactate dehydrogenase (LDH) or it can be converted into Acetyl-CoA through the action of pyruvate dehydrogenase (PDH). Acetyl-CoA can be converted into acetate or it can be used in the Krebs cycle in mitochondria. Acetate and lactate are exported to the intracellular fluid by monocarboxylate transporter 4 (MCT4) and then taken up by developing germ cells trough monocarboxylate transporter 2 (MCT2). The lactate produced by Sertoli cells, can be used for germ cells to ATP production.

In fact, it is characterized as the preferred substrate for developing germ cells, even if these cells possess all the machinery to metabolize glucose (26).

LDH enzyme is responsible for the interconversion of pyruvate into lactate, with the concomitant oxidation/reduction if NADH to NAD^+, a step essential for the continued production of ATP by glycolysis (47). LDH has different isoforms (LDHA, LDHB and LDHC). LDHC is exclusively expressed in tumors (48) and in the testis (49), being particularly abundant in spermatids and spermatozoa (50, 51). A disruption in *Ldhc* gene leads to male infertility and is associated with a decrease in sperm motility and a decline in the levels of ATP in germ cells (52).

In addition to the high amount of lactate being produced by SCs, these cells also produce high amounts of acetate (53). This metabolite seems to have a role in sustaining of the lipid metabolism (particularly phospholipid metabolism) in germ cells (54, 55). In fact, the synthesis of lipids in germ cells is maintained due to the high rates of acetate produced by SCs (53).

1.3.1. Energy sources on Sertoli cell metabolism

There are several substrates that can be used for energy production by SCs, in order to produce ATP. Glucose is the frequently used by different cell types, and SCs are not an exception. Still, some authors described that these cells use other alternative energetic fuels. For instance, the metabolism of lipids has been pointed to be crucial for a proper spermatogenesis, as an inactivation of some genes related to lipid metabolism, disrupted the normal course of spermatogenesis (56).

Glutamine and glycine are other substrates that yield much of the energy required by SCs (27), demonstrating that amino acids can have a role in metabolism in SCs.

Another energetic substrate available in SCs is glycogen. At the end of the 20th century, it was reported the presence of glycogen and glycogen phosphorylase in SCs (57, 58). Later, Vilarroel- Espíndola et al., demonstrated that glycogen has a role during testicular development and it also acts as a modulator of germ cell survival (59).

These multiple, energetic substrates that are available to SCs, demonstrate that they are flexible in metabolites used to produce ATP and thus, to be able to sustain spermatogenesis. Still, this is a subject that deserves more attention in the future.

1.4. Spermatogenesis

Spermatogenesis begins at puberty and continues throughout the entire humans' life. It takes place inside the seminiferous tubules, through a close association between germ cells and SCs (4, 60). All the stages of spermatogenesis are regulated by the niche of the testis, which is mainly established by SCs (23, 24). SCs are vital to spermatogenesis, since they are responsible for the correct nutritional and physical support for developing of germ cells (9).

The spermatogenic event is divided in different biological phases, comprising, among others, a mitotic and meiotic event. Basically, it starts with the differentiation of diploid cells (spermatogonia), through mitosis, into spermatocytes, which suffer a reduction of the chromosome number, trough meiosis, later originating the spermatozoa (haploid cells) (61, 62). Spermatogenesis only occurs due to the controlled environment that maintain the correct proliferation and differentiation of germ cells. To do so, a tight hormonal control must be maintained, with the participation of the hypothalamus and pituitary being imperative. This happens through the interconnected role of various hormonal and paracrine/autocrine regulation factors (63).

Spermatogenesis can be divided into four different phases such as mitosis, meiosis, spermiogenesis and spermiation (11). Initially, it is essential that germ cells pass through the BTB, moving to the basal into the adluminal compartment. Here, the germ cells continue to develop in a defined and immunoprivileged microenvironment (64) to continue this complex process. Briefly, the first event that occur is mitosis. During this process, the undifferentiated type A spermatogonia divides in different ways: in one way, it originates new type A spermatogonia, the cells that keep their function as stem cells; and in other way, after some divisions, some of those cells are differentiated into B spermatogonia (Figure 2). After mitosis, the final product is a preleptotene spermatocyte, that enter in the meiotic phase. This phase is separated in two different periods, Meiosis I and Meiosis II. At the end of this first meiotic division, each daughter cells contains only one partner of the homologous chromosome pairs, being called secondary spermatocyte (n), however, the DNA is still doubled. In Meiosis II, a phase with a very short duration, each secondary spermatocyte produces two spermatids (n), with a haploid number of single chromosomes (65, 66). Finally, for the transformation into highly structured spermatozoa, the spermatids undergo a process called spermiogenesis, in which multiple events that induce morphological transformations (for instance, the establishment of the flagellum, the formation of the acrosome and the elongation of the nucleus) (67). At this point, spermatozoids are released into the lumen of the seminiferous tubule (68) (Figure 2).

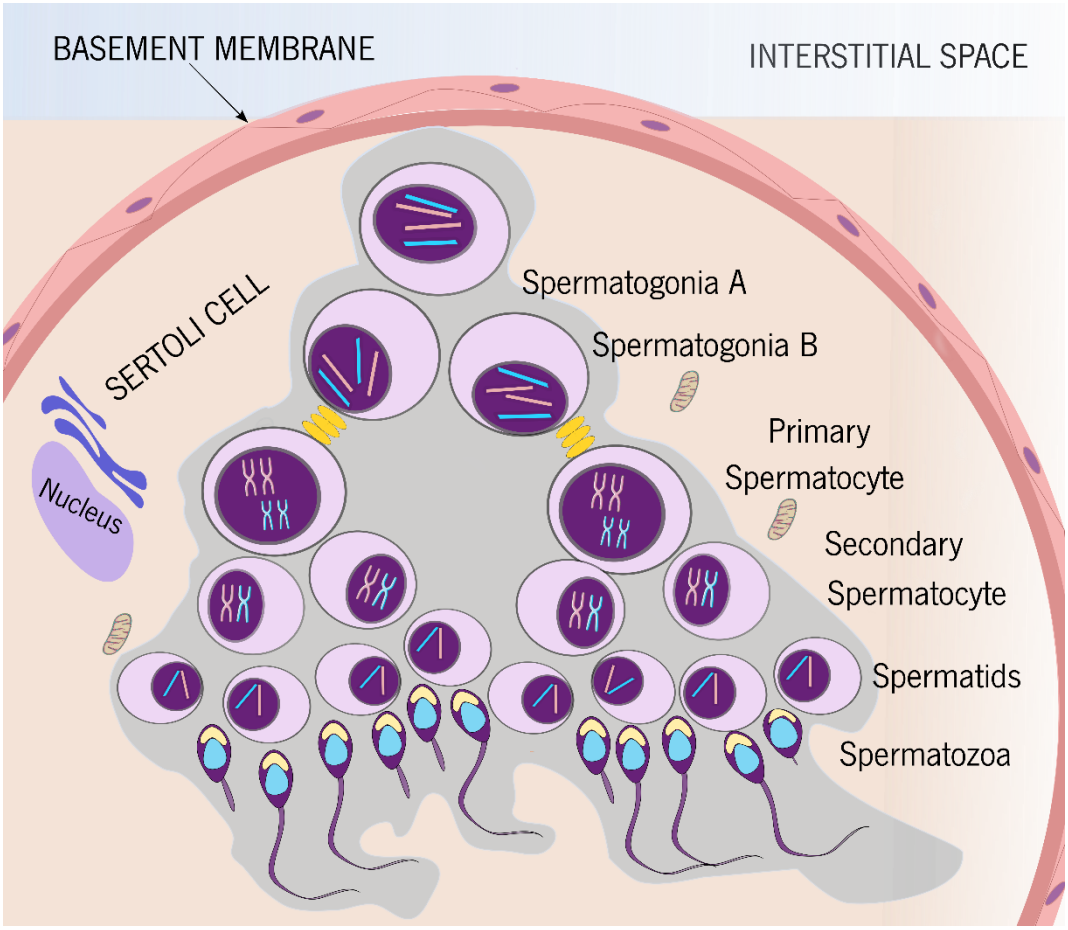

Figure 2. Schematic representation of the principal events during spermatogenesis process in seminiferous tubule. Successive mitosis occurs at a first stage in which each spermatogonia A, is divided and developed into two other cells, called spermatogonia B, which enter meiotic prophase and differentiate in primary spermatocytes. Primary spermatocytes undergo a first meiotic event occur whereas secondary spermatocytes are formed (haploid cells). A second meiotic event happens, and spermatids are formed. Spermatids go through a maturation process where it is formed fully developed spermatozoa. Then, spermatozoa are released in the lumen of seminiferous tubule.

1.4.1. Hormonal regulation of spermatogenesis

Spermatogenesis is tightly regulated by the hypothalamus-pituitary-testis (HPT) axis. The neurons of the hypothalamus produce the gonadotropin releasing hormone (GnRH), that stimulate gonadotroph cells to secret the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). These two pituitary hormones are responsible for the connection between brain and testis (69). Under physiological conditions, LH acts on LCs through LH receptors in the surface of these somatic cells and promotes the biosynthesis of testosterone. Testosterone then diffuses into the seminiferous tubules and systemic

circulation. Indeed, LH levels are positively correlated with testosterone plasma levels (60, 70). Additionally, FSH binds to its receptor in SCs (60) and its main goal is to sustain the male reproductive potential (60, 71).

The HPT axis is tightly regulated through feedback mechanisms to maintain the proper hormonal homeodynamics. In fact, the production of testosterone and 17β- estradiol (E_2) by LCs and the production of inhibin by SCs lead to a negative feedback loop that reduces the secretion of LH and FSH (72) (Figure 3).

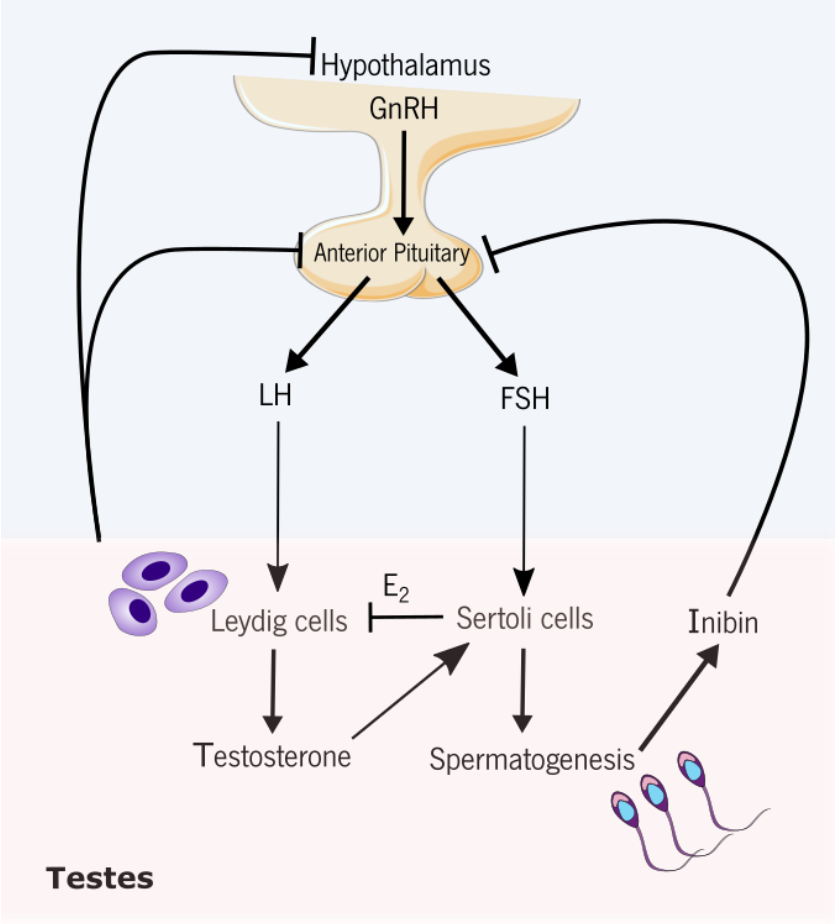

Figure 3. Hormonal regulation of the male reproductive tract. The gonadotropin releasing hormone (GnRH) is synthesized by the hypothalamus, which will stimulate the anterior pituitary to produce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH bind membrane receptor on Leydig and Sertoli cells, respectively, leading to the stimulation of testosterone production and spermatogenesis. High testosterone levels inhibit the release LH and FSH by the anterior pituitary. Inibin production by Sertoli cells regulates FSH and LH production by anterior pituitary in a negative feedback on pituitary. 17β- estradiol (E_2) acts on Leydig cells to inhibit the production of testosterone.

Besides FSH and LH, there are other hormones who deserves special attention, for instance, steroid hormones, thyroid hormones and insulin. Concerning the sex steroid hormones, androgens and estrogens, they are needed for a properly initiation and maintenance of spermatogenesis (73) and are important for development of the reproductive tract (74, 75). Androgens, particularly testosterone, is essential for normal spermatogenesis, in different stages, from initiation, maintenance to reinitiating of the whole process (76). They are necessary to maintain the integrity of the BTB and the assembly of junctional complexes (77, 78).

As well as androgens, estrogens are critical in the development and maintenance of the male reproductive function (4, 79, 80). Its synthesis is assured essentially by the LCs in adult men (81). Moreover, estrogens are able to regulate the HPT axis and thus, indirectly regulate LH and testosterone equilibrium. In fact, estrogens can disrupt the developmental of fetal LCs, leading to deficient proliferation and differentiation of gonocytes. They are also able to inhibit testosterone production by adult LCs (4, 82).

Insulin receptors have also been identified in SCs (83), and it has been described that insulin stimulates the proliferation of LCs, spermatogenesis and spermatic maturation (84). In fact, under insulin deprivation, SCs present an altered expression of metabolism-associated genes involved in the consumption of glucose (more particularly in GLUT1 and GLUT3 receptors), production and export of lactate (42).

Finally, thyroid hormones have an important role in testicular physiology and their deregulation leads to erectile dysfunction issues that could result in a decreased libido or even impotence (85). Thyroid glands produces tri-iodothyronine (T3), that controls the maturation and growth of the testis, by stimulating immature SCs to differentiate (86). These hormones play a critical role in the onset of LCs differentiation and stimulation of steroidogenesis in postnatal rodent testes (87).

1.5. Modulators of testicular metabolism

The modulation of SCs metabolism must ensure a cooperation between testicular cells and the correct function of several metabolic pathways. To achieve that, there is a complex of signals that count with the participation of multiple players, such as hormones, proteins, metabolic products, growth factors, cytokines and so on, that can trigger different signaling cascades. Despite that, other external factors, such as environmental factors, the lifestyle habits, and pathological conditions, that are linked to a disruption of signals and that can lead to infertility.

Over the last years, sirtuins came out as key modulators and targets in physiological and pathological events, such as obesity and cancer (88, 89). Nevertheless, only recently sirtuins were suggested as key metabolic sensors for testicular homeodynamics. Still, the information available is scarce.

1.5.1. Sirtuins

Acetylation and deacetylation are processes required for the post-translational regulation of protein activity. Acetylation is catalyzed by acetyltransferases that transfer an acetyl residue from acetyl-CoA to specific lysine residues in other proteins responsible for multiple cellular processes (e.g. histones). Opposite to acetylation, deacetylation corresponds to the removal of acetyl group from the lysine of acetylated proteins. This processes only happens due to enzymes known as lysine deacetylases (KDACs), particularly sirtuins (90).

Sirtuins belong to a highly conserved family of deacetylases, specifically class III (in a total of four classes), that depend on nicotinamide adenine dinucleotide (NAD^+). This cofactor is essential for electron transfers in an intermediate metabolism that is posteriorly converted into NADH, the reduced form (91). Thus, sirtuins are sensitive to fluctuations in the levels of NAD^+, which will interfere with their activity, and with substrate preference (92). In fact, the ratio NAD+/NADH is the essential piece in multiple metabolic processes such as glycolysis, Krebs cycle and electron transport in mitochondria (93).

Sirtuins respond to different signals like inflammatory signals or hypoxic/oxidative stress (88), metabolic challenges, such as type 2 diabetes and obesity, associated with insulin secretion (94-96). They are also associated with aging and longevity (97, 98), linked to processes like apoptosis and cell survival (99, 100), fatty acid oxidation (101), DNA repair, development and neuroprotection (95) and mitochondrial biogenesis (102).

This family of proteins is homologous to yeast transcriptional repressor, sir2 (90), which is highly conserved from bacteria to humans (103). Indeed, the first evidence on the existence of sirtuins arose when it was demonstrated that the human ortholog of yeast Sir2 can transfer 32P from NAD^+ to bovine serum albumin, indicating the role of Sir2 in mono ADP rybolysation of proteins (104). In the same year, Kaeberlein et al., demonstrated that an overexpression of Sir2 was associated to an increased lifespan (105). Posteriorly, the knowledge of this proteins was amplified and nowadays sirtuins have been found in different subcellular locations and have been classified in a family of seven members (SIRT1 to SIRT7), wherein SIRT1 is the closest phylogenetically to yeast Sir2 (106). Briefly, SIRT1 is predominantly nuclear (107), whereas SIRT2 is located mainly in cytoplasm (108), although it can translocate into the nucleus

as well (109, 110). SIRT3 to SIRT5 are mitochondrial proteins. Nevertheless, SIRT3, during cellular stress, can also translocate from the nucleus to the mitochondria. Finally, SIRT6 and 7 are nuclear sirtuins (111, 112). In terms of activity, SIRT1 to SIRT3 seems to have strong deacetylase activity, while SIRT4 to SIRT7 have weak or no detectable deacetylase activity (113-115). In addition to deacetylation, there are other enzymatic activities for sirtuins. For instance, SIRT4 is mostly responsible for the transfer of ADP-ribose to a specific protein - glutamate dehydrogenase (GDH), rather than releasing the deacylated products (deacetylation) (116, 117) and SIRT5 may act as a demalonylase, dessuccinylase and deglutarylase, in which the acid acyl moieties linked to the lysine residues are removed (114, 118).

The testicular metabolism is not an exception with regard to the maintenance of redox balance in order to guarantee the functioning of all processes of the male reproductive tract, particularly in what concerns to the NAD^+ ratio. In testes, NAD^+ appears to be a vital cofactor and can rewire testicular metabolism events between cytoplasm and mitochondrial compartments (119).

Despite not knowing exactly its role in testicular metabolism, sirtuins are highly expressed in testicular tissue (120), and it seems that the high energy demand of testes is in part related with the restoring of NADH levels.

In 2003, the interest on the role sirtuins in male fertility was first aroused and their potential impact on the regulation of male fertility was addressed. The strategy adopted was to inhibit the activity of sirtuins in mice, creating several null mice transgenic models (121). Since then, many explanatory attempts were done to highlight effectively the role of sirtuins in male fertility.

1.5.1.1. Sirtuin 1 (SIRT1)

Sirtuin 1 is the mammalian homolog of the yeast silent information regulator 2 and is the most studied sirtuin (122). As mentioned above, SIRT1 is a NAD^+ dependent histone deacetylase that has roles in multiple biological processes, resulting in modification of the acetylation status of histones and other proteins (123). Physiologically, SIRT1 seems to have a role in the regulation of energy metabolism, autophagy, apoptosis, inflammation and senescence (124, 125). Chromatin remodeling and DNA repair, with the involvement in cellular processes ranging from stress responses to the activation of survival or death pathway, are also linked to sirtuins (126). For instance, SIRT1 deacetylates specific residues in histones, such as lysine 16 of histone 4 (H4K16), lysine 9 of histone 3 (H3K9), lysine 56 of histone 3 (H3K56) (127). The non-histone protein substrates are diverse, such as: the tumor suppressor p53 that has a role in regulating apoptosis (107, 128) and the nuclear factor kB (NF-kB) that controls inflammation

through deacetylation of p65 subunit, thereby inhibiting NF-kb signaling. The relationship between NF-kb signaling and SIRT1 has repercussions in many inflammatory diseases. When SIRT1 is inhibited, the acetylation levels of the p65 subunit increase, as well as the levels of nitric oxide, which are associated with the inflammation process (129). Moreover, SIRT1 is important as tumor suppressor, with the capacity to deacetylate and inhibit β-catenin transcriptional activity (130). Although, SIRT1 activity in cancer is still on debate, as high levels of SIRT1 could be linked to a resistance to chemotherapy (131, 132). Furthermore, Yeung et al., described the SIRT1 paradox, that consists of the ability of mediating pro-apoptotic or antiapoptotic effects (133).

Regarding the relationship that sirtuins have with cell metabolism, it is already known that SIRT1 has a role in linking diet and cell metabolism. For instance, 5' AMP-activated protein kinase (AMPK), which is a critical regulator of mitochondrial biogenesis in energy deprivation conditions (134), was descried as an enhancer of SIRT1 activity by increasing cellular NAD^+ levels. Higher levels of NAD^+ leads to the deacetylation and modulation of the activity of downstream SIRT1 targets, that include peroxisome proliferator-activated receptor gamma coactivator (PGC-1-α) and the forkhead transcription factor (FOXO1) (135). PGC-1-α is a member of a family of transcriptional regulators that handle the expression of genes related to energy homeostasis, fatty acid oxidation, glucose metabolism and mitochondrial biogenesis (136, 137). In fact, SIRT1 can control mitochondrial biogenesis though regulation of PGC-1-α pathway (138). In the liver, SIRT1 is capable to maintain lipid homeostasis through PPARα mediated β-oxidation of fatty acids, evidencing that it has a role in glucose and lipid metabolism in fasting (139), and suggesting that gluconeogenesis in the liver is regulated by SIRT1 (140). Hence, SIRT1 controls the gluconeogenic/glycolytic pathways trough co-activator PGC-1- α.

Another key feature of SIRT1 is its negative regulation on the expression and phosphorylation of signal transducer and activator of transcription 3 (STAT3). Bernier et al., identified STAT3, which is a contributor to cellular respiration, as an additional SIRT1 target (141).

Nowadays, due to the western lifestyle, diabetes type 2 becomes to be like a pandemic disease, and SIRT1 seems to be involved in this pathology. It is mentioned that SIRT1 positively regulates insulin secretion in pancreatic β-cells by repressing uncoupling protein 2 (UCP2), that bounds directly to the UCP2 promoter. Its function is to uncouple oxygen consumption during respiration from the production of ATP (142).

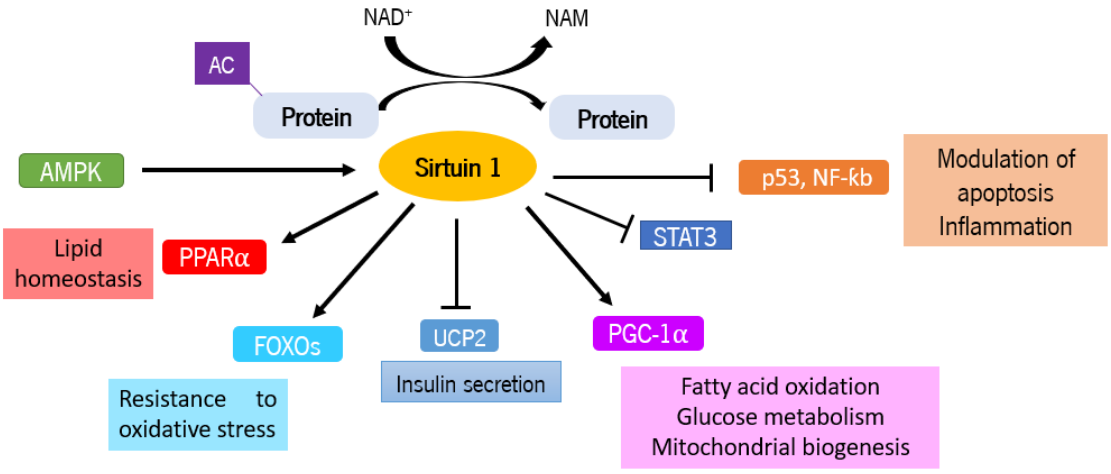

Figure 4. Sirtuin 1 (SIRT1) regulation signaling. Increased expression of SIRT1 results in deacetylation of non-histone and histone substrates, that affect multiple cellular functions. The deacetylase reaction catalyzed by sirtuins is based on the removal of the acetyl group of lysine residue in a reaction that consumes nicotinamide adenine dinucleotide (NAD^+), releasing nicotinamide (NAM). Enhanced levels of 5' AMP-activated protein kinase (AMPK) lead in enhanced SIRT1 activity. SIRT1 activation can mediate PPARα, that has important roles in maintaining lipid homeostasis; forkhead transcription factor (FOXOs) which is important in the regulation of oxidative stress; peroxisome proliferator-activated receptor gamma coactivator 1 -α (PGC1-α) that regulates multiple signaling pathways related with fatty acid oxidation, glucose metabolism mitochondrial biogenesis. Furthermore, SIRT1 regulates insulin secretion by repressing uncoupling protein 2 (UCP2) gene. SIRT1 is also able to regulate apoptosis by deacetylation of p53 and controls inflammation through regulation of the nuclear factor kB (NF-kB) signaling. It also negatively regulates the expression and phosphorylation of signal transducer and activator of transcription 3 (STAT3).

1.5.1.2. SIRT1 in the testicular environment

Due to their role as metabolic sensors, sirtuins, and in particularly SIRT1, might have a relevance in the maintenance of the spermatogenesis (89). Several studies were done, and when the activity of SIRT1 is inhibited, there are immediate percussions on male lifespan. Moreover, SIRT1 knockout (KO) presented a reduction on testes size and sperm quality (121, 143). HPT axis was also affected, with decreased levels of the LH and FSH. So, SIRT1 appear to regulate spermatogenesis at postnatal stages by controlling HPT axis signaling (143). Cakir et al., (144) showed that SIRT1 affects spermatogenesis primarily by

disrupting normal hypothalamus-pituitary complex, since this enzyme is highly expressed at the hypothalamus and its inactivation reduces hypothalamic GnRH expression. To support this information, another study was done, showing that SIRT1 KO mice present a reduced number of mature LCs and, thus, an impairment of steroidogenisis was detected due to the decrease in protein levels of steroidogenic acute regulatory (StAR) protein, the enzyme responsible for a rate-limiting step of steroidogenesis. Anatomically, an absence of tubular lumen formation was observed in the testis in SIRT1 KO mice, which results from a loss of differentiation in SCs, illustrating that loss of SIRT1 could result in arrested spermatogenesis. Concomitantly, Coussens et al., demonstrated that SIRT1 KO mice display decreased sperm counts, with a high aberrant morphology and increased DNA damage (145). In addition, epididymal sperm from SIRT1 KO animals were immature and with reduced motility (146).

Failure of SIRT1 can also be correlated to the unbalance of the oxidative stress, which is one of the main causes of male infertility (147). Reduced levels of SIRT1 are associated to oxidative stress and it is believed that SIRT1 mediates cell apoptosis. Indeed, SIRT1 KO mice exhibit defects in chromatin condensation as well as defects in histone to protamine transition leading to sperm DNA prone to apoptotic/oxidative damage (148). Wu and Bratton showed that reactive oxygen species (ROS) has an impact in the mitochondrial pathway of apoptosis (149). Later on, Rato et al (2016) described that the loss of testicular SIRT1 and SIRT3 results in a lower antioxidant defenses and leads to a decreased mitochondrial function in the testes (119).

More recently, Liu et al. described that SIRT1 KO mice had severe alterations in germ cell development, with early mitotic and meiotic phases not being affected, but with the subsequent spermiogenesis event being compromised. Germ cell exhibited a defective acrosome biogenesis. Additionally, increased acetylation levels of LC3 (a central protein in autophagy) were detected. This affected the recruitment of acrosome biogenesis-related proteins to the acrosomal vesicles, leading to a decrease of autophagy. These data suggest a novel function for SIRT1, by regulating the acrosome biogenesis through SIRT1-mediated LC3 nucleocytoplasmic transportation (150). All these reports revealed that sirtuins have a role in male fertility, particularly in glycolysis, oxidation of fatty acids and oxidative stress, which are crucial pathways for spermatogenesis. They showed also that sirtuins deficiency leads to the formation of deficient spermatozoa. For these reasons, it is off great relevance to fully clarify the role of this protein, which can potentially be of assistance in the development of better in pharmacological treatments for male fertility and extend the reproductive lifespan of males.

1.6. Activators and inhibitors of Sirtuins

Sirtuins, and particularly SIRT1, are linked to the modulation of different signaling cascades. Sirtuins act as metabolic sensors and respond to the fluctuation of NAD^+/ NADH, which is the fuel for the function of multiple metabolic pathways, intrinsically related to several diseases and conditions, such as infertility. Furthermore, the role of some sirtuins, such as SIRT1 and SIRT3, in regulating mitochondrial and cellular energetics makes them a potential target for molecular therapeutics. The modulation of their functional status may have immediate impact on several aspects of cell physiology. So, either natural or synthetic, the inhibitors or activators of sirtuins activity may prove to be an important tool for improving the health status of individuals.

1.6.1. Sirtuin inhibitors

Over the last few years, several sirtuin inhibitors have been developed, and some have target specifically SIRT1, SIRT2, SIRT3 or SIRT5, with some compounds inhibiting more than one sirtuin although with different affinities. Sirtuin inhibitors can be potentially useful as therapeutic strategies. For example, SIRT1 is usually up-regulated in cancer cell lines (151, 152) and thus, its inhibition might repress cancer cell proliferation.

Sirtinol is an inhibitor of SIRT1 and SIRT2, with the capacity of reducing inflammation in capillary endothelial cells of the skin, modulating the expression of dermal cells, and preventing skin disorders (153). This compound is also known to have anticancer potentials, reducing the proliferation of breast cancer (MCF7 cell line) and lung cancer lines (H1299 cell lines) (154).

Another example is cambinol, which is used to inhibit SIRT1 or SIRT2 and SIRT5 (although with less inhibitory effect). It was discovered in 2001 and it is characterized by a weak sirtuin inhibitory activity (155). To overcome that, it has been administrated in a combination of other sirtuin inhibitors, cambinol and EX-527. The results were quite positive. Indeed, Livore et al., demonstrated that SIRT1 and SIRT2 are overexpressed in hepatocellular carcinoma, and that their expression is correlated with tumoral progression and multidrug resistance. When cambinol and EX-527 were administrated together, tumor cell viability and cell migration were reduced, and apoptosis was increased (156).

There are some reports of new compounds that inhibit other sirtuins, such as Nicotinamide and GW5074 that can inhibit SIRT5's desuccinylation activity (157). Furthermore, there are some compounds with the ability to inhibit different sirtuins but with comparable potencies. Dish et al., found that ELT-11c exerts an

inhibitory effect over SIRT1, 2 and 3 (158). More recently, a resveratrol-related compound SDX-437 was described, having a stronger effectiveness against SIRT3 than against SIRT1 (159).

There are some more selective inhibitors, i.e. that present a considerable isoform-specificity. For instance, AGK2 and MIND4 are SIRT2 inhibitors that have protective effects in neurological disorders, being used as pharmacological agents for Parkinson's and Huntington's disease models, respectively (160, 161).

Another aspect to consider is the solubility of the compound. Lain et al., described Tenovin-6, which is water-soluble and has more affinity for SIRT2, then for SIRT1, and less for SIRT3. It was reported as a pharmacological agent for inducing apoptosis in gastric cancer cells (162) and slow down the progression in models of chronic myeloid leukemia (163).

The sirtuin inhibitor EX-527, also known as Selisistat is the only selective inhibitor to SIRT1 used in the clinic environment, that easily penetrates into cells. It was tested both in healthy humans volunteers and in Huntington's disease (HD) patients and no toxicity was detected (164). This later study demonstrated that EX-527 is well tolerated with no adverse effects in circulating levels (165). In sum, SIRT1 inhibition in HD with EX-527 seemed to alleviate the symptoms of this disease (166).

Moreover, a protective effect of EX-527 on cerebral ischemia-reperfusion was observed (167). EX-527 was also used with necrostatin-1, an inhibitor of necroptosis, to investigate what were the percussions at neuroprotective levels. Necroptosis is a type of programmed cell death, that is involved in ischemia-reperfusion-induced brain injury. It was previously known that SIRT1 has a crucial role on neural loss and this study demonstrated it can relieve ischemia, having a neuroprotective potential and thus preventing brain stroke. Concerning neuronal/cognitive disorders, it was further described that EX-527 has a positive effect on depression or anxiety-like behaviors (168).

Additionally to this, it was demonstrated that EX-527 has also benefic effects on acute lung injury (169), and block the amplification of human papillomavirus (170). In cancer, the combination of EX-527 and AGK2, inhibiting SIRT1 and SIRT2 respectively, was able to reduce cell migration by suppressing the HSF1 protein. This protein affects multiple molecular pathways involved in the regulation of cellular migration and cell protection (171-173). It is believed that activation of HSPF1/HSP27 (Heat shock protein 27) is dependent of SIRT1 and 2 pathway and, when these are inhibited, HSF1 ubiquitination and degradation *in vitro* is induced, demonstrating that these SIRTs are important for heat shock response signaling activation (174).

In the fertility field, more specifically in the female reproductive system, the activity of sirtuins was described in early stages of oocyte maturation, in events such as histone deacetylation (175). Indeed, when EX-527 is administrated *in vitro* to mouse oocytes, it causes an increase in ROS production and

abnormal metaphase II plates, demonstrating that SIRT1 has an important role in oocyte maturation (176).

As shown in Table 1, more attention has been given to the pharmacological applications of sirtuins inhibitors, and a new era of therapeutics possibilities has arisen. Still, further studies must be made to provide new selective molecules in this pharmacological field.

1.6.2. Sirtuins activators

Caloric restriction was described to improve the health and extend the lifespan of mammals. This is associated with increased levels of SIRT1 (177), and thus the discovery and the development of new activating drugs has attracted great interest in order to achieve better outcomes. So, over the last few years, attempts were made to increase the pharmacokinetic and pharmacodynamic efficiency of several compounds known to be sirtuin activators.

Resveratrol (RSV) is a natural polyphenol that can be found in red wine and grape skins (178). It was the first sirtuin activator to be described and it was said to increase the lifespan in a range of models, from yeast to worms (179, 180). RSV seems to prevent also neurodegenerative diseases that are correlated with aging (181). It can mimic caloric restriction, preventing the deleterious effects of high-fat diets (182). In mice under a high-fed diet, Person et al., found out that RSV administration improved aortic elasticity, motor coordination and preserved bone mineral density. It also decreased inflammation and apoptosis in vascular endothelium (183).

The fact that RSV has a role in aging and energy spending, aroused interest in the discovery of new molecules, with higher efficacy that this natural polyphenol. One example is SRT1720, which has higher specificity for SIRT1 than SIRT2 and SIRT3 and can counteract multiple metabolic disorders. Indeed, it has been reported that the use of this compound causes a reduction of liver triglyceride content and expression of lipogenic genes in obese an insulin resistant mice, resulting also in an extension of lifespan (184-186). Moreover, it exhibits anti-cancer properties, by inducing apoptosis and a decreased of cell growth in myeloma cells (187). There are also available some nutraceutical formulations with RSV. For instance, ResVida formulation (administration of 150 mg/day) exhibited positive effects in obese men, decreasing circulating levels of glucose and triglycerides, and increasing lipid breakdown (188).

SRT2104 is one of the most studied sirtuin activators. Different studies were made, and some of them demonstrated that it has an effect on lipid metabolization (189), although with no effects in terms of insulin control or improved glucose handling, indicating that SRT2014 might has a modest activity on

SIRT function. It showed some promising results, although it is necessary to improve pharmacokinetics and the bioavailability of this compound upon oral administration (190).

SRT501 has a stronger impact than RSV on SIRT1, enhancing several metabolic signaling pathways, blunting pro-inflammatory pathways, and enhancing mitochondrial biogenesis. In addition, it can lower blood glucose levels and improve insulin sensitivity in patients with type 2 diabetes (191, 192).

Another polyphenol with SIRT activating activity is quercetin, (which can be found in Black mulberry, *Morus nigra*). This compound has hypoglycemic, hypotensive, anti-tumoral and anti-inflammatory effects (193, 194). Still further research is necessary in order to develop new derivatives of quercetin because this compound has low bioavailability. Up until now, two quercetin derivates, diquercetin and 2-chloro-1,4-naphtoquinone-quercetin, were studied. These compounds exhibited an action on SIRT6, which is involved in metabolism and has a role in DNA damage signaling and repair (195, 196). The available data demonstrated that quercetin based derivates are an alternative approach in the regulation of some of these mechanisms. Interestingly quercetin has been reported with a synergy with resveratrol, causing an upregulation of SIRT1 and SIRT2 activities (197).

Curcumin is a natural SIRT activator that belongs to ginger family. It displays lots of benefits on cancer (198, 199), diabetes (200), nonalcoholic fatty liver diseases (201), respiratory diseases (202), anxiety and depression (203). In a very general way, when curcumin is present, the reduction of ATP promoted by curcumin leads to AMPK activation, which in turns increases the NAD^+ levels and consequently leads to the activation of SIRTs, which mediate protective effects against different disorders (204).

The influence of SIRTs activators is still an issue that deserves special attention, particularly when reduced SIRTs levels are associated with some disorders. An example is YK-3-237, that initially was identified as a compound with antiproliferative effects in different cancer cell lines. However, its mechanism of action was unknown (205). Yi et al., were able to describe its SIRT promoting activities, after inducing deacetylation of a triple negative breast cancer (TNBC) cell line carrying different p53 status, a mutant form (mtp53). This resulted in a suppression of cell proliferation and arresting cell growth (206). Moreover, as SIRT1 activation has been shown to have a role in renal fibrosis by protecting the kidney from acute injury, it was described that exposure to YK-3-237 resulted in an enhancement of α-SMA and fibronectin expression (fibroblast activation markers), which in turns aggravates renal fibrosis in a dose-dependent manner (10 μM). This data demonstrates that SIRT1 has an important role in mediating activation of renal interstitial fibroblast (207).

Table 1. Principal sirtuin inhibitors and activators.

Compound	Sirtuin Target	Effects	Reference
Inhibitors			
Sirtinol	Sirtuin 1 Sirtuin 2	Skin disorders Slows down proliferation I breast and lung cancer line	(153, 154)
Cambinol	Sirtuin 1 Sirtuin 2 Sirtuin 5*	Weak effects	(155, 156)
ELT-11c	Sirtuin 1 Sirtuin 2 Sirtuin 3	ND	(158)
MIND4	Sirtuin 2	Huntington's disease	(160, 161)
AGK2	Sirtuin 1 Sirtuin 2	Parkinson's disease and Huntington's disease Suppresses cell migration in cancer	(160, 161, 172, 173)
Tenovin-6	Sirtuin 1* Sirtuin 2 Sirtuin 3**	Induces apoptosis in gastric cancer cells Slows down proliferation in chronic myeloid leukemia cells	(162, 163)
EX-527	Sirtuin 1 Sirtuin 2 * Sirtuin 3 *	Neuroprotective effects Anxiety and depression Suppress cell migration	(166, 168, 173, 174)
Activators			
Resveratrol	Sirtuin 1	Neurodegenerative disorders Positive effects in obesity	(182, 183)
SRT1720	Sirtuin 1 Sirtuin 2* Sirtuin 3*	Effects on metabolic disorders Induces apoptosis Decreases cell growth	(184-187)
SRT2104	Sirtuin 1	Psoriasis Improves lipid parameters	(189, 190)
SRT501	Sirtuin 1	Improves metabolic pathways Enhances mitochondrial biogenesis Improve insulin sensitivity in type 2 diabetes	(191, 192)
Quercetin	Sirtuin 1 Sirtuin 2 Sirtuin 6	DNA damage and repair	(195-197)
Curcumin	Sirtuin 1	Cancer Diabetes Nonalcoholic fatty liver diseases Respiratory diseases Anxiety and depression	(198-202)
YK-3-237	Sirtuin 1	TNBC Renal fibrosis	(206, 207)

Legend: * with less specificity; ** with even lesser specificity; ND- non-defined.

Chapter II

AIM OF THE PROJECT

2. AIM OF THE PROJECT

Over the past years, sirtuins have been recognized as sensors in multiple cellular events, being able to integrate a network of signaling pathways. SIRT1, in particular, when activated/ or inhibited translates into different behaviors that are relevant in the normal functioning of living cells, particularly to their metabolic phenotype. There are several studies made with SIRTs in cancer-related areas, however less attention has been given to their role in non-cancerous cells, particularly in cells that present a "Warburg-like metabolism", like SCs.

YK-3-327 and EX-527 are sirtuin modulators (activator and inhibitor, respectively) of SIRT1. Up to date there is no evidence of the impact of these compounds in testicular metabolism. This work aimed to unravel the actions of these two compounds on testicular cells metabolism, particularly in mouse SCs (mSCs), since spermatogenesis is extremely dependent on the metabolism of these cells, as described previously. Considering that SIRT1 may modulate glycolytic metabolism, we hypothesized that its activation or inhibition may have percussions on testicular metabolism and can control male fertility.

Specifically, we aimed to:

1. Identify SIRT1 expression in the TM4 Sertoli cell line;
2. Evaluate the cytotoxic profile of the exposure to a SITR1 activator (YK-3-237) and inhibitor (EX-527) in the TM4 Sertoli cell line;
3. Evaluate the glycolytic profile of TM4 Sertoli cells after the exposure of EX-527 and YK-3-237;
4. Evaluate the oxidative status of TM4 Sertoli cell after the exposure of EX-527 and YK-3-237;
5. Determine the glycogenic and lipid reserves on TM4 Sertoli cells after the exposure of EX-527 and YK-3-237.

Chapter III

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Chemicals

Fetal Bovine Serum (FBS) was obtained from Millipore (Darmstadt, Germany). Dulbecco's Modified Eagle Medium, Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Bovine Serum Albumin (BSA), trypsin–EDTA, Oil Red O solution, EX-527 and YK-3-237 were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). LDH Citox™ Assay Kit was obtained from BioLegend® (San Diego, CA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye was purchased from Life Tecnhologies (Gaitherburg, MD, USA). RIPAS Lysis Extraction Reagent and BCA Protein Assay Kit were obtained from Thermo Scientific (Whalthan, MA, USA). WesternBrightTM ECL substrate was purchased from Advansta (Menlo Park, CA, USA). Dried milk was obtained from Regilat (Saint-Martin-Belle-Roche, France). EFC™ Western Blotting Reagent was obtained from GE HealthCare Life Sciences (USA).

3.2. Cell culture conditions

Cell line of TM4 mouse Sertoli cells (mSCs) were purchased from ATCC (Manasas,VA, USA). In brief, mSCs were cultured in 75-cm^2 flasks (VWR collection, Amadora, Portugal) at 37°C, 5% CO_2 and maintained in Sertoli culture medium (DMEM:Ham's F12 1:1, supplemented with 10% heat inactivated FBS, 50 µm/mL gentamicin, 1% Pen-Strep, 1% amphotericin B, 15 mM HEPES, 14 mM $NaHCO_3$ and 18 mM Glucose). TM4 is a Sertoli cell line derived from the prepurbertal BALB/c nu/+ mouse exhibiting the capacity to respond to FSH with an increase in cAMP production and does not respond to LH. It is recognized as non-tumorigenic cell line and is negative for ectromelia virus (208).

3.3. Experimental Groups

Sertoli cell were allowed to grow until they reached 90-95% confluence, and then washed thoroughly with phosphate buffered saline (PBS) solution. The medium was replaced by the culture medium supplemented with increasing concentrations of the sirtuin inhibitor (EX-527) or activator (YK-3-237). To evaluate the effect of EX-527 and YK-3-237 we defined five concentrations (1 nM, 10 nM, 100 nM, 1000

nM and 10000 nM) and as a control condition we used culture medium with same volume of DMSO (the vehicle compound where EX-527 and YK-3-237 were dissolved). These concentrations were chosen based on the IC_{50} value of EX-527 (nearly 100 nM). The same range of concentrations were used with YK-3-237 and were chosen based on the maximum inhibitory concentration used in a renal fibrosis case study (10000 nM) (207). Then, the total number of cells was determined using a Neubauer chamber and the cells were collected for protein extraction and enzymatic assays. The extracellular medium was collected for ^{1}H-NMR analysis.

3.4. Sulforhodamine B (SRB) cytotoxicity assay

The cytotoxicity of EX-527 and YK-3-237 was evaluated by the colorimetric SRB assay (209). Briefly, SCs were seeded in a 24-well culture plate, let to grow until reaching 60-70% confluence and then treated with the different concentrations of either the sirtuin activator or inhibitor during 24 hours. After treatment, cells were washed with PBS and fixed in 1% acetic acid in methanol for 1 hour at -20°C. Next, cells were stained with 0,05% (w/v) SRB dissolved in 1% of acetic acid for 1 hour at 37°C. Afterwards, unbound SRB was removed through washing with 1% acetic acid solution. SRB bound to cell proteins was extracted afterwards, with 10 mM Tris solution (pH 10) in a shaker for 10 minutes at room temperature. Then, 100 µL of this solution were transferred to a 96-well culture plate. The optical densities of the resulting media were determined at 490 nm. The percentage of cell proliferation in each treated group was calculated with a normalization to control group.

3.5. Lactate Dehydrogenase (LDH) release assay

LDH release was determined by measuring the extracellular activity using a commercial assay kit following the manufacturers' instructions (LDH Citox™ Assay Kit, BioLegend®, San Diego, CA, USA). To sum up, after 24 hours of the treatment, 100 µL of extracellular media were collected into a 96 well plate, to which were added 100 µL of working solution. LDH assay substrate was added to all samples in a dark environment and this mixture was incubated at 37°C for 15 minutes. After that time, a 50 µL stop solution was added to stop the enzymatic activity and absorbance was read at 490 nm using Bio-Rad model 680 microplate reader. LDH release of each treated group was calculated with a normalization to control group

3.6. MTT viability assay

The metabolic toxicity of SIRT1 inhibitor and activator was assessed through the MTT colorimetric assay (210). In brief, this assay is based on the reduction of MTT to formazan crystals, which are insoluble and have a purple color, that is dependent on cellular mitochondrial dehydrogenases present in viable cells. For that, cells were seeded in a 48-well culture plate and let them grow until reaching 60-70% confluence. Then, cells were treated with the different concentrations of SIRT1 activator and inhibitor in study for 24 hours. After treatment, cells were washed in PBS and 50 µL MTT (5 mg/mL) dissolved in medium without FBS were added to each well. Cells were then incubated at 37°C for 2 hours, in a dark environment. Then, the medium was removed and 250 µL of DMSO were added to each well, to dissolve the formazan crystals formed inside the cells. The plate was placed in a shaker for 10 minutes at room temperature. After that, 100 µL of each well were transferred to a 96 well plate culture and a blank was made with DMSO. The absorbance was read at the wavelengths of 570 nm (considered the optimal wavelength to detect the amount of solubilized formazan) and 650 nm (the reference to detect the base line, also considered as a blank of the assay). Metabolic viability was calculated by the difference of 570 nm and 650 nm. After this adjustment, in each sample was removed the blank (well with only DMSO), and then the values of each treatment group were normalized with the control group.

3.7. Protein extraction and quantification

Total protein was extracted from mSCs using RIPA buffer (PBS 1x, 1% NP-40, 0.5 % Sodium deoxycholate and 0.1% SDS 10%), supplemented with 1% protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A), 100 mM sodium orthovanadate and 100 mM PMSF (phenylmethylsulfonyl fluoride). The cells were kept in ice for 20 minutes with RIPA buffer. After that time, the resulting suspension was centrifuged at 14 000g for 20 minutes. Afterwards, the resulting pellet was discarded. Total protein concentration was determined using Pierce™ BCA Protein Assay Kit according to manufacturer's instructions. Calibration curve was calculated by using different concentrations of BSA as the standards for protein quantification. Optical densities of samples were determined at 560 nm.

3.8. Western Blot

Western Blot (WB) was performed to analyze individual protein level of SIRT1, BAX, BCL-2 and oxidative phosphorylation (OXPHOS) complexes. Protein samples (20 µg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1 % SDS, β-mercaptoethanol, 0.02 % bromophenol blue, pH 6.8) and PBS, denatured for 10 minutes at 55 °C and sonicated for 15 minutes at 4°C. Proteins were fractionated in 12% polyacrylamide gels and electrophoresis was carried out for 100 minutes at 100 V. The proteins were transferred from gels to previously activated polyvinyldene difluoride membranes in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) and then Ponceau solution (NZYtech, Lisbon, Portugal) was used in order to quantify total protein of each sample.

Then, membranes were blocked for 90 minutes in a 5% non-fat milk dissolved in Washing Buffer (1.54 M NaCl, 1M Tris-Base, pH 8.0 and Tween 20) at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies dissolved in 1% BSA. The immune-reactive proteins were detected separately after incubation during 90 minutes at room temperature with the secondary antibodies dissolved in 1% BSA. The antibodies used are listed in Table 2. Membranes were reacted with WesternBright™ ECL and read with the Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK). Regarding the analysis of OXPHOS' protein levels, 20 µg of proteins were mixed with sample buffer, and stirred for 15 minutes at 37°C. The protocol was followed as above, except that proteins were fractionated in 15% polyacrylamide gels and membranes were blocked for 3 hours in a 5% non-fat milk at room temperature. The membranes were then incubated overnight at 4°C with MitoProfile® Total OXPHOS WB Antibody Cocktail (Table 2). Image Lab Software (Bio-Rad, Hemel Hempstead, UK) was used to obtain band densities following standard methods. In all cases, including OXPHOS complexes, the band density was divided by the respective Ponceau staining density and then normalized with the control group value.

3.9. Slot-Blot – Lipid Peroxidation

Lipid peroxidation is a process in which free radicals attack lipids, such as membrane lipids. High lipid peroxidation levels lead to irreparable damages that may result in cellular apoptosis or necrosis programmed cell death (211). The lipid peroxidation levels in TM4 cell line culture from different experimental groups were evaluated using the slot-blot technique, by measuring aldehydic products, such as 4-hydroxynonenal (4-HNE) as described previously by Dias et al., (212). In brief, protein samples were diluted in PBS to a concentration of 0.01µg/mL and used in the slot-blot technique described before

(212). Membranes were incubated overnight at 4°C with a goat anti 4-HNE antibody (Table 2) dissolved in 1% BSA, and then, incubated with a secondary rabbit anti-goat IgG-AP antibody (Table 2) dissolved in 1% BSA, at room temperature during 90 minutes. After incubation with secondary antibody, the membranes were incubated with ECF™ substrate (GE Healthcare, Buckinghamshire, UK) and read using BioRad FX-Pro-plus (Bio-Rad Hemel Hempstead, Uk). Densities from each band were quantified using BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France). Results were expressed as fold variation to the control group.

Table 2. List of Primary and Secondary antibodies used in Western Blot and Slot-Blot technique.

Antibody	Host specie	Molecular weight (kDa)	Dilution	Vendor	Catalog #
Sirtuin 1	Mouse	120	1:1000	Cell Signaling Technology	8469S
Mouse	Goat	-	1:5000	Sigma-Aldrich	A3562
Rabbit	Goat	-	1:5000	Sigma-Aldrich, USA	A3687
BAX	Rabbit	20	1:1000	Cell Signaling Technology	2772S
BCL-2	Rabbit	26	1:1000	Abcam, United Kingdom	Ab196495
OXPHOS	Mouse	20,30,40,48 and 55	1:500	Abcam, United Kingdom	Ab110413
4-HNE	Goat		1:5000	Merck Millipore, Temecula, USA	AB5605
Goat	Rabbit		1:5000	Sigma-Aldrich, USA	A4187

3.10. Mitochondrial Membrane Potential

The mitochondrial membrane potential was evaluated using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Cells were seeded in a 96 well plate culture, with a black plate and clear bottom (Corning Incorporated costar®). When a 60-70 % cell confluence was reached, cells were treated with different concentrations of the SIRT1 inhibitor or activator for 24 hours. After treatment, cells were washed with PBS and incubated in culture medium with JC-1 in a final

concentration of 2 μM (dissolved in 1% FBS). The cells were incubated at 37°C for 30 minutes. The media with JC-1 was then replaced by fresh media without JC-1. Fluorescence of each sample was analyzed at an excitation wavelength of 485 nm and 535 nm and emission wavelength of 530 nm and 590 nm using Cytation™ 3 imaging reader (Biotek Instruments Inc., Winooski,VT) pre-heated at 37°C. The accumulation of JC-1 dye in mitochondria depends on mitochondrial membrane potential. A high concentration of JC-1 forms aggregates that yield red fluorescence at 590 nm, which represent the healthy mitochondria. Furthermore, in unhealthy mitochondria JC-1 only forms monomers, due to its low concentration, that are detected at 530 nm emitting green fluorescence.

3.11. Caspase-3 Activity

The activity of caspase 3 was spectrophotometrically assessed by determining the cleavage of the respective colorimetric substrate, as previously described (213). In brief, proteins (25μg) were incubated with the assay buffer (25mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 100 μM of caspase-3 substrate (ac-DEVD-pNA) for 2 hours at 37°C. The caspase-like activity was determined by detection of the chromophore p-nitroaniline, measured at 405 nm in a spectrophotometer, after the cleavage of the labeled substrate. The method was calibrated with a standard curve calibration of known concentrations of p-nitroaniline. Caspase- 3 activity was calculated with normalization to control group.

3.12. ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra of the extracellular media collected were aquired at 14.1 T, 25°C, using a Brucker Avance 600 MHz spectrometer equipped with a 5 mm QXI probe with a z-gradient (Bruker Biospin, Karlshruhe, Germany), using standard methods (39). Sodium fumarate was used as internal reference (singlet, 6.50 ppm.), in a final concentration of 1 mM, to quantify the following metabolites present in solution (multiplet : ppm): lactate (doublet, 1.33), alanine (doublet, 1.45), acetate (singlet, 1.9), pyruvate (singlet, 2.35), glutamine (quartet, 2.44) and H1-α glucose (doublet, 5.22) were determined when present in medium. The relative areas of ^{1}H-NMR resonances were quantified offline using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis program (Acorn NMR, Inc., Fremont, CA, USA). Metabolite consumption/ production was calculated by measuring the accumulated variation of the metabolite comparing to the initial medium.

3.13. Colorimetric Method For Glycogen Quantification

The glycogen was extracted from cellular pellets collected in section 3.3. First, the pellet was ressuspended in 500 µL PBS, and then 50 µl of this cellular suspension it was added Triton-X 100 (purchased from Sigma Aldrich, St. Louis, MO, USA). After that, 200 µL of resuspended pellet was used and it was added 250 µL of Potassium hydroxide (KOH) and the sample were shaked vigorously in vortex, let stand on ice for 5 minutes and sonicated for 3 minutes and then centrifugated at 16000 g for 20 minutes at 4°C. After that, supernatant was collected, and the samples were neutralized with phosphoric acid (H_3PO_4) and then the samples suffered a second centrifugation (16000 g for 20 minutes at 4°C). To obtain glycogen, 500 µL of supernatant was diluted in 200 µL of ethanol and incubate overnight at 4°C. On the next day, the samples were centrifugated at 16000 g for 20 minutes at 4°C and the supernatant was discarded. The resultant pellet was dissolved in phenol at 6.5% and in concentrated sulfuric acid (H_3SO_4). Afterwards, 250 µL were transferred for a 96-well plate and the absorbance was measured at 490 nm using a microplate reader. The method was calibrated with a standard curve calibration of known concentrations of glycogen. Glycogen was calculated with a normalization to the control group and the results were presented in nmol of glycogen per mg of protein.

3.14. Oil red O Staining (ORO)

To assess intracellular lipid accumulation, cells were stained with Oil Red O staining (ORO). Firstly, cells were seeded in 12-well plate until reach 60-70% of confluence and then treated with different sirtuin activator and inhibitor concentrations for 24 hours. Following this, the cells were fixed with 10% formalin four 1 hour. Then, after raising twice with PBS 1X, cells were incubated for 5 minutes with 60% isopropanol. Then, cells were stained with freshly diluted (in a final concentration of 0.02%) and filtered ORO working solution at room temperature for 20 minutes. After that, cells were washed firstly with 40% of isopropanol and then with PBS 1X (2 at 5 times). For the quantification of lipid accumulation, it was added to the stained samples, 200 µL of 100% isopropanol to each well, and the plates were slowly agitated at room temperature for 10 min for the dissolution of ORO staining. Thereafter, 100 µL of the samples of each well were transferred to a 96-well plate, and the absorbance was measured at 510 nm using a microplate reader. Lipid accumulation of each treated group was calculated with a normalization to the control group.

3.15. Statistical analysis

Statistical significance among the experimental groups was assessed by one-way anova and Two-way anova followed by multiple comparisons through Fisher Least Significant Difference (LSD) method. All experimental data are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism 8 (GraphPad software, San Diego, CA, USA). Possible outliers were removed using ROUT method with a q value of 1%. Result were considered significant when $p < 0.05$.

Chapter IV

RESULTS

4. RESULTS

4.1. SIRT1 protein is expressed in TM4 cell line

In order to investigate the presence of SIRT1 protein in TM4 Sertoli cells, a western blot was performed using a specific anti-SIRT1 mouse antibody that recognized endogenous levels of total SIRT1 protein. The total protein fraction of mouse testicular lysate was used as positive control. Under these conditions we could observe a band that should correspond to the presence of SIRT1 in TM4 Sertoli cells. The expected molecular weight of 120 kDa was detected (Figure 5). Additionally, multiple isoforms were detected.

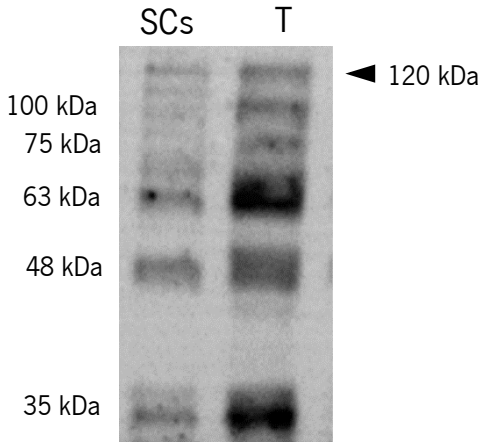

Figure 5. Identification of SIRT1 and its isoforms (35-120 kDa), through Western Blot technique on TM4 Sertoli cells (SCs) and in mouse testicular lysate (T) as positive control. The predicted weight is 120 kDa.

4.2. Sirtuin 1 activation and inhibition do not alter cellular proliferation and metabolic viability of TM4 Sertoli cells

We evaluated the impact of SIRT1 activation and inhibition on mSCs cell proliferation using the SRB assay, which is widely used to investigate proliferation in cell cultures. SRB binds stoichiometrically to proteins and thus the amount of bound dye is directly related for cell mass, which can be extrapolated to measure cell proliferation.

The results showed no differences on cell proliferation when the cells were exposed to EX-527 concentrations (Figure 6, Panel A). However, when SCs were exposed to the sirtuins activator YK-3-237 at a concentration of 1 nM, we could observe a decrease on cell proliferation (0.64±0.10-fold variation to

control) (Figure 6, Panel B). No alterations on cell proliferation were observed when cells were exposed to the other concentrations of this SIRT1 activator.

The SRB assay does not allow to measure metabolic viability. For that purpose, we took advantage of the MTT assay, which relies on measuring cell metabolic activity through determination of mitochondrial function. Slightly variations were observed in the metabolic viability of TM4 Sertoli cells (Figure 6, Panel C and D). When cells were exposed to EX-527 concentrations of 10000 nM (1.09±0.07-fold variation to control) we observed a slight improvement of the metabolic activity when comparing to cells in the control conditions (1.00±0.11) (Figure 6, Panel C). Contrastingly, YK-3-237 tends first to decrease the metabolic viability of TM4 Sertoli cells at 1 nM (0.63±0.05-fold variation to control), but when present in higher concentrations this effect fades, showing no differences between the cells exposed to those concentrations and those in control conditions (Figure 6, Panel D).

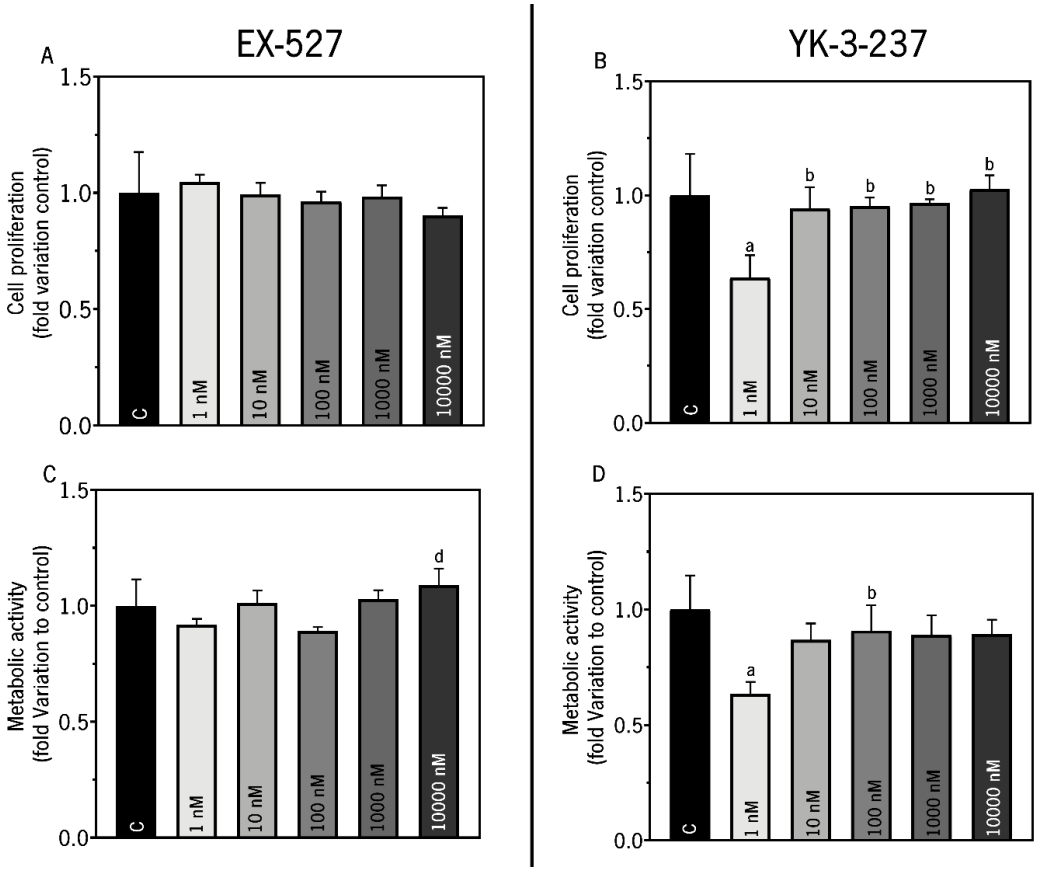

Figure 6. Evaluation of cell proliferation (Panels A and B) and metabolic activity (Panels C and D) on TM4 Sertoli cells after exposure to SIRT1 inhibitor (EX-527) (Panels A and C) or activator (YK-3-237) (Panels B and D). The figure shows pooled data of independent experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a - relative to control group, b - relative to 1 nM and d - relative to 100 nM. Abbreviations: C- Control group.

4.3. Exposure to the SIRT1 inhibitor EX-527 promoted a decrease of LDH leakage on TM4 Sertoli cells

Cellular cytotoxicity was also evaluated by determining LDH (lactate dehydrogenase) leakage assay. LDH is an enzyme that is present within the cell cytoplasm. When cell viability is reduced, there was an increase of LDH levels in the extracellular medium. Our results showed that exposure to the SIRT1 inhibitor EX-527 decreased LDH release into the extracellular medium at concentrations above 1 nM (0.60±0.04-fold variation to control for 10 nM, 0.60±0.04-fold variation to control for 100 nM, 0.60±0.03-fold variation to control for 1000 nM, 0.72±0.09-fold variation to control for 10000 nM, respectively) (Figure 7, Panel A). Extracellular LDH activity, when cells were treated with the SIRT1 activity YK-3-237, showed no significant alteration when comparing to the cells from the control group (Figure 7, Panel B).

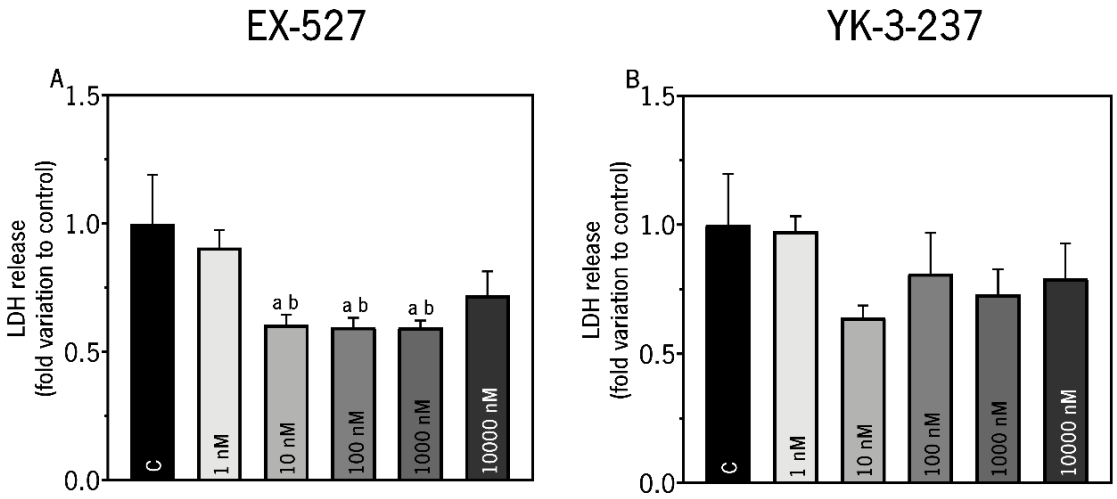

Figure 7. Evaluation of LDH release (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor (EX-527) (Panel A) or activator (YK-3-237) (Panel B). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a - relative to control group, b – relative to 1 nM. Abbreviations: C- Control group.

4.4. SIRT1 inhibition results in the activation of caspase-3 in TM4 Sertoli cells

In order to evaluate the influence of SIRT1 on apoptotic event in TM4 Sertoli cells, we evaluated both the BAX/BCL-2 ratio and the activity of caspase-3. BCL-2 belongs in a Bcl-2 family of proteins that regulates apoptosis, exhibiting an antiapoptotic profile. BAX belongs to a family of proteins which share homology with Bcl-2 however it has a pro-apoptotic profile. Caspase 3 belongs to a family of conserved cysteine

proteases that have a role in apoptosis, which is a normal process for maintain the normal function of a whole organism.

Our results further showed, that there were differences in the caspase activity of the cells treated with EX-527 at 100 nM (2.54±0.67-fold variation to control) (Figure 8, Panel A). No alteration was observed on caspase-3 when cells were exposed to EX-527 at 10000 nM. In the case of SIRT1 activator YK-3-237, as concentration increased, so did caspase 3 activity (Figure 8, Panel B). Nonetheless, with no statistical difference was observed between the Sertoli cells of the experimental groups.

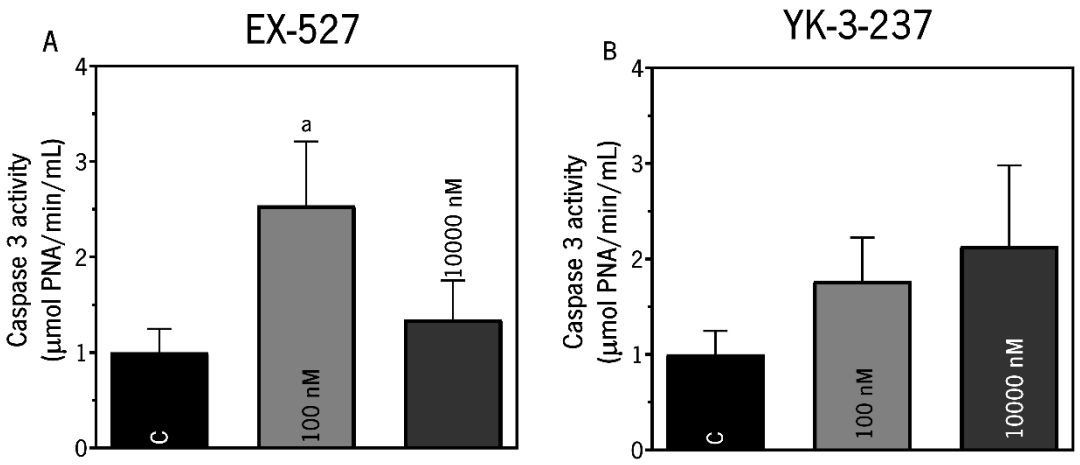

Figure 8. Evaluation of the caspase 3 activity (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor (EX-527) (Panel A) or activator (YK-3-237) (Panel B). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a - relative to control group. Abbreviations: C- Control group.

The ratio between the expression levels of BAX and BCL-2 on the cells exposed to the selected concentrations of the SIRT1 inhibitor EX-527 or the SIRT1 activator YK-3-237 were not statistically altered when compared to that of cells in control conditions (Figure 9, Panel A and B, respectively).

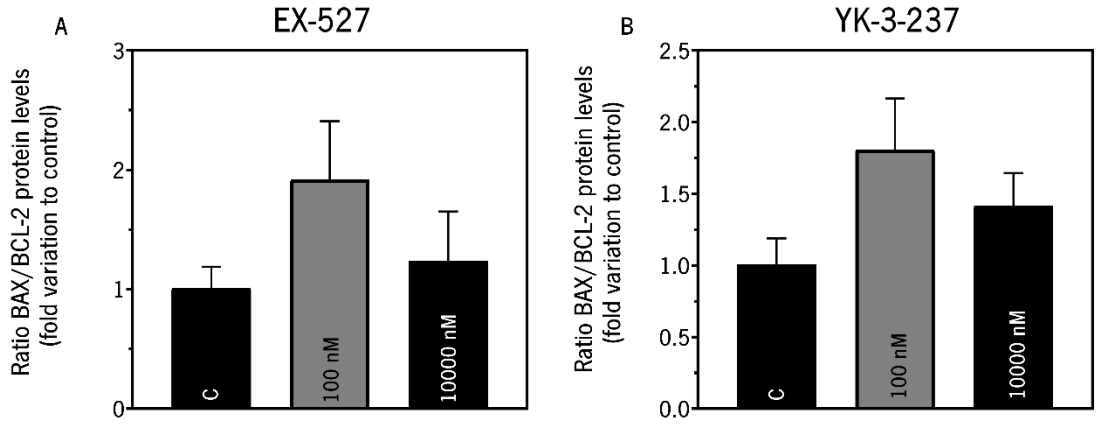

Figure 9. Evaluation of the ratio of BAX/BCL-2 (Panels A and B) on TM4 Sertoli cells after exposure to SIRT1 inhibitor (EX-527) (Panel A) or activator (YK-3-237) (Panel B). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition).

Abbreviations: C- control group.

4.5. SIRT1 modulates glucose catabolism in mouse TM4 Sertoli cells

Glucose catabolism is essential for spermatogenesis, with this hexose being the primary substrate for the production of lactate through glycolysis. In order to determine if SIRT1 can modulate glucose metabolism in mSCs, we evaluated the secretion or consumption of different metabolites that are intimately related to the glycolytic pathway.

In our experimental conditions, SCs from the control group consumed $2.2\times10^{3}\pm8.6\times10^{2}$ nmol/10^{6} cells of glucose. When we exposed these cells to the SIRT1 inhibitor, we observed that glucose consumption was profoundly altered (Figure 10, Panel A), increasing particularly when at the concentrations of 1 nM, 10 nM and 1000 nM ($1.7\times10^{4}\pm2.8\times10^{3}$-fold variation to control, $1.9\times10^{4}\pm3.8\times10^{3}$-fold variation to control and $1.8\times10^{4}\pm3.5\times10^{3}$-fold variation to control, respectively).

The exposure to the SIRT1 activator YK-3-237 also results in altered consumption of glucose by the TM4 Sertoli cells. For concentrations above 1 nM, the uptake of glucose form the extracellular medium is increased to $2.8\times10^{4}\pm2.7\times10^{3}$ – fold variation to control, $1.7\times10^{4}\pm4.3\times10^{3}$ -fold variation to control, $2.9\times10^{4}\pm8.3\times10^{3}$ – fold variation to control, when cells were exposed to concentrations of 10 nM, 1000 nM and 10000 nM of YK-3-237, respectively.

The end product of glycolysis is pyruvate that can follow three main metabolic routes: it can be converted to alanine, reduced to lactate by LDH or be converted to acetyl-coA and used in the Krebs cycle. SCs from the control group produced 5.2×10^{1} nmol/10^{6} cells of pyruvate. When cells were exposed to sirtuin

inhibitor or activator, pyruvate start to be consumed. In one hand, when we exposed these cells to SIRT1 inhibitor, pyruvate consumption was altered (Figure 10, Panel C),with an increase to $3.2\times10^{2}\pm8.2\times10^{1}$- fold variation to control, $5.1\times10^{2}\pm7.6\times10^{1}$- fold variation to control, $2.0\times10^{2}\pm6.1\times10^{1}$-fold variation to control, $3.5\times10^{2}\pm9.7\times10^{1}$-fold variation to control, $3.8\times10^{2}\pm1.2\times10^{1}$- fold variation to control, in concentrations of 1 nM, 10nM, 100 nM, 1000 nM and 10000 nM, respectively. On the other hand, when cells were exposed to YK-3-237, the pyruvate consumption highly increased at 10 nM ($3.9\times10^{2}\pm9.7\times10^{1}$- fold variation to control) and even more at 10000 nM ($9.9\times10^{2}\pm2.2\times10^{1}$- fold variation to control) (Figure 10, Panel D).

Glutamine is an amino acid that can be converted to glutamate and posteriorly to α-ketoglutarate, a substrate that is require in Krebs cycle. Again, as showed on the previous metabolites, glutamine consumption was highly altered when exposed to sirtuin inhibitor and activator. Our results showed, that SCs from the control group consumed $4.5\times10^{2}\pm1.2\times10^{1}$ nmol/ 10^{6} cells of glutamine. When SCs were exposed to sirtuin inhibitor (Figure 10, Panel E), glutamine consumption increased at all concentrations tested ($1.7\times10^{3}\pm1.2\times10^{2}$- fold variation to control, $1.7\times10^{3}\pm1.1\times10^{2}$- fold variation to control, $1.1\times10^{3}\pm1.5\times10^{2}$- fold variation to control, $1.4\times10^{3}\pm3.7\times10^{2}$- fold variation to control and $1.4\times10^{4}\pm1.8\times10^{2}$- fold variation to control, 1 nM, 10 nM, 100 nM, 1000 nM and 10000 nM, respectively).

When SCs were exposed to YK-3-237, the increasing consumption was also noticed as in sirtuin activator. Nonetheless, at concentrations of 10 nM, 1000 nM and 10000 nM the consumption were incredible increasing in comparison to the control group ($2.5\times10^{3}\pm3.0\times10^{2}$- fold variation to control, $1.97\times10^{3}\pm3.4\times10^{2}$ and $3.6\times10^{3}\pm5.1\times10^{2}$- fold variation to control, respectively).

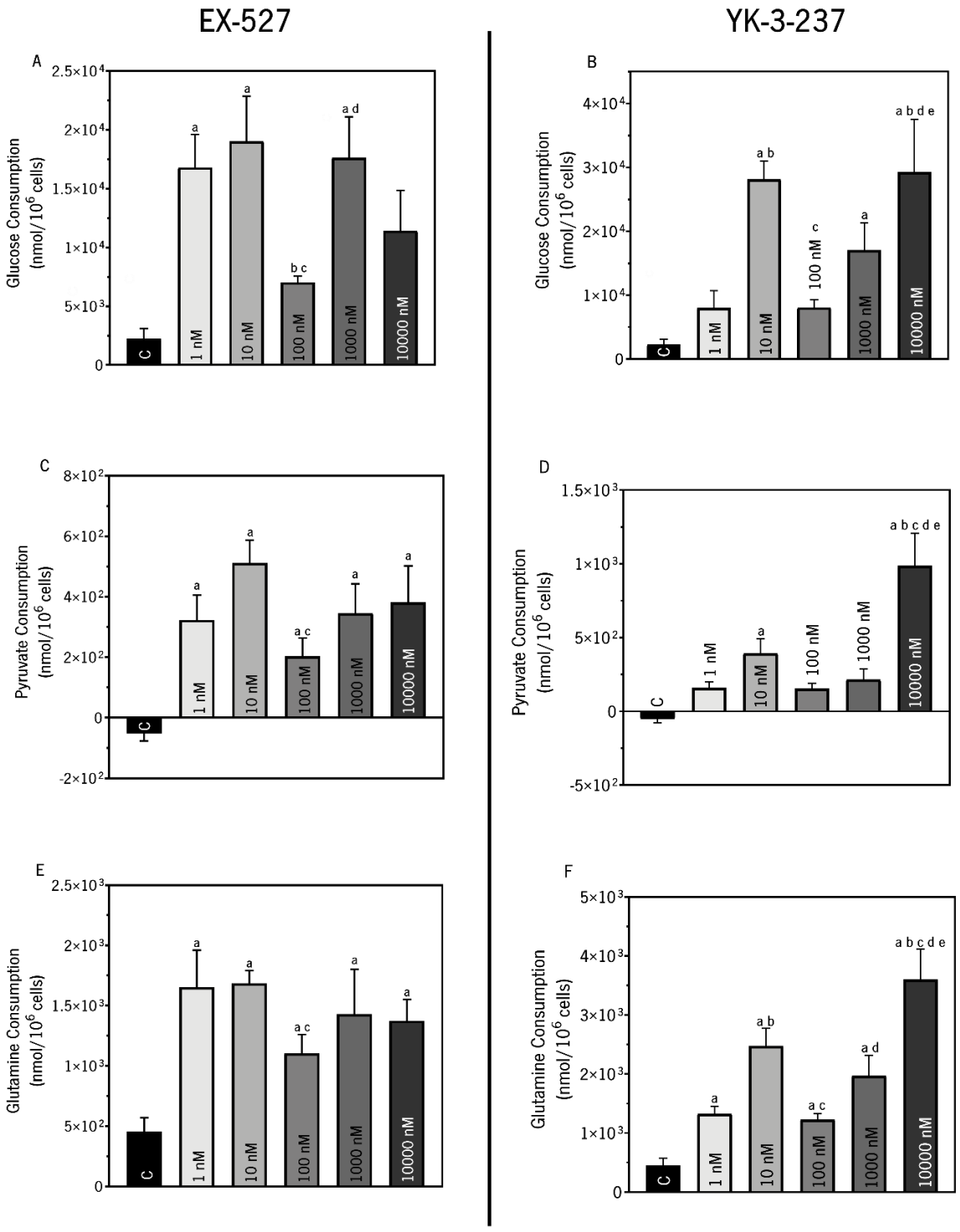

Figure 10. Evaluation of glucose (Panels A and B), pyruvate (Panels C and D) and glutamine (Panels E and F) consumption on TM4 Sertoli cells after exposure to sirtuin inhibitor (EX-527) (Panels A, C and E) and activator (YK-3-237)(Panels B, D and F). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a- relative to control group, b- relative to 1 nM, c- relative to 10 nM, d– relative to 100 nM, e– relative to 1000 nM. Abbreviations: C- Control group.

4.6. Exposure to the highest concentrations of SIRT1 inhibitor EX-527 and activator promoted an increased secretion of alanine and lactate

In virtue of determining if SIRT1 can exert effect on the production of different metabolites, alanine, lactate and acetate production were analyzed, to acquire the information of the direction of the glycolytic flow. Pyruvate, in the presence of L-glutamate, can be converted into L-alanine or in α-ketoglutarate. In our experimental conditions, SCs from the control group produced $7.0\times10^{2}\pm1.2\times10^{1}$ nmol/10^{6} cells of alanine (Figure 11, Panel A). When SCs were exposed to SIRT1 inhibitor, there were no statistical differences, when compared to the control group. Surprisingly, when SCs were exposed to the highest concentration of YK-3-237, alanine production was noticeable increased ($1.7\times10^{3}\pm3.4\times10^{2}$- fold variation to control) (Figure 11, Panel B).

Lactate is considered an important fuel in SCs environment and it results from the pyruvate reduction by LDH. When SCs were exposed to SIRT1 inhibitor no differences were showed, when comparing to the control group ($2.8\times10^{4}\pm4.1\times10^{3}$ nmol/10^{6} cells). However, the highest lactate production was observed at 10000 nM (Figure 11, Panel C). Likewise, when SCs were exposed to sirtuin activator, only at 10000 nM ($4.4\times10^{4}\pm7.7\times10^{3}$- fold variation to control) the lactate production incredibly increased (Figure 11, Panel D).

Pyruvate, despite entering the mitochondria and be converted to acetyl-CoA, it can be converted into acetate. In our experimental conditions, acetate did not suffer any substantial changes, over $2.2\times10^{2}\pm3.8\times10^{1}$ nmol/10^{6} cells produced from the control group. Nevertheless, when SCs were exposed to SIRT1 inhibitor, acetate was little produced at 1000 nM ($6.0\times10^{1}\pm1.1\times10^{1}$- fold variation to control) (Figure 11, Panel E). The exposure of SCs to YK-3-237, acetate was highly produced at 1nM. With increasing concentrations, no alterations were observed, only a decreased of acetate production at 100 nM (Figure 11, Panel F).

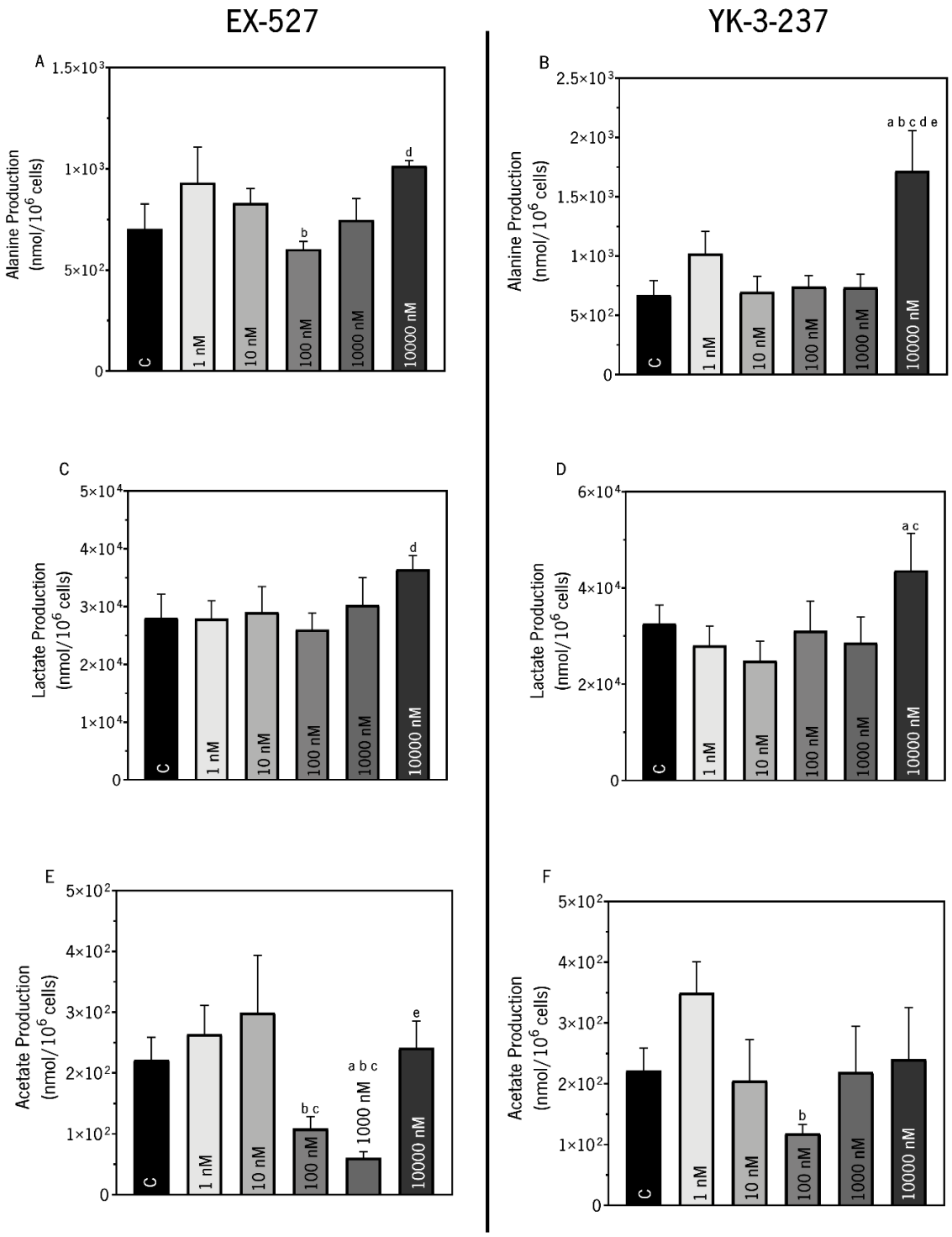

Figure 11. Evaluation of alanine (Panels A and B), lactate (Panels C and D) and acetate (Panels E and F) on TM4 Sertoli cells after exposure of SIRT1 inhibitor (EX-527) (Panels A, C and E) or activator (YK-3-237) (Panels B, D and F). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a- relative to control group, b– relative to 1 nM, c- relative to 10 nM, d– relative to 100 nM, e– relative to 1000 nM. Abbreviations: C- Control group.

4.7. Exposure to SIRT1 activator YK-3-237 or Inhibitor EX-527 did not alter glycogen content on TM4 Sertoli cells

Glycogen stores, when glucose is not available, can be considered as a fuel for a glycolytic pathway. Indeed, glycogen can be converted in glucose-1-phosphate, and subsequentially transformed in glucose-6-phosphate, which in turns proceeds into the glycolytic pathway resulting in pyruvate as an end-product. Glycogen has been described as a possible fuel for SCs metabolism; however, little is known about its specific role.

Our results did not show statistical differences among the intracellular glycogen content of SCs from the various experimental groups, although we can see a slight tendency to a decrease in the glycogen content in cells exposed to rising concentration of the SIRT1 inhibitor EX-527 (Figure 12, Panel A), while in those exposed to the SIRT1 activator YK-3-237 the tendency is to increase, nonetheless not so clear (Figure 12, Panel B).

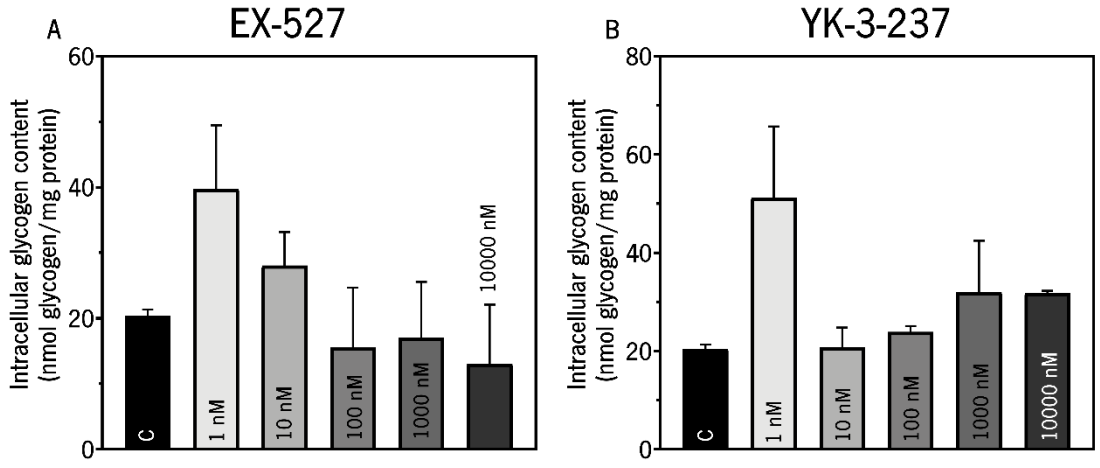

Figure 12. Evaluation of the intracellular glycogen content (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor EX-527 (Panel A) and SIRT1 activator YK-3-237 (Panel B). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=3 for each condition). Significantly different results ($P< 0.05$) are indicated. Abbreviations: C- Control group.

4.8. Exposure of TM4 Sertoli cells to both the SIRT1 inhibitor EX-527 or activator YK-3-237 decreases lipid accumulation in Sertoli cells

To determine the influence of SIRT1 inhibitor EX-527 and activator YK-3-237 on the accumulation of cytoplasmic lipid droplets in TM4 Sertoli cells, ORO staining assay was performed. Overall, both the

exposure to SIRT1 inhibitor EX-527 and activator YK-3-237 led to a decrease on intracellular lipid accumulation. The highest decrease was observed when SCs were exposed to EX-527 (0.60±0.05-fold variation to control and 0.79±0.03-fold variation to control, 100 nM and 10000 nM, respectively) (Figure 13, Panel A), with those exposed to YK-3-237 presenting also significant decrease on the amount of lipid droplets (0.64±0.02-fold variation to control and 0.81±0.05-fold variation to control, 100 nM and 10000 nM, respectively) (Figure 13, Panel B).

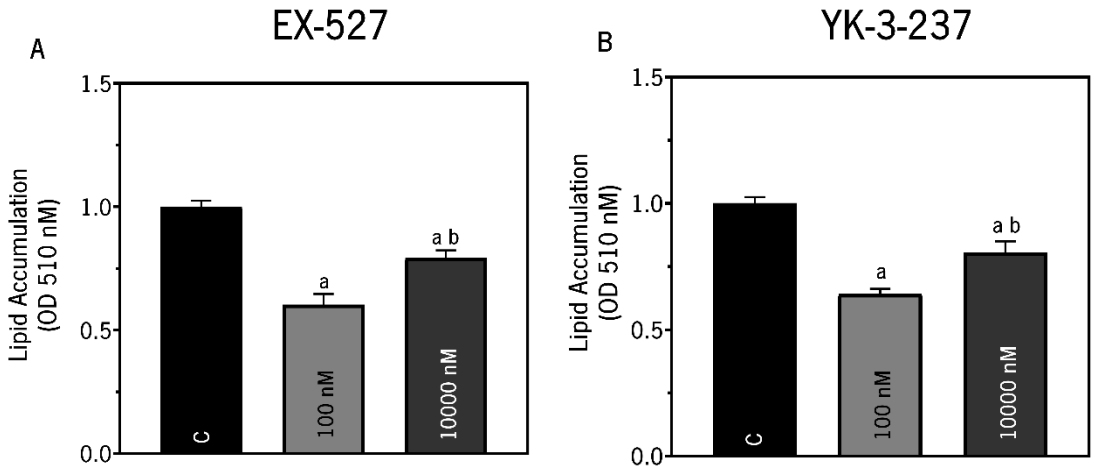

Figure 13. Evaluation of lipid accumulation (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor EX-527 (Panel A) and SIRT1 activator YK-3-237 (Panel B). The results show pooled data of independent experiments and are expressed as mean ± SEM (n=3 for each condition). Significantly different results ($P< 0.05$) are indicated as: a - relative to control group and b – relative to 100 nM. Abbreviations: C- Control group.

4.9. Exposure to the SIRT1 activator YK-3-237 increases the expression of mitochondrial complexes in mouse Sertoli cells

Mitochondria is an organelle essential for ATP production via oxidative phosphorylation (OXPHOS). OXPHOS involves four multi-subunit complexes (I-IV), which are located in the tubular membranes of mitochondrial cristae, while complex V (F_1F_o-ATPase) is localized in cristae bends, driving the ATP synthesis at the expense of an electrochemical proton gradient previously generated. After exposing TM4 Sertoli cells to a selection of concentrations of both the SIRT1 inhibitor and activator, a western blot was made to evaluate the protein levels of the different mitochondrial complexes. Our results showed no significant alterations concerning the expression of the mitochondrial complexes, when cells were exposed to the SIRT1 inhibitor EX-527 (Figure 14, Panel A). Contrastingly, at a concentration of 10000 nM, the

SIRT1 activator YK-3-237 led to a higher expression of all mitochondrial complexes evaluated, with a particularly higher expression of complex V (CV) (Table 3) (Figure 14, Panel B).

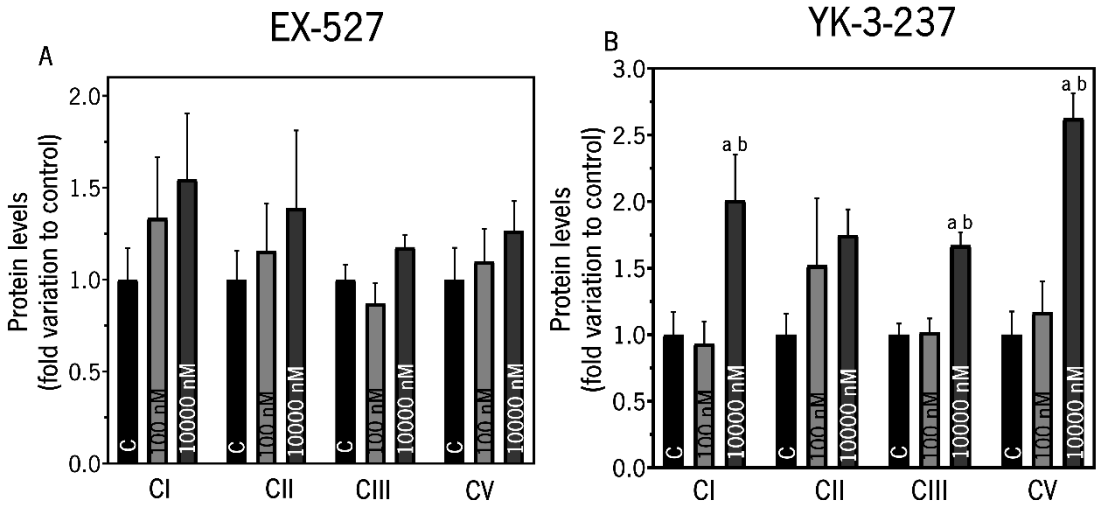

Figure 14. Evaluation of SIRT1 inhibitor EX-527 (Panel A) and SIRT1 activator YK-3-237 (Panel B) in protein expression levels of mitochondria complexes of TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating OXPHOS protein levels. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results (P< 0.05) are indicated as: a - relative to control group and b -relative to 100 nM.

Table 3. Effect of SIRT1 activator YK-3-237 in protein expression levels of mitochondrial complexes on TM4 mouse Sertoli cells from the control group and treated with 10000 nM.

Mitochondrial complexes	Control	YK-3-237 (10000 nM)
CI	1.00±0.21	2.01±0.35
CIII	1.00±0.10	1.67±0.10
CV	1.00±0.21	2.67±0.19

CI- complex I, CIII- complex III and CV- complex V.

4.10. Exposure to the SIRT1 activator YK-3-237 increases mitochondrial membrane potential in mouse TM4 Sertoli cells

JC-1 assay allows to evaluate the mitochondrial transmembrane potential status in live cells. In healthy mitochondria, JC1 forms aggregates, while in unhealthy cells, forms monomers. These two JC1 forms have different fluorescent properties and the ratio of fluorescence JC1 aggregates and JC1 monomers are used as an indicator of mitochondrial health, as it reflects mitochondrial transmembrane potential. Our results showed that when SCs were exposed to the SIRT1 inhibitor EX-527, no alterations were observed on mitochondrial transmembrane potential (Figure 15, Panel A). On the other hand, a higher JC-1 ratio was observed when TM4 Sertoli cells were exposed to YK-3-237 at the concentration of 10000 nM (1.61±0.07-fold variation to control).

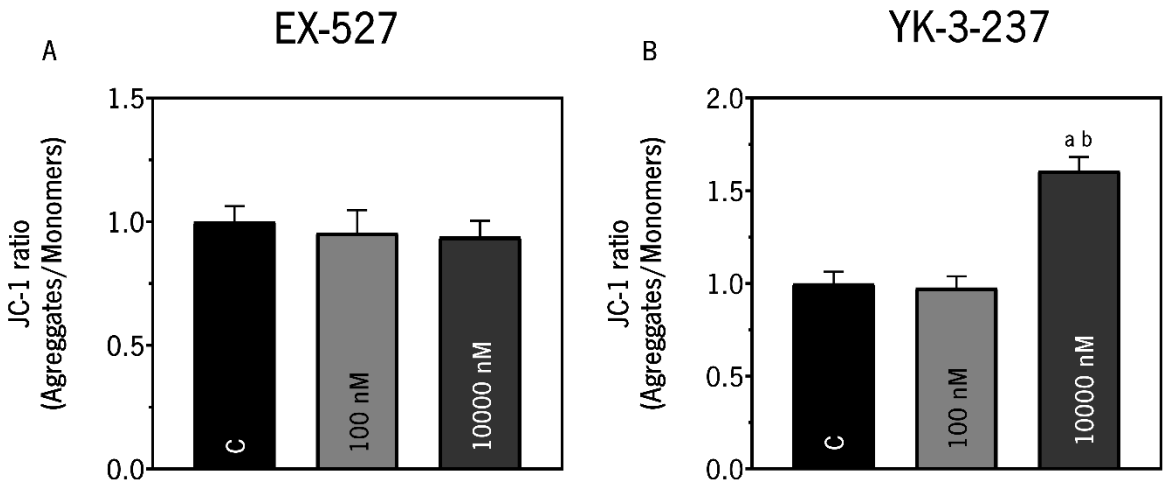

Figure 15. Evaluation of JC-1 ratio (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor EX-527 (Panel A) and SIRT1 activator YK-3-237 (Panel B) in mitochondria. The figure shows pooled data of independent experiments. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P< 0.05$) are indicated as: a - relative to control group and b -relative to 100 nM. Abbreviations: C- Control group.

4.11. Inhibition or activation of SIRT1 increases lipid peroxidation in TM4 Sertoli cells

To assess the oxidative profile of the SCs exposed to the SIRT1 inhibitor EX-527 or activator YK-3-237, we took advantage of the slot-blot technique to quantify lipid peroxidation levels after incubation with increasing concentrations of both compounds (0, 100 and 10000 nM). Exposure to the SIRT1 inhibitor

EX-527 (Figure 16, Panel A) led to an increase lipid peroxidation at concentrations of 100 nM (1.06±0.03-fold variation to control) and 10000 nM (1.07±0.03-fold variation to control). When, mSCs were exposed to YK-3-237 (Figure 16, Panel B) lipid peroxidation levels also increased at all concentrations tested (1.16±0.06-fold variation to control- 100 nM and 1.20±0.03-fold variation to control- 10000 nM).

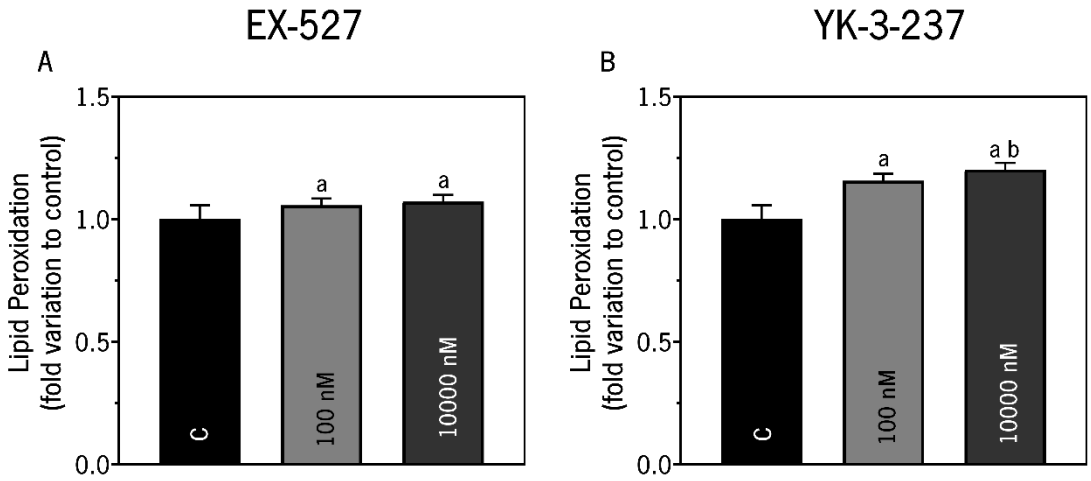

Figure 16. Evaluation of lipid peroxidation (Panels A and B) on TM4 Sertoli cells after exposure of SIRT1 inhibitor EX-527 (Panel A) and SIRT1 activator YK-3-237 (Panel B). Characterization of the lipid peroxidation profile was through 4-hydroxynonenal (4-HNE) quantification in mSCs. The figure shows pooled data of independent experiments and are expressed as mean ± SEM (n=6 for each condition). Significantly different results (P< 0.05) are indicated as: a - relative to control group and b -relative to 100 nM. Abbreviations: C- Control group.

Chapter V

DISCUSSION

5. DISCUSSION

Over the last decade, sirtuins have been considered as key players in multiple cellular events. They are ubiquitously expressed in mammalian tissues and cells and are particularly abundant in the testicular tissue. Indeed, for all SIRT genes, testis is among the adult organs where their mRNAs abundances is the highest, particularly SIRT1 expression (120). Thus, it is reasonable to hypothesize that the presence of endogenous SIRT1 has a specific function on testicular metabolism regulation. SIRT1 is mostly a nuclear protein, however sometimes it can be found in the cytoplasm. Several studies have highlighted the importance of SIRT1 on the testicular environment. It has been reported that this sirtuin controls male fertility through regulation of HPT axis, which impacts LCs and SCs maturation (143), as well as in anatomically aspects of the testes size, and on sperm quality (121).

Nonetheless, there is a gap in understanding the role of SIRT1 in the testicular context, more specifically in testicular metabolism. SCs are known to have a high glycolytic metabolism and no studies were done regarding its regulation by SIRT1 until now. So, our objective was to understand what is the role of SIRT1 on SCs glucose metabolism. The first step was to identify the presence of SIRT1 in mSCs line. We were able to identify, in mSCs and in lysate of mouse testis, the isoform with the expected molecular weight (120 kDa). We also identified multiple isoforms of SIRT1 with distinct sizes and molecular weights. Previous studies have already demonstrated the presence of several SIRT1 isoforms, however, up until now, no data is available in literature about SIRT1 pattern expression in TM4 mSCs. Alternative splicing of SIRT1 can occur during the transcription, which results in isoforms with different molecular weights, but without the catalytic site being affected. Lynch et al., identified in mouse embryonic fibroblasts and in human tissues, particularly in testis, an isoform lacking exon 8, which seems to correspond of the immunoblot band that appeared at the weight around 100 kDa (214). Another isoform well known and documented is the one corresponding to the 75 kDa fragment, which corresponds with the lack of the carboxy-terminus and appeared to be very useful in osteoarthritis. This isoform has been shown to promote chondrocyte survival after exposure to proinflammatory cytokines (215). In our experimental work, this isoform is present in both mouse testis and TM4 cells, being more abundant in testis lysate than in mSCs. Additionally, our results revealed the presence of another immunoblot band at a molecular weight around 60 kDa. This isoform is not very common. It was first described in a study where the authors were trying to enlighten the role of SIRT1 during oocyte maturation. These authors also described another SIRT1 isoform that corresponds to an immunoblot band of 65 kDa (216). Another immunoblot band, at a molecular weight around 48 kDa, appeared in our results, which may be an unknown isoform whose function remains unclear at this point. Finally, an additional isoform was seen in our results,

corresponding to an immunoblot band around 34 kDa. Regarding this isoform, there is information that it might correspond to a fragment in which both exon 2 and 9 are missing. This isoform regulates cancer-related gene expression through the modulation of p53 (217).

As we wanted to evaluate the effects of EX-527 and YK-3-27 on mSCs metabolism, firstly we evaluated the cytotoxicity of each compound (1-10000 nM). Thus, we evaluated cell proliferation through the SRB assay, and metabolic viability by the MMT assay. SIRT1 inhibitor EX-527 did not exert any prejudicial effect on cell proliferation or metabolic viability. Curiously, the higher concentration of EX-527 increased the metabolic viability of these cells. Regarding SIRT1 activator YK-3-237, only the lower concentration decreased mSCs proliferation and metabolic viability. Additionally, we also evaluated LDH release in the cells of each treated group and there was a decrease in mSCs treated with SIRT1 inhibitor EX-527, at concentrations above 1 nM. These results indicate that none of the two compounds, EX-527 and YK-3-237, at the tested concentrations are cytotoxic to mSCs.

Until now, a little is known regarding the intrinsic effect of SIRT1 in metabolism of SCs. It is well reported that SIRT1 can regulate several cellular pathways, such us stress-response pathways with the involvement of PGC-1α. SIRT1 is capable of enhancing oxidative phosphorylation and can trigger antioxidant defenses (138). Previous data from our group showed that in pre-diabetes condition, compromised testicular mitochondrial function is mediated by the PGC1- α/SIRT3 axis and consequently anchored with the decline of respiratory capacity and increase of oxidative stress (218). So, sirtuins dysregulation has direct consequences on male fertility. We hypothesized that SIRT1 modulates mitochondrial activity and oxidative stress regulatory pathway activation. Our results showed that, when mSCs were exposed to the highest concentration of SIRT1 activator, the protein levels of most mitochondrial complexes were increased in TM4 cells. In fact, the highest concentration of YK-327 was the concentration who led to the higher variations on mitochondrial complexes protein levels when comparing to the cells from the control group. The same trend was possible to observe when TM4 cells were exposed to EX-527, however, the alterations did not reach a statistical significance. These results are concordant with the results obtained in JC-1 assay. The higher mitochondrial membrane potential was observed when SCs were treated with the higher concentration of the SIRT1 activator. This data is a good indicator of mitochondrial health, making this compound an attractive solution for molecular therapeutics in the testicular environment. In agreement with this data, there is an example of a study performed in individuals with colon cancer. In this case, when individuals were subjected to chemotherapy, there was an increase activation of SIRT1/PGC-1α, that results in an increment of OXPHOS in colonosphere cultures established from primary colorectal tumors and liver metastases (219).

Nevertheless, an enhanced mitochondrial activity can lead to overproduction of ROS that can make damage all cellular macromolecules, being lipids very susceptible. Testis have a high content of polyunsaturated fatty acids (147), being more prone to lipid peroxidation and potentially interfering with the production of viable spermatozoa (220-222). The treatment with SIRT1 activator increased lipid peroxidation at all assayed concentrations, when comparing to the control group. Still, this damage caused by oxidative stress was not sufficient to translate into an increase of LDH release, which means that mSCs membrane was intact. Additionally, the cells were still metabolically viable and no alteration on cell proliferation was observed in any treated group.

Lipid peroxidation, or other any oxidative damage of cells can lead to apoptosis. Thus, we wanted to determine if the cells exposed to the SIRT1 activator or inhibitor presented an alteration in apoptotic pathways. The balance between the abundance of BAX and BCL-2 in cells may determinate the incidence of apoptosis, in which it is intimately related to the activation of the caspases (223). Our results showed that the exposure to both the SIRT1 inhibitor or activator did not cause alterations on the BAX/BCL-2 ratio in mSCs, while caspase-3 activity showed an increasing tendency, although the alterations in this parameter did not reach statistical significance in all concentrations tested. Previous studies revealed that EX-527 is a promoter of apoptosis in cancer cell lines and other types of cells. Indeed, Kumari et al., reported that sirtuin inhibition can induce apoptosis in platelets (224). Hence, SIRT1 was studied as a possible key factor for cell death control. Their results showed considerable increasing of BAX levels. Moreover, levels of acetylated p53 were also increased, however caspase-3 activity did not seem to be activated when platelet cells were treated with EX-527 at 10000 nM (224), as we also observed in TM4 cells. Hence, in the next works, it would be interesting to investigate if, in our experimental conditions, p53 levels are affected, since apoptosis in the testis is also highly dependent on the acetylation of p53 (225). Another question that would be interesting to answer is which apoptotic pathway might be preferentially adopted, i.e. whether SIRT1 has a regulatory role in the apoptosis event via the extracellular pathway or the intracellular. Yet, this last pathway in our experimental conditions did not seem preferred since the ratio of BAX/BCL-2 was not altered.

Glycolysis and lipid β-oxidation are considered the major metabolic pathways for the catabolism of glucose and lipids to cells produce energy. SCs are not an exception and they exhibit a high glycolytic profile. Lipids and glucose are considered common energy substrates. Since testicular metabolism is still not fully known, and particularly what is the input SIRT1 on the testicular metabolic events, we went to explore the metabolic fingerprint associated with the metabolism of TM4 cells of the various experimental groups. Our results showed that exposure of these cells to both the SIRT1 inhibitor or activator altered dramatically

substrate consumption. Glucose consumption was increased in the mSCs exposed to SIRT1 inhibitor or activator, and pyruvate changed from being exported to the extracellular medium to being consumed in both cases. These results point towards the enhancement of glycolysis. As pyruvate consumption was also enhanced and as this metabolite is at the crossroad of several metabolic pathways, one may suggest that it can be used to produce lactate, alanine or as substrate to the Krebs cycle. Still, no alteration was seen in the excretion of lactate or alanine by mSCs exposed to the SIRT1 activator or inhibitor, with the exception of those exposed to the highest concentrations of YK-3-237 (10000 nM). This suggests that in the cells from the other groups glucose and pyruvate is being metabolized into other end product rather than lactate or alanine.

Moreover, glutamine consumption was also increased in the mSCs of most experimental groups, particularly in the those exposed to the highest concentration of SIRT1 activator, similarly to what was observed for glucose and pyruvate consumption. Previous reports demonstrate that glutamine oxidation is enhanced in cancer cells, particularly when they are exposed to an acidic environment. As cancer cells, SCs adopted an aerobic glycolysis (also called Warburg-like effect) (26), producing and exporting large amounts of lactate/H^+ that contribute to the acidification of the extracellular environment (226). SCs have a high metabolic plasticity and a capacity to adapt themselves to alterations on metabolic availability (227, 228). Moreover, a study using a heterozygous deletion of SIRT1 on mouse embryonic fibroblasts showed an enhancement of glutamine metabolism and cancer development (229).

Hence, to analyze if the enhanced consumption of metabolites observed in the cells of most experimental groups was being used to increase energy storages, we assess both the lipid droplet in TM4 cells exposed to SIRT1 inhibitor or activator. SCs are known to accumulate lipid droplets in their cytoplasm, which can be used as energy reserve, although the real significance of these droplets is not entirely known (16, 230, 231). To determine lipid droplets content in mSCs we used the ORO assay. Our results showed that mSCs treated with SIRT1 activator or inhibitor presented a decreased lipid accumulation. These results suggest that lipid oxidation is regulated by SIRT1 in these cells. Given the decrease of lipid droplets accumulation observed under these conditions, we suggest that mSCs is also being used lipids as an energy source. Recent studies have pointed towards an important role of SIRT1 in glucose and stimulation of fatty acids via activation of PPARα and PGC1α (101). Indeed, fatty acids constitute an important source of energy during different physiological conditions and SIRT1 is involved in a correct coordination of these different energy status. In lower energy status, such as nutrient starvation, SIRT1 is activated (232), while in high fat diet there is a reduction of their expression (233). As referred above, depending on the organ/ tissue, the effects of SIRT1 in metabolism are distinct. For instance, in liver, SIRT1 activation decreases

glycolysis, as well as lipid accumulation by suppressing lipid synthesis (101, 234). In support to this observation, there are studies that demonstrate that mice lacking SIRT1 subjected to a high fat diet are more prone to develop liver steatosis (101, 235, 236), which is the increase of fat amount in the liver. Additionally, if obese mouse models and human were administered a pharmacological SIRT1 activator RSV, liver steatosis was reverted (184, 188, 237-240). Furthermore, it was detected in humans, that SIRT1 mRNA expression is directly corelated with energy expenditure, demonstrating that SIRT1 can modulate the whole body energy (241).

We also went to determine the impact of both the SIRT1 activator or inhibitor on the intracellular levels of glycogen of TM4 cells. As referred above, in liver, caloric restriction diet, shifts metabolism to glycogen breakdown in which SIRT1 is involved (242). Our results showed no statistical differences in the amounts of glycogen in cells from the different experimental groups. Still, we observed a tendency to increase in the glycogen content, particularly in the cells exposed to the SIRT1 activator. The increased glycogen reserves might be related with the increased consumption of glucose and other glyconeogenic substrates, resulting in glycogen stored. Still, we need a higher number of samples (n=6, or more) to confirm this hypothesis.

Chapter VI

CONCLUSION

6. CONCLUSION

Sirtuins are key interveners of a network that intersects different signaling pathways. They are deacetylases that are specifically localized in different cellular organelles, although sometimes they can alter their cellular localization in response to stress or other factors. Nowadays, some lifestyle habits that humankind acquired are considered unhealthy, with repercussions that can be irreparable, and obesity is one those cases. Obesity and sirtuins, specially SIRT1 are closely associated. While it is widely accepted that obesity compromises male fertility, the role of SIRT1 on male fertility is still a matter of debate, with multiple aspects to be unveiled. To clarify some of these issues, we incubated mSCs with two different compounds, a SIRT1 inhibitor (EX-527) and a SIRT1 activator (YK-3-237). No cytotoxic effects were detected, demonstrating that these compounds were not able to alter the stability and integrity of these cells.

Noteworthy, YK-3-237 in a concentration of 10000 nM, revealed to be beneficial in mitochondrial complexes activity resulting in a higher mitochondrial membrane potential. Furthermore, we determined the metabolic fingerprint of the cells when exposed to SIRT1 inhibitor and SIRT1 activator. YK-3-237 modulates glucose catabolism as well as pyruvate and glutamine consumption, allied with an enhanced of the production of key metabolites for spermatogenesis, such as lactate. Additionally, our results demonstrate that treatment with SIRT1 activator resulted in a decrease on mSCs lipid accumulation, revealing that instead of being stored, lipids are used, possibly to obtain energy. To sum up, it is possible to classify SIRT1 as a control point of testicular metabolism, which is so important for a correct spermatogenesis and hence the maintenance of male fertility.

As further perspectives, studies need to be performed to address the contribution of SIRT1 in male fertility, more specifically in concerning their role on SCs physiology and metabolism. It is particularly relevant to determine the importance of the different SIRT1 isoforms observed on mSCs. Moreover, it is also important a deepen knowledge of specific SIRT1 related targets in signaling pathways, such as the role of p53, and how these interactions are linked with the activity of this deacetylase.

Chapter VII

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7. REFERENCES

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