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Effect of *tert*-butyl hydroperoxide in testicular metabolism: a protective role for Regucalcin?

Roberta Vanessa Lopes Martins

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Orientador: Doutora Sara Carina de Lima Correia
Co-orientador: Profª. Doutora Sílvia Cristina da Cruz Marques Socorro

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Resumo

A espermatogénese é o processo biológico complexo que transforma as células estaminais espermatogoniais em espermatozoides, sendo, por esse motivo, a base da fertilidade masculina. Este processo ocorre nos túbulos seminíferos (SeT) e é altamente dependente da cooperação metabólica estabelecida entre as células somáticas, as células de Sertoli (SCs), e as células germinativas. As SCs são conhecidas por serem as reservas metabólicas que fornecem as quantidades adequadas de substratos energéticos às células germinativas. Apesar de consumirem diversos substratos, incluindo aminoácidos como a glutamina, as SCs metabolizam preferencialmente glucose produzindo lactato, sendo este último o substrato de eleição das células germinativas. É amplamente reconhecido que o stresse oxidativo (OS) afeta adversamente a espermatogénese, desregulando o desenvolvimento das células germinativas, e interferindo com a função espermática, assim como perturbando o metabolismo celular. Sabe-se que o hidroperóxido de *tert*-butilo (TBHP) é um indutor de OS ao nível testicular. Para além disso, foi sugerida uma ação metabólica para este composto, tendo sido identificado como um regulador da via das pentoses-fosfato. No entanto, o impacto do TBHP no metabolismo testicular permanece desconhecido.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que tem vindo a ser associada ao controlo da proliferação celular, apoptose, OS e metabolismo. Recentemente, o nosso grupo de investigação demonstrou que a sobre-expressão de RGN modula a metabolização da glucose e da glutamina nas SCs através da regulação da expressão de vários transportadores e enzimas envolvidos na glicólise e na glutaminólise. Além disso, o papel protetor da RGN para a população de células germinativas quando expostas a fatores nocivos tem vindo a ser sugerido. Desta forma, é sensato levantar a hipótese que a RGN possa ter um comportamento semelhante contra as ações desreguladoras do TBHP no metabolismo testicular.

Na presente dissertação foi avaliado o impacto do TBHP no metabolismo testicular da glucose e da glutamina, bem como a influência da RGN na atenuação dos seus efeitos. Para tal, procedeu-se ao isolamento de SeT de ratos transgénicos que sobre-expressam a RGN (Tg-RGN) adultos e dos seus homólogos selvagens (Wt), os quais foram mantidos em cultura durante 24 horas na presença ou ausência de TBHP (250 µM). Posteriormente, a expressão e a atividade de diversos reguladores do metabolismo glicolítico e da glutaminólise foram analisados.

Os resultados obtidos mostraram um aumento no conteúdo intracelular de glucose e de lactato nos SeT quer dos animais Wt quer dos animais Tg-RGN tratados com TBHP. O perfil glicolítico alterado em resposta ao TBHP foi sustentado pela expressão alterada de transportadores de glucose nos animais Wt e Tg-RGN, e pelo aumento da atividade da lactato desidrogenase nos ratos Tg-RGN. Além disso, o TBHP alterou a expressão de alanina transferase e a glutaminólise, embora os efeitos tenham diferido entre os animais Wt e Tg-

RGN. Por sua vez, a sobre-expressão da RGN suprimiu o metabolismo glicolítico nos SeT, independentemente da exposição ao TBHP.
O presente estudo é a primeira evidência que o TBHP é um potente desregulador metabólico testicular. Para além disso, a ação da RGN foi apontada como um possível mecanismo protetor contra os efeitos nocivos do TBHP, enfatizando o papel da RGN como um regulador metabólico na espermatogénese, o que poderá ter importância no contexto da (in)fertilidade masculina. Por fim, as observações aqui alcançadas servem de suporte a trabalho de investigação futuro para clarificar profundamente a relação entre OS, alterações metabólicas e as ações da RGN nos SeT.

Palavras-chave:

Espermatogénese, Glucose, Glutamina, Metabolismo, Regucalcina, Hidroperóxido de *Tert*-Butilo, Túbulos Seminíferos, Stress Oxidativo

Resumo alargado

A espermatogénese é o processo pelo qual ocorre a formação dos espermatozoides. É um processo altamente complexo que se inicia com a diferenciação das células estaminais espermatogoniais. Este processo compreende três fases principais: mitose, meiose e espermiogénese. Durante a fase de proliferação as células estaminais espermatogoniais sofrem uma serie de divisões mitóticas mantendo os níveis de células estaminais espermatogoniais constante ou diferenciando-se em espermatócitos primários. São estes que iniciam a primeira divisão meiótica originando espermatócitos secundários que por sua vez sofrem a segunda divisão meiótica originando espermátides. Durante a espermiogénese ocorre um rearranjo da estrutura celular e a reorganização do citoplasma diferenciando as espermátides em espermatozoides. A fertilidade masculina fundamenta-se numa espermatogénese bem-sucedida, a qual necessário é dependente do suporte das células de Sertoli (SCs)s as células somáticas presentes nos túbulos seminíferos (SeT). AS Scs são responsáveis pela formação da barreira hemato-testicular (BTB) assim como por um conjunto de funções de suporte físico e bioquímico que mantém a linha germinativa. As SCs fornecem às células germinativas as quantidades adequadas de substratos energéticos. Apesar de consumirem diversos substratos, incluindo aminoácidos como a glutamina, as SCs metabolizam preferencialmente glucose produzindo lactato, sendo este último o substrato de eleição das células germinativas. Assim as SCs captam glucose através de transportadores de glucose (GLUTs) e metabolizam-na pela via glicolítica cuja velocidade depende da enzima fosfofrutoquinase 1 (PFK1). No final desta via o piruvato produzido pode ser convertido em lactato por ação da enzima lactato desidrogenase (LDH), ou em alanina por ação da enzima alanina aminotransferase (ALT). O lactato produzido é então exportado para o espaço extracelular pela ação de transportadores de monocarboxilato (MCTs) podendo finalmente ser captado e usado pelas células germinativas. Além da metabolização de glucose as SCs também podem metabolizar aminoácidos como a glutamina. Para isso a glutamina é captada do espaço extracelular pelo transportador de aminoácidos ASC tipo 2 (ASCT2). Após a sua entrada na célula dirige-se a mitocôndria onde é metabolizada pela glutaminase (GLS) podendo os produtos desta reação ter diversos destinos sendo uma delas a síntese de glutationa (GSH) uma enzima presente na regulação dos níveis de espécies reativas de oxigénio (ROS).

É amplamente reconhecido que o stresse oxidativo (OS) afeta adversamente a espermatogénese, desregulando o desenvolvimento das células germinativas, e interferindo com a função espermática, assim como perturbando o metabolismo celular. O hidroperóxido de *tert*-butilo (TBHP) é um hidroperóxido orgânico que induz peroxidação lipídica, alteração da homeostase do cálcio (Ca^{2+}) intracelular, e entre outros o aumento das ROS quando colocado em contacto com as células. A sua metabolização no interior das células é feita por

ação do citocromo P450 ou pela GSH. Sabe-se que o TBHP é um indutor de OS ao nível testicular. Para além disso, foi sugerida uma ação metabólica para este composto, tendo o TBHP sido foi identificado como um regulador da via das pentoses-fosfato. No entanto, o impacto do TBHP no metabolismo testicular permanece desconhecido. A regucalcina (RGN) é uma proteína de ligação ao Ca^{2+} que tem vindo a ser associada ao controlo da proliferação celular, OS, apoptose, OS e metabolismo. Recentemente, o nosso grupo de investigação demonstrou que a sobre-expressão de RGN modula a metabolização da glucose e da glutamina nas SCs através da regulação da expressão de vários transportadores e enzimas envolvidos na glicólise e na glutaminólise. Além disso, o papel protetor da RGN para a população de células germinativas quando expostas a fatores nocivos tem vindo a ser sugerido. Desta forma, é sensato colocar a hipótese que a RGN possa ter um comportamento semelhante contra as ações desreguladoras do TBHP no metabolismo testicular.

Na presente dissertação foi avaliado o impacto do TBHP no metabolismo testicular da glucose e da glutamina, bem como a influência da RGN na atenuação dos seus efeitos. Foram recolhidos testículos de ratos transgénicos que sobre-expressam a RGN (Tg-RGN) e dos seus homólogos selvagens (Wt) com aproximadamente três meses de idade, e procedeu-se ao isolamento de SeT. Os SeT foram mantidos em cultura durante 24 horas na presença ou ausência de TBHP (250 μM). Posteriormente, a expressão e a atividade de diversos reguladores do metabolismo glicolítico e da glutaminólise foram analisados.

Os resultados obtidos mostraram um aumento ao nível do conteúdo intracelular de glucose e de lactato nos SeT quer dos animais Wt quer dos animais Tg-RGN tratados com TBHP. O perfil glicolítico alterado em resposta ao TBHP foi suportado pela expressão alterada de transportadores de glucose em ambos os animais Wt e Tg-RGN e pelo aumento da atividade enzimática da lactato desidrogenase (LDH) nos ratos Tg-RGN. Além disso, o TBHP alterou a expressão de alanina transferase (ALT) e a glutaminólise, embora os efeitos tenham diferido entre os animais Wt e Tg-RGN. Por sua vez, a sobre-expressão da RGN suprimiu o metabolismo glicolítico, independentemente da exposição ao TBHP.

Pela primeira vez foi descrito o TBHP como um potente disruptor do metabolismo testicular

O presente estudo é a primeira evidência que o TBHP é um potente desregulador do metabolismo testicular. Para além disso, a RGN foi apontada como um possível mecanismo protetor contra os efeitos nocivos do TBHP, enfatizando o papel da RGN como um regulador metabólico na espermatogénese, o que poderá ter importância no contexto da (in)fertilidade masculina.

Abstract

Spermatogenesis is the complex biological process that transforms spermatogonial stem cells into spermatozoa, and, thus, is the basis of male fertility. It takes place in the seminiferous tubules (SeT) and is highly dependent on the metabolic cooperation established between the somatic Sertoli cells (SCs) and germ cells. SCs are known as the metabolic stores supplying germ cells with adequate amounts of energy substrates. Despite consuming several subtracts, including amino acids like glutamine, SCs prioritize the metabolization of glucose with the production of lactate, the preferred substrate of germ cells. It is widely known that oxidative stress (OS) adversely affects spermatogenesis, disrupting the development of germ cells, and interfering with sperm function, as well as disturbing cell metabolism. Tert-Butyl hydroperoxide (TBHP) is a well-known OS inducer in the testis. Also, TBHP metabolic action have been suggested. It was identified as a regulator of the pentose phosphate pathway. However, the impact of TBHP in testicular metabolism remains unknown.

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that has been associated with the control of cell proliferation, apoptosis, OS and metabolism. Recently, our research group have demonstrated that RGN overexpression modulates glucose and glutamine handling by SCs by regulating the expression of several transporters and enzymes involved in glycolysis and glutaminolysis. Furthermore, the protective role of RGN for the germ cell population upon exposure to damaging factors has been suggested. So, is tis liable to hypothesize that RGN may have a similar behavior against TBHP actions disrupting testicular metabolism.

In the present dissertation, the impact of TBHP on testicular glucose and glutamine metabolism and the influence of RGN in attenuating its effects were evaluated. Isolated SeT from adult wild-type (Wt) and transgenic rats overexpressing regucalcin (Tg-RGN) were maintained in culture for 24 hours in the presence or absence of TBHP (250 μM). After that, the expression and activity of several regulators of glycolytic metabolism and glutaminolysis were analysed.

The results obtained showed an increase in the intracellular content of glucose and lactate in the SeT of both Wt and Tg-RGN animals treated with TBHP. The altered glycolytic profile in response to TBHP was underpinned by the altered expression of glucose transporters in both Wt and Tg-RGN, and increased lactate dehydrogenase activity in the Tg-RGN rats. Moreover, TBHP altered alanine transferase expression and glutaminolysis though the effects differed between Wt and Tg-RGN animals. In turn, RGN overexpression suppressed the glycolytic metabolism in SeT, regardless of TBHP treatment.

The present study is the first evidence that TBHP is a potent testicular metabolic disruptor. Furthermore, RGN action was identified as a possible protective mechanism against the damaging effects of TBHP. These findings also emphasize the role of RGN as a metabolic regulator in spermatogenesis, which could have importance in the context of male

(in)fertility. Finally, the outcomes achieved herein support further research work to deep clarify the relationship between OS, metabolic alterations and the RGN actions in the SeT.

Keywords

Glucose, Glutamine, Metabolism, Oxidative Stress, Regucalcin, Tert-Butyl Hydroperoxide, Seminiferous Tubules, Spermatogenesis

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ALT	Alanine transaminase
AMH	Anti-Mullerian hormone
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
BSA	Bovine serum albumin
BTB	Blood-testis-barrier
Ca^{2+}	Calcium
CAT	Catalase
DHT	5α-dihydrotestosterone
E_2	17β-estradiol
ERs	Estrogen receptors
F1,6-BP	Fructose-1,6-bisphosphate
F6-P	Fructose-6-phophate
FSH	Follicle stimulating hormone
G6-P	Glucose-6-phosphate
GLS	Glutaminase
GLUTs	Specific glucose transporters
GnRH	Gonadotropin-releasing hormone
GOD	Glucose oxidase
GPER	Membrane G-protein coupled estrogen receptor
GPT	Glutamate pyruvate transaminase
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
LCs	Leydig cells
LDH	lactate dehydrogenase
LH	Luteinizing hormone
LO	Lactate Oxidase
MCTs	Monocarboxylate transporters
OS	Oxidative stress
PFK1	Phosphofrutokinase-1
POD	Peroxidase

PVDF	Polyvinylidene difluoride
RGN	Regucalcin
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SCs	Sertoli cells
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SeT	Seminiferous tubules
SOD	Superoxide dismutase
SRY	Sex-determining region Y
T	Testosterone
T3	Triiodothyronine
TBHP	Tert-butyl hydroperoxide
TCA	Citric acid cycle
TIGAR	TP53-induced glycolysis and apoptosis regulator
WB	Western blot
Wt	Wild-type

I. Introduction

1. Brief overview of testicular and epididymal anatomy

The testicles are two oval glandular organs situated in the scrotum, a fibromuscular structure similar to a sac composed by two layers, one of skin and other of dartos muscle [1, 2]. They are suspended by the spermatic cord [1-3]. Each testis is surrounded by the *tunica albuginea*, a dense fibrous capsule that in turn is involved by the *tunica vaginalis*. Connective tissue septa divide the organ into lobules that hold the seminiferous tubules (SeT, Figure I.1) [4]

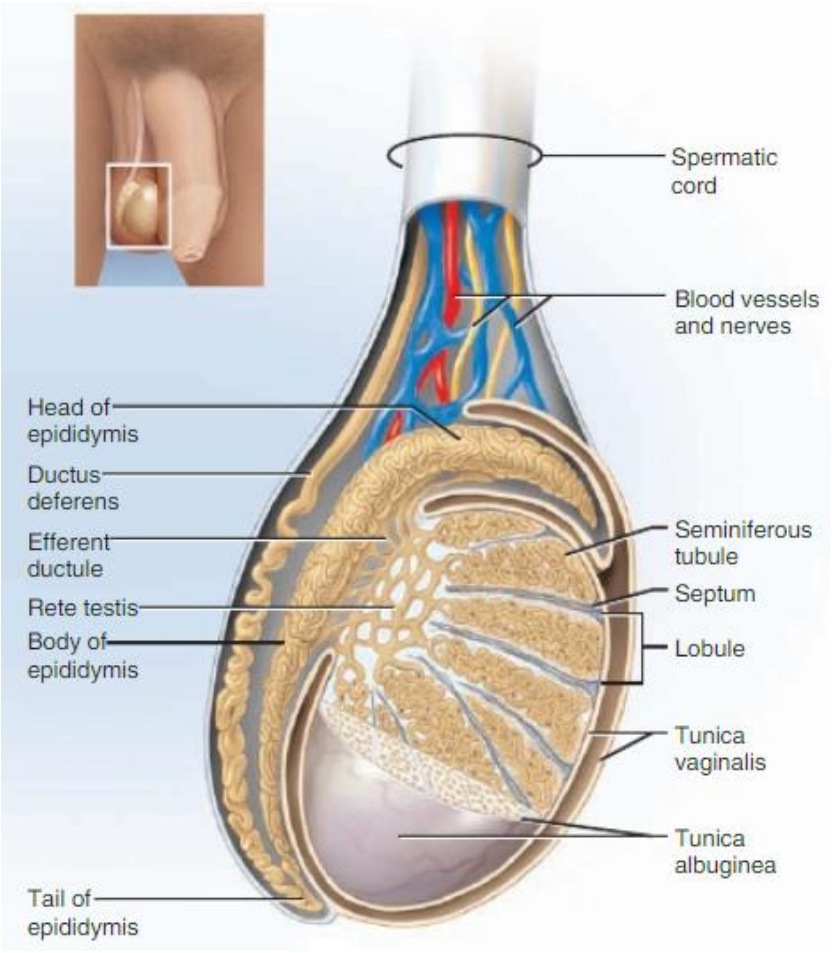

Figure I.1 Schematic representation of the mammalian testis, epididymis, and spermatic cord. Testicles are suspended by the spermatic cord and involved by the scrotum, a structure similar to a bag, which internally is composed by the *tunica vaginalis* and *tunica albuginea*. Various septa extending from the *tunica albuginea* divide the testis in lobules containing the seminiferous tubules. The seminiferous tubules converge to the *rete testis* that is connected to the efferent ductules, which, in turn, are connected to the head of the epididymis. The three major regions of the epididymis are represented (head, body and tail), followed by the *ductus deferens* (adapted from [4]).

The exterior of the SeT is surrounded by the mesenchymal cells that include the peritubular cells. These cells are contractile elements producing peristaltic waves across the SeT [5, 6]. The interstitium also contains blood, lymphatic vessels and various cell types, including fibroblasts, leukocytes, macrophages and endocrine cells, the Leydig cells (LCs), which are responsible for testosterone (T) production [7, 8]. During fetal development, LCs are the somatic cells that support testis cord assembly [9], and play an important role in the scrotal descent of the testis [10]. Adult LCs are involved in the regulation of spermatogenesis [11].

The SeT are constituted by the lamina propria and a germinal epithelium [12-15]. The interior of the SeT contains the germ cells, that form several concentric layers that are penetrated by a single somatic cell type, the Sertoli cells (SCs) [16].

SCs are columnar cells that extend from the base of the SeT until the lumen (Figure I.2) conferring germ cells and SeT structural support and organization [17]. SCs are also responsible for the creation of the blood-testis-barrier (BTB) by forming tight, adherens- and gap-junctions with each other, though the tight junctions are the major component of BTB [18]. The BTB divides the seminiferous epithelium into two compartments, the basal and the adluminal (Figure I.2) [19]. Also, it provides an immunoprivileged microenvironment that prevents the passage of small molecules and the detection of germ cells antigens avoiding an inflammatory immune response [20, 21].

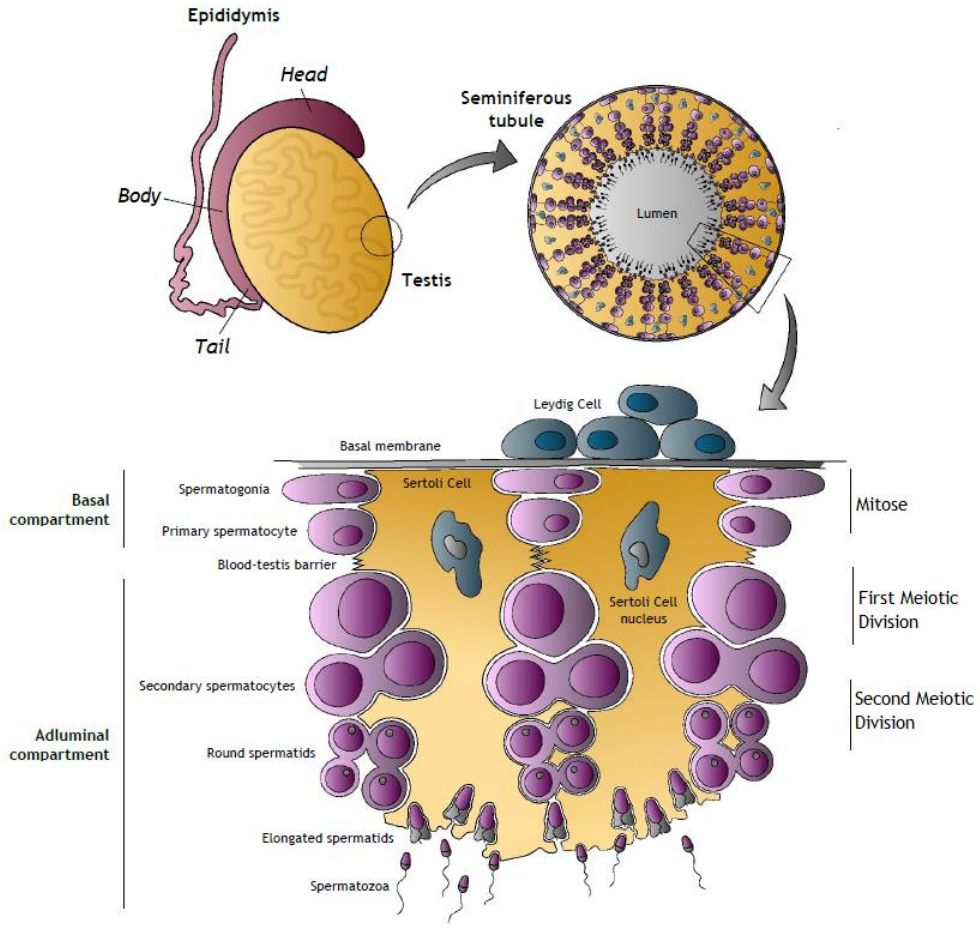

Figure I.2 Schematic representation of the testicular histology and spermatogenic process. Spermatogenesis occurs inside of the seminiferous tubules involving an intimal relationship between the different cell types within the tubules. The relationship between Sertoli cells and germ cells is crucial for a successful spermatogenesis. Firstly, germ cells suffer mitotic divisions or spermatocytogenesis, maintaining the number of stem cells constant. Then, primary spermatocytes suffer the first meiotic division originating secondary spermatocytes. The second meiosis produces the haploid cells, spermatids. Afterwards, spermiogenesis leads to generation of spermatozoa and its release in the lumen (adapted from [22]).

Most importantly the activity of SCs and their biochemical support are crucial for the development and maturation of the germ cells SCs actions and their communication with germ cells will be detailed in a separate topic during spermatogenesis [17]. The terminal ends of the SeT are connected with the *rete testis* trough the *tubuli recti* which in turn are linked to the efferent ductules [23, 24]. Efferent ductules are the link between the SeT and the epididymis (Figure I.1). The main role of the efferent ductules is the fluid reabsorption and the deliver of SeT secretions to the epididymis [23, 24].

The epididymis is an highly compartmentalized organ that can be divide into *caput* (head), *corpus* (body) and *cauda* (tail) (Figure I.1). Each region has been shown to have a different function concerning sperm maturation and its fertilizing capacity [25-27]. However, has been assumed that the *caput* and *corpus* regions perform early and late sperm maturation events, respectively, while the *cauda* mainly stores the functionally mature spermatozoa [27]

2. Testicular physiology: an overview of spermatogenesis

The mammalian testis are responsible for the very crucial process for male fertility, the spermatogenesis, the set of events that culminates with the production of male gametes [28]. Thus, testis activity results into two types of products: i) the exocrine products, such as testicular fluid and spermatozoa; and the ii) endocrine products, such as sex steroid hormones.

a. A look into the cellular basis of spermatogenic process

Spermatogenesis is a complex, dynamic and continuous process that occurs in the SeT, the functional unit of the testis. In this process, a spermatogonial stem cell suffers a process of differentiation and maturation resulting in an exceptional number of mature male gametes, the spermatozoa [29]. In men, this process takes about 64 days to occur, and is followed by a period of 8-17 days in transit throughout the epididymis [30].

Before spermatogenesis starts, which occurs at puberty, there is a long period of preparation during the fetus and infant phase. Throughout embryogenesis the primordial germ cells migrate to the genital ridge and become surrounded by primordial SCs forming the gonocytes that are kept in G0/G1 phase until the maturation of the germinative germ cells, an event that occurs only after birth [31, 32].

There are three different types of spermatogonial cells: type-A, intermediate and type-B [28]. Type A spermatogonia are undifferentiated and serve as a lifetime supply of germ cells. Type B spermatogonia is a daughter cell from a mitotic division of type A spermatogonia, after they reached a fixed number of mitotic divisions. Type B spermatogonia are committed to differentiate and start migrating into the SeT, enlarge and become primary spermatocytes continuing the spermatogenesis process [4, 28].

Spermatogenesis includes three different sequential processes until the production of spermatozoa (Figure I.2): i) mitotic divisions of spermatogonia (spermatocytogenesis) to maintain constant the population of stem cells, and then differentiation of type B spermatogonia into primary spermatocyte; ii) meiosis, which allows the exchange of genetic material between homologous chromosomes of primary spermatocyte producing the secondary spermatocyte after the first meiotic division; the second meiotic division produces the haploid cells, spermatids; iii) spermiogenesis or the differentiation that transforms round spermatids into elongated spermatids, and, then, the elongated spermatids into spermatozoa, which are finally released in the lumen of the SeT (spermiation) [28, 33-35]. Spermiation is a time-consuming and complex process occurring over several days. During spermiation the mature spermatids are released into the SeT prior to their passage to the epididymis. This process is critical because determines the number of sperm entering the epididymis, and thus the content of sperm present in the ejaculated [36].

As already mentioned, spermatogenesis is highly dependent on the SCs function. These cells provide the necessary nutritional and physical support for the germ cells, producing the seminiferous fluid, and also, having a role in spermiation [37, 38].
The endocrine cells in the exterior of SeT, the LCs, also have an important role in spermatogenesis. The T produced by the LCs has a big impact in this process regulating the spermatogenic output together with follicle stimulating hormone (FSH) [39]. T is required for spermatogenesis because of its role regulating several processes, namely, the maintenance of the BTB, meiosis, the Sertoli–spermatid adhesion and sperm release [40].
At the end of spermatogenic process, the spermatozoa produced are released in the lumen of the SeT as non-functional gametes. Spermatozoa full fertilization capacity, progressive motility and ability to fertilize an oocyte, are acquired in a maturation process occurring throughout the passage in the different epididymal regions [41].

b. Seminiferous tubules epithelium: Sertoli and germ cells

The importance of SCs for testicular function is established since embryonic development. Testis development takes place during the fetal life, driven by the genetic program in the Y chromosome, namely the *SRY* (sex-determining region Y) gene, which supports the differentiation of fetal precursorcells into SCs [42]. Thus, SCs are the first testicular cell-type to differentiate in the developing testis, with every other cell depending upon them [43]. SCs influence the differentiation of both the primordial germ cells and LCs [42, 44]. During fetal life, SCs secrete the anti-Mullerian hormone (AMH) that is responsible for the degeneration of Mullerian ducts, thus, avoiding the regression of the anlagen, uterus and fallopian tubes [45, 46], and allowing the development of male excurrent ducts, besides testis. [45, 47]
After the formation of the testis, a rapid proliferation of the primordial germ and SCs occurs, which is maintained till puberty. At this stage, mitosis of SCs stops, the establishment of tight-junctions between SCs takes place, and spermatogenesis initiates [17]. Male fertility ensured by a successful spermatogenesis requires very complex and dynamic processes of interactions between germ cells and SCs in the epithelium of the SeT. As stated before, the evolution of this epithelium is long-time process that begins in the early stages of fetal development. When completely formed, the SeT are lined by a stratified epithelium composed by SCs and germ cells, with mature SCs occupying 17-20 % of all volume [48]. Because of the very close relationship between SCs and germ cells, and the high-depence of these, there is a maximum number of germ cells that can be supported by each single SC. This ratio is species specific but has been shown to be constant within a single species [17].
A considerable amount of data calls the SCs as “supporting or nurse cells” because of their major role in germ cells development. SCs provide the physical support, protection, and all the nutrients and growth factors that allow germ cell survival [49-52]. SCs also have a critical role in the self-renewal of germ cells [51]. Indeed, they produce a panoply of products that modulate the SeT environment with impact in spermatogenesis. These products can be

divided into several categories [49, 53]: (i) transporters or bioprotective proteins secreted in high abundance, such as metal ion transporters, like transferrin and ceruloplasmin [49, 53]; (ii) proteases and proteases inhibitors important in tissue remodeling [49, 53]; (iii) glycoproteins that form the basement membrane and can act as growth factors or paracrine factors, such as collagen type IV and laminin [49, 53]; (iv) regulatory glycoproteins in low levels but still carry out their biochemical roles[49, 53].

SCs are also responsible for the production and secretion of the SeT fluid. This fluid consists in a number of products such as transport and binding-proteins, proteases, protease inhibitors, hormones and growth factors that have a role in spermatogenesis. Moreover, this fluid maintains the nutritional and hormonal microenvironment necessary for the germ cells viability and differentiation, providing the suitable *milieu* for the development of spermatozoa [54] The SeT fluid also acts as a vehicle for transport of spermatozoa from the testis to the ducts

c. Hormonal Regulation of Spermatogenesis

The regulation of a complex process like spermatogenesis depends on assortment of a set of hormones through endocrine, paracrine, juxtacrine and autocrine communication pathways [55]. The major player in this regulation is the hypothalamus that coordinates the so-called hypothalamic-pituitary-gonadal axis (Figure I.3). The hypothalamic gonadotropin-releasing hormone (GnRH) upon binding and activating its receptor regulates the activity of pituitary secreting the gonadotropins luteinizing hormone (LH) and FSH [56, 57]. LH and FSH act on LCs and SCs, respectively, with their action being mediated by specific transmembrane associated G-protein coupled receptors, LH-R and FHS-R (Figure I.3) [56, 58].

LH stimulates the LCs to produce T, which acts as a paracrine factor in the SeT. T together with FSH stimulate SCs to produce regulatory molecules that stimulate the progression of spermatogenesis [40, 55, 58]. SCs products can be divided into three categories: (i) molecules that facilitate the transport of iron and hormones like transferrin and androgen-binding protein (ABP); (ii) proteases and proteases inhibitors involved in tissue remodeling; (iii) structural components of the basement membrane and nutrients needed for a successful spermatogenesis [40, 55, 58].

The hypothalamic-pituitary-gonadal axis is controlled by the negative feedback mechanisms driven by T, which inhibits the release of GnRH and LH (Figure I.3) [59]. Inhibin is also responsible for a negative feedback mechanism (Figure I.3). This polypeptide belongs to the TGF-β superfamily of growth and differentiation factors and is a secretory product of SCs highly regulating FSH levels [60, 61]

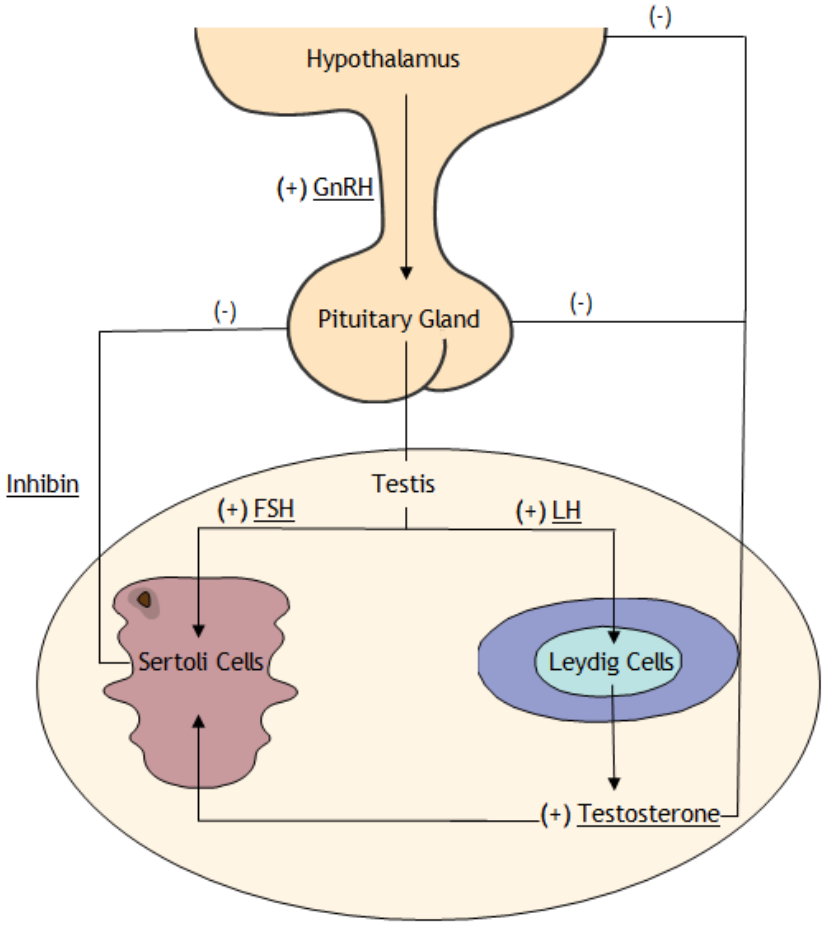

Figure I.3 Schematic representation of hormonal regulation of the spermatogenesis. The release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the activity of Sertoli cells whereas LH simulates Leydig cells, inducing the production of testosterone (T). T induce a negative feedback (-) on the hypothalamus and pituitary gland decreasing the levels of GnRH and LH. Sertoli cells secret the glycoprotein inhibin that selectively induces a negative feedback (-) in the secretion of FSH.

Alongside with androgens and FSH, in the last years, it has been shown that estrogens also have a critical role in spermatogenesis and in male fertility [62, 63]. The classical estrogen receptors (ERs) are expressed in many of the testicular cells and can be divide in two subtypes, ERα and ERß. These receptors belong to the family of nuclear hormone receptors acting as ligand-activated transcription modulators [64, 65]. The absence of ERα was associated with abnormal spermatogenesis and male infertility, with this fact being demonstrated for the first time in knockout mice for a functional ERα [62, 64]. Estrogens actions in the testis, namely, in SCs, also have been associated with the membrane G-protein coupled estrogen receptor, the GPER [66].

Besides responding to estrogens, the mammalian testis can biosynthesize these class of steroid hormones. Estrogens are produced by the activity of cytochrome P450 aromatase (P450arom) that catalyzes the irreversible transformation of androgens. Aromatase is present in LCs and SCs, but also in germ cells [67]. All these cell types also have ERs, which may

explain the role of estrogens in several physiological process in male reproductive tract such like germ cell survival, spermatogenesis and spermiation [22, 65, 68]. Moreover, estrogens can have a direct effect in the production of T by the LCs down-regulating the expression of steroidogenic enzymes [69].

3. Sertoli and germ cells metabolism

The SCs are committed with important roles in different stages of the germ line development and spermatogenesis, being one of their major roles the provision of energy and nutritional support. Glucose is considered the universal energy substrate used by nearly all cells, including the germ cells. However, despite the fact that all germ cells possess the machinery of the glycolytic pathway, some of them depend on the nutritional support provided by SCs [70, 71]. Within the SeT, SCs metabolize several substrates, and used glucose to produce pyruvate that, then, is converted into lactate, which is secreted to the SeT environment and used by germ cells [72-74].For this reason, only 25 % of the pyruvate produced by SCs is oxidized during the citric acid cycle (TCA) [75].

Despite lactate has been pointed out as the main energy source for germ cells, there are evidence of its other roles, namely, an anti-apoptotic effect [76], and an association with the stimulation of RNA and protein synthesis in spermatids [70].

As mentioned above, germ cells have the capacity to use glucose as energy source, but their activity in this aspect is very low [77]. Spermatogonia, because are localized outside the BTB, may utilize the glucose from the blood as energy source [70]. However, spermatocytes and spermatids suffer a rapid decline in the adenosine triphosphate (ATP) content requiring the pyruvate and lactate produced by SCs [78]. On the other hand, spermatozoa have a high glycolytic capacity, exhibiting two characteristics: (i) highest glycolytic activity and lowest TCA cycle activity compared with all germ cells, (ii) the energy production is compartmentalized because the oxidative phosphorylation and glycolysis occurs in different pieces. This high activity is needed for hyperactivated motility, capacitation and *in vivo* fertilization [70, 79, 80].

To support the energetic needs of the germ cells, SCs sustain a high glycolytic flux exhibiting a Warburg-like metabolic behavior like the one observed in cancer cells [77]. However, without having the primary deleterious characteristic as cancer cells, the mitotic proliferation [77].

Glucose, from the plasma, is transported through the BTB and enters the SCs via specific glucose transporters, the GLUTs [79]. Fourteen functional isoforms of GLUTs have been identified and grouped in three classes [81]. GLUT1, GLUT2, GLUT3 and GLUT8 are the ones that have been identified in the testis [82, 83]. Immunohistochemical techniques allowed to identify GLUT1, GLUT2 and GLUT3 in the rat testis [84]. In SCs the expression of GLUT1, GLUT3 and GLUT8 mRNA was reported [82]. Recently, the presence of GLUT1, GLUT2 and GLUT3 proteins was identified in rat SCs [85]. GLUT1 and GLUT3 possibly work together to maintain the high-energy rate needed [82]. GLUT2 is described to participate in the handling of the dietary sugars having a role in the uptake of glucose by SCs [85]. GLUT8 only identified in the endoplasmic reticulum membrane of SCs apparently is not involved in glucose uptake [86].

After entering the cell, glucose suffers a series of enzymatic reactions being metabolized in glycolysis (Figure I.4), the process that originates pyruvate and two molecules of ATP [87]. With higher or lesser extent, glycolysis occurs in all cells in the body. Firstly, glucose is phosphorylated into glucose-6-phosphate (G6-P) by the action of the enzyme hexokinase or glucokinase [88], and then G6-P undergoes an isomerization reaction originating fructose-6-phophate (F6-P) which is phosphorylated into fructose-1,6-bisphosphate (F1,6-BP) by the action of the enzyme phosphofrutokinase-1 (PFK1) [89]. PFK1 catalyzes a rate limiting step of glycolysis being considered the most important glycolytic enzyme in this pathway. High levels of ATP inhibit the activity of PFK1 leading to an accumulation of F6-P, which also increases G6-P levels resulting in the inhibition of the hexokinase. Adenosine monophosphate (AMP) can reverse this inhibition increasing ATP levels [90].

Most of the pyruvate produced by SCs is converted into lactate by the action of the enzyme lactate dehydrogenase (LDH) with the oxidation/reduction of NADH to NAD+ [91, 92]. The lactate produced is then exported to the SeT microenvironment by the specific membrane-bounded transporters, the monocarboxylate transporters (MCTs) [93, 94]. There are several MCTs isoforms, but MCT4 is the one responsible for the exportation of lactate, and was known to be expressed by SCs (Figure I.4)[95].

Alanine transaminase (ALT) or glutamate pyruvate transaminase (GPT) is the enzyme that catalyzes the reversible transamination of L-alanine and α-ketoglutarate to form pyruvate and L-glutamate. [96] (Figure I.4). It plays an important role as an intermediary in the metabolism of glucose and amino acids [97] because the pyruvate formed can then be reduced to L-lactate by LDH [98].

It has been shown that in conditions of glucose deprivation, SCs can alter their metabolism and continue to delivery lactate to the germ cells ensuring their survival [99]. This glucose deprivation can activate specific adaptive responses such as the activation of AMP-activated protein kinase (AMPK) [99]. AMPK is activated when the levels of ATP are low [100]. Once activated AMPK suppresses biosynthetic pathways and activates ATP-generating pathways like fatty acid oxidation and glycolysis [99]. The SCs can satisfy their energetic necessities by oxidative substrates mostly through ß-oxidation of fatty acids or through catabolism of some amino acids like glutamine [75, 77, 101].

Glutamine is one of the most abundant nonessential amino acids. It enters the cells by the amino acids transporter the ASCT2 [102, 103]. Once inside the cell glutamine goes to the mitochondria and suffers hydrolysis to generate glutamate and ammonium by the action of the mitochondrial enzyme, glutaminase (GLS) [104]. The produced glutamate can be further catabolized in the TCA cycle or participate in the glutathione (GSH) synthesis [102] (Figure I-4).

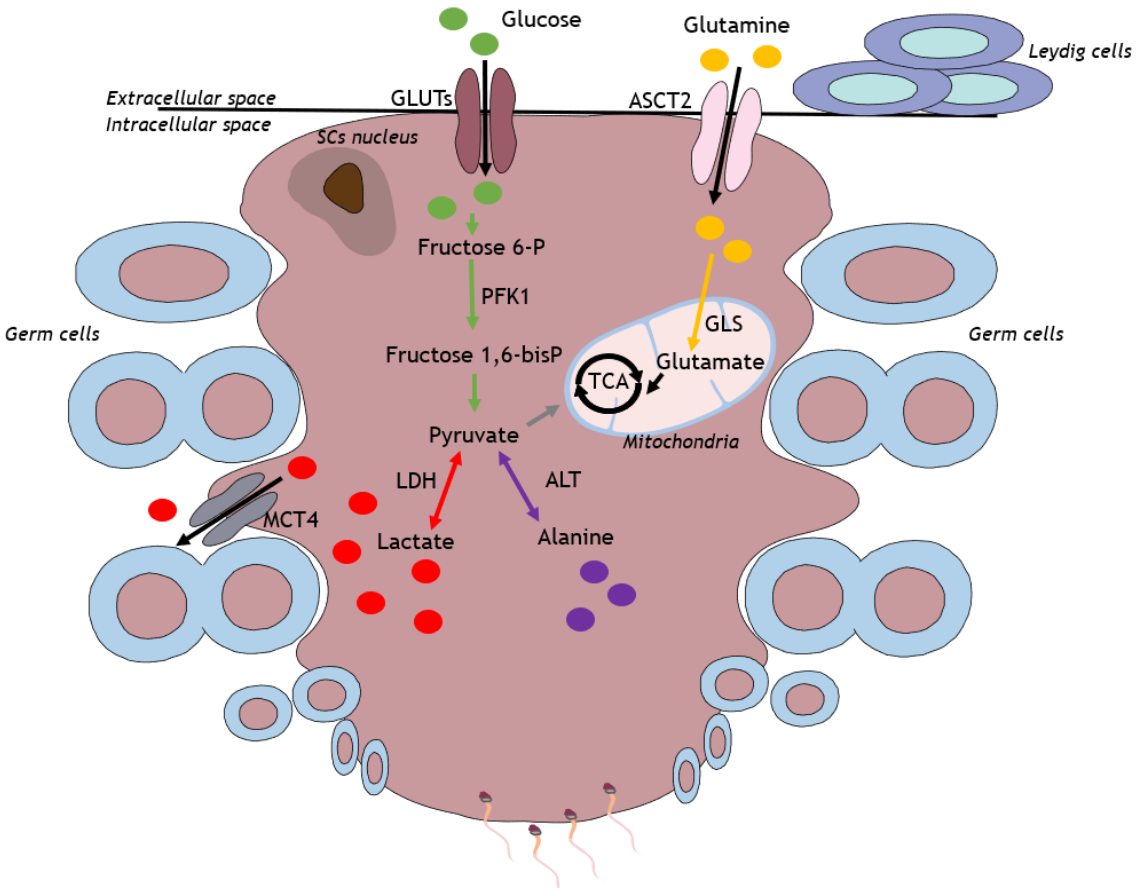

Figure I.4 Schematic representation of the glucose and glutamine metabolizing pathways in Sertoli cells (SCs). Glucose enters the Sertoli cells by specific glucose transporters (GLUTs), especially GLUT1, GLUT2 and GLUT3. Inside the cell, glucose is converted into pyruvate by glycolysis (green arrows) through the action of different enzymes. Phosphofrutokinase-1 (PFK1) regulates this pathway by the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, a rate limiting step of glycolysis. The pyruvate resultant from glycolysis can have three possible destinies: i) go directly to the mitochondria entering the citric acid cycle (TAC) cycle (grey arrow); ii) be converted into alanine through the action of the enzyme alanine transaminase (ALT, purple arrow); or iii) be converted into lactate through the action of the enzyme lactate dehydrogenase (LDH, red arrow). The produced lactate is exported into the extracellular space through monocarboxylate transporters, specifically by the MCT4. Alternatively, the pyruvate necessary for lactate production can came from the activity of ALT since the alanine-pyruvate interconversion is a reversible reaction. SCs can also metabolize glutamine (yellow arrows), that enters the cell by amino acids transporter ASCT2, and is converted into glutamate by the action of the enzyme glutaminase (GLS). The glutamate produced can enter in TCA.

Several hormones that regulate spermatogenesis and others, have been unraveled to control the SCs' metabolism, namely, FSH, triiodothyronine (T3), 17β-estradiol (E_2), 5α-dihydrotestosterone (DHT) and insulin [105]. Also, several studies have shown that metabolic disorders are associated with male infertility [71] [106]. This includes insulin resistance, diabetes and obesity, which have been shown to be closely related with male infertility [107]. Metabolic syndrome, defined as the presence of three or more of the following conditions: abdominal obesity, elevated fasting glucose, elevated triglycerides, low high-density lipoprotein (HDL), cholesterol and elevated blood pressure has also been associated with men infertility [108]. The cumulative evidence linking infertility syndromes with metabolic disorders has placed [106] SCs´ metabolism in a core position as a modulator of male fertility, as their metabolic actions boost the development of germ cells.

4. Oxidative stress as a damaging factor for spermatogenesis and male fertility

Reactive oxygen species (ROS) are byproducts of oxygen metabolism produced via the electron transfer chain system present in the mitochondria [109]. During mitochondrial respiration, ROS are generated by the incomplete reduction of molecular oxygen during the process of oxidative phosphorylation [110]. The redox reactions are present in almost all fundamental processes of cells like the regulation of the endoplasmic reticulum function. However, an imbalance between prooxidant and antioxidant factors causes the event known as oxidative stress (OS) [111, 112]. When ROS production greatly exceeds the capacity of endogenous cellular antioxidant defense system, OS occurs [113]. Thus, ROS play a dual role being beneficial and harmful for the living systems [114]. ROS can be generated in response to exogenous agents or endogenous processes [115], like radiation, temperature, drugs, toxins, minerals, infections, among others [109].

Oxidative stress is a harmful factor and a major cause of male infertility with damaging effects on the development of germ cells and sperm function [115]. Even with the poor intratesticular vascularization and low oxygen tension, spermatogenesis and steroidogenesis are very susceptible to suffer damage because of the highly abundance of unsaturated fatty acids and ROS generating systems present in the testis [116]. ROS can damage biological macromolecules like proteins, lipids and nucleic acids. Also, ROS can disturb the sperm membrane, reducing motility and capacity to fuse with the oocyte, or injure directly the DNA [117, 118]. OS increases the levels of glucocorticoids, which causes a decrease in T levels inhibiting the testicular expression of antioxidant enzymes [119].

To counteract the effects of ROS, the testis exhibit antioxidant defense mechanisms that include antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and catalase (CAT) [120]. SOD can be found in two different types, the cytoplasmic type, copper/zinc superoxide dismutase (Cu/Zn-SOD) and a mitochondrial type, manganese superoxide dismutase (Mn-SOD) [121]. Furthermore, testis can exhibit non-enzymatic antioxidants like ascorbic acid, reduced glutathione, α-tocopherol, carotenoids, urate, vitamins A and E, albumin, taurine, hypotaurine, among others [114, 122]. The oxidant protection of sperm continues in the epididymis and other males ducts, with the seminal plasma containing antioxidant properties that protect spermatozoa during storage [3, 114].

Oxidative stress also is related with metabolic alterations. Several studies with cadmium, an OS inducer, have shown metabolic alterations in several marine species [123-125].

Organic hydroperoxides that have been substantially used as a prooxidant agents to induce OS in various systems *in vitro* concur to lipid peroxidation [126, 127]. *Tert*-butyl hydroperoxide (TBHP) is one of these organic hydroperoxides widely used in studies of OS in the testis [128-130]. TBHP can be metabolized by cytochrome P450 in the mitochondria generating ROS that can induce lipid peroxidation. On the other hand, TBHP can be detoxified by GSH. Besides

that, it can alter the intracellular calcium (Ca^{2+}) homeostasis and induce the leakage of LDH [131, 132]. Also, TBHP is described to have the capability of deregulating the pentose phosphate pathway [133]. The relationship between TBHP exposure and testicular metabolic alterations is largely unknown, and further research is needed on this subject to clarify the interplay between OS and metabolism.

5. Regucalcin protein' actions: a focus on reproduction

The intracellular Ca^{2+} concentration is important in diverse cellular functions in the testis like: gene transcription, meiosis, post meiotic differentiation and sperm function [134]. Ins the SeT, germ cells can generate Ca^{2+} currents and during the spermatogenesis these currents increase, suggesting an important role for this ion during germ cell differentiation [135, 136]. In response to T and FSH, the major hormones in the regulation on spermatogenesis, stimulated SCs show an increase of intracellular Ca^{2+} [137, 138], which is important for the maintenance of the integrity of the BTB and for the enzymatic activity [139]. Also, the regulation of Ca^{2+} homeostasis and maintenance of intracellular Ca^{2+} levels seem to be related to the modulation of steroidogenesis in the LCs [140]. Ca^{2+} is required even in post-spermatogenesis events of spermatozoa like capacitation, acrosome reaction, and motility [141, 142].

Regucalcin (RGN) is a Ca^{2+}-binding protein first described in 1978 [143], composed by 299 amino acid residues and with an estimated molecular weight of 33 kDa [144]. The *RGN* gene is localized in the X chromosome and is highly conserved during evolution from invertebrates to vertebrates, which lead author to claim a basic and important biological function for RGN [145, 146]. Fujita and co-authors named RGN as senescence marker protein 30 because of its molecular weight and the marked diminished expression with aging [147-149].

RGN has been identify in several tissues such as liver [150, 151], kidney [152, 153], bone [154], brain [155, 156], breast [157, 158], heart [159, 160], lung [161], ovary [162], prostate [163-165], submandibular gland [166] and testis [139]. Inside the testis RGN is expressed in LCs, SCs and in all the germ line [139].

The expression of RGN is regulated by several factors, such as the transcription factor AP-1, ß-catenin, nuclear factor I-AP1, RGN gene promoter region-related protein, insulin, aldosterone [146], Ca^{2+}, thyroid and parathyroid hormones, and OS [167]. The effect of sex steroid hormones modulating RGN expression has been assessed in various tissues and cell lines, establishing RGN as an androgen-target gene in the male reproductive tract [157]. The androgen DHT has been showed to up-regulate RGN expression in the rat testis [139]. Moreover, RGN is also regulated by estrogens since the administration of 17ß-estradiol (E_2) in the rat liver lead to an increase in the expression of RGN mRNA [168]. Also, it has been described that E_2 induces a decrease in the expression of RGN in rat kidney cortex [169] and in rat mammary gland and prostate [163]. RGN regulation by both E_2 and DHT was also shown in MCF-7 and LNCaP cells [164]. Contrarily to other Ca^{2+}-binding proteins like calmodulin, RGN do not contain the typical structural Ca^{2+}-binding EF-hand motif [147, 170]. Nevertheless, RGN plays a very important role in regulating the activity of Ca^{2+} pumps (Figure I.5) at cell membrane and endoplasmic reticulum keeping the intracellular Ca^{2+} homeostasis [171] and regulating the uptake of Ca^{2+} by mitochondria [165].

RGN modulates the activity of Ca^{2+} pumps and the uptake of Ca^{2+} by mitochondria, by enhancing Ca^{2+}-ATPase activity and suppressing the expression of the L-type Ca^{2+} channel and

calcium-sensing receptor mRNA [172]. Using $^{45}Ca^{2+}$ in epididymal tissue cultures, it was found a diminished Ca^{2+} influx in the epididymis of transgenic rats overexpressing RGN (Tg-RGN), which indicates in the existence of reduced Ca^{2+} concentrations in the epididymal lumen of these animals. Analysis of sperm parameters of Tg-RGN rats revealed lower sperm counts, but higher percentage of viable and morphologically normal sperm, suggesting that RGN has a role in sperm maturation [172].

RGN is also involved in the regulation of diverse proteins of intracellular signaling pathways like kinases, phosphatases, phosphodiesterase, synthases and proteases [173]. Besides being a cytoplasmatic protein, RGN can translocate to the nucleus and inhibit DNA and RNA synthesis, which has been linked with the control of cell death and proliferation [163, 173](Figure I.5). Furthermore, RGN regulates the expression of cell cycle and apoptosis regulators (Figure I.5), providing a resistance to apoptosis by inhibiting the increase of intracellular calcium concentration $[Ca^{2+}]_i$ [172], and also inhibits caspase 8 activity [174], improves Akt pathway activity and increases the expression of antiapoptotic agents like Akt-1 and Bcl-2 [175]. Other studies showed that RGN-knockout mice were more susceptible to tumor necrosis factor (TNF-α) and Fas-mediated apoptosis in comparison with their wild-type (Wt) counterparts [176, 177]. In the testis, a tight control of germ cells survival and apoptosis is crucial maintaining the proper ratio between germ cells and SCs, and prerequisite necessary for a successful spermatogenesis [178]. A previous work of our research group using SeT cultures of Tg-RGN animals, demonstrated that RGN suppresses thapsigargin- and actinomycin D-induced apoptosis in the SeT by modulating the expression and activity of target regulators of apoptosis [179]. A reduced activity of caspase-3 and increased expression of anti-apoptotic Bcl-2 protein were found in the SeT of Tg-RGN rats. Also, the mRNA expression of p53 and p21 were significantly diminished in SeT of Tg-RGN treated with thapsigargin or actinomycin D [179]. Thus, RGN has a beneficial role in male reproduction, since its overexpression can protect germ cells from apoptosis induced by damaging factors [179]. Another *in vivo* study supported the protective effect of RGN in the testis. Radiation-induced testicular damage had minimized effects in the testis of Tg-RGN rats [180]. RGN overexpression was concomitant with lower rate of apoptosis evidenced by the reduced activity of caspase-3, lower levels of caspase-8, and increased Bcl-2/Bax ratio in the testis of Tg-RGN animals comparatively to their Wt littermates. [180].

Concerning OS, RGN has been identified as an antioxidant protein inhibiting the activity of several enzymes responsible for the generation of ROS or improving the antioxidant defenses (Figure I.5) [115, 181]. Previous studies using OS inducers in Tg-RGN animals showed that these animals displayed higher GST activity and an increase of the overall antioxidant capacity [115]. Interestingly, and also supporting its antioxidant activity, RGN was identified as a gluconolactonase, an enzyme implicated in the biosynthesis of L-ascorbic acid [182]. Moreover RGN was shown to enhance the activity of SOD, GPx and GST [115, 183]. Studies in RGN knockout mice have demonstrated that these mice have higher levels of OS in comparison with their Wt counterparts [184]. Remarkably, the epididymis of Tg-RGN rats

presented higher antioxidant potential and diminished lipid peroxidation [172] (Figure I-5), which was suggested to be related with the lower incidence of morphology defects in the spermatozoa of Tg-RGN [172, 180].

More recently, RGN also has been associated with the control of cell metabolism, what is not entirely surprising since Ca^{2+} signals play a known role in the regulation of different metabolic pathways [145]. Studies using Tg-RGN rats showed bone loss in femoral tissue and altered bone morphology, mineral content and density and decreased polar strength strain index and cortical thickness [154].

Furthermore, RGN was implicated in liver metabolism causing hyperlipidemia with ageing, manifested by the increased serum triglyceride, total cholesterol, HDL-cholesterol and free fatty acid concentrations [185, 186]. RGN overexpression stimulated glucose utilization in the liver since Ca^{2+} increases the activity of several enzymes in glucose metabolism, suggesting an action in the control of gluconeogenesis and glycolysis [145]. However, the prostate of Tg-RGN animals displayed lower glucose consumption, diminished expression of GLUT3 and PFK1, as well as lower levels of lactate and decreased MCT4 expression and LDH activity, which indicated that RGN would inhibit glycolytic metabolism [187].

Very recently, following the results in other tissues, the role of RGN modulating the metabolism of SCs was investigated. Results obtained by Silvia Socorro research group demonstrated that primary SCs cultures from Tg-RGN animals, despite consuming less glucose, produced and export higher lactate amounts than their Wt counterparts. The increase of lactate production was explained by the increased expression of ALT and the higher consumption of glutamine [85]. Also, in bone marrow cell cultures an increase in the consumption and lactate production was observed in the presence of RGN [188].

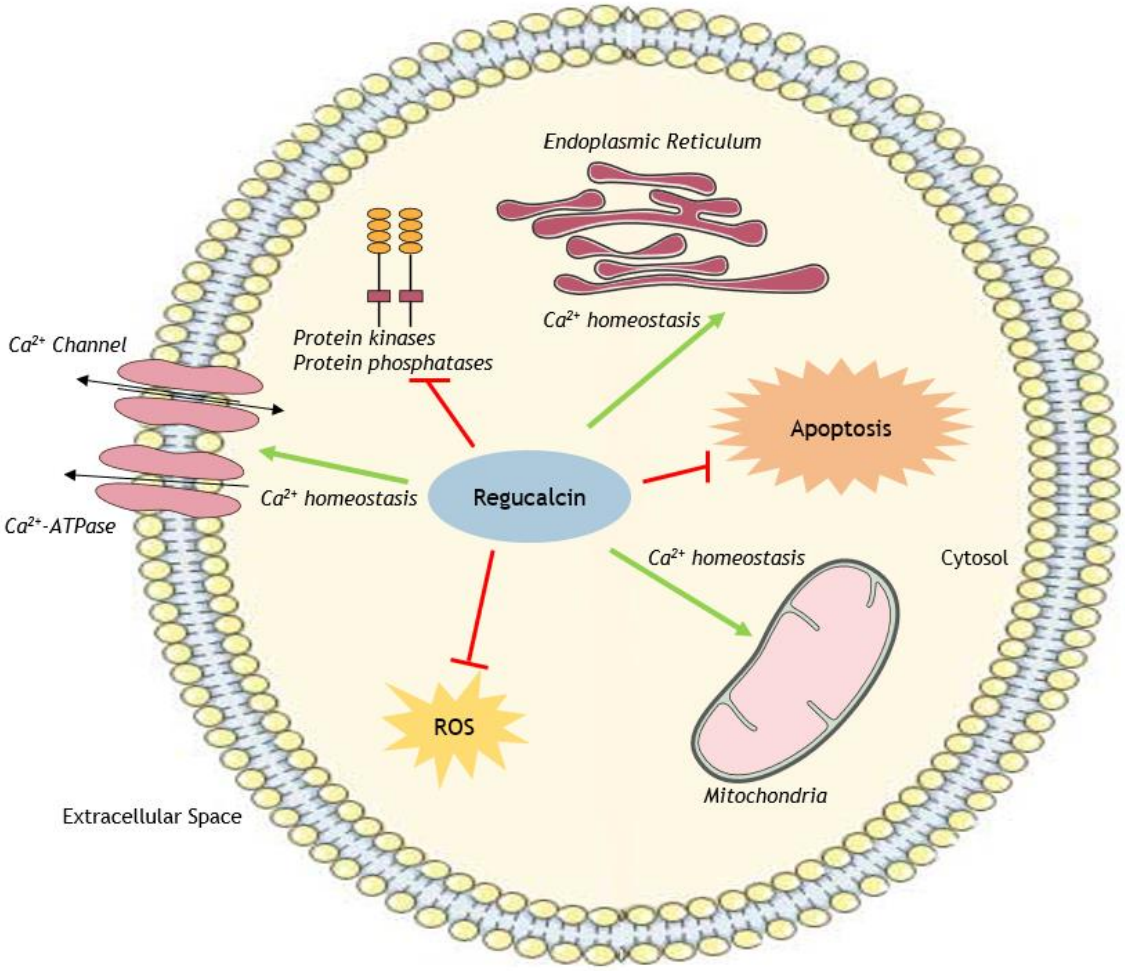

Figure I.5 Schematic representation of the regucalcin' (RGN) actions in intracellular signalling pathways. RGN plays an important role in the modulation of intracellular Ca^{2+} concentrations by regulating the activity of several Ca^{2+} channels present at plasma membrane, mitochondria and endoplasmic reticulum (green arrows). RGN has a suppressive effect on the activity of protein kinases and phosphatases. RGN also has antioxidant activity regulating the production of reactive oxygen species and can translocate to the nucleus and suppress apoptosis (red arrows).

The information gathered from previous studies has indicated that RGN overexpression modulates glucose and glutamine handling in the SCs through the regulation of the expression of several transporters and enzymes involved in glycolysis and glutaminolysis. However, the knowledge about the RGN's influence in the metabolism of testicular cells still is in the beginning.

II. Aim of this dissertation

Mammalian spermatogenesis is a continuous and highly coordinated process that takes place inside the SeT. It involves a series of cell division and germ cell maturation culminating with the production of spermatozoa, and thus, being the basis of male fertility. To maintain a high proliferative rate, the germ cells need appropriate nutritional and physical support. SCs, the somatic cells within the SeT, are responsible for the fulfilment of these requirements. SCs uptake glucose from the extracellular medium metabolizing it into lactate, which is then provided to the developing germ cells. Besides glucose, SCs can also metabolize other energy substrates like alanine and glutamine.

Oxidative stress is a harmful factor for spermatogenesis and a major cause of male infertility. It is widely known that OS adversely affects the spermatogenic process, disrupting germ cells development and proper sperm function. Moreover, it has been described that several OS inducers act simultaneously as metabolic disruptors. TBHP is an organic hydroperoxide known for its capability to induce OS in the testis with effects affecting male reproductive capacity. Also, TBHP was identified as a regulator of the pentose phosphate pathway. However, the impact of TBHP in testicular metabolism remains unknown.

Regucalcin is a Ca^{2+}-binding protein, that besides its role in the regulation of intracellular Ca^{2+} homeostasis has been associated with the modulation of cell proliferation, apoptosis, OS and metabolism. RGN is broadly expressed in the testis, being identified in all the germ line, as well as in SCs. Previous studies of our research group demonstrated the role of RGN protecting germs cells upon exposure to damaging stimuli, such as chemical inducers of apoptosis, oxidants agents, and radiation. Within this context, it is liable to predict that RGN may have a similar behavior against the actions of TBHP disrupting testicular metabolism.

The present work aimed to analyze the putative TBHP's actions as a metabolic disruptor in the SeT and determine the influence of RGN attenuating its effects. For this purpose, SeT from Tg-RGN rats and their Wt littermates were maintained in culture for 24 hours in the presence or absence of TBHP (250 µM), and the metabolic response was characterized. The following metabolism parameters were evaluated:

- Intracellular glucose and lactate content;
- Expression and activity of several transporters and enzymes involved in the regulation of glycolytic metabolism and glutaminolysis.

III. Materials and methods

1. Animals and Tissue Collection

Three-month-old Wt and Tg-RGN Sprague-Dawley rats (*Rattus norvegicus*) were used in this study. The Tg-RGN rats were originally generated by Yamaguchi M. by oocyte transgene pronuclear injection [189] and obtained from Charles River (Barcelona, Spain). Wt animals were purchased from Japan SLC (Hamamatsu, Japan).

The rats were maintained with food and water *ad libitum* in a constant room temperature (20 ± 2°C) on a 12-hour cycle of lighting and darkness. The animals were handled in compliance with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised on 1996) and the European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU).

Wt and Tg-RGN rats (n≥5 in each group) were euthanized by cervical dislocation under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) administered intraperitoneally at a dose of 100µL per 100g of body weight. The testes were removed, trimmed free of fat and washed in cold phosphate-buffered saline (PBS).

2. SeT Culture

Under sterile conditions, using for that purpose a laminar flow chamber, the testes were placed in petri dishes with pre-warmed DMEM:F-12 culture medium (Sigma-Aldrich) supplemented with 20mg/L gentamicin sulfate, 0.1mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µg/L bovine serum albumin (BSA) at 33 °C. The tunica albuginea was cut and peeled back to expose the SeT. The dissection of the SeT was performed with the aid of a magnifier (Nikon SMZ645 Zoom Steriomicroscope, EUA) with fibre optic lighting (LG 100G) and surgical supplies precision. Multidishes of 12 wells (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark) were filled with 2mL of culture medium per well and in each well were placed about 10 fragments of SeT with approximately 1cm long (the damaged ones were discarded). The SeT were cultured in an atmosphere of 5% CO_2 at 33°for 24h in the presence or absence of TBHP at a concentration of 250 µM, a well-known inducer of OS [115]. At the end of the experimental time the SeT were recovered from the medium, snap-frozen in liquid nitrogen and stored at -80°C.

3. Total Protein Extraction and Quantification

Total protein was isolated from cultured rat SeT using RIPA buffer (1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 %SDS in PBS) supplemented with a protease inhibitors cocktail (Sigma-Aldrich).

SeT were homogenized and centrifuged at 14000 rpm for 20 minutes at 4 °C in a Hettich Mikro 200R centrifuge (Hettich zentrifugen, Germany). The supernatant containing the protein was collected and kept on ice. The total protein concentration was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and using BSA as a standard.

4. Western Blot

Total proteins (50 µg of each protein extract) were denatured at 100°C for 5 minutes and resolved in a 12% gel by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at 200 V for 55 minutes. Then, proteins were transferred to a PVDF (polyvinylidene difluoride) membrane at 750 mA for 1h30 minutes at 4°C. Membranes were incubated overnight at 4°C with a rabbit anti-GLUT-1 (1:500, CBL242, Millipore, MA, USA); rabbit anti-GLUT-3 (1:500, sc-30107, Santa Cruz Biotechnology); rabbit anti-PFK-1(1:1000, sc-67028, Santa Cruz Biotechnology); rabbit anti- LDHA (1:10000, Ab52488, Abcam, Cambridge, MA, USA); mouse anti-ALT(1:200, sc-374501, Santa Cruz Biotechnology); rabbit anti-MCT-4 (1:1000, sc-50329, Santa Cruz Biotechnology); rabbit anti-ASCT-2 (1:500, V501, Cell Signaling Technology, Danvers, MA, USA); rabbit anti-GLS (1:1000, ab93434, Abcam, Cambridge, MA, USA); rabbit anti-AMPKα1/2 (1:500, #2532, Cell Signaling Technology, Danvers, MA, USA); primary antibodies. A mouse anti-ß-actin monoclonal antibody (1:10000, A1978, Sigma-Aldrich) was used for protein loading control in all Western Blot (WB) analysis. A goat anti-mouse IgG-HRP (1:40000, sc-2005, Santa Cruz Biotechnology); mouse IgGκ light chain binding protein (m-IgGκ BP) (1:5000, sc-516102, Santa Cruz Biotechnology); goat anti-rabbit IgG-HRP (1:5000, sc-2004, Santa Cruz Biotechnology) and goat anti-rabbit IgG-HRP (1:5000, #7074, Cell Signaling Technology, Danvers, MA, USA were used as secondary antibodies.

The membranes were incubated with ECL substrate (Bio-Rad) for 5 minutes and scanned with the ChemiDoc™ MP Imaging System (Bio-Rad). The band densities were obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective ß-actin band density.

5. LDH Enzymatic Activity

The enzymatic activity of LDH was determined using a commercial kit (Spinreact, Girona, Spain) according the manufacturers' instructions.

LDH is the enzyme responsible for the reduction of pyruvate by NADH. The rate of decrease in concentration of NADH, measured spectrophotometrically, is proportional to the catalytic concentration of LDH present in the sample. An initial absorbance was read at 340 nm, and after the kinetic reaction starts the absorbance was repeatedly read with 1-minute intervals subsequently for 3 minutes.

The enzymatic activity was calculated by measuring the variation on the absorbance and calculated as U/µg of protein.

6. Intracellular Metabolites Extraction

A methanol/chloroform/water extraction was used to extract polar and non-polar metabolites from SeT as described by Sellick et al. 2010 [190]. About 100 mg of SeT were snap-frozen in liquid nitrogen and macerated, followed by the addition of 1 mL of cold methanol (HPLC grade, 4°C) and 250 µL of sterile cold water. The samples were vortexed for 60 seconds. Chloroform (500 µL) was then added and the samples vortexed again for 60 s plus sonication during 15 minutes at 4°C. After that, 500µL of chloroform and sterile cold water were added to the samples, which were vortexed for 60s and centrifuged at 5000g for 15 min at 4 °C. The upper layer was collected, frozen, lyophilized, and then diluted in 10 µL of sterile water for quantification of intracellular glucose and lactate concentrations.

7. Quantification of Intracellular Glucose and Lactate Content

The concentration of both intracellular glucose and lactate was determined using a commercial kit (Spinreact)as previously described [85].

Glucose Oxidase (GOD) is responsible for the oxidation of glucose in gluconic acid with the formation of hydrogen peroxide (H_2O_2). This can be detected by a chromogenic oxygen acceptor, the phenol-aminophenazone, that in the presence of peroxidase (POD) form a colored quinone compound.

Lactate Oxidase (LO) is responsible for the oxidation of lactate in pyruvate and H_2O_2, which in the presence of POD, 4- aminophenazone (4-AP) and 4-chlorophenol form a red quinone compound.

Briefly, the intensity of the color formed is proportional to the content of the glucose/lactate present in the sample by reading the absorbance after 10 and 5 minutes of incubation, respectively, at 505 nm. Both methods were calibrated using glucose and lactate positive controls included in the kit. The quantification of glucose and lactate present in the samples was in compliance with manufacturer´s instructions. Results were expressed as nmol/g of SeT tissue.

8. Statistical Analysis

The statistical significance of differences between the different experimental groups was evaluated by unpaired t-test, using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The differences were considered significant when $p < 0.05$. All the experimental data are shown as mean ± SEM.

IV. Results

1. Intracellular content of glucose and lactate were altered in the presence of TBHP in Wt and Tg-RGN animals

To maintain the high metabolic needs required for germ cells differentiation, SCs use glucose as the main energetic source and convert into pyruvate and lactate, supplying germ cells with the required energetic metabolites [191]. This metabolic cooperation within SeT might be maintained to ensure the proper germ cell development. This study investigated the effect of the pro-oxidant agent TBHP disrupting the glycolytic metabolism in SeT, and if the *in vivo* overexpression of RGN counteracts the presence of the noxious stimulus. For this purpose, we started measuring the intracellular content of glucose and lactate in the SeT of four experimental groups: Wt control animals, Wt animals treated with TBHP (250 μM), Tg-RGN control animals and Tg-RGN animals treated with TBHP (250 μM), after a culture period of 24 hours.

The intracellular concentration of glucose tended to be increased in the SeT of Wt animals treated with TBHP when compared to the control group (Figure IV.1A), though due the high SEM error the difference was not statistically significant. The content of glucose was significantly increased in the SeT of Tg-RGN animals treated with TBHP when compared with the Tg-RGN control group (232.4 ± 51.04 vs. 78.84 ± 32.71, $p<0.05$, Figure IV.1A). Noteworthy, the content of glucose in the SeT of Tg-RGN control group was significantly lower when compared with the corresponding Wt control (78.84 ± 32.71 vs. 452.7 ± 92.20, $p<0.01$, Figure IV.1A). Even after treatment with TBHP, Tg-RGN animals maintained lower levels of glucose compared with Wt animals in the same experimental conditions (232.4 ± 51.04 vs. 1948 ± 799.9, $p<0.05$, Figure IV.1A).

The same pattern was observed for the intracellular content of lactate. The SeT of TBHP-treated Wt animals displayed increased lactate concentration when compared with the control group (1282.0 ± 125.8 vs. 647.2 ± 160.0, $p<0.05$, Figure IV.1B). Also, lactate content was increased in the SeT of TBPH-treated Tg-RGN animals (279.3 ± 78.23 vs. 62.39 ± 36.69 in the control group, $p<0.05$, Figure IV.1B). Lastly, the SeT of both control and treated-Tg-RGN groups displayed decreased lactate when compared with the Wt groups (62.39 ± 36.69 in Tg-RGN control vs. 647.2 ± 160.0 in Wt control, $p<0.05$; 279.3 ± 78.23 in TBHP-treated Tg-RGN vs. 1282.0 ± 125.8 TBHP-treated Wt, $p<0.001$, Figure IV.1B).

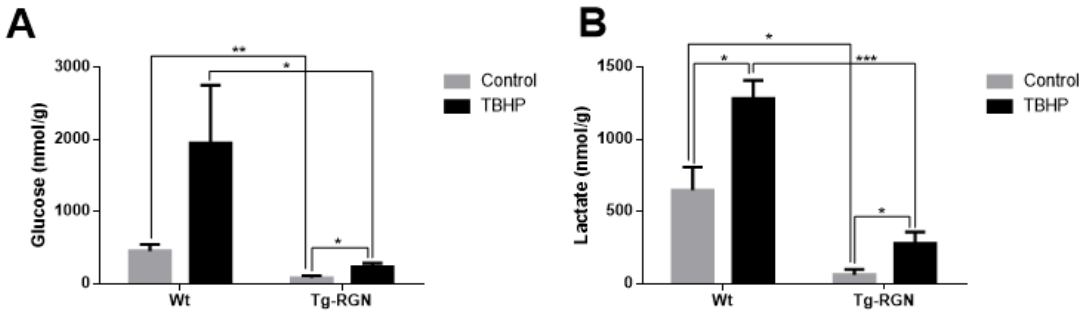

Figure IV.1. Intracellular content of glucose (A) and lactate (B) in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. (n≥4 in each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2. Glycolytic metabolism regulators in the SeT of Wt and Tg-RGN animals in the presence of TBHP

a. TBHP increased GLUT1 and GLUT3 expression in the SeT of Wt animals and GLUT3 in Tg-RGN

To provide lactate for the germ cells, SCs require several glycolytic enzymes and transporters. Glucose enters the SCs by the activity of GLUTs [79], whose specific isoforms have been characterized concerning their biochemical features, tissue-specific expression pattern, substrate specificity and proper kinetic characteristics [192]. We analyze the expression of GLUT1 and GLUT3 as they have been reported to play the major role in SCs metabolism [82].

The protein levels of GLUT1 were significantly increased in the SeT of Wt animals treated with TBHP when compared to the SeT of Wt animals (1.370 ± 0.09593 vs. 1.000 ± 0.03877, $p<0.01$, Figure IV.2A). No effect was perceived on the expression levels of GLUT1 in the SeT of TBHP-treated Tg-RGN animals (Figure IV.2A). Consequently, the expression levels of GLUT1 in the SeT of TBHP-treated Tg-RGN animals were significantly lower compared with the Wt treated-animals (0.5114 ± 0.1334 vs. 1.370 ± 0.09593, $p<0.01$, Figure IV.2A).

Also, GLUT3 expression was significantly increased in the SeT of Wt animals in response to TBHP treatment (1.807 ± 0.1646 vs. 1.000 ± 0.3372 in the control group, $p<0.05$, Figure IV.2B). In this case, this effect was followed in the SeT of Tg-RGN animals. TBHP-treatment increased GLUT3 expression from 1.782 ± 0.3655 in control Tg-RGN to 4.053 ± 0.6527 in the TBHP-treated group ($p<0.05$, Figure IV.2B). Moreover, GLUT3 protein levels were significantly higher in the SeT of TBHP-treated Tg-RGN animals compared with the Wt-treated group (4.053 ± 0.6527 vs. 1.807 ± 0.1646, $p<0.05$, Figure IV.2B).

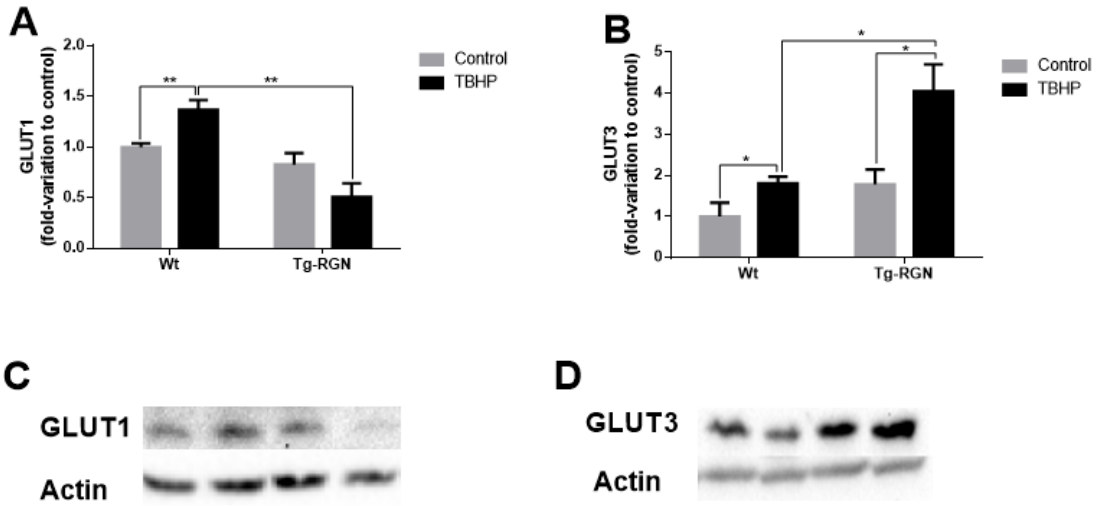

Figure IV.2 GLUT1 (A) and GLUT3 (B) expression in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with □-actin ($n\geq4$ in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$, ** $p < 0.01$. Representative immunoblots for GLUT1 and GLUT3 are shown in panels C and D, respectively.

b. TBHP diminished the expression levels of PFK1 and MCT4 in the SeT of Wt animals whereas PFK1 expression was increased in Tg-RGN

PFK1 is the first enzyme catalyzing a rate-limiting step in the glycolytic pathway [193], being responsible for the conversion of F6-P into F1,6-BP [89]. PFK1 expression was significantly decreased in the SeT of Wt animals treated with TBHP when compared with the SeT of Wt group control (0.7160 ± 0.09795 vs. 1.000 ± 0.05311, $p<0.05$, Figure IV.3A). However, PFK1 expression levels were significantly increased in the SeT of Tg-RGN animals treated with TBHP when compared with the control group (1.472 ± 0.1273 vs. 0.9152 ± 0.1040 $p<0.01$, Figure IV.3A).

The end product of glycolysis pyruvate is converted to lactate that is exported to the extracellular medium by the activity of MCTs [194]. MCT4 is the MCTs isoform associated with the export of lactate was shown to be expressed in the SCs [95, 195]. TBHP significantly decreased the expression levels of MCT4 in the SeT of Wt animals (0.7832 ± 0.08402 vs. 1.000 ± 0.06494 in Wt control group, $p<0.05$, Figure IV.3B). No statistically significant differences were observed on the expression levels of MCT4 in response to TBHP treatment in the Tg-RGN rats (Figure IV.3B). Overall, MCT4 expression was significantly lower in the SeT of Tg-RGN rats (0.6117 ± 0.1230 in Tg-RGN control vs. 1.000 ± 0.06494 in Wt control, $p<0.05$; 0.5137 ± 0.1380 in TBHP-treated Tg-RGN vs. 1.000 ± 0.06494 Wt control, $p>0,05$, Figure IV.3B).

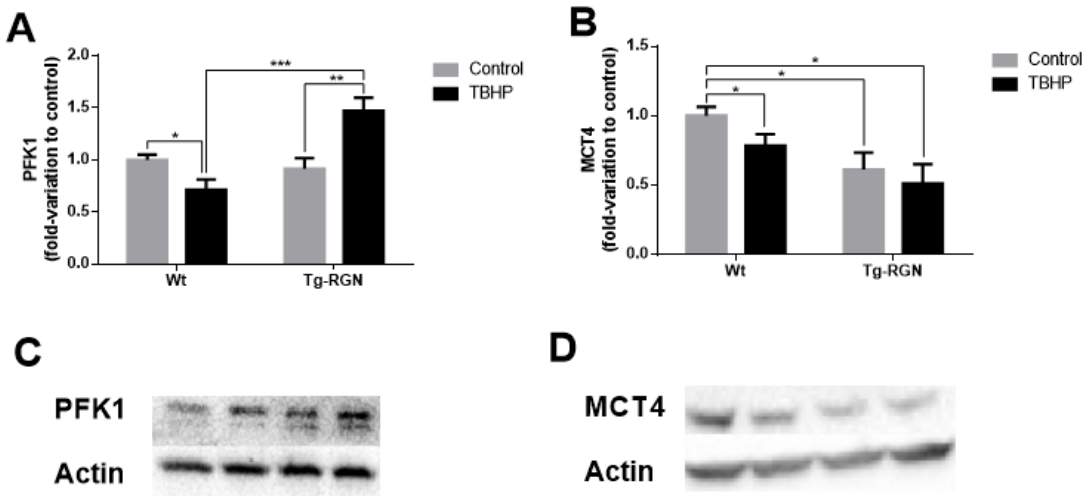

Figure IV.3 PFK1 (A) and MCT4 (B) expression in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with □-actin ($n\geq4$ in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$. Representative immunoblots for PFK1 and MCT4 are shown in panels C and D, respectively.

c. LDH expression levels had no significant differences among experimental groups but LDH activity was increased in the SeT of Tg-RGN animals in the presence of TBHP

LDH is the enzyme responsible for the reversible conversion of pyruvate into lactate [196], the main energy substrate used by the germ cells [197]. Therefore, it is of uttermost importance to evaluate the protein expression levels and enzymatic activity of LDH in the SeT of Wt and Tg-RGN animals, in the presence or absence of TBHP.

The expression levels of LDH were not significantly different among the different experimental groups, not depending on TBHP treatment or RGN overexpression (Figure IV.4A). However, the activity of LDH was found to be significantly higher in the SeT of TBHP-treated Tg-RGN animals when compared with the control group (3.553 ± 0.3603 vs. 1.991 ± 0.2270 μg/μL, $p<0.05$, Figure IV.4B). This response do not occurred in the Wt animals, with TBHP inducing no effect on LDH activity. (Figure IV.4B). In this way, LDH activity was higher in the SeT of TBHP-treated Tg-RGN animals compared with Wt treated-animals (3.553 ± 0.3603 vs. 2.001 ± 0.1739 μg/μL, $p<0.05$, Figure IV.4B).

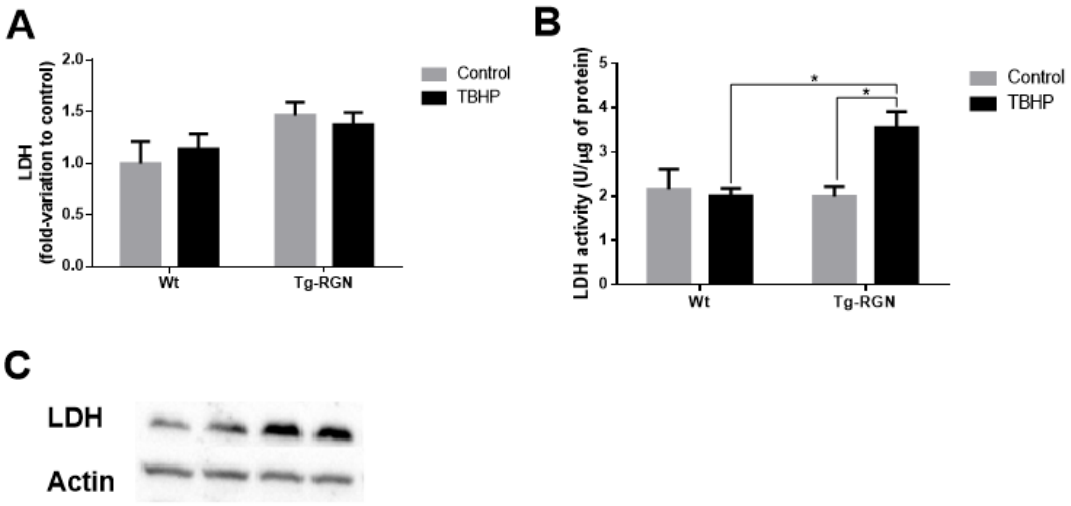

Figure IV.4 LDH expression (A) and activity (B) in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with □-actin (n≥4 in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$. A representative immunoblot is show in panel C.

d. TBHP increased the expression levels of ALT in the SeT of Wt animals whereas ALT expression was diminished in Tg-RGN

Besides the activity of LDH, pyruvate can be obtained by other pathways including its metabolization from alanine in a reaction catalyzed by the ALT enzyme, that plays an important role as an intermediary in the metabolism of glucose and amino acids [97]. ALT expression levels were significantly increased in the SeT of TBHP-treated Wt animals (1.802 ± 0.3177 vs. 1.000 ± 0.1879 in the control, $p<0.05$, Figure IV.5A). Contrastingly, TBHP treatment significantly decreased ALT expression in the SeT of Tg-RGN animals (0.6945 ± 0.1294 vs. 1.018 ± 0.09122 in Tg-RGN control group, $p<0.05$). Inherently, Tg-RGN treated animals displayed lower ALT expression compared with the SeT of Wt animals treated with TBHP (0.6945 ± 0.1294 vs. 1.802 ± 0.3177, $p<0.01$, Figure IV.5A).

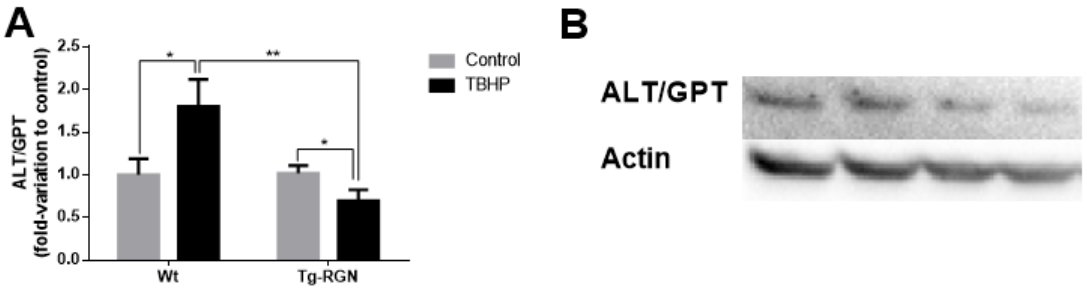

Figure IV.5 ALT/GPT expression (A) in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 µM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with ¤-actin ($n\geq4$ in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$, ** $p<0.01$. A representative immunoblot is show in panel B.

3. TBHP increased AMPK expression in the SeT of Wt animals

AMPK is a central regulator of energy homeostasis that is activated in situations of glucose deprivation. [198]. Once activated AMPK suppresses biosynthetic pathways and activates ATP-generating pathways [99, 199].

TBHP-treatment strongly increased AMPK expression levels in the SeT of Wt animals (2.549 ± 0.3583 vs. 1.000 ± 0.1484, $p<0.01$, Figure IV.6A). No significant differences were observed on the expression levels of AMPK in response to TBHP treatment in the Tg-RGN animals (Figure IV.6A). Thus, AMPK expression in the SeT of TBHP-treated Tg-RGN animals was significantly lower compared with the Wt treated-animals (1.580 ± 0.1858 vs. 2.549 ± 0.3583, $p<0.05$, Figure IV.6A). Interestingly, the AMPK content was higher in the SeT of Tg-RGN rats when compared with the Wt counterparts (1.698 ± 0.08185 vs. 1.000 ± 0.1484, $p<0.01$, Figure IV.6A).

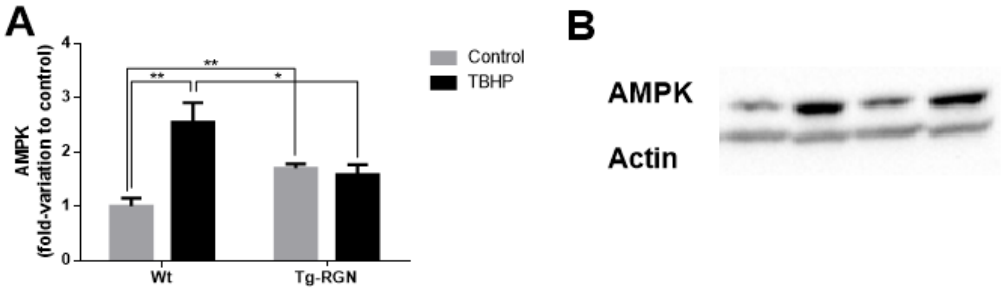

Figure IV.6 AMPK (A) in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with □-actin (n≥4 in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$, ** $p<0.01$. Immunoblot is show in panels B.

4. GLS expression was increased in the SeT of Wt animals and decreased in Tg-RGN animals in the presence of TBHP

Glutamine is one of the most abundant nonessential amino acid presents in cells. Although SCs preferentially use glucose, it has been shown that the single oxidation of glutamine can yield as much as the energy required by SCs. GLS is the mitochondrial enzyme responsible for the metabolization of glutamine into glutamate and ammonia [200]. GLS protein expression was significantly augmented in the SeT of Wt animals in response to TBHP treatment (1.564 ± 0.2437 vs. 1.000 ± 0.1262 in the Wt control group, $p<0.05$, Figure IV.7A).

However, the GLS expression levels were significantly decreased in the SeT of TBHP-treated Tg-RGN animals when compared to the control group (0.6705 ± 0.03396 vs. 1.059 ± 0.09323, $p<0.01$, Figure IV.7A). As a consequence, the GLS expression levels were significantly lower in the SeT of treated-Tg-RGN animals compared with the Wt animals in the same condition (0.6705 ± 0.03396 vs. 1.564 ± 0.2437, $p<0.01$, Figure IV.7A).

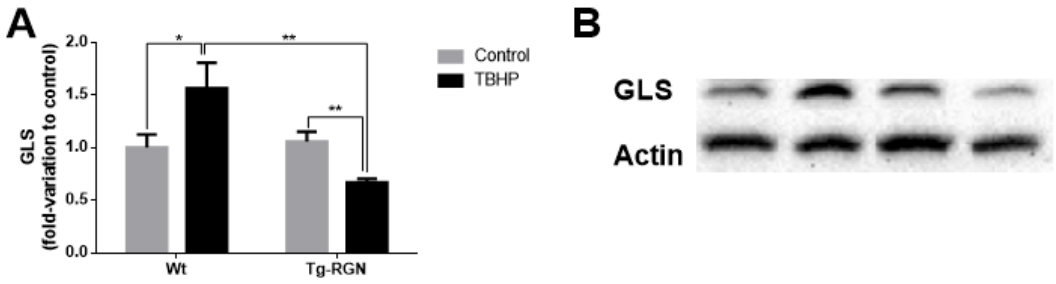

Figure IV.7 GLS (A) expression in the SeT of Wt and Tg-RGN animals cultured in the presence or absence of TBHP (250 μM) for 24 hours. Data are presented as mean ± S.E.M. after normalization with □-actin (n≥4 in each group). Results are expressed as fold-variation relatively to Wt untreated-SeT. * $p < 0.05$, ** $p<0.01$. A representative immunoblot is show in panels B.

V. Discussion

Oxidative stress is a major concern for male fertility. ROS generated by exogenous or endogenous processes have an important physiological role in the testis [201], however when the antioxidant defense system fails, or ROS production is abnormally enhanced, OS occurs. This condition affects the spermatogenic process by disrupting germ cells development and proper sperm function [115]. OS can also induce metabolic alterations, which can affect the appropriate nutritional support required by germs cells being in this way harmful for male fertility.

Several studies showed that TBHP is an OS inducer [115, 131, 132] and its capability deregulating metabolism, namely the pentose phosphate pathway, also has been described [133]. In a previous study, we have shown that TBHP can induce alterations in the antioxidant defense system in rat SeT and augment the apoptotic rates [115]. Moreover, we demonstrated that RGN can counteracts these effects [115]. Our research group also have shown that RGN can modulate the metabolism of SCs [85], as well as modulating the antioxidant enzymes increasing protection against OS [115].

In this dissertation, we investigated the effect of TBHP as a metabolic disruptor, and the role of RGN maintaining the testicular metabolic profile. We started by analyzing the glycolytic metabolism in the SeT of Tg-RGN and Wt animals in the presence or absence of TBHP. The results obtained showed that the intracellular content of glucose tended to be increased upon treatment with TBHP both in Wt and Tg-RGN animals (Figure IV.1A). Similar results were obtained for the intracellular content of lactate, mirroring what was observed for glucose (Figure IV.1B). These findings are supported by previous reports showing that lactate production is increased under OS conditions [202]. In the scenario of SeT, this is quite relevant as lactate has been reported as an anti-apoptotic factor for germ cells [203]. Also, it was described that the addition of exogenous lactate limits the loss of germ cells and lowers the number of degenerating germ cells [204]. Therefore, it is liable to assume that the increased lactate levels can be a response to counteract apoptosis induced by TBHP.

Overall, regardless of TBHP treatment, the SeT of Tg-RGN rats displayed lower intracellular content of glucose and lactate, suggesting that RGN can suppress glycolytic metabolism. Accordingly, an *in vitro* study using primary SCs cultures showed that the SCs isolated from the testis of Tg-RGN animals displayed less intracellular glucose than those of Wt homologous [85]. Moreover, the role of RGN suppressing glycolytic metabolism was reported already in the prostates of Tg-RGN animals, which showed reduced glucose and lactate contents [187]. RGN *in vivo* overexpression was also associated with lower levels of glucose in the liver and brain [187].

Glucose enters the cell by specific transporters, the GLUTs [79]. Several GLUTs isoforms have been identified, but GLUT1 and GLUT3 have been assigned as those working together in glucose incorporation in the SeT [82].

In our experimental conditions, TBHP increased GLUT1 expression in the SeT of the Wt animals, and GLUT3 both in Wt and Tg-RGN animals (Figures IV.2A and IV.2B). These results were in agreement with the higher intracellular glucose content measured in the SeT after

treatment (Figure IV.1A). Moreover, the increased expression of GLUT1 and GLUT3 in response to OS induced by TBHP followed other previous studies showing the augment of these transporters expression in brain and trophoblast cells in stressful conditions of hypoxia with increased ROS levels [205-207].

Interestingly, the SeT of TBHP-treated Tg-RNG animals showed less expression of GLUT1 then the Wt in the same condition, which was accompanied by lower intracellular content of glucose also (Figures IV.1A and IV.2A). Contrastingly, GLUT3 expression was higher in the SeT of TBHP-treated Tg-RGN animals (Figure IV.2B). Also, it was described before that the SCs isolated from the testis of Tg-RGN rats displayed higher GLUT3 expression of GLUT3 [85].

After entering the cells, glucose suffers a series of reactions until being converted to pyruvate, the end product of glycolysis. PFK1 is considered the gatekeeper of glycolysis as it catalyzes the conversion of F6-P into F1,6-BP [89]. Exposure to TBHP altered differentially the expression levels of PFK1 in the SeT of Wt and Tg-RGN. PFK1 expression was diminished in the SeT of Wt animals upon treatment but increased in Tg-RGN (Figure IV.3A). An increase of ROS was shown to enhance the gene expression of p53, which, in turn, can activate other genes increasing the generation of ROS. This leads to a feedback loop culminating in apoptosis [208]. However, other studies have described that p53 can also activate a protein responsible for lowering the ROS, the TP53-induced glycolysis and apoptosis regulator (TIGAR). The increase of TIGAR expression was shown to inhibit PFK1 by altering the concentration of fructose 2,6-bisphosphate [209]. TIGAR protein is present in testis and its expression was increased in testicular ischemia-reperfusion injury [71]. This can explain the decreased levels of PFK1 found in the SeT of TBHP-treated Wt animals. Relatively to the increased levels of PFK1 in the SeT of TBHP-treated Tg-RGN animals no definitive conclusion can be drawn at the moment [210].Nevertheless, increased expression levels of PFK1 were described in the SCs of Tg-RGN animals [85], though here no significant difference was found between Wt and Tg-RGN control groups, which could be related with the fact that we are analyzing the whole SeT.

In the testis, namely in SCs, the pyruvate generated from glycolysis mainly is converted into lactate by the activity of LDH. TBHP treatment did not change the expression levels of LDH (Figure IV.4A), but increased LDH activity in the SeT of Tg-RGN (Figure IV.4B). It is known that TBHP can induce the leakage of LDH from the cell [131], which somehow can explain that no differences were found on LDL expression between control and treated-animals.

Increased LDH activity in the TBHP-treated Tg-RGN supports the augmented lactate content found in the SeT of these animals (Figure IV.1B). As discussed before, lactate is an anti-apoptotic factor for the germ cells, thus, increased LDH activity and augmented lactate in the SeT of TBHP-treated Tg-RGN animals would be viewed as a protective mechanism driven by RGN to counteract the apoptotic effects of TBHP.

Despite increasing lactate content in the SeT of both Wt and Tg-RGN animals, TBHP do not affected LDH expression and only augmented LDH activity in Tg-RGN, which indicates that the lactate being accumulated in the SeT of Wt animals can be obtained from pyruvate generated

by other sources than glycolysis. Indeed, pyruvate can be obtained from alanine by the action of ALT. Note of worthy, TBHP increased ALT expression in the SeT of the Wt animals but not in Tg-RGN (Figure IV.5A), which have lactate production justified by the increased LDH activity. Therefore, these results indicate that the higher intracellular lactate content measured in the SeT of Wt animals after TBHP treatment (Figure IV.1B), can result from the activity of ALT. Also, several studies have reported an increase in the ALT levels under OS conditions [211, 212]. In opposition to Wt, Tg-RGN rats are known to maintain lower levels of OS in the SeT upon TBHP treatment [115], which also supports the increased activity of ALT to generate lactate in these group. To further confirm this possibility would be determinant in the future to measure the intracellular contents of alanine and pyruvate.

The lactate produced is mainly exported to the extracellular space by MCT4. MCT4 expression was decreased in the SeT of Wt animals after TBHP treatment (Figure IV.3B), which further supports the measured higher content of lactate (Figure IV.1B). The results obtained for the MCT4 expression in the SeT of Wt and Tg-RGN also followed the previous findings in rat prostate, which showed a reduced expression of this MCT under over expression of RGN [187]. MCT1 is another MCT isoform involved in lactate transport, which is known to be expressed in mice and rat testis [93]. Thus, it cannot be excluded that MCT1 is being mediating the lactate handling in the SeT of both Wt and Tg-RGN rats.

AMPK is a central regulator of glycolytic metabolism and energy supply that is activated under certain conditions as a cell adaptive response. When activated AMPK suppresses the biosynthetic pathways and activate the ATP-generating pathways like glycolysis [99]. TBHP treatment augmented AMPK expression in the SeT of Wt animals (Figure IV.7A), but no effect was observed in the Tg-RGN group. AMPK is activated when the ATP levels are low [100], but can also be activated by an increase of ROS [213], switching the cellular metabolism to catabolic pathways, and maintaining the metabolic homeostasis [214]. Evidences exist that OS activates AMPK with the up-regulation of glycolysis [213, 214]. The results obtained suggest that the increase of OS after TBHP treatment can promote the augmented expression of AMPK in the SeT of Wt animals, which do not happened in the SeT of TBHP-treated Tg-RGN because they present lower levels of OS [115]. Nevertheless, it would be fundamental determined if these alterations on AMPK expression are underpinned by its altered activity.

Glutamine is one of the most abundant nonessential amino acids that also can be an energy substrate by its conversion into glutamate and in ammonium by the action of the mitochondrial enzyme GLS [104]. TBHP increased GLS expression levels in the Set of Wt group whereas decreasing it in Tg-RGN (Figure IV.6A), which suggest that TBHP also disrupted the glutaminolysis profile and the normal energy supply to germ cells. Further research is needed, namely, quantifying glutamine in the SeT to confirm this fact. Also, would be interesting to analyze the expression levels of ASCT2, the amino acids transporter responsible for the up-take of glutamine.

Besides being an energy substrate, glutamine is also associated with OS homeostasis. It is a precursor of GSH a critical non-enzymatic antioxidant widely present in different cell types

[215]. GLS activity is important in this process as it converts glutamine in glutamate, which is required for GSH synthesis [216]. For this reason, inhibition of GLS has been shown related with increased OS [216]. Even though GLS expression was increased in the Wt-treated animals and decreased in Tg-RGN further research is needed to clarify the effect of this compound in the activity of GLS and the role of RGN in this pathway.

VI. Conclusion and Future Perspectives

The present dissertation evidenced the activity of TBHP as a metabolic disruptor in testicular cells, as well as pointing out RGN as a protective factor against some of the damaging effects of this compound.

TBHP disrupted the glycolytic metabolism in rat SeT tubules in culture increasing the intracellular content of glucose and lactate, by altering the expression of several modulators of glycolytic metabolism, an effect that was also observed under conditions of RGN overexpression. However, the higher expression levels of RGN were associated with the suppression of glycolytic metabolism regardless of TBHP treatment. Moreover, RGN appeared to reduce some of the TBHP effects deregulating the same metabolic players. For example, Tg-RGN animals displayed increased LDH activity, which supports the higher lactate content. This can be related with lower apoptosis of germ cells as lactate, besides being an energy substrate, also is an anti-apoptotic factor. Overall, the findings obtained herein highlight the RGN role as a metabolic regulator in SeT, which could have importance in the context of spermatogenesis and male (in)fertility.

Although the increase in ROS production and OS have been already linked with a metabolic deregulation that affects the nutritional support of cells, this was the first study demonstrating the metabolic actions of the OS inducer TBHP in SeT.

Besides affecting glycolysis, TBHP also altered the expression of ALT and glutaminolysis. However, and interestingly, the observed effects were distinct in Wt and Tg-RGN animals. ALT expression levels were increased in response to TBHP in Wt animals whereas decreasing in Tg-RGN, which may suggest a mechanism to decrease the effects of TBHP. In fact, the clarification of the precise mechanisms by which TBHP alters ALT activity is an aspect of uttermost importance to be addressed in the future. The altered ALT expression also indicated that the lactate present in the SeT could be generated from pyruvate obtained via alanine. Thus, the understanding of other pathways related whit lactate production in SeT should deserved further attention.

Concerning glutaminolysis, namely GLS expression, the same pattern of response seen for ALT was observed. GLS expression was increased in the Wt-treated animals whereas decreasing in Tg-RGN. Taking in account that GLS produces glutamate that is a precursor for the synthesis of GSH, an antioxidant factor responsible for the detoxification of TBHP, becomes evident that further research is needed to clarify the effect of this compound in the activity of GLS. Also, a deep analysis of glutamine metabolism evaluating the expression of other regulators and enzyme activities in this pathway would be crucial to clarify the regulatory and protective mechanism that would be driven by RGN.

Male fertility has become a serious health concern over the last years and OS is widely known as a major damaging factor for spermatogenesis, being a strong inducer of germ cells death. Therefore, evaluating the actions of disruptors of the "healthy" OS is continuously needed, and its liaison with metabolism is a research goal of paramount relevance. This study is a useful basis opening new perspectives of research to a better understanding of the relationship between OS, metabolic alterations and RGN actions in the SeT. Ultimately, it

would contribute to the development of new and more efficient fertility-preserving strategies and treatments.

VII.References

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