UNIVERSIDADE DA BEIRA INTERIOR
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Effects of 17β-Estradiol and Endocrine Disruptor Methoxychlor in Spermatogonial Stem Cells: a Protective Effect of Regucalcin?

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"Live as if you were to die tomorrow.
Learn as if you were to live forever."

— *Mahatma Gandhi*

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Resumo

Os disruptores endócrinos químicos (EDCs) englobam um conjunto de compostos presentes no meio ambiente, de origem natural ou produzidos pelo homem, e que interferem com a função endócrina através da alteração do metabolismo, síntese e/ou mecanismos de ação hormonal. Nos últimos anos, os estrogénios têm-se revelado importantes reguladores do destino das células germinativas, embora, o efeito destas hormonas na espermatogénese continue a ser alvo de controvérsia. Ainda assim, a informação existente suscita a preocupação relativamente aos efeitos dos EDCs com comportamento estrogénico. O metoxicloro (MXC) é um inseticida extremamente utilizado no sector agrícola, tratando-se de um EDC que atua mimetizando as ações dos estrogénios (xenoestrogénio). Apesar da sua capacidade em influenciar o sistema reprodutor masculino já ter sido demonstrada, não se conhece qual o impacto deste EDC na população de espermatogónias estaminais (SSCs). As SSCs constituem a população de células estaminais adultas presentes no testículo, tendo capacidade de auto-renovação e altas taxas de diferenciação, o que faz com que a sua atividade biológica seja o alicerce da espermatogénese. Assim, qualquer distúrbio que possa ter um efeito nefasto na população de SSCs terá um impacto quantitativo e qualitativo na produção de espermatozóides e, consequentemente, na fertilidade masculina.

A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que tem sido associada com o controlo da proliferação celular, stress oxidativo, apoptose e metabolismo. Além disso, foi sugerido o papel protetor desta proteína sobre as células germinativas expostas a fatores nocivos, como por exemplo indutores de stress oxidativo e apoptose, congelamento e radiação. Deste modo, é presumível que a RGN possa ter um comportamento semelhante contra as ações dos EDCs nas SSCs.

Na presente dissertação, foi estudado o impacto do 17β-estradiol (E_2) e do MXC no metabolismo glicolítico e na sobrevivência/apoptose das SSCs, assim como a influência da RGN na possível atenuação destes efeitos. Com este intuito, uma linha celular de espermatogónias estaminais de rato (GC-6spg), transfetada de modo a sobrexpressar RGN (GC-6spg/RGN), foi mantida em cultura. Após confirmação da sobrexpressão da RGN através de *Western blot* e imunofluorescência, as células GC-6spg/RGN e as células transfetadas somente com o "vetor vazio" (GC-6spg/Mock) foram expostas a 100 nM de E_2 ou 25 μM de MXC durante 48 horas. De seguida, avaliou-se o consumo de glicose e a produção lactato, assim como a expressão e atividade de reguladores do metabolismo glicolítico e da apoptose através de ensaios espectrofotométricos e *Western blot*.

Os resultados obtidos mostraram um aumento da atividade glicolítica nas células GC-6spg que sobrexpressavam RGN (GC-6spg/RGN), comparativamente com células com expressão basal (GC-6spg/Mock), inclusivamente na presença de E_2 ou MXC, tendo sido observado um aumento no consumo de glicose e produção de lactato. Por sua vez, o tratamento com E_2 não afetou o

metabolismo glicolítico das células GC-6spg. No entanto, no caso do tratamento com MXC foi constatado um considerável aumento no fluxo glicolítico, efeito que foi atenuado pela sobrexpressão de RGN.

No que diz respeito à apoptose, as células GC-6spg/RGN apresentaram uma diminuição na apoptose, comparativamente com as GC-6spg/Mock, nomeadamente pela diminuição do rácio entre as proteínas Bax (proapoptótica)/Bcl-2 (anti-apoptótica), expressão da p53 e atividade da caspase-3. O tratamento com E_2 pareceu diminuir a taxa apoptótica das células GC-6spg, ao passo que no tratamento com MXC as taxas de apoptose estavam aumentadas. De um modo geral, a sobrexpressão da RGN contrariou os efeitos do E_2 e do MXC na apoptose destas células.

O presente estudo é o primeiro a evidenciar a modulação do metabolismo e da apoptose das SSCs por fatores hormonais, nomeadamente o E_2 e o EDC com propriedades xenoestrogénicas, MXC. De facto, o MXC alterou consideravelmente o estado apoptótico e o metabolismo das células GC-6spg, ao passo que o tratamento com E_2 apresentou efeitos moderados. Além disso, a RGN foi identificada como um possível fator de proteção contra os efeitos nocivos do MXC nas células GC-6spg. Apesar de ainda numa fase inicial, os resultados obtidos enfatizam o possível impacto negativo que a exposição ao MXC pode ter na população celular de SSCs, com eventual comprometimento da fertilidade masculina.

Palavras-Chave

Apoptose, Espermatogónias estaminais, Metabolismo, Metoxicloro, Regucalcina, 17β-estradiol

Resumo Alargado

Os disruptores endócrinos químicos (EDCs) englobam um conjunto de compostos presentes no meio ambiente, de origem natural ou produzidos pelo homem, e que interferem com a função endócrina através da alteração do metabolismo, síntese e/ou mecanismos de ação hormonal. Tendo em conta o seu modo de ação fisiológica os EDCs podem ser classificados em quatro categorias diferentes: xenoandrogénios e xenoestrogénios, que mimetizam a ação dos androgénios e estrogénios, respetivamente; e antiandrogénios e antiestrogénios, os quais antagonizam a ação destas hormonas. No caso dos xenoestrogénios, estes são assim compostos químicos que interferem com os mecanismos endócrinos mimetizando a ação estrogénica, podendo ligar-se aos recetores de estrogénio (ERs) como agonistas. De entre a diversidade de substâncias com estas características temos como exemplo os fitoestrogénios, flavonóides, químicos industriais, bisfenilpoliclorados, éteres bifenil polibromados, alguns medicamentos sintéticos como o dietilestilbestrol, plastificantes como o bisfenol A, filtros UV, conservantes, pesticidas e inclusivamente metais como o cádmio. A exposição a estes compostos é uma constante na nossa atividade diária, seja através da ingestão de alimentos e águas contaminadas, assim como pelas partículas presentes no ar, não excluindo outras vias como o contacto com a pele.

Nos últimos anos, os estrogénios têm-se revelado importantes reguladores do destino das células germinativas, embora, o efeito destas hormonas na espermatogénese continue a ser alvo de controvérsia. Se há estudos que identificam os estrogénios como fatores de sobrevivência, outros há que reportam a sua ação como indutores da apoptose na linha germinativa. Ainda assim, a informação existente suscita a preocupação relativamente aos efeitos dos EDCs com comportamento estrogénico.

O metoxicloro (MXC) é um inseticida extremamente utilizado no sector agrícola, tratando-se de um EDC que atua mimetizando as ações dos estrogénios, sendo portanto classificado como um xenoestrogénio. Alguns estudos têm descrito a capacidade do MXC em influenciar o sistema reprodutor masculino, nomeadamente ao nível da viabilidade, motilidade e número de espermatozóides, chegando mesmo a causar a inibição da espermatogénese em alguns casos. No entanto, não se conhece qual o impacto deste EDC na população de espermatogónias estaminais (SSCs). As SSCs constituem a população de células estaminais adultas presentes no testículo, tendo capacidade de auto-renovação e altas taxas de diferenciação, o que faz com que a sua atividade biológica seja o alicerce da espermatogénese. Assim, qualquer distúrbio que possa ter um efeito nefasto na população de SSCs terá um impacto quantitativo e qualitativo na produção de espermatozóides e, consequentemente, na fertilidade masculina.

A proteína de ligação ao cálcio (Ca^{2+}) regucalcina (RGN) regula o transporte de Ca^{2+} através da membrana plasmática e dos organelos celulares, nomeadamente, mitocôndria, reticulo

endoplasmático e inclusivamente o núcleo, controlando assim os níveis intracelulares deste ião. A RGN tem também a capacidade de interagir com enzimas dependentes do Ca^{2+}, tais como tirosina cinases, fosfatases, fosfodiesterases e óxido nítrico sintase, influenciando assim também a sinalização intracelular. As funções da RGN têm sido associadas ao controlo da proliferação celular, stress oxidativo, apoptose e metabolismo. Além disso, estudos *in vivo* e *in vitro* têm sugerido que a RGN pode ter um papel protetor sobre as células germinativas expostas a fatores nocivos, como por exemplo indutores de stress oxidativo e apoptose, congelamento e radiação. Deste modo, é presumível que a RGN possa ter um comportamento semelhante contra as ações dos EDCs nas SSCs.

Na presente dissertação, foi estudado o impacto do 17β-estradiol (E_2) e do MXC no metabolismo glicolítico e na sobrevivência/apoptose das SSCs, assim como a influência da RGN na possível atenuação destes efeitos. Com este intuito, uma linha celular de espermatogónias estaminais de rato (GC-6spg), transfetada de modo a sobrexpressar RGN (GC-6spg/RGN), foi mantida em cultura. Após confirmação da sobrexpressão da RGN através de *Western blot* e imunofluorescência, as células GC-6spg/RGN e as células transfetadas somente com o "vetor vazio" (GC-6spg/Mock) foram expostas a 100 nM de E_2 ou 25 μM de MXC durante 48 horas, de modo a mimetizar as elevadas concentrações intratesticulares desta hormona observadas em indivíduos inférteis. No caso do MXC, a concentração escolhida teve por base outros estudos que também avaliaram o efeito de exposição a este composto em modelos celulares. Nos diferentes grupos experimentais com estimulação com E_2 ou MXC e controlos, foi avaliado o consumo de glicose e a produção lactato, assim como a expressão e atividade de reguladores do metabolismo glicolítico e da apoptose através de ensaios espectrofotométricos e *Western blot*.

Os resultados obtidos mostraram um aumento da atividade glicolítica nas células GC-6spg que sobrexpressavam RGN (GC-6spg/RGN), comparativamente com células com expressão basal (GC-6spg/Mock), inclusivamente na presença de E_2 ou MXC, tendo sido observado um aumento no consumo de glicose e produção de lactato. Por sua vez, o tratamento com E_2 não afetou o metabolismo glicolítico das células GC-6spg. No entanto, no caso do tratamento com MXC foi constatado um considerável aumento no fluxo glicolítico, efeito que foi atenuado pela sobrexpressão de RGN.

No que diz respeito à apoptose, as células GC-6spg/RGN apresentaram uma diminuição na apoptose, comparativamente com as GC-6spg/Mock, nomeadamente pela diminuição do rácio entre as proteínas Bax (proapoptótica)/Bcl-2 (anti-apoptótica), expressão da p53 e atividade da caspase-3. O tratamento com E_2 pareceu diminuir a taxa apoptótica das células GC-6spg, ao passo que no tratamento com MXC as taxas de apoptose estavam aumentadas. De um modo geral, a sobrexpressão da RGN contrariou os efeitos do E_2 e do MXC na apoptose destas células.

O presente estudo é o primeiro a evidenciar a modulação do metabolismo e da apoptose das SSCs por fatores hormonais, nomeadamente o E_2 e o EDC com propriedades xenoestrogénicas,

MXC. De facto, o MXC alterou consideravelmente o estado apoptótico e o metabolismo das células GC-6spg, ao passo que o tratamento com E_2 apresentou efeitos moderados. Além disso, a RGN foi identificada como um possível fator de proteção contra os efeitos nocivos do MXC nas células GC-6spg. Apesar de ainda numa fase inicial, os resultados obtidos enfatizam o possível impacto negativo que a exposição ao MXC pode ter na população celular de SSCs, com eventual comprometimento da fertilidade masculina.

Abstract

Endocrine disrupting chemicals (EDCs) are a set of compounds, either natural or produced by man, that interfere with the endocrine function by altering hormone metabolism, synthesis, and mechanism of action. In the last years, estrogens have emerged as important regulators of germ cell fate, although, the beneficial or detrimental effects of these hormones in spermatogenesis remains controversial, which raised the concern about the EDCs with estrogenic behavior. Methoxychlor (MXC) is an insecticide extensively used in the agricultural sector, which displays endocrine disrupting activity by mimicking estrogens actions (xenoestrogenic). Although it has been proved that MXC can affect the male reproductive function, little is known regarding the impact of this EDC in the spermatogonial stem cell (SSCs) population. SSCs are the adult stem cell population in the testis, having self-renewal capability and high differentiation rates, and its biological activity is the foundation of spermatogenesis. Therefore, any threat disturbing SSCs population can have a detrimental impact on the spermatogenic output and male fertility.

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that has been associated with the control of cell proliferation, oxidative stress, apoptosis, and metabolism. Furthermore, the protective role of RGN for the germ cell population upon exposure to damaging factors, such as oxidative stress, apoptosis inducers, freezing, and radiation has been suggested. So, it is highly likely to hypothesize that RGN may have a similar behavior against EDCs actions in SSCs.

In the present dissertation, the impact of 17β-estradiol (E_2) and MXC on SSCs glycolytic metabolism and survival/apoptosis and the influence of RGN in attenuating their effects were evaluated. For this purpose, a rat spermatogonial stem cell line (GC-6spg) transfected to overexpress RGN (GC6-spg/RGN) was cultured. After confirming RGN overexpression by means of Western blot analysis and immunofluorescence, GC6-spg/RGN cells and mock-transfectants (GC-6spg/Mock) were exposed either to 100 nM of E_2 or 25 μM of MXC for 48 hours. Glucose consumption and lactate production, as well as, the expression and activity of glycolytic metabolism and apoptosis regulators were evaluated by spectrophotometric assays and Western blot analysis.

The results obtained showed an increased glycolytic activity in GC-6spg cells overexpressing RGN (GC-6spg/RGN) compared to the mock-transfectants, regardless of E_2 or MXC treatments, as indicated be the augmented glucose consumption and lactate production. E_2 treatment did not affect the glycolytic metabolism of GC-6spg cells, though, in the case of MXC exposure, an enhanced glycolytic metabolism was shown. Nevertheless, RGN overexpression diminished the effect of MXC.

Concerning apoptosis, it was found that GC-6spg/RGN cells displayed diminished apoptosis compared with mock-transfectants, namely, by the observed diminution of Bax

(proapoptotic)/Bcl-2 (antiapoptotic) protein ratio, p53 expression and caspase-3 activity. E_2 also seems to decrease the apoptotic rate of GC-6spg cells whereas upon MXC treatment apoptosis was increased. Interestingly, overall, RGN overexpression tended to counteract E_2 and MXC effects over apoptosis.

The present study is the first evidence that SSCs metabolism and apoptosis can be modulated by hormonal factors, namely E_2 and the EDC with xenoestrogenic properties, MXC. Indeed, MXC was shown to greatly change the apoptotic status and metabolism of GC-6spg cells, with E_2-treatment displaying mild effects. Furthermore, RGN was identified as a possible protective mechanism against the damaging effects of MXC in GC-6spg cells. Although preliminary, the obtained findings also highlight for the impact that MXC exposure might have disrupting the SSCs population and compromising male fertility.

Keywords

Apoptosis, Metabolism, Methoxychlor, Regucalcin, Spermatogonial stem cells, 17β-estradiol

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List of Abbreviations

Aal	Aaligne
ABP	Androgen binding-protein
AcGFP1	*Aequorea coerulescens* green fluorescent protein
AhR	Arylhydrocarbon receptor
Apr	Apaired
AR	Androgen receptor
As	Asingle
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth-factor
BTB	Blood-testis barrier
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate
CO_2	Carbon dioxide
CREB-1	cAMP responsive element-binding protein 1
DHT	5α-dihydrotestosterone
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E_2	17ß-estradiol
ECL	Enhanced chemiluminescence
EDC	Endocrine disrupting chemical
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor 1
ERR	Estrogen related receptor
ERα	Estrogen receptor α
ERαKO	Estrogen receptor α knockout
ERß	Estrogen receptor ß
ERßKO	Estrogen receptor ß knockout
Fas-L	Fas ligand
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC-6spg	Gonadal cell-6 spermatogonia
GC-6spg/Mock	Gonadal cell–6 spermatogonia transfected with empty vector
GC-6spg/RGN	Gonadal cell–6 spermatogonia transfected with regucalcin
GDNF	Glial cell-derived neurotrophic factor
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
GPER	G protein-coupled estrogen receptor
G418	Geneticin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF-1	Insulin growth factor 1

IL1α	Interleukin-1α
IRES	Internal ribosomes entry site
LDH	Lactate dehydrogenase
LC	Leydig cell
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LIF	Leukemia inhibitor factor
MCS	Multiple cloning site
MCT	Monocarboxylate transporter
MEM	Minimum Essential Medium
miRNA	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
MXC	Methoxychlor
NAD	Nicotinamide adenine dinucleotide
NR	Nuclear receptor
PDGF	Platelet-derived growth factor-BB
PFK1	Phosphofructokinase 1
PGC	Primordial germ cell
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome-proliferator activated receptor
P_{PGK}	Murine phosphoglycerate kinase promoter
$Puro^r$	Puromycin resistance gene
PVDF	Polyvinylidene difluoride
PXR	Pregnane X receptor
RGN	Regucalcin
RIPA	Radioimmunoprecipitation assay
SC	Sertoli cell
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SeT	Seminiferous tubule
SSC	Spermatogonial stem cell
T	Testosterone
TCA	Tricarboxylic Acid
TGF	Transforming growth factor
Tg-RGN	Transgenic rats overexpressing regucalcin
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor α

I. Introduction

1. General Overview of mammalian Spermatogenesis

a. The Spermatogenic Process

Testis, or the male gonad, is the essential organ of the male reproductive system, producing testosterone and spermatozoa, thus having endocrine and exocrine functions, respectively (1). Each testis has an ovoid shape and is located in the scrotum in order to provide a cooler environment compared to body temperature (1-2 ºC below) to support spermatogenesis (2). The testicles are enclosed by *tunica albuginea*, a white and inextensible fibrous capsule that runs inside of the testis walls, forming septa, which separates the testicular tissue into lobules (figure 1) (1). The functional testicular tissue within the lobules is called testicular parenchyma and is constituted by the seminiferous tubules (SeT), Leydig cells (LCs) and Sertoli cells (SCs) (1). The SeT, surrounded by a wall of collagen fibers and myofibroblastic cells (*lamina propria*) containing peritubular cells (3), are the site where spermatogenesis occurs. The SeT epithelium is separated from the *lamina propria* by a basal membrane and includes all stages of germ cell differentiation, as well as the somatic SCs (3). SeT are convoluted tubules ending in straight portions that build up the rete testis, which establishes communication with the efferent ductules (1). The interstitial compartment, residing between SeT, contains a great diversity of cells and structures such as LCs, leukocytes, macrophages, mesenchymal cells, nerves and blood vessels (3). On the posterior surface, the testicles are associated with the epididymis and spermatic cord, the latter incorporating the ductus deferens. The epididymis is a highly compartmentalized organ to where the efferent ductules converge, and can be divided into three distinct regions, the *caput*, *corpus* and *cauda* (figure 1) (4).

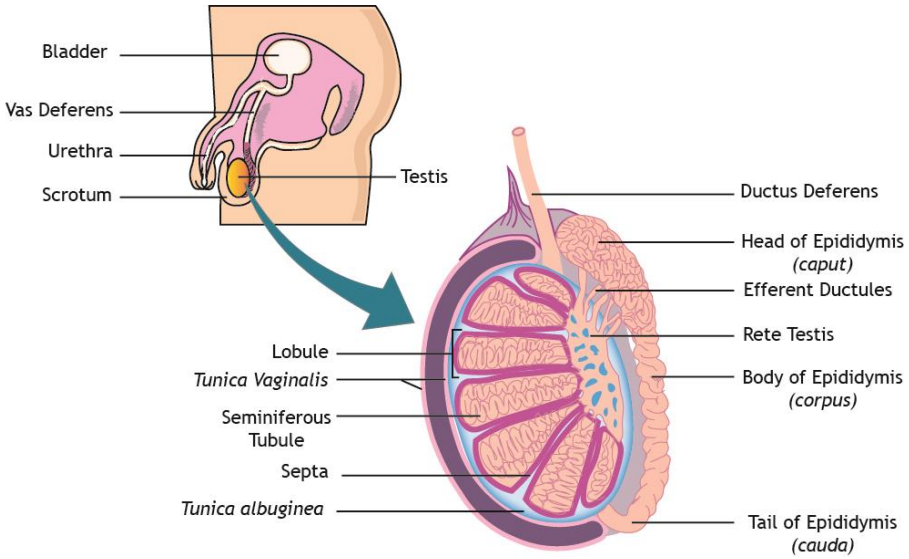

Figure 1. Schematic representation of the mammalian testis and associated structures. Testis is covered by *tunica vaginalis* (externally) and *tunica albuginea* (internally), the latter runs inside the testis wall forming septa, which subdivide the testis in lobules. Seminiferous tubules (SeT) are present within the lobules, converging to the rete testis. Rete testis is connected to efferent ductules which, in turn, are connected to the head of epididymis. The three major structures of the epididymis are represented (*caput, corpus* and *cauda*), followed by the ductus deferens.

Mammalian spermatogenesis is a complex and extremely coordinated process involving cell division and differentiation of spermatogonial stem cells (SSCs) that culminates with the production of male gametes, the spermatozoa (3).

During the period of gestation, fetal SCs surround and aggregate with primordial germ cells (PGCs) to form the seminiferous cords (5). PGCs attach to the basal membrane of the SeT and start calling SSCs. These cells are able to divide mitotically and colonize the testis (3). SSCs are large cells with a big round nucleus and a large amount of cytoplasm until puberty, when spermatogenesis takes place (3). SSCs self-renewal and its differentiation into spermatogonia have a key role in the maintenance of spermatogenesis cycle (6). The spermatogenic process begins at the basal compartment and moves towards the lumen of the SeT (6).

Each spermatogenic cycle involves three main phases: proliferation of spermatogonia by mitotic division, spermatocytes meiosis and differentiation of haploid spermatids (6).

Following the beginning of spermatogenesis, different stages of germ cell differentiation surrounded by the SC cytoplasm are found within the seminiferous epithelium (figure 2) (3).

Spermatogonia are diploid cells and, as aforementioned, divide by mitosis originating two cell types (A and B). Type A spermatogonia remains close to the tubule wall as stem cells, and maintaining the spermatogonia population in the testis and thus, having a determinant role preserving male fertility (7). Type B spermatogonia are committed to differentiate. These cells enter meiosis, an essential step in germ cell development, that allows diploid cells (2n) become haploid (n) (3). Meiosis takes place in two consecutive cell divisions, each one comprising prophase, metaphase, anaphase, and telophase (I and II) (3). Prophase I starts with a replication of deoxyribonucleic acid (DNA) in B spermatogonia, they detached from basal membrane and are now called primary spermatocytes. This primary spermatocytes are diploid with a doubled chromatin (3). Each primary spermatocyte divides into two secondary spermatocytes, these last are haploid round cells with the sister chromatids paired and heterochromatic nuclei (8). Secondary spermatocytes enter second meiotic division, the sister chromatids are separated and four round spermatids are generated, staying within the seminiferous epithelium during a short period of time (1-2 days) (8). Spermatids are haploid cells with a single chromatin, a round and homogenous nucleus and an early forming acrosome vesicle (3).

At this stage, spermatids are ready to proceed to the final stage of spermatogenesis, spermiogenesis, i.e. the differentiation of round to elongated spermatids and afterwards into mature spermatozoa (3).

The seminiferous cycle ends with the release of mature elongated spermatids into the tubular lumen, being now called spermatozoa or sperm (3). This process is called spermiation.

Spermiation is a critical determinant of the quantity of sperm that go into the epididymis and therefore present in the ejaculate (9).

In theory, one spermatogonia origins four spermatids, but, due to germ cell loss during meiosis in man (10), only two spermatids are originated by spermatogonia (11). The mean daily sperm production (DSP) is about 6 x 10^6 sperm per gram testis tissue and day (11).

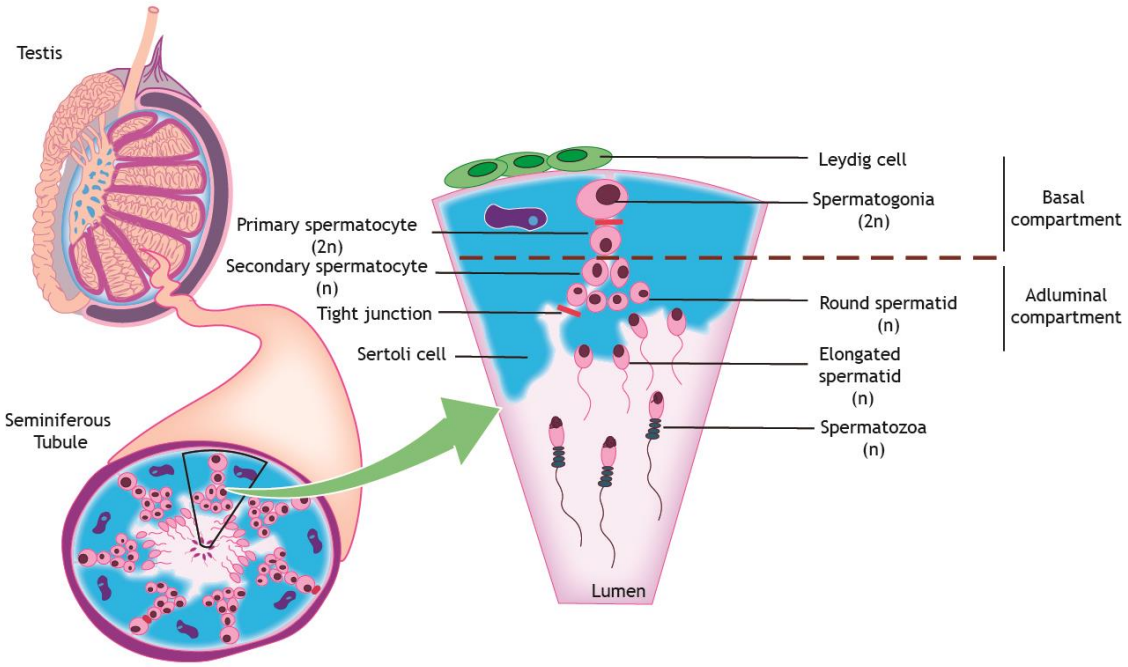

Figure 2. Schematic representation of the testicular histology and mammalian spermatogenesis. Spermatogenesis occurs in the seminiferous tubules (SeT) in close contact to the only somatic cell type within the tubules, the Sertoli cells (SCs); Leydig Cells (LCs) reside in the interstitial space between tubules. This process begins with the differentiation of spermatogonia into primary spermatocytes, which then pass the tight junctions formed by adjacent SCs, evolving into secondary spermatocytes. The secondary spermatocytes originate the spermatids, which then suffer a process called spermiogenesis originating the spermatozoa.

b. Spermatogonial Stem Cells (SSCs)

SSCs are the adult stem cell population of the testis, having self-renewal capability and high differentiation rates. The biological activity of SSCs provide the foundation of spermatogenesis.

As it was referred, spermatogonia are subdivided into A (without heterochromatin) and B spermatogonia (abundant heterochromatin) (figure 3) (12). Type A spermatogonia presents two subtypes, Apale and Adark spermatogonia, differing in their nuclear appearance (13). The first one is characterized by a round to ovoid, pale nucleus due to slightly packed euchromatic DNA and they can proliferate either into Apale subtypes or type B spermatogonia, on the other hand, Adark spermatogonia is characterized by a dark nucleus with a central brighter area (13). Both are stem cells, however, Adark spermatogonia has only a basal mitotic activity (13). In the spermatogonial compartment it is possible to observe 3 different subtypes of Apale spermatogonia: Asingle (As), Apaired (Apr) and Aaligne (Aal) (12). The first ones are the stem cells of spermatogenesis and, upon division, their daughter cells either migrate, separate and become new stem cells, or stay together (incomplete

cytokinesis) and become Apr spermatogonia (connected by an intercellular bridge) (12). About half of stem cells goes through self-renewing divisions and the other half divides to form Apr spermatogonia (12). Apr spermatogonia divide and form chain of 4, 8 or 16 Aal spermatogonia (12). The latter differentiate into A1 spermatogonia, the first generation of differentiating spermatogonia. A1 spermatogonia go through several divisions (A2, A3, A4 and intermediate), finally resulting in B spermatogonia (12). Primary spermatocytes are originated by B spermatogonia, through mitotic division. It may be of a great importance that A spermatogonia express high levels of telomerase since the mitotic activity of SSCs is continuous and becomes higher with aging (12).

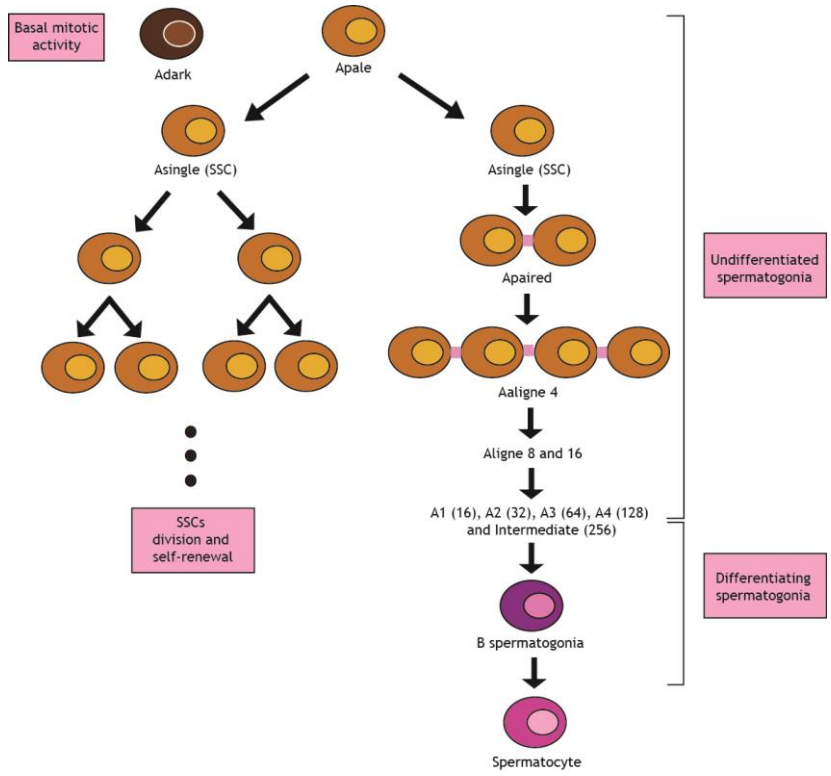

Figure 3. Spermatogonial stem cells (SSCs) division, self-renewal and differentiation. The hierarchic division of SSCs starts with two types: Adark and Apale. The first one has only a basal mitotic activity while Apale spermatogonia can proliferate into Apale subtypes - Asingle (As), Apaired (Apr) and Aaligne (Aal) - and, lately, into type B spermatogonia. About half of As spermatogonia undergoe self-renewal divisions, and the other half proceed to the differentiation process. Finally, primary spermatocytes are originated from B spermatogonia.

The function of SSCs is supported within specialized microenvironments known as “niches” which provide extrinsic stimuli that regulate self-renewal and differentiation through both architectural support and growth factor stimulation (14).

In mammalian testes, SCs are the major contributors to the SSC niche (15-17) but also peritubular myoid cells and LCs have a role (16, 17). Peritubular myoid cells surround the SeT and provide physical support to these structures. Furthermore, myoid cells have contractile features facilitating the transport of spermatozoa and testicular fluid in the lumen of tubule (18).

Both SCs and peritubular myoid cells contribute to the formation and support of SSCs niches. Glial cell-derived neurotrophic factor (GDNF), a member of transforming growth factor (TGF) super-family and a major growth factor produced by these cells, has impact on SSCs self-renewal (19-21), and proliferation (20). The GDNF co-receptors, GFRA1 and tyrosinekinase transmembrane protein Ret, have been indicated as SSCs markers (20). GDNF binds to GFRA1, which mediates the phosphorylation of Ret and the activation of multiple signaling pathways, stimulating the proliferation of SSCs via Ras/ERK1/2 pathway and activation of transcript factors, such as cyclic adenosine monophosphate (cAMP) responsive element-binding protein one (CREB-1) (20).

The accumulation of Apr and Aal spermatogonia was observed in regions of SeT near LCs clusters, suggesting that these cells also may contribute to the SSCs niches (16).

Gonadotropins were found to play a major role in SSCs niches dependently on the developmental stage, since gonadotropin releasing hormone (GnRH) release during postnatal development impairs SSC proliferation and in adult males SSCs proliferation increases when GnRH is suppressed (22).

c. Germ Cells Metabolism and Apoptosis: Mechanisms and Regulation

Alterations in germ cell proliferation/differentiation, survival and energy metabolism have a profound impact on the reduction of sperm counts and quality, commonly leading to infertility. To understand how the metabolism (figure 4) and apoptosis (figure 5) of germ cells can be modulated, firstly, it is necessary to characterize the molecular mechanisms underlying these biological processes.

The glycolytic process has been conserved among different species across evolution. However, some enzymes have testis-specific isoforms that are expressed largely on some spermatogenic cells rather than others (23, 24) In the earliest stage of development, spermatogonia possess all the enzymes needed to perform glycolysis, and thus these cells preferentially use glucose as energy source. The same behavior is observed in spermatozoa (23, 25). However, spermatocytes and spermatids, despite possessing all glycolytic enzymes, have their glycolytic apparatus inactivated and for that reason, use lactate as their primary energy source (26, 27). Why germ cells differ in their metabolic needs still is a matter not completely understood, but it might be related to the position within the SeT. The blood-testis barrier (BTB), established by the tight junctions formed between adjacent SCs (28), separates spermatogonia and primary spermatocytes on the outer portion of the SeT and secondary spermatocytes and spermatids in the inner portion of these tubules (28). In this way, spermatogonia and primary spermatocytes can have access to the glucose flowing in the blood, whereas secondary spermatocytes and spermatids depend exclusively upon SCs' metabolism to be provided with energetic substrates (26).

The glucose uptake by SCs is modulated by the facilitated-diffusion glucose transporter family (GLUTs). These transporters can be divided into three subfamilies: class I (GLUT1-4), class II (GLUT5, 7, 9, 11) and class III (GLUT6, 8, 10, 12) (29). GLUT1 and GLUT3 are known to play an essential role in SCs metabolism (30) and GLUT8 has been reported not to be involved in glucose uptake since it has been identified in the endoplasmic reticulum membrane but not in plasma membrane of SCs (31). GLUT3 is the most abundant GLUT in the testis, being expressed by all cell types in the SeT (32).

The process of glycolysis consists in several glucose conversion steps with pyruvate as the end product, and generating two molecules of adenosine triphosphate (ATP) (33). Firstly, glucose is phosphorylated (with ATP consumption) into glucose-6-phosphate, then into fructose-6-phosphate and, lastly, into fructose-1,6-biphosphate (34). The first reaction is catalyzed by the enzyme hexokinase and, the last one by phosphofructokinase 1 (PFK1) (34). High levels of ATP can inhibit PFK1 activity and when it occurs there is an accumulation of glucose-6-phosphate in the cell, thus resulting in the inhibition of hexokinase activity (34). This mechanism allows the regulation of glycolysis rates, since when the cell has an adequate quantity of metabolic energy, the glucose conversion is inhibited (34).

The preferred energetic substrate of developing germ cells, lactate, is produced in the cytosol of SCs (figure 4) through the reduction of pyruvate, with the concomitant oxidation of nicotinamide adenine dinucleotide (NADH) to NAD+, a reaction catalyzed by lactate dehydrogenase (LDH) (35).

The produced lactate becomes available to the germ cells by the activity of monocarboxylate transporters (MCTs), which are largely responsible for the transport of lactate and other monocarboxylates across the plasma membrane of several cells (36). Fourteen types of MCTs (1-14) have been characterized based on their sequence homology, but only four (1-4) have been proven to transport monocarboxylates (36). In SCs, it has been observed the high expression of MCT4, the main isoform required for lactate export (37). Sperm are known to express MCT1 and MCT2; MCT1 is detected in all germ cells with spermatogonia being a predominant expression site, while MCT2 is specifically detected in the tail of elongated spermatids and spermatozoa (38). Germ cells metabolize lactate converting it to pyruvate through LDH activity and then converting pyruvate into carbon dioxide (CO_2) and ATP through tricarboxylic acid (TCA) cycle, being lactate a modulator of energy homeostasis in these cells (25, 35).

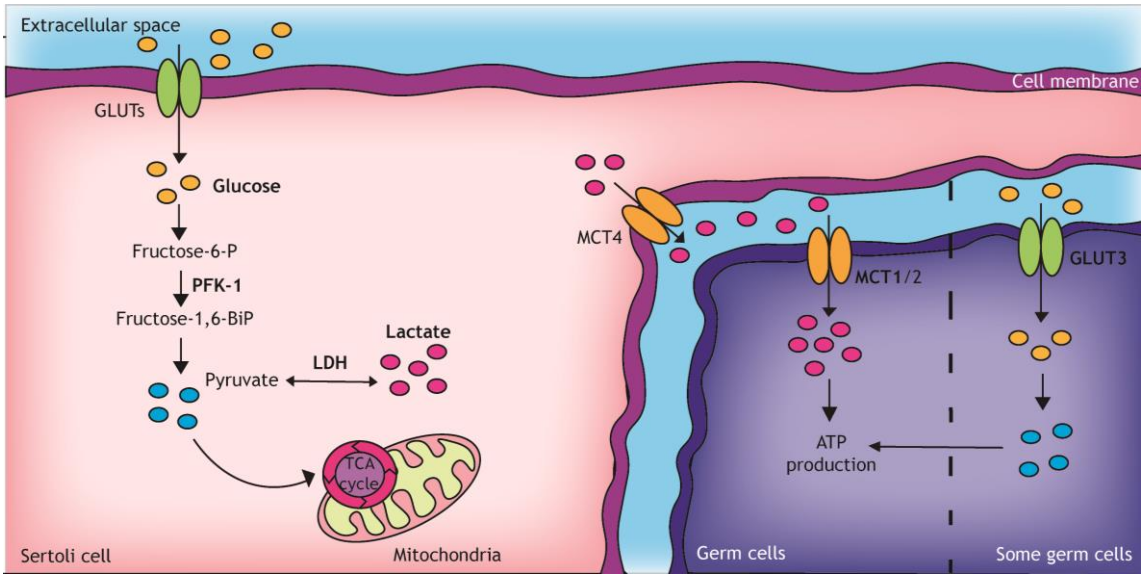

Figure 4. Sertoli cells (SCs) and germ cells metabolism: a teamwork process. Glucose is uptaken by SCs via glucose transporters (GLUTs) and converted to pyruvate by glycolysis, through the action of several enzymes. Phosphofructokinase-1 (PFK1) catalyzes the conversion of fructose-6-phosphate (Fructose-6-P) into fructose-1,6-bisphosphate (Fructose-1,6-BiP). Pyruvate, the end product of glycolysis, can either go through mitochondria, where it is used to regenerate acetyl-CoA by tricarboxylic acid (TCA) cycle, or be reduced into lactate by lactate dehydrogenase (LDH). Afterwards, lactate is exported across the SCs membrane through monocarboxylate transporter 4 (MCT4), and enters in the germ cells through MCT1 or MCT2, then is metabolized and converted into pyruvate through LDH and, finaly, pyruvate is converted into carbon dioxide (CO_2). Germ cells that are located outside the blood barrier (spermatogonia p.e.) might utilize the glucose from blood as an energy source, as well as the spermatozoa located in the lumen of SeT.

Endocrine factors, such as sex steroid hormones (5α-dihydrotestosterone, DHT, 17β-estradiol, E_2) (39), follicle-stimulating hormone (FSH), insulin and insulin growth factor I (IGF-1), have been shown to control the lactate production by SCs (40). Also, locally produced paracrine/autocrine factors (TGF-β, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), tumor necrosis factor α (TNF-α) and interleukin-1α (IL1α) positively affect the lactate production by SCs, being involved in cell-cell communication in the testis (41-44). These factors have specific targets, more precisely: glucose uptake, total LDH activity and *Ldha* gene transcription (41-44).

Besides the fulfillment of the metabolic needs, a fine balance between germ cell proliferation, differentiation and apoptosis is necessary to diminish the risk of testicular diseases and infertility.

Apoptosis is a critical process in the quantitative and qualitative control of germ cells. Germ cell apoptosis has been shown to play an important role in controlling sperm output in many species, and massive germ cell death occurs under physiological conditions during the earlier stages of spermatogenic process (constitutive apoptosis). But also in the adult testis, the fine control of apoptosis is critical for maintenance of spermatogenesis and male fertility, since germ cells are very sensitive to damaging conditions, such as, heat shock, ionizing radiation, growth factor deprivation and chemotherapeutic agents. Therefore, apoptosis is a relevant

mechanism for elimination of damaged germ cells avoiding passage of defects to the future generations (45, 46).

Cell shrinkage, DNA fragmentation and externalization of phosphatidylserine at cell membrane are some of the hallmarks of the process of programmed cell death by apoptosis (47). This process may be triggered by two distinct pathways: the extrinsic (receptor-mediated) and the intrinsic (mitochondrial) (47). The enzymes that execute apoptosis are specific proteases, the caspases. Firstly, caspases are synthesized as inactive zymogens (procaspases) and then, in response to death stimuli, they become active (47). Through dimerization, the initiator caspases (8 and 9) are auto-activated, then cleaving the effector caspases (3, 6 and 7) and leading to their activation (47). The activation of death receptor located in cell membrane, namely Fas (CD95/Apo-1) and tumor necrosis factor receptor 1 (TNFR1), triggers the extrinsic pathway inducing the activation of procaspase-8 (47). On the other hand, the intrinsic pathway could be activated by different stimuli, such as DNA damage, oxidative stress, starvation and autophagy (47). These stimuli lead to the activation of proapoptotic members of the Bcl-2 protein family, namely, Bax, decreasing the ratio between anti-apoptotic (Bcl-2 and Bcl-xL) and proapoptotic proteins (Bax). Bax is then translocated to the mitochondria, which loss its membrane potential (permeabilization), culminating on cytochrome c release (47). Cytochrome c interacts with dATP, cytosolic apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, forming the apoptosome complex (47). The activation of effector caspase-3 has been considered a remarkable end-point of apoptotic cell death, since both pathways converge at this point (figure 5) (47). Additional pathways include the perforin/granzyme pathway, which triggers apoptosis via granzyme B or granzyme A (48) and the p53 pathway, that is required for cell growth, an regulation of apoptosis induced by genotoxic and non-genotoxic stresses (49).

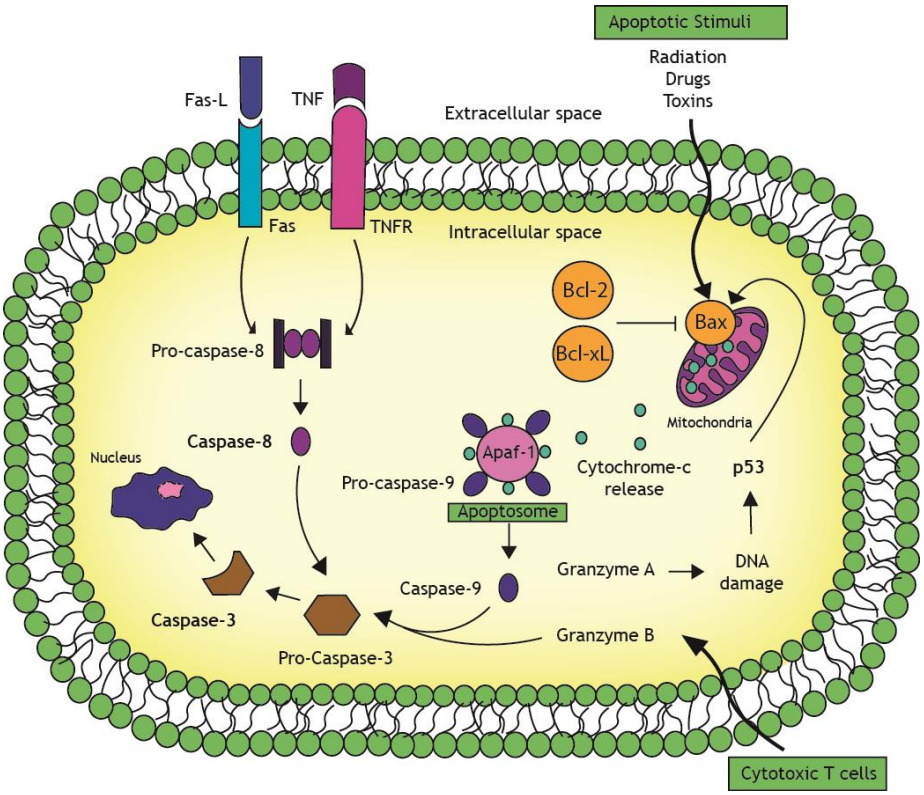

Figure 5. Programmed cell death and its players. Apoptosis may be triggered by two distinct pathways: the extrinsic (receptor-mediated) and the intrinsic (mitochondrial). The receptors triggering the extrinsic pathway (e.g. Fas and TNFR) are located at the plasma membrane and are activated by its ligands (Fas-L and TNF, respectively) triggering the activation of the initiator caspase-8. The Intrinsic pathways is activated by a variety of apoptotic stimuli. The ratio of proapoptotic (Bax)/anti-apoptotic (Bcl-2-Bcl-xL) signals are augmented, leading to the cytochrome-c release by mitochondria. The cytochrome-c, the pro-caspase-9 and the protease activating factor (Apaf-1) form the apoptosome, activating the initiator caspase-9. Besides these pathways, immune cells (cytotoxic T cells) can trigger the process of apoptosis by the release of granzymes (A and B). All the pathways converge on pro-caspase-3 that, after cleavage, become the active effector caspase-3, the end and irreversible point of apoptosis.

As previously referred, early on fetal development, PGCs migrate to the developing gonad for further differentiation. It has been shown that the cells with an aberrant migration in addition to excess generated cells undergo apoptotic cell death. The process of apoptosis in those cells is largely dependent on Bcl-xL and Bax (50). In fact, the balance between Bcl-2/Bcl-xL and Bax is extremely important on the regulation of the apoptotic process. In Bax knockout mice or mice overexpressing Bcl-2 or Bcl-xL, the early wave of apoptosis is eliminated and an accumulation of spermatogonia and spermatocytes is observed, leading to infertility (51). Similarly to the effects observed by Bax overexpression, Bcl-xL deficient rats demonstrated increased germ cell death, but those expressing lower levels of Bcl-2 display normal spermatogenesis (52). When gonocytes differentiate into spermatogonia an extremely increase in apoptosis rates is observed, involving caspase-3,-8 and -9 and the involvement of both extrinsic and intrinsic pathways (53).

The quantity, function and efficiency of SCs appear to be limiting to germ cell numbers, being the survival of germ cell directly related to the number of SCs and probably to their secretory

capability (15). Thus, SCs up-regulate Fas ligand (Fas-L) to eliminate Fas-positive germ cells, which cannot be supported adequately (54). Fas receptor and Fas-L are expressed in the testis and it has been shown that the upregulation of Fas receptor is associated with spermatocyte apoptosis (55). Altered meiotic and postmeiotic germ cell maturation might be associated with an upregulation of Fas gene expression (56). Fas/Fas-L system may be involved in the quality control mechanism of the produced gametes, since postmeiotic germ cell arrest has been associated with an increased Fas expression in germ cells (56).

The involvement of gonadotropins in apoptosis regulation has been studied. In mammalian testis, FSH, luteinizing hormone (LH) and testosterone (T) have all been shown to regulate germ cell survival (57). Furthermore, estrogens have been demonstrated to regulate cell apoptosis at several points (47, 58). Both intrinsic and extrinsic pathways are induced after a decrease in FSH and testosterone levels, proving that FSH and T inhibit death signals for the germ cells (59).

d. Hormonal Control of Spermatogenesis

The process of spermatogenesis requires the action of a complex set of steroid hormones and peptides, being all of them essential to the normal function of the seminiferous epithelium. Their actions are performed by autocrine, paracrine, endocrine and juxtacrine signaling mechanisms, under the hypothalamic-pituitary-gonadal axis control of spermatogenesis (figure 6) (60). These hormones have a key role in the proliferation and function of somatic testis cells and, consequently, in the regulation of germ cell development (61, 62). SCs and LCs are the direct targets for hormone action, and their coordinated actions are paramount for male fertility. The hypothalamic-pituitary-gonadal axis is activated by the secretion of GnRH by the hypothalamus. GnRH stimulates pituitary to release gonadotropins (63). Anterior pituitary secretes glycoproteic hormones, FSH and LH, that have direct effects in the testis stimulating somatic cells to support spermatogenesis (64). These hormones interact with specific G protein coupled receptors, FSH receptor (FSHR) present in SCs (65) and LH receptor (LHR) present either in LCs and spermatogenic cells (66).

The primary role of FSH is stimulate SCs proliferation during prepubertal development, it is important since the number of germ cells is directly correlated with the number of SCs (67). FSH stimulates SCs to produce several growth factors and proteins that influence spermatogenesis, such as the androgen-binding protein (ABP) and inhibin (68).

LH regulates the steroidogenic activity of LCs and synthesis of T, that diffuses into the SeT, stimulating the activity of SCs together with FSH (68).

This axis is tightly regulated by negative feedbacks that maintain the ideal concentration of hormones and other essential factors for spermatogenesis. When the levels of T are elevated it induces a negative feedback that inhibits the release of GnRH and LH (69). In response to

high levels of FSH, SCs produce the hormone inhibin, a protein member of the transforming growth factor ß superfamily that acts suppressing the release of FSH from the pituitary (70).

Androgens are widely recognized as the main regulators of male reproductive function, with the androgenic actions being absolutely required for successful spermatogenesis. However, testis also have the ability to irreversibly convert androgens into estrogens, which until the last few years, were considered "female hormones". More recent studies, have demonstrated the relevance of these hormones in the male reproductive tract (71-76). The conversion of androgens into estrogens, such as E_2 is dependent on the presence of a microsomal enzymatic complex – aromatase (Cyp19a1) (77). Aromatase is composed of cytochrome P450 (P450arom), a specific glycoprotein, and an ubiquitous reductase (77). In 1993, aromatase expression in adult testicular germ cells was first reported (78), with germ cells contributing to ~62% of total testicular aromatase (79). SCs also express aromatase being able to synthesize E_2, a feature that is more associated with the immature cells (80).

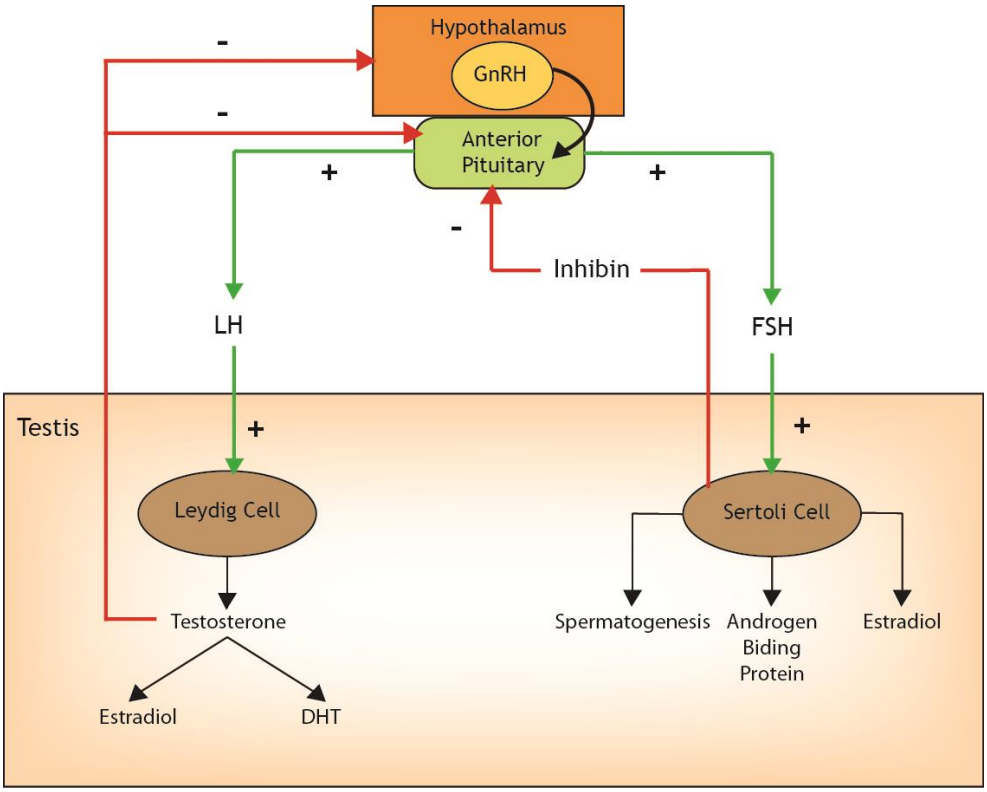

Figure 6. Hormonal regulation of spermatogenesis. Hypothalamus release the gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH), having a positive feedback on Sertoli cells (SCs) and Leydig cells (LCs), respectively. In turn, SCs produce 17ß-estradiol (E_2), androgen binding protein (ABP), inhibin and regulate spermatogenesis while LCs essentially produce androgens, such as testosterone (T), which by peripheral aromatization originates E_2. A negative feedback (-) by T on the hypothalamus and pituitary regulates the levels of GnRH, LH and FSH, although its main action is to decrease secretion of LH. FSH secretion is also subject of a negative feedback (-) by inhibin secreted by SCs.

2. 17ß-estradiol (E_2) Effects on Germ Cells Fate and Fertility

Sex steroids are master regulators of germ cell survival being the programmed cell death by apoptosis, a critical process in regulating the size and quality or the germ line. However, the beneficial or detrimental effects of estrogens in spermatogenesis are a matter of discordance between authors.

Estrogens interact with two subtypes of nuclear receptors (estrogen receptor α, ERα and estrogen receptor ß, ERß) (71) and with a membrane bound receptor G protein-coupled estrogen receptor (GPR30 or GPER) (72). The most well know function of estrogens in male reproductive function is the regulation of fluid absorption in the efferent ducts and rete testis. This function has been demonstrated by studies using ERα knockout (ERαKO) mice showing fluid resorption impairment (75). ERα is required in efferent ductule epithelium for fluid resorption (73) and for the maintenance of sperm motility and morphology (74). The ERß knockout (ERßKO) mice did not showed the dramatic reproductive changes seen in ERαKO mice, being the reproductive phenotype in ERαKO/ERßKO mice similar to ERαKO (75, 76). These data lead some authors to assume that ERα is functionally dominant in males.

The responsiveness to estrogenic hormones together with the fact that testicular cells can synthesize E_2 clearly supported the physiological role of estrogens on reproductive function.

High levels of estrogens were found in the testicular interstitial fluid (81), raising the hypothesis that these hormones have an active role in male reproductive function, and inciting studies on this thematic. Furthermore, estrogens are demonstrated to directly affect LCs through the inhibition of T production (82).

ERs have been detected in multiple cell types, including SCs and in some germ cells (83). ERß inactivation has been associated with the increased number of spermatogonia by more than 50% in neonatal mice (84). Interestingly, studies have revealed that SCs do not mediate the effects of estrogens on gonocytes development, suggesting a direct action of estrogens on germ cells (85).

Studies using rats during the neonatal period, in which E_2 was administered (5-11 days), showed that the number of undifferentiated and differentiating type A spermatogonia were increased at day 15 (86). These studies suggest a stimulatory role for estrogen is spermatogonial division, but it is difficult to infer whether this effect is direct or it is due to perturbation of the hormonal signals from pituitary (86). However, Miura et al (87) suggested a direct stimulatory effect of estrogen on these cells, since they demonstrated that E_2 significantly increased germ cell DNA synthesis and mitotic division. Despite E_2 treatment *in vivo* and *in vitro* induced spermatogonial mitosis, germ cells did not progress into meiosis (87). Therefore the spermatogonial mitosis induced by E_2 may not be for sperm formation, but for SSCs self-renewal (87).

Estrogens have also been shown to stimulate gonocytes proliferation, the precursor cells of spermatogonia, also called prespermatogonial cells. This stimulation was demonstrated to be dose-dependent, since proliferation was stimulated by a 1 μm of E_2 but not by higher doses (88). Aromatase activity in SCs is high during the neonatal period when gonocytes are proliferating and differentiating in spermatogonia and these cells have been shown to contain ERß, enforcing the existence of a direct action of estrogen in stimulating precursor germ cell mitosis (89).

Therefore, the studies presented above lead to believe that E_2 have a stimulatory role in germ cell development, more precisely in SSCs, increasing their proliferation rates. It was also shown that it only occurs with low doses, being this effect compromised at high doses of E_2. However, there are a lack of information in what concerns to the effects of high concentrations of E_2 in SSCs.

Contrasting with the beneficial actions of estrogens in spermatogenesis and sperm maturation are the evidences of the deleterious effects of these hormones in male reproductive function. Clinical studies have shown that increased intratesticular production of estrogens is linked to germ cell apoptosis and consequent spermatogenic failure. Increased E_2 levels have been detected in the testis of idiopathic infertile patients (90, 91).

Fetal period is critical for sex differentiation and testis morphogenesis. Fetal exposure to E_2 was shown to affect the development of testis (cord formation alteration), decrease the number of spermatogenic cells, such as gonocytes, SCs, LCs and decrease T levels (85). Also, fetal exposure to endocrine disrupting chemicals (EDCs) has been proved to generate transgenerational defects in spermatogenic capacity and sperm viability (92, 93). Increased apoptosis of spermatogenic cells and decreased sperm number and motility were observed, and this disruption was transmitted through four generations (92, 93).

3. Endocrine Disrupting Chemicals (EDCs) as Damaging Factors for Spermatogenesis and Male Fertility

EDCs are defined as the environmental xenobiotics that have the capability to alter and/or disrupt normal endocrine hormone signaling, by altering hormone production, bioavailability, metabolism or mechanism of action, thus interfering with the normal development of male and female reproductive systems (94).

The chemical origin, source or physiological mode of action of EDCs allows the classification of these compounds into different categories. Regarding chemical origin there are two types of EDCs, those that occur naturally, such as phytoestrogens and natural estrogens and those that are synthesized (95). In what concerns their source, EDCs can be divided into i) natural and artificial hormones ii) drugs with hormonal side effects, iii) industrial and household chemicals and iv) side products of industrial and household processes (96). EDCs can be also

classified into four categories depending on their physiological mode of action: xenoandrogens, xenoestrogens, antiandrogens and antiestrogens. The latter antagonize the androgen receptor (AR) and ERs, respectively, blocking the activation of these hormone receptors (97).

a. Mechanisms of Action

The mechanisms through which EDCs exert their effects both at endocrine and systemic level are very complex. In a simplistic way, it is possible to divide the mode of action of EDCs into five main mechanisms (figure 7).

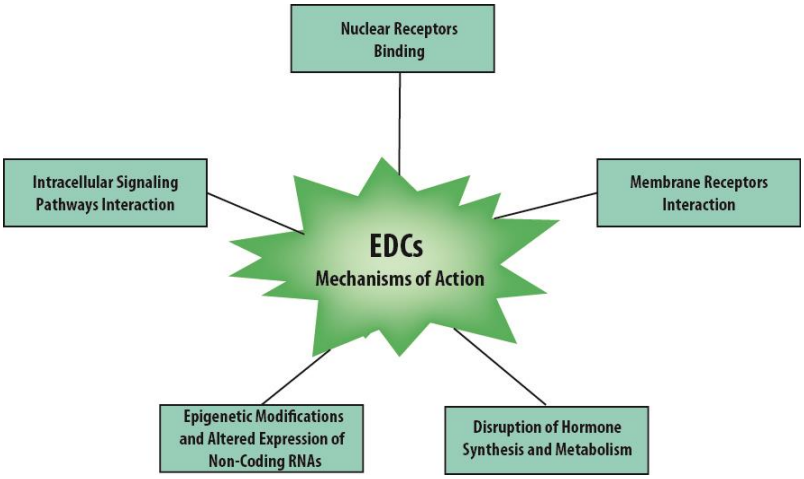

Figure 7. Schematic Representation of the EDCs' Mechanisms of Action.

i. Nuclear Receptors Binding

EDCs can interact with nuclear receptors (NRs), leading either to their activation or blockage. NRs are a class of specific receptor proteins that act as transcription factors, regulating gene expression in target cells and tissues (98, 99). NRs bind to the hormone-responsive elements (consensus DNA sequences in the promoter region of target genes) or interact with other co-activators and co-repressors proteins to achieve their functions (100-102).

It is well established that EDCs interact with the classical steroid NRs (ERs and AR), however, EDCs can also bind another members of the nuclear receptor family, such as estrogen-related receptors (ERRs), the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the peroxisome-proliferator activated receptor (PPAR) (103, 104).

Regarding ERRs, there are three different types: ERRα, ERRß and ERRγ. They have a high level of similarity and identity in both DNA-binding and ligand-binding domain with ERs. However, E_2 has not the ability to bind any of these ERRs. ERRs can bind to the functional estrogen-response elements in ER target genes, which suggests a possible overlap between ERRs and ERs actions (105, 106). AhR, CAR, and PXR are a group of NRs that have been associated with

xenobiotic metabolism and transport by regulating the expression of cytochrome P450 enzymes (107, 108). The PPAR family of NRs are present in reproductive tissues and its activation by EDCs has been shown leading to developmental effects (109). Similarly to ERRs, there are 3 isotypes of PPAR: PPARα, PPARß and PPARγ. The PPAR pathway modulates receptors and genes involved in cellular differentiation, adipogenesis and hormone and energy homeostasis. They are activated by binding of natural ligands such as polyunsaturated fatty acids and eicosanoids or in other hand, by synthetic ligands (110).

The activation of all of these previously-mentioned receptors and the up and down-regulation of their target genes affects the normal action of endogenous androgens and estrogens, by interfering with their actions with their target receptors, AR and ERs, respectively. Thus, leading to consequences at reproductive and hormonal levels (102).

ii. Membrane Receptors Interaction

EDCs can also interact with plasma membrane receptors, such as mERα, mERß and GPER. The mERα and mERß are the classical nuclear ERs, ERα and ERß, respectively, which have been shown to be translocated to the plasma membrane via mechanisms that remain unknown (111). This interaction activates second messenger-triggered signal cascades, not depending on the regulation of gene transcription and having rapid nongenomic actions (111, 112). An example of a nongenomic mechanism mediated by mERα and mERß is the rise of intracellular calcium (Ca^{2+}) levels driven by a rapid increase in Ca^{2+} influx, which can promote changes in intra- and extra-cellular processes, cell motility and rapid hormone secretion (113). GPER has been shown to be ubiquitously expressed and its activation is an alternative estrogen signaling pathway that may be used by EDCs to deregulate the hormonal balance with an impact in a broad range of tissues (114, 115).

iii. Intracellular Signaling Pathways Interaction

Besides the actions mediated by the nuclear and membrane receptors, estrogens can interact with other protein targets in the cytosol. The well-known mitogenic effects of estrogens have been linked to the activation of the Src/Ras/ERK pathway (116). The interaction of ERs with c-Src kinase is determined by binding of estrogens to the cytosolic ERs, changing the conformation of the kinase to an active state and leading to activation of the Src/Ras/ERK signaling cascade (116).

iv. Epigenetic Modifications and Altered Expression of Non-coding RNAs

As previously mentioned EDCs can interact with NRs and alter the array of genes expressed in a specific cell at a given moment. Furthermore, EDCs can influence the cell epigenetic panorama, through DNA methylation and histone modification, leading to an altered expression of target genes (117, 118). Moreover, it has been established that early life exposure to EDCs altering gene expression via epigenetic mechanisms is a feature that can be heritable in successive generations - transgenerational epigenetic inheritance (119, 120).

EDCs actions over gene transcription have also been linked with their effects on small non-coding RNAs of which several male reproductive cells are dependent. An example of this dependence is related with PGCs in the mammalian testis. PGCs differentiation has been shown to be dependent on the expression of specific micro-ribonucleic acids (miRNAs), which was shown to be disrupted in testicular germ cell tumors and mouse SCs after exposure to an EDC with xenoestrogen activity (121).

v. Disruption of Hormone Synthesis and Metabolism

Another mechanism through which EDCs can exert their effects is the interference with the hormone synthesis and metabolism. For example, aromatase activity can be modulated by several class of environmental pollutant compounds, which can induce or inhibit its activity (122-124). Besides aromatase, EDCs can also inhibit other p450 enzymes that are involved in the metabolism of T and estrone in the liver (125). The most affected families of p450 enzyme are CYP1, CYP2, and CYP3, which are responsible for drug and steroid metabolism (125). Finally, EDCs can affect the neuroendocrine homeostasis, which can lead to several perturbations, such as disturbance of GnRH levels (126). These altered levels of GnRH can be induced by these chemical substances which can affect endogenous steroid production through negative and positive feedbacks (126).

b. Xenoestrogens in Endocrine Disruption: the Case of Methoxychlor (MXC)

As previously mentioned, xenoestrogens are chemicals that interfere with the endocrine processes by mimicking estrogens action, thus, binding to ERs as agonists and displaying estrogenic properties (127). Among the substances demonstrated to have estrogenic effects are included phytoestrogens, flavonoids, industrial chemicals, polychlorinated biphenyls, polybrominated biphenyl ethers, some synthetic pharmaceuticals that are used in medical treatment such as diethylstilbestrol, plasticizers such as bisphenol A, UV filters, preservatives, pesticides and several metals such as cadmium (128). It has been proved that either through contaminated dietary intake or air environment, for example, diesel exhaust

particles, we are exposed to complex mixtures of pollutants, which have already demonstrated estrogenic activity (129).

It is now accepted that xenoestrogens exposure, especially in early-life, and long-term exposure, can induce reproductive disorders at sexual differentiation and disrupt the development and function of the reproductive organs and gene expression (128).

Methoxychlor (MXC) is a chlorinated hydrocarbon insecticide with a double ring structure (figure 8), has a composition of 1,1,1-trichloro-2,2-bis(methoxyphenyl)ethane, with the chemical formula $C_{16}H_{15}Cl_3O_2$ (130) and presents xenoestrogenic activity (ERα agonist) (131). It was first synthesized in 1893, and its commercial production began in 1946, being approved for use as an insecticide on 87 agricultural crops and on dairy cattle, beef cattle, goats, sheep and swine (131).

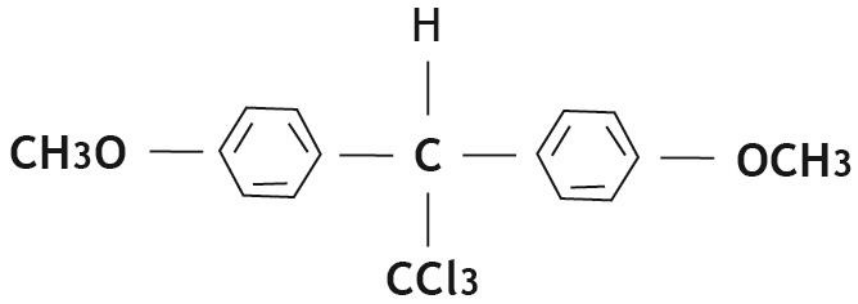

Figure 8. Chemical structure of methoxychlor. A double ring chlorinated hydrocarbon, with the chemical formula $C_{16}H_{15}Cl_3O_2$

Embryonic days 8 to 14 (E8-E14) are a critical period for sex differentiation and testis morphogenesis. The exposure of pregnant rats to MXC (100 or 200 mg/kg/day) during this period produces transgenerational defects in spermatogenic capacity and sperm viability, associated with increased spermatogenic cell apoptosis and decreased sperm number and motility, which are transmitted through four generations (93).

Adult male rats fed with 3% MXC diet for 45 days have shown suppression of spermatogenesis, although, in this study SCs and spermatogonia appeared normal (132). In another study, MXC also has been shown to inhibit spermatogenesis, nevertheless SCs, spermatogonia and spermatocytes showed degenerative changes (130). Furthermore, some of SeT did not contain any cellular element with the exception of spermatogonia (130). Perinatal oral treatment of rats with MXC (150 mg/kg/day) reduced testicular size, decrease the number of spermatogonia, SCs and decrease the level of serum LH and FSH in those animals at adults (133).

Most studies were carried out using higher toxicological doses of MXC than those found in realistic conditions (over 100 mg/kg/day). Research on the effects of environmentally relevant doses of MXC (less than 2 mg/kg/day) is required. In this context, Pole et al. exposed pregnant mice (F0) to MXC by intraperitoneal injections (1mg/kg/day) during gestation and lactation periods, then F1 males were assessed (134). This study shown that 1 mg/kg/day MXC

exposure disturbed the testicular development, decreasing serum T levels and increasing E2 levels (134).

There are several studies evidencing the effects of MXC exposure *in vivo*. However, few reports of *in vitro* studies are found, especially in what concerns the SCCs population, which are the "founders" of spermatogenesis.

4. The Regucalcin (RGN) Protein: a Protective Factor for Germ Cells?

Germ cell proliferation and differentiation needs to be meticulously regulated and this regulation might be done by several hormonal and non-hormonal factors. The Ca^{2+} ion and its intracellular balance have been shown to be part of these regulators (135). Ca^{2+} fluxes are generated by germ cells when spermatogonia develop into early spermatids (136) and the spermatozoa capacitation, motility and acrosome reaction are dependent on Ca^{2+} to properly occur (137).

Regucalcin (RGN), also known as senescence marker protein 30, is a Ca^{2+}-binding protein involved in Ca^{2+} homeostasis, and a multi-functional protein. This 299 amino acid protein with ~33 kDa of molecular weight is encoded by the *Rgn* gene and resides in the nucleus, cytoplasm, peri-nuclear space (138) and in mitochondrial fractions (139). On the evolutionary point of view, RGN has an highly conserved sequence (140), which is supportive of its fundamental biological role.

RGN regulates the Ca^{2+} transport across the cell membrane and between the cytoplasm and the nucleus and its organelles, controlling intracellular Ca^{2+} levels (141). This protein is also able to interact with several intracellular signaling pathways through Ca^{2+} dependent enzymes regulation (tyrosine kinases, phosphatases, phosphodiesterases, and nitric oxide synthase) (142-144).

RGN expression and function was initially associated with non-reproductive tissues, as liver, renal cortex (145), brain (146), heart (147), bone (148), lung (149) and submandibular gland (150). Recently, the expression of RGN in reproductive tissues was demonstrated, being described on the testis, epididymis, seminal vesicle and prostate of rat, as well as in human testis (151). In the rat testis, RGN was shown to be expressed in several germ cell types, namely spermatogonia, spermatocytes, spermatids and spermatozoa, with human testis displaying a similar expression pattern (151). RGN expression is regulated by several factors, such as Ca^{2+} sex steroid hormones (152-154), aldosterone (155) and insulin (141).

Given that Ca^{2+} signaling plays a major role in gametogenesis, a role for RGN in male reproductive function has been suggested (156), and recent reports have been disclosing RGN actions.

Besides its function in the modulation of intracellular Ca^{2+}, cytoprotective functions have also been assigned to RGN. Studies of our research group and others have linked the activity of RGN with the control of cell survival and apoptosis, and its antioxidant properties also have been described.

SeT from transgenic rats overexpressing RGN (Tg-RGN) and wild-type were cultured in the presence/absence of apoptosis inducers (157). It was demonstrated that comparatively with controls Tg-RGN SeT showed a diminished expression and activity of caspase-3, alongside with the increased expression of Bcl-2, and augmented Bcl-2/Bax ratio, in the presence of apoptosis inducers (157), indicating a resistance to apoptosis under conditions of RGN overexpression. Messenger RNA (mRNA) expression of proapoptotic p53 and cell cycle inhibitor p21 were also strongly increased (157). In another study, the same animal models were used and SeT were cultured in the presence/absence of pro-oxidant stimuli (158). SeT from Tg-RGN displayed a significantly higher antioxidant capacity both in control and experimental conditions (158). Furthermore, the activity of caspase-3 was significantly increased in SeT of wild-type rats, which was not observed in Tg-RGN animals (158). Curiously, the generation of radioresistant cell lines using fractionated irradiation is accompanied by a concomitant overexpression of RGN (159), which suggests that RGN expression increases in response to cell damage, likely as a protective mechanism. Indeed, the protective effect of RGN against radiation-induced damage in testicular cells was described. Tg-RGN animals, exposed to a single dose of X-rays (6 Gy), displayed higher gonodosomatic index, and sperm viability and motility relatively to their wild-type counterparts, as well as a higher frequency of normal sperm morphology, a diminished incidence of head-defects and a lower rate of apoptosis (160). These findings strongly support the involvement of RGN in the anti-apoptotic response, which is further corroborated by the observed enhanced expression of RGN in the testis of irradiated rats (160).

Considering that the most well-established role of RGN is related to the maintenance of intracellular Ca^{2+} homeostasis in many types of cells, another study analyzed sperm parameters, antioxidant potential and Ca^{2+} flux in rat epididymis of Tg-RGN (161). Tg-RGN rats displayed an altered morphology of epididymis, lower sperm counts and motility, a diminished rate of Ca2+ influx; also the sperm viability and the frequency of normal sperm were higher in Tg-RGN animals comparatively with wild-type (161). These results suggested the role for RGN in sperm maturation, since it demonstrated the importance of maintaining Ca^{2+} level in the epididymal lumen (161). Very recently, Pillai et al. characterized the localization of RGN in water buffalo (*Bubalus bubalis*) spermatozoa (162), and studied the effects of this multi-functional protein in spermatozoa cryopreservation (163), and capacitation (164). Two new RGN isoforms of 44 kDa and 48 kDa were detected in buffalo spermatozoa along with the reported 34, 28 and 24 kDa isoforms, with the 34 kDa isoform being spermatozoa membrane-associated (162). Furthermore, RGN was detected in the acrosomal region of the caudal and ejaculated spermatozoa while in testicular spermatozoa

RGN is present in the acrosomal region and also in the cytoplasm (162). Supplementing sperm cryopreservation medium with different concentrations of RGN (20, 40 and 60 µg/ml) showed that 40 µg/mL RGN had a cryoprotective effect inducing a significant increase in the post-thaw progressive motility of spermatozoa, and *zona pellucida* binding (163). In the same animal model, the addition of recombinant RGN to capacitating media significantly reduced the percentage of capacitated spermatozoa comparatively (164). Moreover, the degradation of RGN observed in the seminal plasma and its suppressive effects on *in vitro* capacitation of spermatozoa, indicated the possible anti-capacitation role in the reproductive tract (164).

Studies demonstrating RGN influence on the regulation of several transporters and glycolytic enzymes lead to believe that RGN may have a role in the control of the glycolytic metabolism. Lower glucose levels and diminished expression of GLUT3 and PFK1 were found in the Tg-RGN prostate, comparing to the wild-type group (37). Thus, the glycolytic metabolism seems to be suppressed under RGN overexpression. This suppression on glycolytic metabolism was accompanied with a decrease in lactate levels in consequence of a diminished LDH expression and activity (37). In bone marrow cell cultures, the opposite has occurred, and an increase in the consumption of glucose and lactate production was observed when RGN is up-regulated (165).

A downregulation of RGN expression is induced by E_2 administration in rat prostate and mammary gland (152), however, the stimulation of breast cancer cell line MCF-7 with E_2 caused an up-regulation of RGN expression (153). Though in the prostate cancer cell line LNCap, DHT was shown to down-regulate RGN expression (153), in rat SeT the inverse effect was shown (154).

Recently, histopathological and *in vivo* evidence had pointed RGN as a protective molecule in carcinogenesis also.

II. Aim of the Dissertation

EDCs are environmental compounds, either natural or produced by man, that interfere with the endocrine function by altering hormone metabolism, synthesis, and mechanism of action. Among the variety of EDCs, it has been shown that several of these compounds can mimic (xenoestrogens, xenoandrogens) or antagonize (antiestrogens, antiandrogens) the action of sex steroid hormones. Therefore, it has been demonstrated that the exposure to EDCs in utero and at early life stages can perturb hormonal signaling causing birth defects, behavioral disorders, disrupted spermatogenesis, and also cancer. Moreover, it has been shown that some of these modifications may be passed down to the future generations.

In the last years, estrogens have emerged as important regulators of germ cell fate, although, the beneficial or detrimental effects of these hormones in spermatogenesis remains controversial. MXC is an insecticide extensively used in the agricultural sector displaying xenoestrogenic activity, which has been proved to affect the male reproductive system. Nevertheless, little is known regarding the impact of EDCs in the SSCs population. This crucial cell type in the testis is the foundation of spermatogenesis, maintaining the germ cell cycle and, thus, sustaining male fertility. Thus, any factors that may affect SSCs can compromise the spermatogenic output and cause male infertility.

RGN is a Ca^{2+}-binding protein, which besides its role in the regulation of intracellular Ca^{2+} and Ca^{2+}-dependent enzymes, has been associated with the control of cell proliferation, oxidative stress, apoptosis, and metabolism. Previous studies of our research group have shown that RGN is expressed in several germ cells types, namely spermatogonia, spermatocytes, spermatids, and spermatozoa. Furthermore, our research findings demonstrated the protective role of RGN over the germ line upon exposure to damaging factors, such, as apoptosis-inducers, oxidant agents, and radiation. Following this rational it is highly likely to assume that RGN may have a similar behavior against EDCs actions.

The present dissertation aims to study the impact of E_2 and MXC on SSCs glycolytic metabolism and survival/apoptosis, and determine the influence of RGN in attenuating their effects. For this purpose, a rat spermatogonial stem cell line (GC-6spg) transfected to overexpress RGN (GC6-spg/RGN) was used as the study model. GC6-spg/RGN cells and mock-transfectants (GC-6spg/Mock) were exposed either to 100 nM of E_2 or 25 μM of MXC and, glucose consumption and lactate production, as well as, the expression and activity of glycolytic metabolism and apoptosis regulators were evaluated.

III. Materials and Methods

1. Vector Construction

In order to obtain rat SSCs overexpressing RGN, a recombinant vector with a RGN insert was constructed. Briefly, an 897 bp DNA fragment encompassing the open reading frame of human RGN was obtained using liver cDNA as template. Reverse transcriptase – polymerase chain reaction (RT-PCR) was carried out using specific primers (forward primer, 5-tgagctagcctgcgaccatgtcttccatta-3; reverse primer, 5-cctgctcgagtcccgcataggagtagggac-3) with *Nhe* I e *Xho* I restriction sites to allow the directional cloning of RGN into the expression vector. The amplified complementary DNA (cDNA) was purified, digested with *Nhe* I e *Xho* I restriction enzymes, and cloned into pGEM-T Easy Vector for subcloning (Promega). For this purpose, XL1-Blue competent cells (200249, Stratagene) were transformed with the ligation product, and left to grow overnight at 37ºC in LB agar plates containing ampicillin (100 µg/mL). Colonies were screened for the presence of the pGEM-T/RGN vector, and subsequently were used to inoculate ampicillin-LB medium and grown overnight at 37ºC under orbital agitation. Plasmid DNA was extracted using Wizard SV Plus Minipreps (Promega) and digested with *Nhe* I e *Xho* I restriction enzymes to confirm the presence of insert. After sequencing to confirm the identity of the amplicon, orientation and reading frame, the pGEM-T/RGN vector was digested with *Nhe* I e *Xho* I. The *Nhe* I-*Xho* I restriction fragment encoding the RGN protein sequence was then subcloned into the pIRES2-AcGFP1 vector (figure 9, Clontech Laboratories). The procedures of cloning were the same described above, except that the antibiotic used was kanamycin (50 µg/mL).

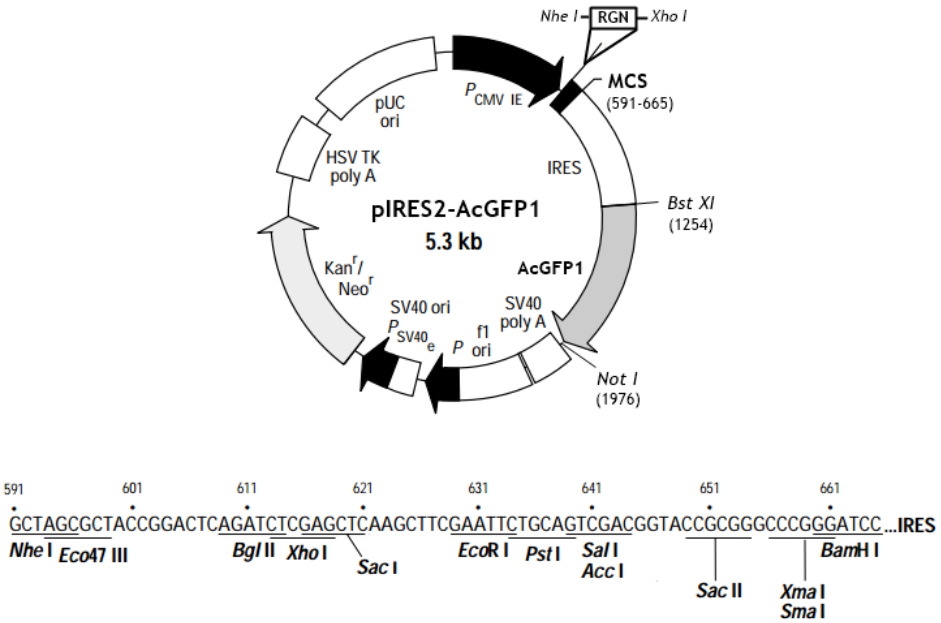

Figure 9. Restriction Map and Multiple Cloning Site (MCS) of pIRES2-AcGFP1 Vector. pIRES2-AcGFP1 contains the internal ribosomes entry site (IRES) of the encephalomyocarditis virus between MCS and the *Aequorea coerulescens* green fluorescent protein 1 (AcGFP1) coding region. The position of the *Nhe I*-*Xho I* restriction fragment encoding the RGN protein sequence is indicate into the MCS of the pIRES2-AcGFP1 vector (adapted from (166), Clontech Laboratories).

2. SSCs Culture

The rat cell line with SSC characteristics, GC-6spg (gonadal cell-6 spermatogonia), was generated by Doctor Ans Van Pelt, Academic Medical Center (AMC), The Netherlands.

GC-6spg cells were maintained in Minimum Essential Medium (MEM, 21430-020, Invitrogen) with 1x MEM containing: 0.12 % sodium bicarbonate (S8761, Sigma Aldrich), 4 mM L-glutamine (25030-024, Invitrogen), 100 µg/mL penicilline/streptomycine (15140-122, Invitrogen), nonessential amino acids 100x (11140-035, Invitrogen), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 15630-056, Invitrogen) and 40 µg/mL gentamicine (15710-049, Invitrogen).

The standard 1x MEM GC-6spg cell culture medium was supplemented with 2.5 % fetal calf serum (FCS, Australia; 10131-019, TermoFisher), 10 ng/mL platelet-derived growth factor-BB (PDGF, P4306, Sigma-Aldrich), 10 ng/mL leukemia inhibitory factor (LIF, CA-92590, Millipore), 20 µM forskolin (F3917, Sigma Aldrich), 200 µg/mL geneticin (G418, A6789, PanReac AppliChem), 10 ng/mL bFGF (F0291, Sigma-Aldrich), 1 µM E2 and 50 µg/mL puromycin 10 mg/mL (P8833, Sigma-Aldrich).

GC-6spg cells were cultured at 32 ºC in an atmosphere of 5% CO_2. Cultures were used for transfection.

3. Stable Transfection

GC-6spg cells were cultured for 24h in Matrigel-coated 35-mm wells (six-well plate) to improve cell attachment. Cells were co-transfected with 1.79 µg of RGN/pIRES2-AcGFP1 vector, or pIRES2-AcGFP1 empty-vector, and 1.79 µg of pLVX-Puro Vector (PT4002-5, Clontech Laboratories) using 5.8 µl of FuGENE 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) per well. The pLVX-Puro vector (figure 10) was used to allow further selection of co-transfected cells since this vector contains a puromycin resistance gene. Among the variety of transfection reagents, FuGENE 6 is a non-liposomal reagent that produces high levels of transfection with minimal cytotoxicity for many eukaryotic cell lines (167).

For cells selection, puromycin (P8833, Sigma-Aldrich) was added to the culture medium after 7 days at a concentration of 5 µg/ml. Selection medium was refreshed twice a week. Cells surviving the puromycin-selection were passed every 3 days with 0.5 mM ethylenediamine tetraacetic acid (EDTA) at room temperature and cultured further in selection medium without Matrigel (to avoid interference of including growth factors with those used subsequently in cell cultures).

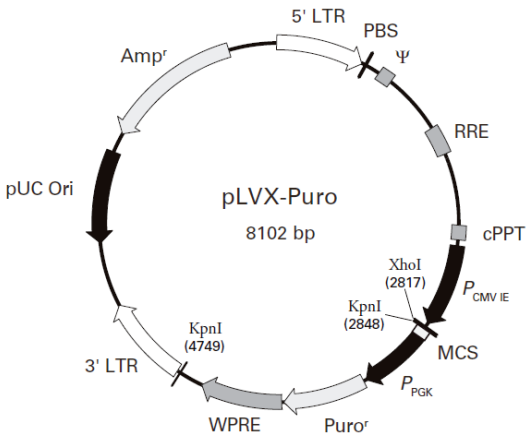

Figure 10. pLVX-Puro Vector Map. pLVX-Puro contains a puromycin resistance gene (Puro^r) under the control of the murine phosphoglycerate kinase promoter (P_{PGK}) for the selection of stable transfectants ((168), Clontech Laboratories).

The transfection was confirmed by fluorescence microscopy technics, evaluating the presence of green fluorescent cells due to AcGFP-1 expression. RGN overexpression in GC-6spg cells-transfected cells with a fragment encoding the RGN protein (GC-6spg/RGN) compared to the GC-6spg mock-transfectants (GC-6spg/Mock, basal expression) was determined by Western Blot analysis.

4. E_2 and MXC Treatments

To perform the E_2 (E258, Sigma-Aldrich) and MXC (36161, Sigma Aldrich) treatments, GC-6spg/RGN and GC-6spg/Mock were cultured in standard culture medium as described above until they reach 60 % of confluence. The culture medium, 10x MEM (M3924, Sigma-Aldrich) used was identical to the maintenance medium except for the presence of phenol red, and the FCS was stripped by dextran coated charcoal (C6241, Sigma-Aldrich).

Approximately 3 000 000 of GC-6spg/RGN and GC-6spg/Mock cells were cultured in each T-25 flask, in the presence or absence of 100 nM of E_2 or 25 µM of MXC, for 48 hours at 32 ºC in an atmosphere of 5% CO_2. The 100 nM dose of E_2 mimics the elevated concentrations of estrogens found in infertile patients (58) and the 25 µM dose of MXC was chosen based on previous studies showing effects of this compound (169, 170).

At the end of the experiment, GC-6spg cells were recovered and stored at -80ºC until protein isolation.

5. Protein Extraction and Quantification

Total proteins were isolated from E_2- and MXC-treated GC-6spg/RGN and GC-6spg/Mock cells, and controls using the radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA)

supplemented with 1% protease-inhibitors cocktail (Sigma-Aldrich) and 10% of phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The cell lysates were homogenized, centrifuged at 14000 *rpm*, 20 min, 4 ºC, in a Hettich Mikro 200R centrifuge, and the supernatant containing proteins was collected and kept on ice. Afterwards, protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. The proteins were stored at -80 ºC.

6. Quantification of Glucose and Lactate

The glucose consumption and lactate production were evaluated through spectrophotometric assays using commercial kits (Spinreact, Girona, Spain) following the manufacturer's protocols. For this purpose, cell culture medium of GC-6spg/RGN and GC-6spg/Mock cells was collected at 0h and 48h of treatment with E_2 (100 nM) and MXC (25 µM).

For glucose quantification in culture medium, kit R1 buffer (92 mmol/L Tris, pH 7.4 and 0.3 mmol/L phenol) was used to dissolve R2 powdered enzyme mix (15000 u/L glucose oxidase, 1000 u/L peroxidase, 2.6 mmol/L 4-aminophenazone). In a 96 well-plate, 100 µL of this working solution were added to 1 µL of each sample of each experimental condition (n=6/group); 100 µL working solution (blank); 100 µL working solution + 1 µL of the aqueous glucose calibration solution (standard). The plate was incubated for 10 min at 37 ºC and the absorbance values were read at 505 nm (violet).

The lactate content was evaluated by a similar methodology. R1 buffer (50 mmol/L PIPES, pH 7.5, 4 mmol/L 4-chlorophenol) was used to dissolve R2 powdered enzyme mix (800 u/L lactate oxidase, 2000 u/L peroxidase, 0.4 mmol/L 4-aminophenazone). In a 96 well-plate, 100 µL of the working solution were added to 1µL of each sample each sample of each experimental reagent per well, for each well of each condition (n=6); 100 µL working reagent (blank); 100 µL working reagent + 1 µL of the aqueous lactate calibration solution (standard). The plate was incubated and mixed for 5 min. at 37 ºC and the absorbance values were read at 505 nm (violet).

The glucose consumption and lactate production by GC-6spg/RGN and GC-6spg/Mock cells in response to the E_2 and MXC were calculated in comparison with the glucose and lactate content at 0 hours, and normalized for the total number of cells in each experimental group.

7. Western Blot

25 µg of protein extract from each experimental group were mixed with a previously-prepared loading buffer (10% 2-Mercaptoethanol) and denatured at 100 ºC. Proteins were resolved in a 12.5 % gel by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were incubated overnight at 4 ºC with mouse anti-RGN (1:1000, Ab67336, Abcam), rabbit

anti-LDH (1:10000, Ab52488, Abcam, Cambridge, MA, USA), rabbit anti-PFK1 (PFK1) (1:1000, sc-67028, Santa Cruz Biotechnology), rabbit anti-Bax (1:1000, 2772, Cell Signalling Technology), rabbit anti-Bcl2 (1:1000, 2876, Cell Signalling Technology), and rabbit anti-p53 (1:1000, sc-6243, Santa Cruz Biotechnology) primary antibodies. A mouse anti-ß-actin (1:10000, 5441, Sigma-Aldrich) antibody was used as for protein loading control in all western blot analyses. Goat-anti-rabbit IgG-HRP (1:40000, NIF1317; Santa Cruz Biotechnology) or goat anti-mouse IgG + IgM-HRP (1:40000, Santa Cruz Biotechnology) were used as secondary antibodies. The membranes were incubated with enhanced chemiluminescence (ECL) substrate (Bio-Rad) for 5 min. and immune-reactive proteins were scanned with the ChemiDoc™ MP Imaging System (Bio-Rad). Bands density was assessed according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective ß-actin band density. Results are presented as fold-variation relatively to the control group (GC-6spg/Mock without any treatment).

8. Caspase-3 Activity

Protein samples of each experimental condition were used to determine caspase-3 activity. In a 96-well plate, 5 µL total protein extracts were incubated with 85 µL of assay buffer (20mM HEPES pH 7.4; 2 mM EDTA; 0.1 % 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate, CHAPS; 5 mM dithiothreitol DTT) and with 2 mM of caspase-3 substrate (Ac-DEVD-pNA; Sigma-Aldrich). Blanks were performed without protein. The incubation was undertaken overnight at 37 ºC and the absorbance values were read at 405 nm, which are proportional to the production of the yellow product p-nitro-aniline (pNA) after cleavage of Ac-DEVD-pNA by caspase-3. The amount of generated pNA was obtained by extrapolation with a standard curve of free pNA at different concentrations, and is directly proportional to the activity of caspase-3.

9. Statistical Analysis

The statistical analysis of all data obtained were performed with the GraphPad Prism v6.01 software (GraphPad Software, San Diego, California, USA). Statistically significant differences between the tested groups were obtained by Unpaired Student's T-Test and one-way ANOVA followed by Bonferroni multiple comparison test as applicable. The differences were considered significant when $P<0.05$ ($p<0.05$ = (*), $p<0.01$= (**), $p<0.001$ = (***) and $p<0.0001$ = (****)). Experimental data are shown as mean ± SEM.

IV. Results

1. Confirmation of Stable Transfection and RGN Overexpression in SSCs

The transfection rate of GC-6spg cells was analyzed by fluorescence microscopy techniques and confirmed every passage. pIRES2-AcGFP1 vector constitutively expresses the Green Fluorescent protein (GFP) allowing immediate identification of transfected cells. GC-6spg co-transfected with pIRES2-AcGFP1/RGN/pLVX-Puro (GC-6spg/RGN) demonstrated high transfection rates, ranging from 60-100%. In figure 11, GC-6spg co-transfected with pIRES2-Ac-GFP1 empty vector/pLVX-Puro (GC-6spg/Mock, A) and GC-6spg/RGN (B) representative photomicrographs are shown.

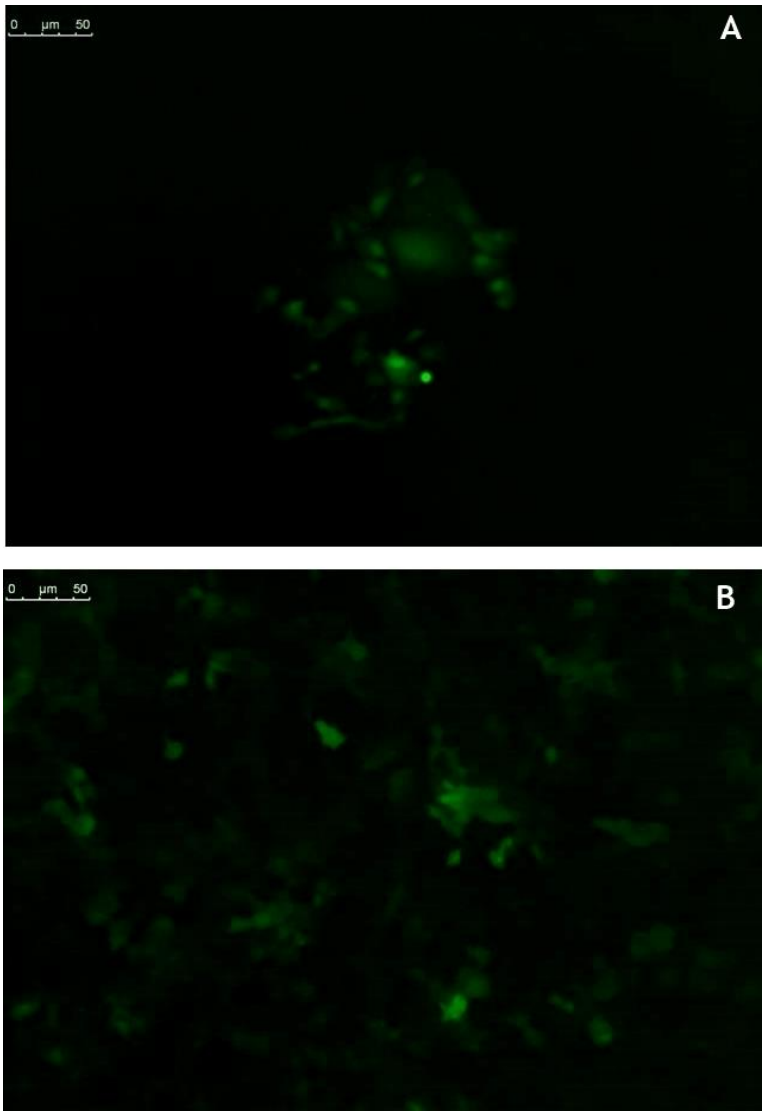

Figure 11. Representative images of GC-6spg cells co-transfected with pIRES2- AcGFP1/ pLVX-Puro (GC-6spg/Mock, A) or with pIRES2-AcGFP1/RGN/pLVX-Puro (GC-6spg/RGN, B). GC-6spg/RGN cells showed high transfection rate, seen by the elevated percentage of GFP positive cell.

To confirm if RGN was overexpressed in GC6-spg/RGN cells comparatively to GC-6spg/Mock transfectants, a Western Blot Analysis was performed. As expected RGN expression levels (figure 12) were increased in GC-6spg/RGN cells when compared to the GC-6spg/Mock control group (approximately 2.7-fold variation, $p<0.01$).

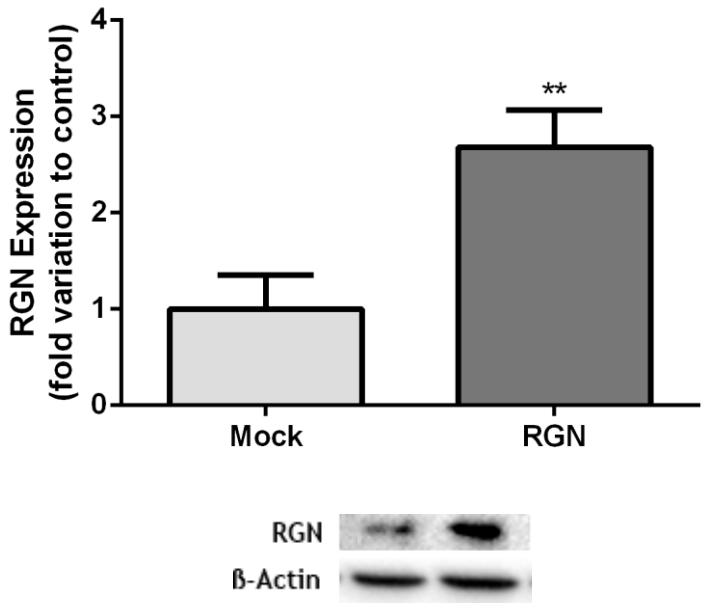

Figure 12. Expression of Regucalcin (RGN) protein in GC-6spg cells co-transfected with an empty-vector (Mock transfectants) and GC-6spg cells co-transfected with the pIRES2-AcGFP1/RGN/pLVX-Puro vector (RGN transfectants). Results are expressed as fold-variation comparatively to the control (Mock). Error bars indicate mean ± S.E.M ($n\geq4$ in each group) after normalization with ß-actin. ** $p<0.01$. Representative blots are shown.

2. Metabolic Alterations in SSCs in the Presence or Absence of E_2 or MXC

a. Glucose Consumption and Lactate Production

Although lactate produced by SCs has been indicated as the preferential energy source of germ cells, it is known that SSCs may also utilize glucose from the bloodstream since they are located at the periphery of SeT outside of the BTB (26). Glucose consumption and lactate production were quantified to understand how glucose is metabolized by SSCs, and how/if its metabolism is altered upon exposure to E_2 and MXC. The differences in glucose consumption and lactate production between GC-6spg/Mock and GC-6spg/RGN transfectants cultured in the presence or absence of E_2 or MXC for 48 hours were assessed.

Glucose consumption (figure 13A) was found to be significantly increased in GC6-spg/RGN transfectants with or without E_2 or MXC treatment when compared to GC-6spg/Mock (RGN: 5.52 ± 0.06; RGN E_2: 6.60 ± 0.06; and RGN MXC: 7.78 ± 0.06 vs. 3.50 ± 0.26 pmol/cell in Mock transfectants; $p<0.0001$). E_2 did not affect glucose consumption in mock-transfected cells (Mock E_2) when compared with the respective control GC-6spg/Mock (Mock), whereas MXC was shown to increase glucose consumption in GC-6spg/Mock cells (Mock MXC: 8.58 ± 0.18 vs. Mock: 3.50 ± 0.26 pmol/cell, $p<0.0001$). Nevertheless, GC-6spg/RGN cells either treated with E_2 or MXC displayed increased glucose consumption when compared to GC-6spg/RGN (RGN E_2: 6.60 ± 0.06 and RGN MXC: 7.78 ± 0.06 vs. 5.52 ± 0.06 pmol/cell in RGN; $p<0.0001$). However, a decreased glucose consumption was observed in MXC-treated GC-6spg/RGN cells compared to GC-6spg/Mock MXC (RGN MXC: 7.78 ± 0.06 vs. Mock MXC: 8.58 ± 0.18 pmol/cell, $p<0.01$), an effect not seen with E_2, where the inverse occurred (RGN E_2: 6.60 ± 0.06 vs. Mock E_2: 3.59 ± 0.35 pmol/cell, $p<0.0001$).

Lactate production results (Figure 13B) followed glucose consumption, being significantly increased in GC6-spg/RGN transfectants (with or without E_2/MXC) when compared to GC-6spg/Mock (RGN: 5.37 ± 0.27; RGN E_2: 6.12 ± 0.42; RGN MXC: 16.18 ± 0.68 vs. 1.84 ± 0.33 pmol/cell in Mock transfectants; $p<0.0001$). GC-6spg/Mock cells treated with E_2 (Mock E_2) did not displayed any variation in lactate production compared with the respective control (Mock). However, lactate production was increased in E_2-treated GC-6spg/RGN cells compared to GC-6spg/Mock E_2 (RGN E_2: 6.12 ± 0.42 vs. Mock E_2: 2.38 ± 0.34 pmol/cell, $p<0.0001$).

As observed for glucose consumption, lactate production was significantly increased in GC-6spg/Mock MXC-treated cells respectively to GC-6spg/Mock (Mock MXC: 18.44 ± 0.53 vs. Mock: 1.84 ± 0.33 pmol/cell, $p<0.0001$). Lactate production was also increased by MXC in GC-6spg/RGN when compared to the untreated GC-6spg/RGN (RGN MXC: 16.18 ± 0.68 vs. RGN: 5.37 ± 0.27 pmol/cell, $p<0.0001$). However, a decreased lactate production was observed in MXC-treated GC-6spg/RGN cells compared to GC-6spg/Mock MXC (RGN MXC: 16.18 ± 0.68 vs.

Mock MXC: 18.44 ± 0.53 pmol/cell, p<0.05). No differences were obtained in the case of GC-6spg/RGN E_2 compared to GC-6spg/RGN.

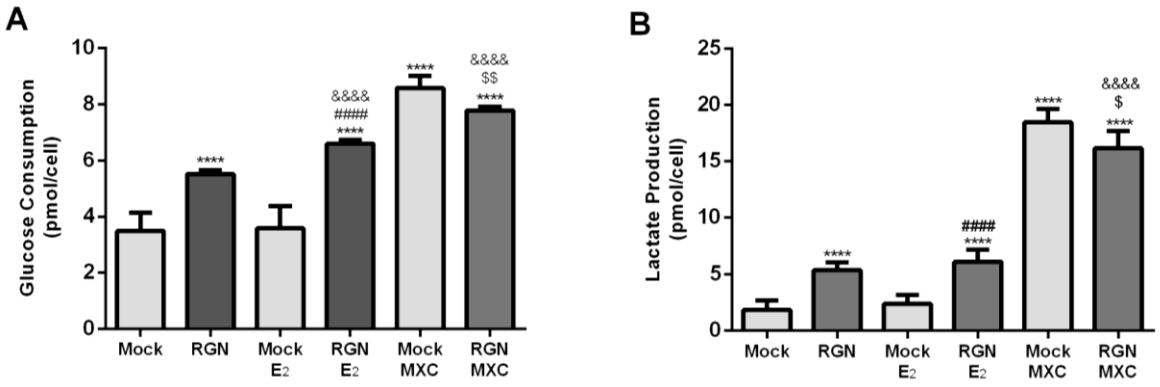

Figure 13. Glucose consumption (A) and lactate production (B) in Mock and regucalcin (RGN) GC-6spg transfectants cultured for 48h, in the presence or absence of 25 µM of methoxychlor (MXC) or 100 nM of estradiol (E_2). Results are expressed as mean ± S.E.M. (n≥5 in each group). (*) statistically significant difference when compared with Mock group; (#) statistically significant difference when compared with Mock E_2 group; ($) statistically significant difference when compared with Mock MXC group; (&) statistically significant difference when compared with RGN group (**** p<0.0001; #### p<0.0001; $ p<0.05; $$ p<0.01; &&&& p<0.0001).

b. Expression Levels of Glycolytic Enzymes

Glycolysis starts with glucose phosphorylation into glucose-6-phosphate, then into fructose-6-phosphate and, lastly, into fructose-1,6-biphosphate (171). The fructose-1,6-biphosphate is produced in a reaction catalyzed by phosphofructokinase 1 (PFK1), an extremely important regulatory enzyme that determines the flux through the glycolytic pathway (171).

No significant differences were observed in the expression levels of PFK1 (figure 14A) in consequence of RGN overexpression. Despite being apparently decreased, PFK1 expression in GC-6spg/RGN cells was not statistically different from GC-6spg/Mock transfectants. Also, no significant differences were found in response to E_2 treatment, the expression of PFK1 in GC6-spg/Mock, GC6-spg/Mock E_2, and GC6-spg/RGN E_2 was similar. In the case of MXC treatment, no significant difference was observed between GC-6spg/Mock and GC-6spg/Mock MXC. However, PFK1 expression was demonstrated to be augmented in the GC6-spg/RGN MXC group when compared to GC-6spg/RGN control (RGN MXC: 1.31 ± 0.16 vs. RGN: 0.50 ± 0.02 fold variation, p<0.01) and when compared to GC6-spg/Mock MXC (RGN MXC: 1.31 ± 0.16 vs. Mock MXC: 0.68 ± 0.02 fold variation, p<0.01).

Another central player in the glycolytic metabolism is the LDH enzyme that is responsible for the reversible conversion of the end-product of glycolysis pyruvate into lactate. LDH is an important modulator of energy homeostasis in germ cells (35), with the majority of germ cells located in the adluminal compartment of SeT metabolizing lactate converting it into pyruvate, which is then redirected to the mitochondria (25). However, SSCs cells reside outside the BTB, and it is likely to assume that they can uptake glucose from the bloodstream, and eventually drive it to the glycolytic process producing lactate, as SCs do.

LDH expression levels (figure 14B) were shown to be significantly diminished in cells overexpressing RGN (GC-6spg/RGN) when compared to their GC-6spg/Mock counterparts (RGN: 0.67 ± 0.10 vs. Mock: 1.00 ± 0.08 fold variation, $p<0.05$). Both E_2 and MXC significantly increased LDH expression as indicated by the comparisons between GC-6spg/Mock E_2 and GC-6spg/Mock MXC with GC-6spg/Mock transfectants (Mock E_2: 1.50 ± 0.20 and Mock MXC: 1.37 ± 0.10 vs. 1.00 ± 0.08 fold variation in Mock; $p<0.05$). RGN overexpression counteracted the effects of E_2 and MXC, diminishing LDH expression levels to those of the untreated Mock transfectants (RGN E_2: 0.80 ± 0.08 vs. Mock E_2: 1.50 ± 0.20 fold variation, $p<0.01$ and RGN MXC: 0.79 ± 0.08 vs. Mock MXC: vs. 1.37 ± 0.10 fold variation, $p<0.001$). LDH expression levels were kept constant in cells overexpressing RGN in all study conditions.

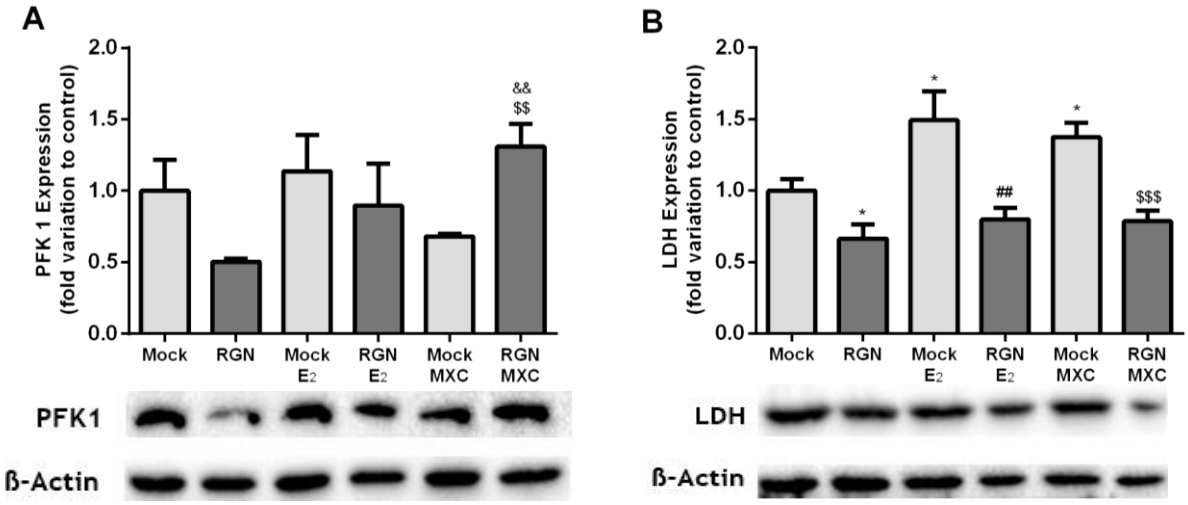

Figure 14. Protein expression of PFK1 (A) and LDH (B) in Mock and regucalcin (RGN) GC-6spg transfectants cultured for 48h, in the presence or absence of 25 µM of methoxychlor (MXC) or 100 nM of estradiol (E_2). Data are presented as mean ± S.E.M. (n≥5 in each group). Results are expressed as fold-variation comparatively to control in absence of E_2 and MXC (Mock); (*) statistically significant difference when compared with Mock group; (#) statistically significant different when compared with Mock E_2 group; ($) statistically significant difference when compared with Mock MXC group; (&) statistically significant difference when compared with RGN C group (* $p<0.05$; ## $p<0.01$; $$$ $p<0.001$; && $p<0.01$). Normalization was performed with ß-actin. Representative blots are shown.

3. SSCs Apoptosis in Response to RGN Transfection and E_2 and MXC Treatment

a. Bax/Bcl-2 Signaling and p53 Expression

In the intrinsic pathway of apoptosis, the outer mitochondrial membrane permeabilization and the consequent cytochrome c release is considered a "point of no return" (172). The balance between Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) regulators is extremely important in the regulation of the apoptotic process by promoting or inhibiting permeabilization of mitochondria membrane (172). Furthermore, it has been established that this balance can affect the output of spermatogenic process (51, 52).

Bax expression levels (figure 15A) were decreased in cells overexpressing RGN (GC-6spg/RGN) compared with cells with basal expression of this protein (RGN: 0.37 ± 0.18 vs. Mock: 1.00 ± 0.11 fold variation, $p<0.01$). The decreased Bax expression in cells overexpressing RGN (GC-6spg/RGN) is maintained in E_2 treatment (RGN E_2: 0.75 ± 0.08 vs. Mock E_2: 1.24 ± 0.13 fold variation; $p<0.05$). Despite the visible increase in Bax expression between control untreated group (GC-6spg/Mock) and E_2-treated cells (GC-6spg/Mock E_2), no significant difference was perceived. On the contrary, MXC treatment significantly enhanced Bax expression (Mock MXC: 1.62 ± 0.15 vs. Mock: 1.00 ± 0.11 fold variation, $p<0.05$). MXC effects inducing Bax were abolished by RGN overexpression (RGN MXC: 0.81 ± 0.13 vs. Mock MXC: 1.62 ± 0.15 fold variation, $p<0.01$).

The anti-apoptotic protein Bcl-2 (figure 15B) had its expression increased in GC-6spg/RGN cells compared to GC-6spg/Mock transfectants (RGN: 1.6 ± 0.19 vs. 1.00 ± 0.07 fold variation in Mock). Despite E_2 treatment alone did not affect Bcl-2 expression, in the case of RGN overexpression a significant increase was observed in response to E_2 (RGN E_2: 2.46 ± 0.39 vs. Mock E_2: 0.76 ± 0.15 fold variation, $p<0.01$). In the case of MXC treatment, the GC-6spg/Mock MXC group was shown to significantly express more Bcl-2 protein than the GC-6spg/Mock (Mock MXC: 1.70 ± 0.16 vs. Mock: 1.00 ± 0.07 fold variation, $p<0.01$). Again, RGN overexpression counteracted the MXC effects (RGN MXC: 0.74 ± 0.23 vs. Mock MXC: 1.70 ± 0.16 fold variation, $p<0.05$). Also, Bcl-2 expression in GC-6spg/RGN MXC was diminished in comparison to GC-6spg/RGN (RGN MXC: 0.74 ± 0.23 vs. RGN: 2.46 ± 0.39 fold variation, $p<0.05$).

The balance between Bax (proapoptotic) and Bcl-2 (anti-apoptotic) proteins dictates cell susceptibility to apoptosis. The Bax/Bcl-2 protein ratio (figure 15C) was decreased in GC-6spg cells overexpressing RGN (RGN: 0.23 ± 0.18 vs. 1.00 ± 0.09 fold variation in Mock transfectants, $p<0.01$). The effect of RGN overexpression is maintained under E_2-treatment; GC-6spg/RGN E_2-treated cells displayed a decreased Bax/Bcl-2 ratio relatively to GC-6spg/Mock transfectants (RGN E_2: 0.36 ± 0.23 vs. 1.00 ± 0.09 fold variation in Mock, $p<0.05$).

E_2 treatment significantly increased the Bax/Bcl-2 ratio (Mock E_2: 1.62 ± 0.14 vs. Mock: 1.00 ± 0.09 fold variation, $p<0.05$), an effect strongly suppressed in the presence of RGN overexpression (RGN E_2: 0.36 ± 0.23 vs. Mock E_2: 1.62 ± 0.14 fold variation; $p<0.01$). No differences were observed with MXC treatment alone. However, Bax/Bcl-2 ratio was significantly increased by MXC in GC-6spg/RGN cells (RGN MXC: 1.09 ± 0.18 vs. RGN: 0.23 ± 0.18 fold variation, $p<0.05$).

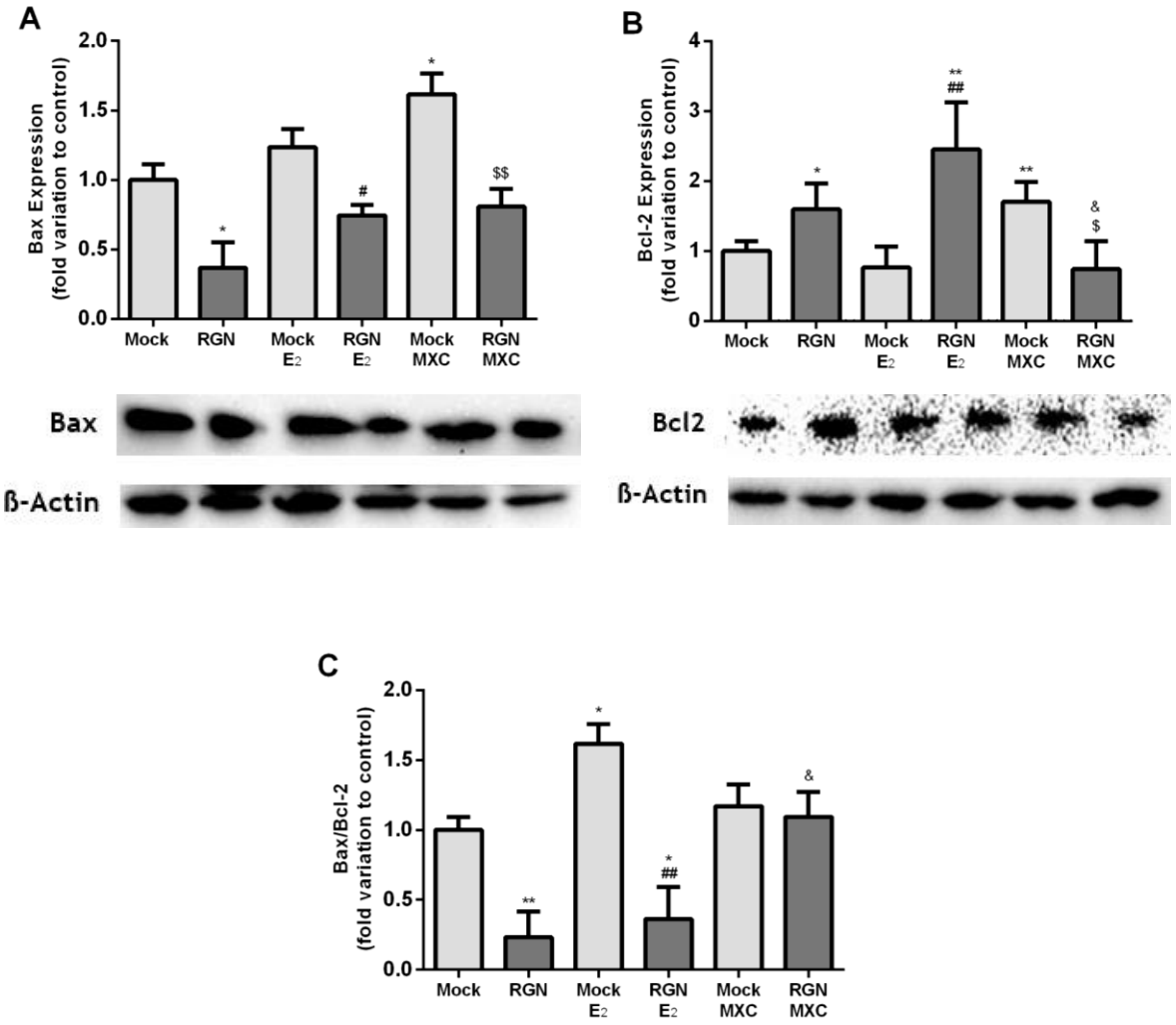

Figure 15. Protein expression of Bax (A) and Bcl-2 (B), and Bax/Bcl-2 protein ratio (C) in Mock and regucalcin (RGN) GC-6spg transfectants cultured for 48h, in the presence or absence of 25 μM of methoxychlor (MXC) or 100 nM of estradiol (E_2). Data are presented as mean ± S.E.M. (n≥4 in each group). Results are expressed as fold-variation comparatively to control in absence of E_2 and MXC (Mock); (*) statistically significant difference when compared with Mock group; (#) statistically significant difference when compared with Mock E_2 group; ($) statistically significant difference when compared with Mock MXC group; (&) statistically significant difference when compared with RGN group (* $p<0.05$; ** $p<0.01$; # $p<0.05$; ## $p<0.01$; $ $p<0.05$; $$ $p<0.01$; & $p<0.05$). Normalization was performed with ß-actin. Representative blots are shown.

The expression levels of the pro-apoptotic tumor suppressor protein p53 (figure 16), were also evaluated in GC-6spg/Mock and GC6-spg/RGN cells with or without MXC or E_2 treatments.

GC-6spg cells overexpressing RGN (GC-6spg/RGN) showed lower expression levels of p53 compared to mock transfectants (RGN: 0.42 ± 0.05 vs. 1.00 ± 0.095 fold variation in Mock

transfectants, $p<0.01$); a feature maintained in E_2 and MXC treatments (RGN E_2: 0.36 ± 0.06 and RGN MXC: 0.51 ± 0.07 vs. 1.00 ± 0.10 fold variation in Mock transfectants; $p<0.01$).

Both E_2 and MXC treatments also significantly diminished p53 expression levels (Mock E_2: 0.61 ± 0.062, $p<0.05$ and Mock MXC: 0.31 ± 0.028, $p<0.001$ vs. 1.00 ± 0.10 fold variation in Mock transfectants). No significant differences were observed between GC-6spg/RGN and GC-6spg/RGN MXC or between GC-6spg/RGN and GC-6spg/RGN E_2. Cells overexpressing RGN (GC-6spg/RGN) showed lower levels of p53 expression in the case of E_2 and higher levels in the case of MXC treatment, comparing to their control counterparts (RGN E_2: 0.36 ± 0.06 vs. Mock E_2: 0.61 ± 0.06 fold variation; RGN MXC: 0.51 ± 0.07 vs. Mock MXC: 0.31 ± 0.028 fold variation; $p<0.05$).

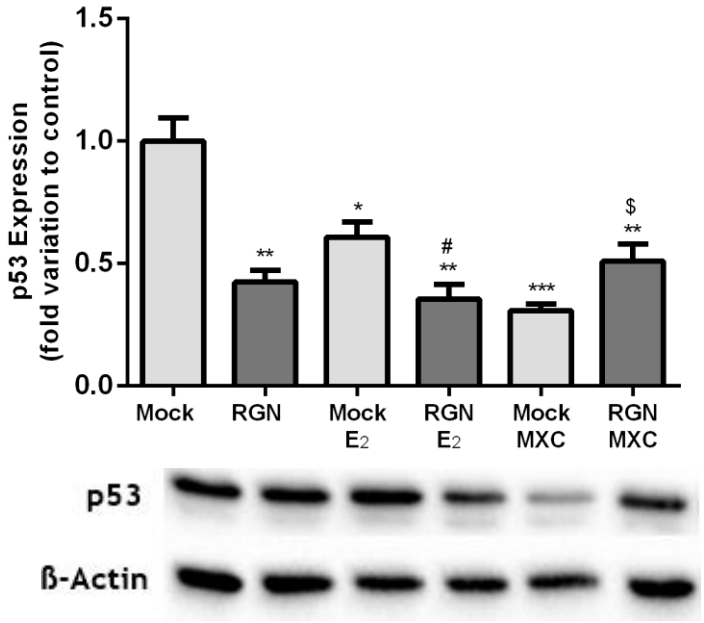

Figure 16. Protein expression of p53 in Mock and regucalcin (RGN) GC-6spg transfectants cultured for 48h, in the presence or absence of 25 μM of methoxychlor (MXC) or 100 nM of estradiol (E_2). Data are presented as mean ± S.E.M. ($n \geq 4$ in each group). Results are expressed as fold-variation comparatively to control in absence of E_2 and MXC (Mock C); (*) statistically significant difference when compared with Mock C group; (#) statistically significant difference when compared with Mock E_2 group; ($) statistically significant difference when compared with Mock MXC group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; # $p<0.05$; $ $p<0.05$). Normalization was performed with ß-actin. Representative blots are shown.

b. Caspase-3 Activity

The activation of the effector caspase-3 has been considered a remarkable end-point of apoptotic cell death, since both intrinsic and extrinsic pathways of apoptosis converge at this point (47).

Caspase-3 activity (figure 17) was shown to be significantly decreased in GC-6spg/RGN and GC-6spg/RGN E_2 cells compared to the GC-6spg/Mock transfectants group (RGN: 0.77 ± 0.07, $p<0.05$ and RGN E_2: 0.63 ± 0.02, $p<0.001$ vs. 1.00 ± 0.06 fold variation in Mock transfectants).

Also, E_2 treatment diminished caspase-3 activity (Mock E_2: 0.71 ± 0.06 vs. 1.00 ± 0.06 fold variation in Mock transfectants).

In contrast, MXC strongly increased caspase-3 activity (Mock MXC: 1.30 ± 0.09 vs. 1.00 ± 0.06 fold variation in Mock transfectants, $p<0.05$). No significant differences were observed between RGN overexpressing cells exposed to E_2 and RGN transfectant cells with no exposure. However, caspase-3 activity was significantly higher in GC-6spg/RGN treated with MXC than in GC-6spg/RGN (RGN MXC: 1.05 ± 0.05 vs. RGN: 0.77 ± 0.07 fold variation, $p<0.01$); although not restoring the expression levels in the GC-6spg/RGN groups, RGN overexpression significantly lowered caspase-3 activity in MXC treatment (RGN MXC: 1.05 ± 0.05 vs. Mock MXC: 1.30 ± 0.09 fold variation, $p<0.05$).

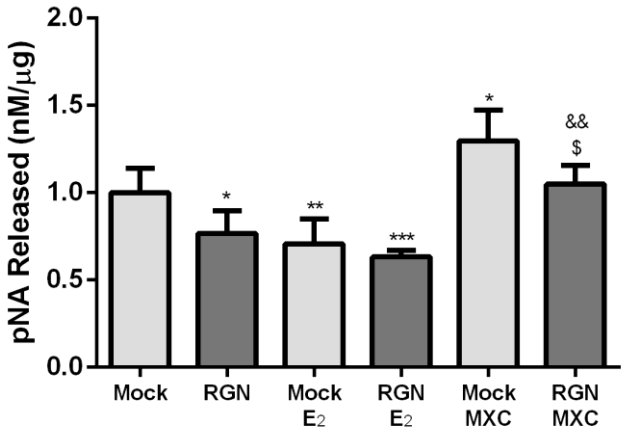

Figure 17. Caspase-3 activity of Mock and regucalcin (RGN) GC-6spg transfectants cultured for 48h, in the presence or absence of 25 μM of methoxychlor (MXC) or 100 nM of estradiol (E_2). Data are presented as mean ± S.E.M. (n≥5 in each group). Results are expressed as fold-variation comparatively to control in absence of E_2 and MXC (Mock C); (*) statistically significant difference when compared with Mock C group; ($) statistically significant difference when compared with Mock MXC group; (&) statistically significant difference when compared with RGN group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; $ $p<0.05$; && $p<0.01$).

V. Discussion

EDCs exposure *in utero* and during early life can perturb hormonal signaling causing birth defects, behavioral disorders, disrupted spermatogenesis and even cancer. Moreover, it has been shown that EDCs effects can be passed down to the future generations. In the testis, the population of SSCs are the responsible for the transmission of genetic information, and play a relevant role as the foundation of spermatogenesis and male fertility. The self-renewal capability and the high differentiation of SSCs presupposes a high metabolic activity and a meticulous regulation of cell cycle entrance and apoptosis in order to provide the adequate spermatogenic output. Nevertheless, there are no reports focused directly on assessing the mechanisms behind regulation of SSCs metabolism and apoptosis. Furthermore, the response of SSCs to hormonal or environmental stimuli is a matter that remains completely unknown. Like stem cells, an unpredictable behavior may be implied and studies specifically evaluating hormone/EDCs effects on this particular testicular cell type are of utmost importance.

To start fulfilling this lack of knowledge, the present thesis investigated the effect of E_2 and the xenoestrogen MXC in apoptosis and metabolism of GC-6spg cells, a rat cell line with SSC's characteristics. Moreover, we hypothesize that the RGN protein may have a protective role against the likely damage actions of these hormonal factors. For this purpose, we firstly generated GC-6spg cells transfected to overexpress RGN (GC-6spg/RGN), which showed an increased RGN protein expression of, approximately, 3-fold compared with the mock-transfectants (GC-6spg/Mock, figure 12). RGN has been shown to be expressed in several germ cell types of human and rat testis, namely spermatogonia, spermatocytes, spermatids and spermatozoa (151) but the results presented herein are the first report of its presence in SSCs.

Secondly, GC-6spg/RGN and GC-6spg/Mock cells were exposed to 100 nM E_2 or 25 µM MXC, and the apoptosis and metabolism analyzed.

GC-6spg cells exposed to E_2 (GC-6spg/Mock E_2) did not presented alterations in glycolytic metabolic, either in glucose consumption (figure 13A) or lactate production (figure 13B), which was consistent with the unaltered PFK1 expression (figure 14A). However, LDH expression (figure 14B) was considerably augmented in response to E_2 treatment, which do not sustains the unaltered lactate production relatively to control. It is not possible to drive a definitive explanation for this fact at the moment, but it could be suggested that LDH is not actively producing lactate. LDH catalyzes the reversible reaction of metabolizing pyruvate into lactate, and it has been shown that the LDH isoform LDHC, which is highly expressed in germ cells, is the responsible for the conversion of lactate into pyruvate (25). Following this rational, it is possible to consider that E_2 is specifically regulating the LDHC isoform, and that GC-6spg cells are using lactate to produce pyruvate that is then redirected to the mitochondria. Anyway, it cannot be excluded the possibility that despite the increased LDH expression, the LDH activity could be unaltered.

On the contrary MXC stimuli, considerably increased glucose consumption (figure 13A) and lactate production (figure 13B) in GC-6spg cells, which was supported by the increased LDH

expression. The increased glycolytic metabolism is a typical feature of cancer cells. Moreover, MXC has been indicated as carcinogenic in female reproductive tissues, such as breast (173) and ovary (174). Therefore, the accentuated difference in glucose consumption and lactate production verified in MXC-treated cells suggests that this EDC establishes of a high glycolytic metabolism, an effect not observed in E_2-treated cells, which can be altering SSCs to a malignant phenotype.

Interestingly, RGN overexpression tended to counteract the MXC effects. The increase in glucose consumption (figure 13A) and lactate production (figure 13B) induced by MXC is higher in mock-transfected cells that in GC-6spg/RGN MXC. In other words, the glucose consumption and lactate production in MXC-treated cells were lower under conditions of RGN overexpression. Moreover, the observed increase in LDH expression (figure 14B) in response to MXC was abolished in GC-6spg cells overexpressing RGN, with the expression levels returning to that of control (Mock). These findings suggest the protective role of RGN against the possible damage of MXC by enhancing glycolytic metabolism.

RGN overexpression itself increased the glucose consumption (figure 13A) and lactate production (figure 13B) in GC-6spg cells, regardless of E_2, or MXC stimulation, and despite no significant difference was observed in PFK1 expression (figure 14A), and LDH expression (figure 14B) was significantly lower. It would be determinant to confirm whether the diminished of expression of LDH is being counterbalanced by its augmented activity, sustaining the high lactate production. It is also important to notice that the increase in glycolytic flux upon MXC-treatment was much higher than that verified by RGN overexpression. Thus, it is liable to assume that the effects of RGN are moderated whereas the effects of MXC are exacerbated and probably damaging. The effects of RGN overexpression increasing glucose consumption and lactate production were also demonstrated in bone marrow cell cultures, which was linked with the physiological effect of enhanced osteoclast activity (165).

Resembling cancer cells, SSCs are constantly in division, which implies a high metabolic demand. The augmented glucose consumption in GC-6spg/RGN cells suggests that RGN can be boosting glucose uptake, accelerating their glycolytic metabolism, and sustaining the high proliferation rates. In turn, it may indicate higher differentiation of SSCs and empowerment of spermatogenic process, which could lead to higher sperm counts, improved quality of spermatozoa and/or enhanced fertility. The *in vivo* findings in the Tg-RGN rats (knock-in animals overexpressing RGN) further corroborated this idea, since these animals displayed augmented sperm viability and lower incidence of morphology defects, despite showing lower sperm counts (161) Considering germ cells metabolism, it is known that, in general, they are not lactate producers, instead they use the lactate produced by SCs to sustain their metabolic needs (35). Moreover, it is established that lactate is the preferred energetic substrate of germ cells (25). However, the germ cells outside the BTB, as is the case of SSCs, can have access to glucose present in bloodstream, and can uptake it directing to the glycolytic

metabolism. Indeed, the results obtained demonstrated that GC6-spg cells produce lactate, a feature that is stimulated by RGN overexpression, as well as by MXC stimulation.

The effect of RGN overexpression on apoptosis of GC-6spg cells was evaluated by analyzing the expression and activity of key regulators of apoptotic pathways. RGN overexpression decreased the activity of the apoptosis effector caspase-3 (figure 17), concomitantly, with diminished Bax/Bcl-2 ratio (figure 15C), which suggests an anti-apoptotic role for RGN in SSCs. The tumor suppressor protein p53 has a critical role in the regulation of the Bcl-2 family members, and also upregulates the expression of cyclin-dependent kinase inhibitor, p21, arresting cell cycle at G1 phase and promoting apoptosis in response to a variety of stress stimuli (175). Thus, the lower expression levels of p53 (figure 16) in GC-6spg/RGN cells further supports the anti-apoptotic action of RGN, and other studies also report the diminished expression of p53 in the prostate of Tg-RGN rats (37). Indeed, the function of RGN suppressing apoptosis has been described in other *in vivo* and *in vitro* study models. The SeT of Tg-RGN rat showed diminished expression and activity of caspase-3 in the presence of chemical apoptosis inducers, alongside with the increased expression of antiapoptotic Bcl-2, and the augmented Bcl-2/Bax ratio, indicating a resistance to apoptosis under conditions of RGN overexpression (157). Also, a study generating radioresistant pancreatic cell lines showed a concomitant overexpression of RGN (159), which suggested that RGN expression increased in response to cell damage, likely as a protective mechanism. Indeed, a study of our research group described the protective effect of RGN against radiation-induced damage in testicular cells. Tg-RGN animals exposed to X-rays displayed higher sperm viability and motility relatively to their wild-type counterparts, as well as a higher frequency of normal sperm morphology, a diminished incidence of head-defects and a lower rate of apoptosis (160). These findings strongly support the involvement of RGN in the anti-apoptotic response, which is further corroborated by the observed enhanced expression of RGN in the testis of irradiated rats (160). Taking into account the wide effects of RGN overexpression protecting testicular cells against chemical-inducers of apoptosis and radiation-induced damage, it is highly expected a similar response in GC6-spg/RGN cells in the presence of damaging stimuli.

In the case of E_2 treatment, caspase-3 activity (figure 17) was decreased in of GC-6spg/Mock E_2 cells, alongside with the diminished expression of the tumor suppressor and anti-proliferative p53 protein (figure 16). These results sustain that E_2 has anti-apoptotic effects in SSCs. However, the Bax/Bcl-2 ratio (figure 15C) was increased by E_2, lead to believe that the intrinsic pathway of apoptosis is being inhibited at some point. Many mechanisms would be involved, namely a family of proteins called inhibitors of apoptosis proteins (IAPs) that, after caspases' activation, can bind to them avoiding cleavage (176).

Considering the sensibility of the germ line is not surprisingly that augmented rates of apoptosis have been identified in the testes of subfertile and infertile men, which also display high E_2 intratesticular concentrations (177, 178). Also, the altered expression patterns of a panoply of apoptosis related genes have been described in human testes with defective

spermatogenesis (47, 179, 180). The 100 nM of E_2 used in the present study resembles the high E_2 intratesticular concentrations found in the testes of subfertile and infertile men, and have been shown to augment germ cell apoptosis in SeT cultures *ex vivo* (58), whereas in the particular case of SSCs apoptosis was diminished. Curiously, the same E_2 concentration was shown to decrease apoptosis of SCs, which leads us to hypothesize that also in the context of apoptosis SSCs have a behavior similar to the diploid somatic cells. Indeed, SSCs are diploid cells outside the protection of the BTB and thus, is it likely that they should be more resistant to damage than haploid germ cells.

Contrastingly with the E_2 effect, MXC stimuli increased caspase-3 activity (figure 17), though no differences were observed in Bax/Bcl-2 ratio (figure 15C). This suggests that the observed increase in caspase-3 activity is not due to the intrinsic pathway of apoptosis, and maybe possibly activated by the extrinsic pathway, which is also sustained by the highly decreased expression of p53 (figure 16), since this protein is a direct regulator of Bax/Bcl-2. Nevertheless, apoptosis of GC-6spg cell was increased by MXC-treatment, which indicates that this EDC may disturb the SSCs population affecting the normal course of spermatogenesis. Importantly, and supporting the idea of its protective role, RGN overexpression attenuated the effect of MXC increasing caspase-3 activity (figure 17).

The augmented apoptosis indicated by the increased activity of caspase-3 in response to MXC treatment is not entirely in accordance with the hypothesis of MXC as a carcinogen in SSCs by altering their metabolism towards the highly glycolytic phenotype typical of cancer cells. A known feature of cancer cells is the development of strategies to escape or suppress programmed cell death. Nevertheless, the highly decreased expression of tumor suppressor p53 in MXC-treated cells allow assuming an initial tumorigenic phase.

In sum, RGN overexpression was shown to stimulate the glycolytic metabolism of SSCs, which may help sustaining their high metabolic needs. Herein, we identified SSCs as lactate producers, similarly with SCs, which suggests a model of lactate shuttle between SSCs, SCs and other germ cells (figure 18). This shuttling of lactate between neighbor cells with different metabolic requirements has been reported in other cell types, namely in the tumoral microenvironment, where the cancer cells next to blood vessels uptake the metabolites in bloodstream and produce new metabolites in order to supply the others more distant from blood vessels (181). The first ones export lactate through the MCT4 and the latter import lactate through MCT1/2 (lactate shuttle) (181). A similar mechanism has been proposed between SCs and germ cells (figure 18) (25). However, RGN overexpression was capable of attenuating the exacerbate and potentially harmful effect of MXC stimulating the glycolytic metabolism of SSCs.

Both E_2 and RGN had antiapoptotic effects over SSCs whereas MXC induced apoptosis. Also in the apoptotic cell death, and similarly with the response seen in metabolism, RGN overexpression counteracted the proapoptotic behavior of MXC.

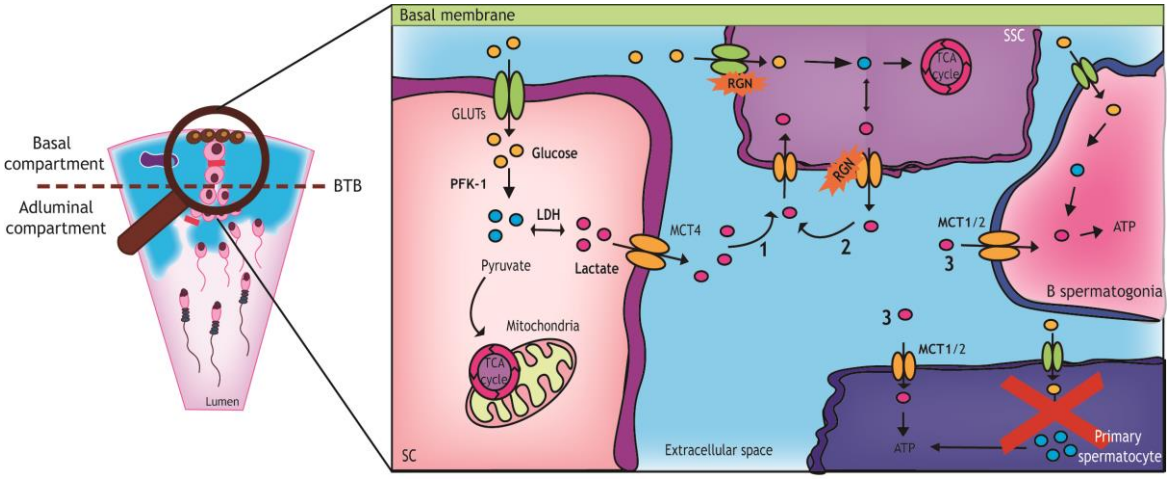

Figure 18. Lactate shuttle and regucalcin (RGN) role in spermatogonial stem cells (SSCs) metabolism. Lactate produced by Sertoli cells (SCs) is exported to the extracellular space through monocarboxylate transporter 4 (MCT4), and enters in: i) spermatogonial stem cells (SSCs, Lactate shuttle 1, not demonstrated yet) and ii) in other germ cells through MCT1 or MCT2 (lactate shuttle 3). In the present work, SSCs were demonstrated to produce lactate, leading to believe that they can participate in the lactate shuttle 3 together with SCs, as well as in lactate shuttle 2 (self-sustain). The germ cells represented are located outside the blood testicular barrier (spermatogonia p.e.) and might utilize the glucose from blood as energy source. In SSCs, RGN was shown to increase glucose consumption and lactate production (represent in orange). GLUTs, glucose transporters; PFK-1, phosphofructokinase-1; TCA cycle, tricarboxylic acid cycle; ATP, adenosine triphosphate.

VI. Conclusions and Future Perspectives

The fine regulation of spermatogenesis is strictly dependent on sex steroid hormones. Recently, estrogens have emerged as important regulators of germ cell fate. However, the beneficial or detrimental effects of estrogens in spermatogenesis are controversial. Moreover, EDCs with xenoestrogenic properties, including MXC have been shown to affect male reproduction. However, the molecular mechanisms underlying EDCs actions leading to pathologies of the reproductive system are not well characterized, specifically their impact on SSCs.

The present dissertation demonstrated the presence of RGN in SSCs and was the first study evidencing the effect of E_2 and MXC on SSCs metabolism and apoptosis, as well as pointing out RGN as a protective factor for the damaging effects of these compounds.

Herein, it was shown that RGN increased the glycolytic activity and diminished apoptosis of SSCs. MXC-treatment greatly stimulated the metabolism of SSCs and enhanced their apoptotic status, whereas E_2-treatment displayed none or mild effects. These findings highlight for the impact that MXC exposure might have disrupting the SSCs population and compromising male fertility. Interestingly, overall, RGN overexpression tended to counteract MXC effects on glycolytic metabolism and E_2 and MXC effects over apoptosis supporting the protective role of RGN in spermatogenesis. Although preliminary, these findings are an important piece of work in the context of EDCs actions and male infertility. In the future, the full comprehension of the molecular mechanism responsible for RGN actions in SSCs will be of uttermost importance, as a strategy to alleviate the effects of damaging factors. A greater number of players of the glycolytic and apoptotic pathways still need to be evaluated to fully understand these regulatory and protective mechanisms. Also, the proposed model of metabolic interaction/cooperation between SSCs and other germ cells and SCs located in the neighborhood needs to be studied. Thus, co-cultures of the different cell types should be performed in order to have a more realistic physiological environment.

Ultimately, after solving the questions aroused, this study can be a useful base for *in vivo* approaches and for the development of new and more efficient fertility-preserving strategies and treatments.

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