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The impact of endocrine disruptors on spermatogonia survival: the case of methoxychlor

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Dedicatória

Aos meu pais, avós e irmã

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Resumo

Os desreguladores endócrinos (EDCs) são xenobióticos que têm a capacidade de interferir na síntese, secreção e metabolismo hormonal, podendo assim afetar o sistema reprodutor. Hoje em dia estamos ubiquamente expostos a estes compostos e por diversas vias, tais como ingestão, inalação e absorção dérmica. O metoxicloro (MXC) é um pesticida organoclorado com persistência moderada no meio ambiente, capaz de afetar adversamente a espermatogénese, desregulando o desenvolvimento das células germinativas, e interferindo com a função espermática. De entre as células germinativas, as espermatogónias do tipo B são a primeira população celular diferenciada da linha germinativa e as iniciadoras do processo espermatogénico. No entanto, pouco se sabe sobre o papel do MXC na modulação das vias de sobrevivência das espermatogónias do tipo B.

Na presente dissertação, foi estudado o impacto do MXC na sobrevivência/apoptose assim como nas defesas antioxidantes das espermatogónias. Para tal, uma linha celular de espermatogónias do tipo B, as GC-1spg, foi colocada em cultura na presença (5, 10, 25, 50 e 100 μM) e ausência de MXC, durante 48 horas. O efeito do MXC na viabilidade das células GC-1spg foi analisado através de ensaios de bromido de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT). A expressão proteica de reguladores chave da apoptose, dos recetores de estrogénios alfa e beta (ERα e ERβ, respetivamente) e do recetor de androgénios (AR) foi analisada pela técnica de *Western Blot* e a atividade de enzimas antioxidantes e da caspase-3 por ensaios colorimétricos.

Os resultados obtidos demonstram que o MXC diminui a viabilidade das células GC-1spg de uma forma dependente da concentração, aumentando a atividade das enzimas antioxidantes superóxido dismutase e glutationa peroxidase, e da caspase-3. Apesar do aumento da razão Bax (proteína pró-apoptótica)/Bcl-2 (proteína anti-apoptótica), verificou-se uma diminuição da expressão da caspase-8 e da p53 nas GC-1spg expostas ao MXC. O tratamento com MXC diminuiu a expressão de ambos os recetores de estrogénios (ERα e ERβ) e do AR nas GC-1spg.

O presente estudo evidencia a modulação da apoptose nas células GC1-spg pelo MXC, sendo o primeiro a demonstrar a expressão do ERβ nesta linha celular GC-1spg e a mostrar o impacto do MXC na expressão destes recetores de esteroides sexuais. Através da investigação dos mecanismos e vias pelos quais os EDCs exercem os efeitos nefastos na saúde reprodutora masculina, o presente estudo abre horizontes para a identificação de alvos para desenvolvimento de novas estratégias de preservação da fertilidade masculina e eventuais tratamentos.

Palavras-Chave

Apoptose; Espermatogónias tipo B; Metoxicloro; Recetores de esteroides sexuais; Sobrevivência; Stress oxidativo

Resumo alargado

Os desreguladores endócrinos (EDCs) são xenobióticos que têm a capacidade de interferir na síntese, secreção e metabolismo hormonal, podendo assim afetar o sistema reprodutor. Os EDCs podem ser classificados como agonistas, mimetizando hormonas esteróides sexuais endógenas, como é o caso dos xenoestrogénios/xenoandrogénios ou antagonistas, bloqueando a ação destas hormonas, os denominados anti-estrogénicos/anti-androgénicos. O metoxicloro (MXC) é um pesticida organoclorado com persistência moderada no meio ambiente e está descrito como sendo capaz de mimetizar as ações dos estrogénios. O MXC apresenta uma maior afinidade para o recetor de estrogénio alfa (ERα) do que para o ER beta (ERß). Alguns estudos têm descrito as consequências da exposição ao MXC no sistema reprodutor masculino, nomeadamente, a redução do tamanho dos testículos, distúrbios na espermatogénese, atrofia do epidídimo e diminuição da viabilidade e do número de espermatozoides.

Em condições fisiológicas normais, a eliminação das células germinativas ocorre espontaneamente através de um processo denominado apoptose. A apoptose consiste na morte celular programada, de forma controlada e ordenada, que permite manter o equilíbrio entre a proliferação e degeneração. A exposição aos EDCs pode exacerbar a apoptose destas células levando ao comprometimento da função testicular. Além disso, o tecido testicular e o sistema reprodutor masculino são particularmente suscetíveis a stress oxidativo (OS). O OS ocorre quando existe um desequilíbrio entre a produção e remoção de espécies reativas de oxigénio (ROS), incapaz de ser regulado pela atividade de enzimas antioxidantes.

De entre as várias células germinativas, as espermatogónias do tipo B são a primeira população celular diferenciada, iniciando o processo espermatogénico. Assim, qualquer ameaça à sua sobrevivência pode ter consequências nefastas para a fertilidade masculina. Além disso, a sua localização fora da barreira hematotesticular torna-as mais suscetíveis à ação de fatores exógenos, como é o caso dos EDCs. É já conhecido que o MXC afeta adversamente a espermatogénese, desregulando o desenvolvimento das células germinativas, e interferindo com a função espermática. No entanto, pouco se sabe sobre o papel dos EDCs na modulação das vias de sobrevivência de espermatogónias do tipo B, em específico.

Na presente dissertação, foi estudado o impacto do MXC na sobrevivência/apoptose, assim como nas defesas antioxidantes das espermatogónias. Para tal, uma linha celular de espermatogónias do tipo B, as GC-1spg, foi colocada em cultura na presença (5, 10, 25, 50 e 100 μM) e ausência de MXC, durante 48 horas. O efeito das várias

concentrações de MXC na viabilidade das células GC-1spg foi analisado através de ensaios de bromido de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT) e as concentrações de 10 e 25 μM de MXC foram selecionadas para as análises posteriores. As alterações na expressão proteica de reguladores chave da apoptose (Bax, Bcl-2, p53, caspase-8) e de recetores hormonais (ERα, ERβ, recetor de androgénios, AR) foram analisadas pela técnica de *Western Blot*. A atividade das enzimas antioxidantes superóxido dismutase (SOD) e glutationa peroxidase (GPx), assim como da caspase-3, o ponto sem retorno da via apoptótica, foi avaliada por ensaios colorimétricos.

Os resultados obtidos demonstram que o MXC diminui a viabilidade das células GC-1spg de uma forma dependente da concentração. Na presença de MXC, foi observado um aumento da atividade das enzimas antioxidantes SOD e GPx, assim como da caspase-3. Embora não tenha sido observada nenhuma alteração nos níveis de expressão da proteína pró-apoptótica Bax, o tratamento com MXC aumentou a razão Bax/Bcl-2, diminuindo notoriamente a expressão da proteína anti-apoptótica Bcl-2. No entanto, verificou-se uma diminuição da expressão da caspase-8 e da p53. O tratamento com MXC demonstrou diminuir a expressão do ERα, ERβ e AR nas GC-1spg.

O presente estudo evidencia a modulação da apoptose das células GC1-spg pelo MXC. Além de afetar a viabilidade celular, o MXC teve efeito pró-apoptótico, possivelmente pela indução da via intrínseca. O MXC parece estar a estimular as defesas antioxidantes das células GC-1spg, o que é compreensível visto que já foi descrito como indutor de OS, e este aumento na atividade das enzimas antioxidantes pode representar um mecanismo de combate à excessiva formação de ROS.

Este estudo é o primeiro a demonstrar a expressão do ERβ em células GC-1spg e a mostrar o impacto do MXC na expressão destes recetores de esteroides sexuais.

Curiosamente, observou-se uma diminuição da expressão de todos os recetores hormonais indicados após exposição ao MXC, sendo que mais estudos devem ser efetuados para explorar este efeito.

Embora preliminares, os resultados obtidos enfatizam o possível impacto negativo que a exposição ao MXC pode ter na população celular de espermatogónias do tipo B. Estes resultados são extremamente relevantes, explorando os mecanismos e vias pelos quais os EDCs exercem os seus efeitos nocivos na saúde reprodutora masculina. Desta forma, abrem horizontes para a identificação de alvos para o desenvolvimento de novas estratégias de preservação da fertilidade masculina e eventuais tratamentos.

Abstract

Endocrine disruptors (EDCs) are xenobiotics that have the ability to interfere with hormone synthesis, secretion and metabolism, thus affecting the reproductive system. Today we are ubiquitously exposed to these compounds through various pathways, such as ingestion, inhalation and dermal absorption. Methoxychlor (MXC) is an organochloride pesticide with moderate persistence in the environment capable of adversely affecting spermatogenesis, deregulating the development of germ cells, and interfering with sperm function. Among germ cells, type B spermatogonia are the first differentiated germline cell population and the initiator of the spermatogenic process. However, little is known about the role of MXC in modulating the survival pathways of type B spermatogonia.

In the present dissertation, the impact of MXC on survival/apoptosis, and on the antioxidant defences of spermatogonia, was studied. For this purpose, a type B spermatogonia cell line, the GC-1spg, was cultured in the presence (5, 10, 25, 50 and 100 μM) and without MXC for 48 hours. The effect of MXC on GC-1spg viability was analysed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assays. Moreover, protein expression of key regulators of apoptosis, estrogen receptors alpha and beta (ERα and ERβ, respectively) and androgen receptor (AR) was analysed by *Western Blot* and the activity of antioxidant enzymes and caspase-3 by colorimetric assays.

The obtained results demonstrated that MXC decreases the viability of GC-1spg cells in a concentration-dependent manner, increasing the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase, and caspase-3. Despite the increase in the Bax (pro-apoptotic protein)/Bcl-2 (anti-apoptotic protein) ratio, there was a decrease in caspase-8 and p53 expression in GC-1spg exposed to MXC. In addition, MXC treatment decreased the expression of both estrogen receptors (ERα and ERβ) and AR in GC-1spg.

The present study demonstrates the modulation of GC1-spg apoptosis by MXC, being the first to demonstrate the expression of ERβ in GC-1spg and to show the impact of MXC on the expression of these sex steroid receptors. By investigating the mechanisms and pathways by which EDCs exert harmful effects on male reproductive health, the present study widens the horizons for the identification of targets for developing new strategies for preserving male fertility and eventual treatments.

Keywords

Apoptosis, Methoxychlor, Oxidative stress, Sex steroid receptors, Survival, Type B spermatogonia

Índice

1. Introduction 1
    1.1. Brief overview of testicular anatomy and physiology 1
    1.2. The mammalian spermatogenic process 3
    1.3. Hormonal regulation of spermatogenesis 4
    1.4. Mechanisms involved on germ cell death/survival 6
        1.4.1. Oxidative stress 6
        1.4.2. Apoptosis 8
    1.5. Generalities on endocrine-disrupting chemicals 11
        1.5.1. Classification 11
        1.5.2. Mechanism of action 12
        1.5.3. Pesticide threatening to male fertility: the case of methoxychlor 13
2. Aim of the dissertation 16
3. Materials and methods 17
    3.1. Cell line 17
    3.2. Cell culture and methoxychlor treatment 17
    3.3. Cell viability assays 17
    3.4. Total protein extraction and quantification 18
    3.5. Superoxide dismutase assay 18
    3.6. Glutathione peroxidase assay 19
    3.7. Caspase-3 like activity assay 19
    3.8. Western blot analysis 20
    3.9. Statistical analysis 20
4. Results 21
    4.1. Methoxychlor treatment decreased the viability of GC-1spg in a dose-dependent manner 21
    4.2. GC-1spg antioxidant defences are prompted upon exposure to methoxychlor 22
    4.3. GC-1spg apoptotic status after methoxychlor treatment 23
        4.3.1. The enzymatic activity of caspase-3 was increased in methoxychlor-exposed GC-1spg 23
        4.3.2. Methoxychlor exposure modulated apoptosis-related protein expression levels in GC-1spg 23

4.4. Estrogen and androgen receptors are expressed in GC-1spg cells, being downregulated by methoxychlor 27
5. Discussion 29
6. Conclusions and future perspectives 35
7. References 37

List of Figures

Figure 1 – The mammalian testis and associated structures.....2
Figure 2 – Testicular histology and spermatogenic process.....4
Figure 3 – Hormonal regulation of the spermatogenesis.....5
Figure 4 – Production of reactive oxygen species by membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NADPH dehydrogenase.....7
Figure 5 – Programmed cell death.....11
Figure 6 – Percentage of viable GC-1spg cells after exposure to several concentrations of methoxychlor for 48 h, evaluated by the MTT assay.....21
Figure 7 – Glutathione peroxidase and superoxide dismutase activity in GC-1spg cells cultured for 48 h, in the presence or absence of 10 and 25 μM methoxychlor.....22
Figure 8 – Caspase-3 activity in GC-1spg cells cultured for 48 h, in the presence or absence of 10 and 25 μM methoxychlor.....23
Figure 9 – Protein expression of the Bcl-2 family proteins, Bax and Bcl-2, in GC-1spg cells cultured for 48 h, in the presence or absence of 25 μM methoxychlor.....24
Figure 10 – Protein expression of caspase-8 in GC-1spg cells cultured for 48 h, in the presence or absence of 25 μM methoxychlor.....25
Figure 11 – Protein expression of p53 in GC-1spg cells cultured for 48 h, in the presence or absence of 25 μM methoxychlor.....26
Figure 12 – Protein expression of estrogen receptors, ERα and ERß, and androgen receptor in GC-1spg cells cultured for 48 h, in the presence or absence of 25 μM methoxychlor.....27

List of abbreviations

AhR	Aryl hydrocarbon receptor
Apaf-1	Apoptotic protease-activating factor 1
AR	Androgen receptor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting-domain death agonist
BTB	Blood-testis barrier
Ca^{2+}	Calcium
CAR	Constitutive androstane receptor
CARD	Caspase-recruitment domain
CAT	Catalase
cBID	Cleaved Bid
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1- propanesulfonate,
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper zinc superoxide dismutase
CYP	Cytochrome P450
DDT	Dichloro-diphenyl-trichloroethane
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E2	17-ß estradiol
EDCs	Endocrine-disrupting chemicals
EDTA	Ethylenediamine tetraacetic acid
ER	Estrogen receptor
ERR	Estrogen related receptors
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
G6PD	Glucose-6-phosphate dehydrogenase
GC-1spg	Gonadal cell-1 spermatogonia
GnRH	Gonadotropin-releasing hormone

GPER	G protein-coupled estrogen receptor
GPx	Glutathione peroxidase
GSSH	Oxidized glutathione
GST	Glutathione-*S*-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
HPTE	2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane
LCs	Leydig cells
LH	Luteinizing hormone
LHR	Luteinizing hormone *receptor*
mER	Membrane estrogen receptors
Mn-SOD	Manganese superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MXC	Methoxychlor
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NRs	Nuclear hormone receptors
O2•	Superoxide anions
OH•	Hydroxyl
OS	Oxidative stress
pNA	p-nitro-aniline
PPAR	Peroxysome proliferator activated receptor
PXR	Pregnane X receptor
RIPA	Radioimmunoprecipitation assay
RIPK1	Receptor-interacting protein kinase 1
RNA	Ribonucleic acid
ROO•	Peroxyl
ROS	Reactive oxygen species
SCs	Sertoli cells
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SeTs	Seminiferous tubules
SOD	Superoxide dismutase
T	Testosterone
TNF	Tumour necrosis factor
TNF-R1	Tumour necrosis factor receptor type 1

TRADD	TNFR1-associated death domain protein
TSPY1	Testis-specific protein Y-linked 1
USP7	Ubiquitinated ubiquitin-specific peptidase 7

1. Introduction

1.1. Brief overview of testicular anatomy and physiology

The male reproductive system includes the testes, the system of ducts that store and transport sperm, the accessory glands that produce secretions which constitute the semen and the penis (1). The testes are located within the scrotum, which serves as a protective envelope and helps to maintain the testicular temperature approximately 2 °C below abdominal temperature (2). Mammalian testes (Figure 1) have to fulfill two major functions: the generation of gametes and the production and controlled release of sex steroids, fundamental to the normal development of male internal and external genitalia (2,3). Both testes are covered by two tunics. The outer *tunica* is the *tunica vaginalis* and their visceral layer covers the surface of each testis, except where the testis attaches to the epididymis and spermatic cord (4). The parietal layer of the *tunica vaginalis* covers more tissue than the previous one, extending superiorly onto the distal part of the spermatic cord (4). The separation between the visceral and parietal layers is filled with fluid, allowing the movement of the testis in the scrotum (4). *Tunica albuginea* is inner the visceral layer of the *tunica vaginalis*, extending into the testis as fibrous septa to allow the formation of approximately 250 to 300 pyramidal lobules, each of which containing coiled seminiferous tubules (SeTs) (4).

The anatomical organization of the testicles reflects its functional role allowing the separation between the endocrine and the gamete compartments without compromising the coordination between them. They consist of two compartments: the interstitial (place of steroidogenesis) and the SeT (the functional unit where spermatogenesis happens) (3,5). The most frequent cell type in interstitial compartment is the Leydig cell (LCs), which secretes testosterone (T), the most important male sexual hormone (5,6). Macrophages are also commonly observed there, particularly on the surface of the SeT in close apposition to the areas of tubules enriched for undifferentiated spermatogonia (6). The SeT compartment is further divided into three areas: the convoluted SeT, which is lined by the seminiferous epithelium undergoing spermatogenesis, the intermediate region, which is a conical portion lined by an epithelium composed of Sertoli cells (SCs) only, and the straight SeT, which is a straight canal connecting to the rete testis and lined by a low cuboidal epithelium (6). These are covered by *lamina propria*, which consists of a basal membrane, a layer of collagen and the peritubular cells (myofibroblasts) (7). The SeTs in the adult are composed of SCs and germinative cells. SCs line the basement membrane and are commonly known as “nurse cells”, playing a pivotal role in the regulation of spermatogenesis (8,9). Their central functions are: formation of the

blood-testis barrier (BTB), providing structural and nutritional support to the developing germ cells, phagocyting residual bodies and degenerated germ cells, the production and release of regulatory factors and the establishment of a localized immune privileged environment (9). The establishment of the BTB is due to the tight junctions formed between SCs and prevent the passage of proteins from the interstitial space into the lumen of the SeT (9). In addition, these cells have been shown to be responsible for the movement of germ cells from the base toward the lumen and for the release of mature sperm into the SeT lumen (9). These cells also actively phagocytose damaged germ cells and residual bodies, which are portions of the germ cell cytoplasm not used in the formation of spermatozoa (9).

Testicular secretion composed of spermatozoa and testicular fluid leave the highly coiled SeTs and enter the short, straight tubule. These tubules empty into a network of tubules still within the testis, the rete testis, which in turn are linked to the efferent duct. The efferent duct lead to an organ lying outside the testis, the epididymis (Figure 1) (10). Sperm leave the testis as non-functional gametes and it is only during passage through the epididymis that they acquire the ability to move progressively, and to capacitate, eventually gaining the ability to fertilize (11). The microenvironment is established by the highly active absorptive and secretor activities of the cells that line the epididymal epithelium. Consequently, spermatozoa are rendered mature in the head-body epididymis and are then stored in the tail region of this tissue (12).

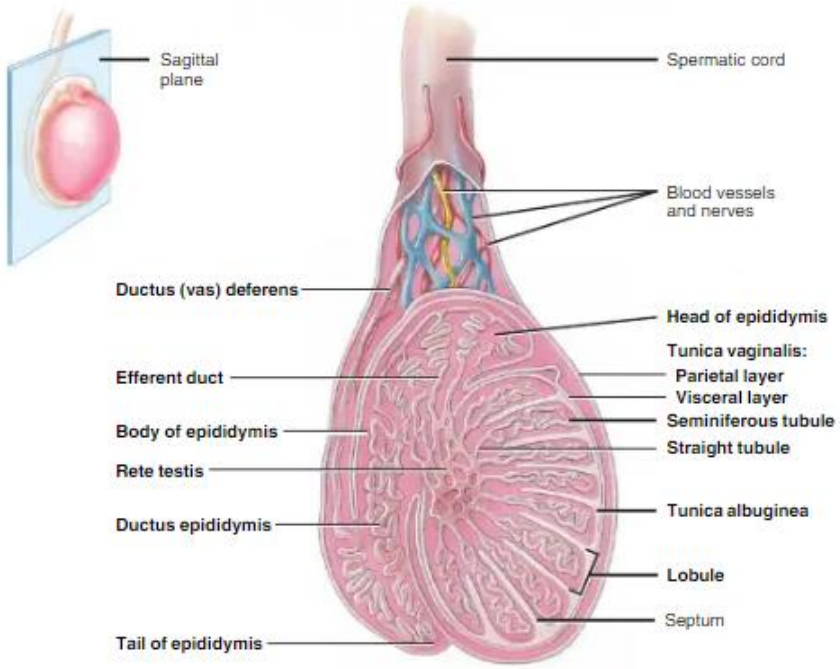

Figure 1. The mammalian testis and associated structures. The testis is encapsulated by *tunica vaginalis* (externally) and *tunica albuginea* which results in the formation of pyramidal lobules containing the seminiferous tubules (SeTs). SeTs converge to the rete testis that is connected to the efferent ducts. The three major structures of the epididymis are represented: head (*caput*), body (*corpus*) and tail (*cauda*) (adapted from (13)).

1.2. The mammalian spermatogenic process

Male fertility requires the production of large numbers of spermatozoa by the testis, through a complex and highly coordinated process known as spermatogenesis (4). Spermatogenesis occurs in the epithelium of the SeTs and requires the involvement of a complex assortment of peptides and hormones (14,15). This process is a continuum of cellular differentiation with three major phases: proliferation and differentiation of spermatogonia, meiosis, and spermiogenesis which represents a complex metamorphosis of round haploid germ cells into the highly specialized structure of the spermatozoon (16,17). The overall duration of spermatogenesis is calculated as around 35 days in mouse, 50 days in rat, 37–43 days in different monkey species and at least 64 days in man (7). A highly complex series of molecular events, requiring proper interactions between SCs, germ cells, epithelial tubular cells, and the integrity of the BTB, is needed for a successful spermatogenesis (17). Spermatogonial stem cells, which lie at the basement membrane, are the foundation of the spermatogenic process (18). These cells replicate mitotically to both guarantee the germ cell line (spermatogonia A) and give rise to new populations (spermatogonia B), committed to differentiate and move along the seminiferous epithelium (18). The B spermatogonia divide into preleptotene spermatocytes, also diploid cells (19). Afterwards, preleptotene spermatocytes enter leptotene stage, being only these primary spermatocytes that traverse the BTB to enter the adluminal compartment for further development, and then, undergo the first division of meiosis yielding secondary spermatocytes (Figure 2) (17,20). Spermatocytes are found in all stages, because meiosis is a prolonged period of spermatogenesis that extends over approximately 14 days in the mouse (21). Round spermatids are produced through the second meiotic division. Once spermatids are formed, cell division stops and spermiogenesis starts giving rise to elongated spermatids. During spermiogenesis, alterations occur in the male gamete nuclear proteins, cellular size, cellular shape, the position and size of pro-acrosomal granules and the localization of the centrioles (22). This process culminates in the release of elongated spermatids into the lumen of the SeT as immature spermatozoa, the so-called spermiation (17,20).

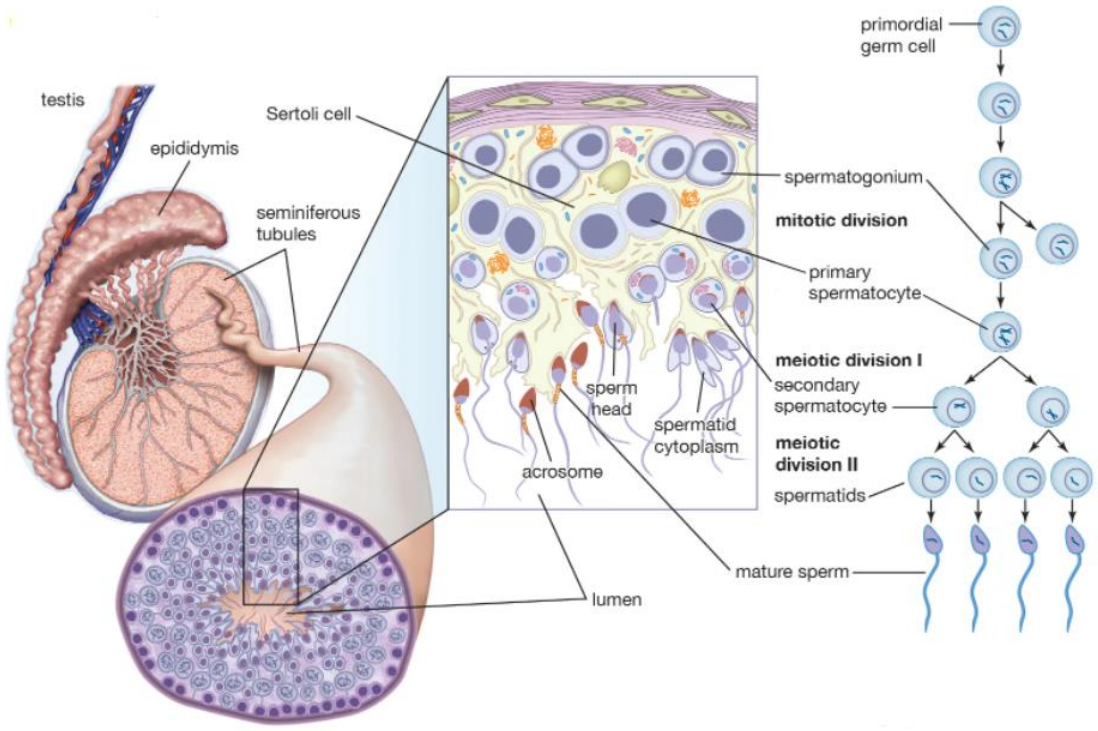

Figure 2. Testicular histology and spermatogenic process. Spermatogenesis occurs inside of the seminiferous tubules (SeTs) in close contact to the Sertoli cells (SCs); Leydig Cells (LCs) reside in the interstitial space between tubules. The relationship between SCs and germ cells is crucial for a successful spermatogenesis. This process begins with the differentiation of spermatogonia into primary spermatocytes. Then, primary spermatocytes suffer the first meiotic division originating secondary spermatocytes. The secondary spermatocytes originate the spermatids by second meiotic division, which then suffer a process called spermiogenesis originating the spermatozoa (adapted from (23)).

1.3. Hormonal regulation of spermatogenesis

Spermatogenesis is regulated by a complex array of endocrine, paracrine and autocrine regulatory cross-talk that involves SCs, LCs, peritubular and germ cells (24). The major player in the hormonal regulation of spermatogenesis is the hypothalamus–pituitary–testis axis, where the hypothalamus releases the gonadotropin-releasing hormone (GnRH) into the hypophyseal-portal circulation, with its pulsatile signals stimulating gonadotrophic cells of the anterior pituitary to secrete the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The dysfunction of this axis leads to infertility (15,25). The testicular target cells of LH are the LCs present in the interstitial space, and those of FSH are the SCs present within the SeTs. They mediate their individual actions on spermatogenesis through their cognate receptors, FSHR and LHR, both plasma membrane associated G-protein coupled receptors. LH stimulates T production by LCs, and FSH, in synergy with T, stimulates SCs to produce regulatory molecules and nutrients needed for the maintenance of spermatogenesis. Hence, both T and FSH regulate spermatogenesis indirectly through SCs (26). FSH binds to specific receptors in the SCs and stimulates the production of androgen-

binding protein, being necessary for the initiation of spermatogenesis through the stimulation of spermatogonial proliferation and meiosis (2,22). However, full maturation of the spermatozoa appears to require not only a FSH effect but also T. Indeed, the major action of FSH on spermatogenesis may be via the stimulation of androgen-binding protein production, which allows a high intratubular concentration of T to be maintained (2). T inhibits LH secretion in two ways. It acts on the hypothalamus to decrease the amplitude of GnRH bursts, which results in a decrease in the secretion of gonadotropins and acts directly on the anterior pituitary gland to decrease the LH response to any given amount of GnRH. The major inhibitory signal, exerted directly on the anterior pituitary gland, is the protein hormone inhibin. This is a logical completion of a negative feedback loop such that FSH stimulates SCs to increase both spermatogenesis and inhibin production, and inhibin decreases FSH release (Figure 3) (1).

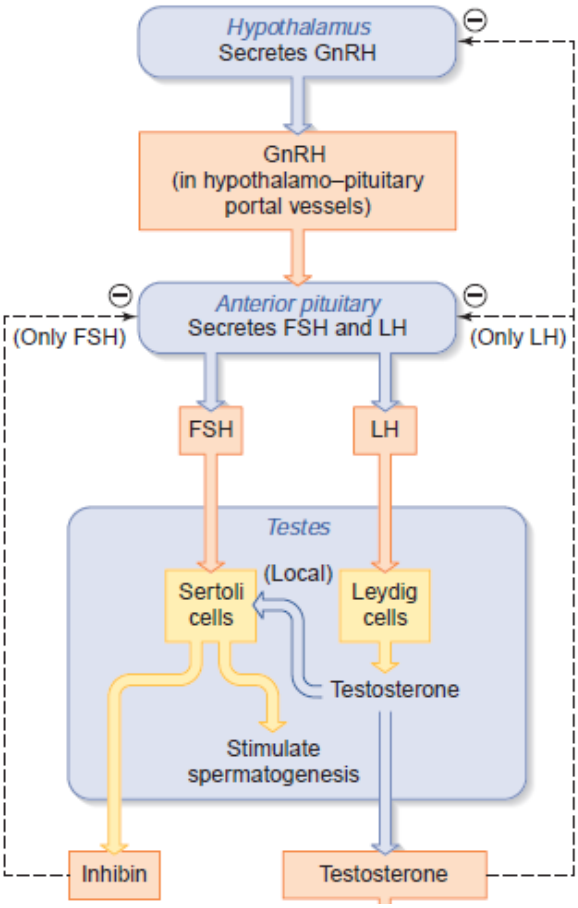

Figure 3. Hormonal regulation of the spermatogenesis. Hypothalamus release the gonadotropin releasing hormone (GnRH), which stimulates the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the activity of Sertoli cells and LH simulates Leydig cells, inducing the production of inhibin and testosterone, respectively. Inhibin induce a negative feedback on pituitary gland and testosterone in pituitary gland and hypothalamus, decreasing the levels of GnRH and LH (adapted from (1)).

The output of spermatogenesis and the number of spermatozoa produced also depends on other basic process, namely cell death processes.

1.4. Mechanisms involved on germ cell death/survival

1.4.1. Oxidative stress

Oxygen is required for energy metabolism for the survival and normal functions of most eukaryotic organisms. Along the respiratory chain, oxygen is also partially reduced, at low ratio, into superoxide, a basic free radical that can be converted eventually into other forms of reactive oxygen species (ROS) (27). Oxidative stress (OS) is defined as a condition in which the antioxidant scavenging system of the cell is overwhelmed by the overproduction of ROS, resulting in a state of oxygen paradox. That is free radicals are required for cellular processes, but, at increased concentrations, can also interfere with essential metabolic processes. OS is a major cause of sperm cell dysfunction and a major contributor to the aetiology of male infertility owing to impairment of both the structural and functional integrity of spermatozoa (28).

In the testis, during normal spermatogenesis, spermatozoa gradually reduce the size of their cytoplasm while SCs phagocytise it. However, an arrest in spermiogenesis results in excess cytoplasm around the midpiece with the production of excessive amounts of ROS, which, in turn, leads to OS (29). Sperm with excess cytoplasmic residues in the mid-piece are known as immature sperm. Additional cytoplasm in the mid-part of the sperm contains glucose-6-phosphate dehydrogenase (G6PD) enzyme (30). The retained cytoplasm allows the formation of nicotinamide adenine dinucleotide phosphate (NADPH) from retained G6PD via the hexose monophosphate shunt. NADPH leads to the generation of ROS by means of two pathways (29,30). The first includes a membrane-bound NADPH oxidase enzyme that uses oxygen as a source to produce the superoxide anion, which can further produce other highly reactive molecules such as hydrogen peroxide. The second consists of NADPH dehydrogenase, also known as diaphorase, which is responsible for oxidation–reduction (redox) reactions in the mitochondria and the generation of ROS (29). ROS, produced by exogenous and endogenous factors, are highly reactive oxidizing free radical agents and include superoxide anions (O2•), hydrogen peroxide (H_2O_2), peroxyl (ROO•), and hydroxyl (OH•) radicals (28,31). These molecules play a key role altering male reproductive functions (31).

To counteract the damaging effect of ROS, germ cells are endowed with extensive antioxidant defence mechanisms, including the antioxidant enzymes

superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-*S*-transferase (GST), and non-enzymatic antioxidants (e.g. glutathione, vitamin C, and vitamin E) (32). SOD plays a central role in protecting against OS by catalysing the reduction of superoxide anions to hydrogen peroxide and oxygen. The resultant hydrogen peroxide is further resolved to oxygen and water via the action of catalase (Figure 4). There are two types of SOD, a cytoplasmic type, copper zinc superoxide dismutase (Cu*/*Zn-SOD), and a mitochondrial type, manganese superoxide dismutase (Mn-SOD), both of which acting as antioxidant enzymes (33). GPx reduces hydrogen peroxide to water to limit its harmful effects (Figure 4) (34).

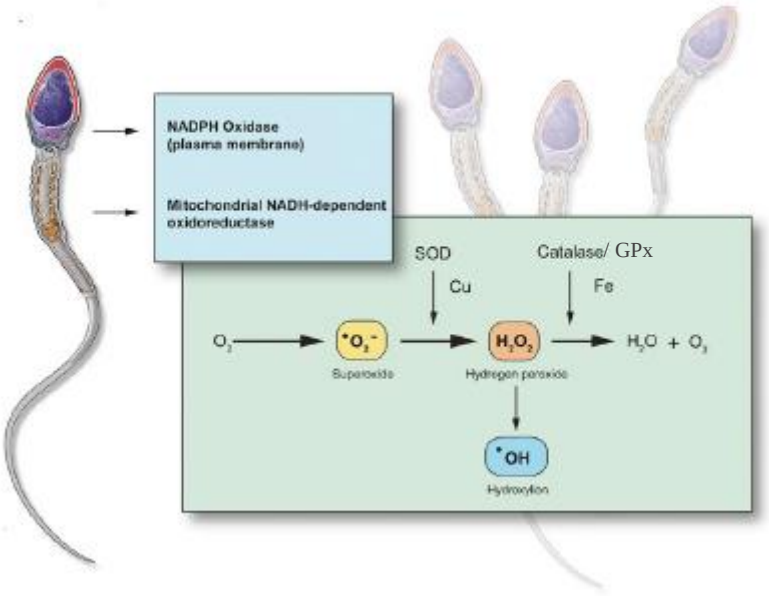

Figure 4. Production of reactive oxygen species (ROS) by membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NADPH dehydrogenase. NADPH oxidase enzyme that uses oxygen to produce the superoxide anion, which can further produce other highly reactive molecules such as hydrogen peroxide. NADPH dehydrogenase is responsible for redox reactions in the mitochondria and the generation of ROS. Superoxide dismutase (SOD) protect against oxidative stress by catalysing the reduction of superoxide anions to hydrogen peroxide and oxygen. The hydrogen peroxide is further resolved to oxygen and water via the action of glutathione peroxidase (GPx) and catalase (adapted from (29)).

Environmental exposures, lifestyle modifications, technological advancements, alcohol consumption, smoking of cigarettes and physical stress are among the prime exogenous causes of ROS production and multiple mechanisms involving metabolism in the cell membrane, mitochondria, peroxisomes, and endoplasmic reticulum can produce endogenous ROS (31). Environmental chemical components can induce OS by triggering redox-sensitive pathways subsequently leading to various malaise, such as inflammation and cell death. These contaminations deteriorate semen parameters, DNA integrity via disrupting LCs and SCs function, hormone biosynthesis, gene expression and epigenetic modifications (31).

The major sources of ROS in sperm include activated leukocytes in the seminal plasma and the mitochondria in the spermatozoa. Low levels of ROS are required for several redox-sensitive physiological processes, such as sperm capacitation and hyperactivation, although supraphysiological ROS levels impede sperm membrane fluidity and permeability (28). High levels of ROS neutralize antioxidants in the seminal plasma and can increase the possibility of infertility not only directly by inducing OS, but also indirectly by acting through the hypothalamic axis of hormone release (30,31). ROS reduce male sex hormone levels and disrupt the hormonal balance that regulates male reproductive functions, causing infertility. They not only interfere in the communication between testis and the hypothalamic-pituitary unit, but also disrupt the cross-talk between the hypothalamic-pituitary-gonadal axis with other hypothalamic hormonal axes (31).

Sperm cells are highly sensitive to oxidative damage. These cells have cytoplasmic membranes rich in unsaturated fatty acids, and thus they are faced with the lipid peroxidation under the influence of ROS. Sperm cells are incapable of repairing damage by OS because they suffer from lack of essential cytoplasmic enzymes. Finally, lower motility and death of sperm occurs due to the loss of adenosine triphosphate (ATP) caused by lipid peroxidation followed by axonemal injury (30).

1.4.2. Apoptosis

Apoptotic cell death is a programmed, controlled process of cellular self-destruction for the greater benefits of the organism that requires protein synthesis and specific cellular signals and proteins (35,36). It is the process by which a cell stops growing and dividing and instead enters a process that ultimately results in the controlled cell-death without spillage of its contents into the surrounding environment, unlike what occurs in necrosis (37). It plays a crucial role in embryogenesis, normal tissue turnover, immune development and defence, and protection against tumorigenesis (35). This regulated cell death-process serves several important functions in the testis, a few of which include maintaining appropriate germ cell/SCs ratio, removing defective germ cells (75% of the produced male germ cells are discarded through the process of apoptosis) and maintenance of overall quality control in sperm production (38,39). In certain pathological circumstances, a huge increase in germ cell apoptosis occurs, which can lead to male idiopathic infertility. Apoptosis has been observed often in spermatocytes, less in spermatogonia, and rarely in spermatids (39).

This cell death pathway is activated when a cell is no longer needed or has sustained serious damage and the initiation of this process is dependent on the activation of a series of cysteine-aspartic proteases known as caspases (35,37).

Apoptosis also involves characteristic changes within the nucleus. The nucleus undergoes condensation as endonucleases are activated and begin to fragment nuclear DNA into oligonucleosomes. Additionally, apoptosis is characterised by a loss of mitochondrial function (40). Death signals are relayed through signalling pathways which lead to activation of caspases responsible for the execution of cell destruction (36). There are two main apoptotic pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway (Figure 5).

The intrinsic pathway of mammalian apoptosis is regulated by the Bcl-2 family of proteins, the adaptor protein apoptotic protease-activating factor 1 (Apaf-1) and the cysteine protease caspase family. The Bcl-2 family of proteins has a crucial role in intracellular apoptotic signal transduction. This gene family includes both anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic proteins (Bax, Bad). The anti-apoptotic regulators are localized in the mitochondrial outer membrane, the endoplasmic reticulum and the perinuclear membrane (41). Bcl-2 has been shown to protect cells from diverse death-inducing stimuli (42). Bax facilitates the mitochondrial release of cytochrome c into the cytoplasm where it binds to Apaf-1, a mammalian cell-death gene that transmits apoptotic signals from mitochondrial damage to activate caspases (43). This association initiates the assembly of the so-called apoptosome, which recruits the initiator pro-caspase-9 to activating recruitment domain (CARD), allowing autoactivation and then proteolysis. The process in turn activates downstream executor caspases for cleavage of cellular substrates leading to cell death (Figure 5)(40,41,43).

Caspases are expressed as catalytically inactive proenzymes. Caspases with CARDs, which include caspase-1, -2, -4, -5, -9, -11 and -12, are most probably activated through an intracellular activating complex exemplified by the cytochrome c/Apaf-1/caspase-9 complex (41). On the other side, caspases with the death-effector domain, which include caspase-8 and caspase-10, are activated by interacting with the intracellular domains of death receptors, such as the Fas and tumour necrosis factor (TNF) receptors. This additional pathway involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell (44). The extrinsic apoptotic pathway is a pathway triggered by engagement of receptors of the TNF receptor family (death receptors), with their respective protein TNF family ligands (40,43). The death receptors in the extrinsic pathway are all anchored to the cell membrane by their transmembrane regions. Upon interaction with an extracellular ligand, membrane receptors relay death signals into intracellular space via their cytoplasmic death domains (36). The two most prominent members of the death receptors are tumour necrosis factor receptor type 1 (TNF-R1) and Fas (CD95). Whereas TNF-R1 mediates cell death inflammatory responses, Fas is involved in the killing of target cells by

cytotoxic T cells and in the activation-induced cell death of T cells. Fas ligand engagement of Fas on the target cell results in the recruitment of caspase-8 via an adaptor protein (Fas-associated death domain protein; FADD) to form a death-inducing signalling complex, increasing the local concentration of pro-caspase and promoting the mutual auto-activation. The activation of initiator caspases results in the processing of the downstream effector caspases-3, -6 and -7 (Figure 5) (40,43).

There is evidence that the two pathways are linked and that molecules in one pathway can influence the other. Some cells do not die in response to the extrinsic pathway alone and require an amplification step that is induced by caspase-8 (43). In this situation, capase-8 targets the BH3-only protein Bid (BH3-interacting-domain death agonist) for cleavage and generate the activated fragment t-Bid. T-Bid then directly activates proapoptotic multi-domain proteins to induce mitochondrial outer membrane permeability, so this co-engages the intrinsic pathway (43). Alternatively, apoptosis can be induced by an internal stimulus such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic calcium (Ca^{2+}) and severe OS are some triggers of the initiation of the intrinsic mitochondrial pathway resulting from exposure to radiation or toxins as well as in response to metabolic or cell cycle perturbations (35,43). These events stimulate the tumour suppressor protein p53 to recruit Bax to the outer mitochondrial membrane, leading to apoptotic cell death (40,43). Besides, programmed cell death can be mediated by granzyme B/perforin released by cytotoxic T cells. Granzyme B is an aspartyl serine protease located in the granules of cytotoxic T and NK cells. Upon signalling via the T cell receptor, cytotoxic T cells release granzyme B and perforin, a protein which forms pores in the membrane of the target cell and allows granzyme B to enter. Once in the cytoplasm of the target cell, granzyme B can cleave and activate caspase-3 directly (Figure 5) (40). The extrinsic, intrinsic, and granzyme B pathways converge on the same end-point, the cleavage of caspase-3,thus resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (44).

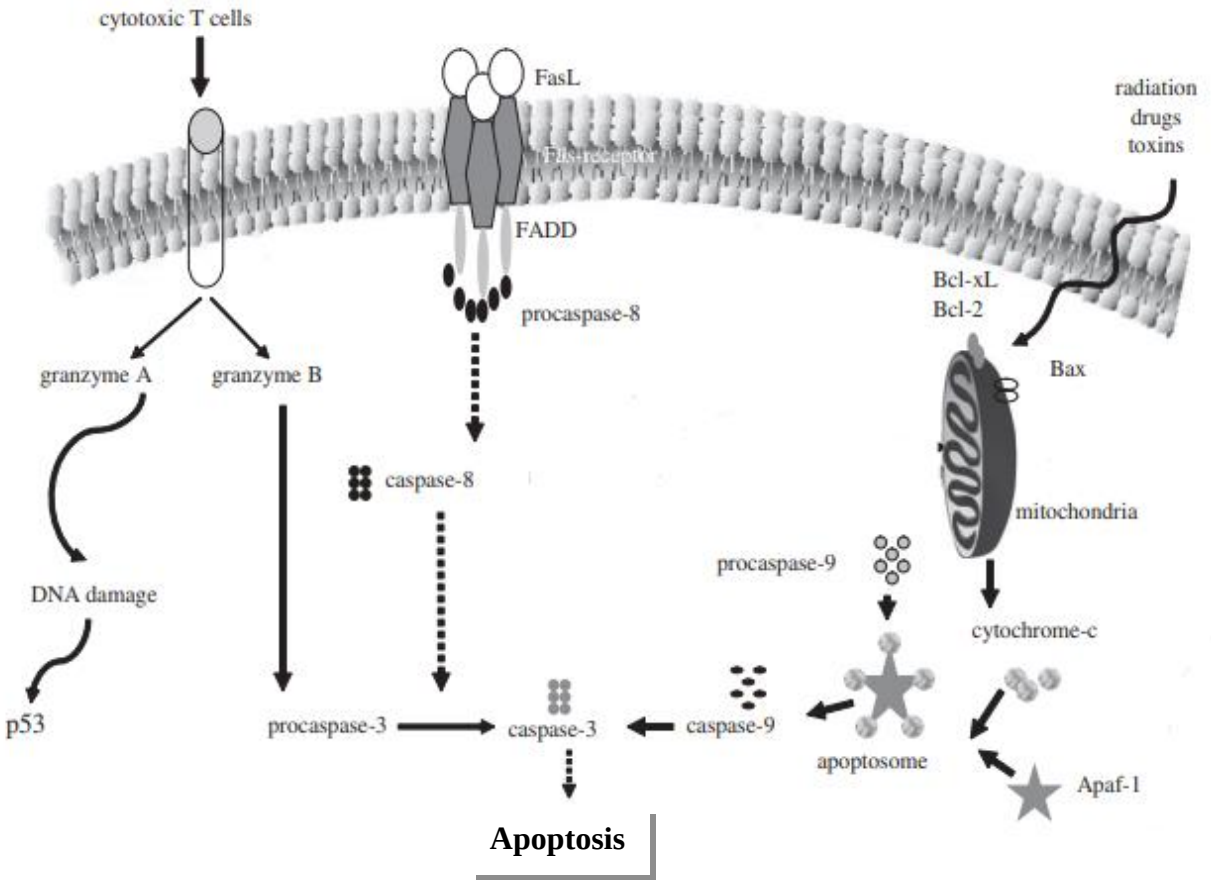

Figure 5. Programmed cell death. There are two major apoptotic pathways: the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway. Extracellular ligand binding (FasL and tumor necrosis factor, TNF) to death receptors (FasR and TNF receptor, TNFR) triggers the extrinsic pathway resulting in the direct activation of initiator caspase-8. The intrinsic pathway is initiated in response to apoptotic stimuli (radiation, drugs, toxins, etc.) leading to the activation of Bax. Bax is translocated to the mitochondria with consequent release of cytochrome c, which together with apoptotic protease activating factor 1 (Apaf-1), forms the apoptosome and activates caspase-9. Cytotoxic T cells can trigger the process of apoptosis by the release of granzymes (A and B). All the pathways converge on procaspase-3 that, after cleavage, become the active effector caspase-3, the end and irreversible point of apoptosis (adapted from (45)).

1.5. Generalities on endocrine-disrupting chemicals

1.5.1. Classification

The term endocrine-disrupting chemicals (EDCs) has been applied to classify chemicals, or chemical mixtures, that interfere with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (46,47). The group of molecules acting as EDCs is highly heterogeneous and includes compounds that are often distantly related to endogenous ligands in terms of size or chemical structure (47). Sometimes, the metabolite(s) of the EDC is(are) more hazardous than the parent compound itself. Although, there are some structural

features that are indicative of endocrine disruption, it is also generally not possible to determine whether a compound is an EDC based on its structure (46).

EDCs can occur naturally, like natural chemicals found in human and animal food and can be synthesized, like synthetic chemicals used as industrial solvents or lubricants and their by-products, plastics, plasticizers, pesticides and some pharmaceutical agents (46). Therefore, EDCs can be grouped according to their origin: natural and artificial hormones, drugs with hormonal side effects, industrial and household chemicals, side products of industrial and household processes (46). Also, these compounds can be classified as agonists affecting the endocrine system of an organism by mimicking natural hormones (e.g. xenostrogenic, xenoandrogenic), or antagonize their action (e.g. anti-estrogenic, anti-androgenic) (47).

1.5.2. Mechanism of action

The first and most described mechanism underlying exposure to EDCs is the binding of these chemicals to nuclear hormone receptors (NRs) to exert its action (48). As supra-mentioned, EDCs can act either as agonists or antagonists, thus enhancing, dampening, or blocking the action of hormones (49). They also alter the number of NRs in different cell types and the concentration of circulating hormones (50). NRs constitute a conserved family of proteins classified as hormone-dependent transcription factors which modulate genetic expression in response to activation by small lipophilic ligands. Consequently, they exert long-term control of their target cells' phenotype. These receptors impact numerous genes and are involved in a network of intricate signaling pathways (51–53). They are divided into steroid receptors, nonsteroid receptors, and orphan NRs that have no known endogenous ligand (54). The steroid receptors are the estrogen receptors α and β (ERα and ERβ) and the androgen receptor (AR) (48,54). EDCs can also bind other members such as the subfamily of pregnane X receptor (PXR), the peroxisome proliferator activated receptor α and γ (PPARα, PPARγ), the constitutive androstane receptor (CAR), the estrogen related receptors (ERRα, β and γ in mammals) and aryl hydrocarbon receptor (AhR) (48,55).

Less understood are potential interactions of EDCs on the first line membrane receptors, associated with non-genomic effects. In the case of ERs, the membrane forms (mERs) include mERα, mERβ, and G protein-coupled estrogen receptor (GPER) (56). EDCs with xenoestrogenic activity can bind to mER and produce rapid changes in signaling, similar to 17-ß estradiol (E2) (57). An example of a nongenomic mechanism mediated by mERα and mERβ is the increase of intracellular 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 (58).

Hormone availability is dependent on hormone biosynthesis, transport of the hormone to the target tissue, levels of hormone binding proteins, and hormone catabolism. Steroid hormone catabolism is particularly affected by EDCs, since many of the xenobiotic-metabolizing enzymes are involved in both these processes (55). Activation of AhR leads to increased expression of xenobiotic metabolizing enzymes, cytochrome P450s (e.g. CYP1A2, CYP3A4, CYP1A1, and CYP1B1), that are responsible for the hydroxylation of E_2. Several EDCs have been reported to alter intracellular aromatase activity, which converts T to E_2, being a direct AhR target gene (59). Thus, activation of AhR by EDCs can lead both to increased degradation of steroid hormones as well as to higher estradiol production (55)(59).

In parallel with these classical pathways, it appears that EDCs not only involve genetics but also epigenetic mechanisms. The epigenetic effects of EDCs refer to heritable changes in gene function in the absence of DNA sequence alterations (50). Epigenetic effects are mediated by transcription factors that repress or enhance the transcription of specific genes. The main epigenetic mechanisms include DNA methylation, post–translational modifications of histone proteins and non–coding RNA. These epigenetic processes control tissue development by controlling gene expression (34).

Among the deleterious effects of EDCs, exerted by this great variety of mechanisms, their reproductive toxicity is of utmost concern. EDCs have been related to reduced fertility in both male and female, breast, endometrial and testicular cancer, birth defects of reproductive organs, changes in the onset of puberty and alteration of the ratio of male to female births (59).

1.5.3. Pesticide threatening to male fertility: the case of methoxychlor

Methoxychlor (MXC), 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane, is an organochlorine pesticide, structurally similar to dichloro-diphenyl-trichloroethane (DDT), used to control insects on agricultural crops, livestock, animal feeds, barns, home, gardens and on household pets (60–62). MXC is absorbed at the level of the gastrointestinal tract and is metabolized in the liver by O-demethylation to polar mono- and bis-phenolic metabolites (61,63). Its persistence in the environment is overwhelming, since significant amounts of MXC and MXC-deriving metabolites can be measured in human tissues, long after stopping its use (63). Despite its accumulation in some living organisms, including algae, bacteria, snails, clams, and some fish, in most

of them MXC is converted into other substances that are rapidly released, so MXC does not usually build up in the food chain. However, low levels are sometimes detected in foods obtained from areas where MXC has been used (64). MXC have a weak estrogenicity, it is at least several thousand fold less potent than endogenous estrogens (63,64). It is known to act as an agonist of ERα and ERβ and as an antagonist of AR (63,64), binding much less avidly to ER-ß than to ER-α (60). The MXC biphenolic metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) has approximately 100-fold more affinity to ERα than MXC (61). MXC is a known EDC to cause the reduction of LH-stimulated T production in rodent LCs (65). The antiandrogenic effects of MXC and its metabolite HPTE are mediated via direct inhibition of T biosynthetic enzymes. MXC and HPTE directly inhibit T production in rat LCs via inhibiting CYP11A1 activity (65).

It is known that at doses between 20 and 500 mg/kg body weight/day, MXC induced abnormalities in male reproductive system including delayed sexual maturity, decrease in testis weight, atrophy of epididymis, prostate and seminal vesicles, impaired steroidogenesis, decreased epididymal sperm counts, and viability (66). In another study, rats treated with 10 or 100 mg/kg MXC daily displayed reduced serum T levels and delayed rat LCs regeneration (45). Perinatal and juvenile oral treatment with MXC was also shown to reduce testicular size and SCs number (46). Moreover, the spermatogenic potential based on number of spermatogonia and number of spermatids per testis was reduced and the ratio of spermatid number per spermatogonia increased (46). Embryonic testis treated *ex vivo* with 2 and 20 mM MXC showed abnormal cord formation and swelling (67). This swollen appearance was also seen with 3 mM and 6 mM of the MXC metabolite HPTE. The swollen cord appearance in testis organs was due to an increased number of cells in cord cross sections and reduction in interstitial cell number (67).

Elimination of spermatogenic cells via apoptosis occurs spontaneously under normal physiologic conditions (39). Also, the testicular tissue and male reproductive system are particularly susceptible to OS (39). Environmental exposures, as is the case of EDCs, can exacerbate OS and arouse germ cells' apoptosis leading to chemical-induced testicular impairment. In animals orally administered with MXC (50 mg/kg body weight), an increase in the levels of cytosolic cytochrome c and procaspase-9 was shown as early as 6 h following exposure (62). This was accompanied with time-dependent elevations in the levels of Fas, FasL, pro- and cleaved caspase-3 (62). Also, in doses between 50-200 mg/kg/day and for 15 consecutive days, MXC decreased the activity of 3ß-hydroxysteroid dehydrogenase and 17ß-hydroxysteroid, SOD, CAT and GPx, while H_2O_2 production and caspase-3 activity were increased (66). These studies

conclude that MXC has the capability of inducing testicular apoptosis and OS in adult rats.

The reproductive toxicity of EDCs is undeniable, being exerted by diverse mechanisms and mainly culminating on the disturbance of germ cells survival. Despite all the evidence associating EDCs to male infertility, little is known about the effect of EDCs, particularly MXC, on specific testicular cell populations.

2. Aim of the dissertation

EDCs are a group of compounds present in the environment that interfere with the synthesis, secretion, and metabolism of natural hormones. These compounds can be classified as agonists by mimicking natural hormones (xenoestrogenic, xenoandrogenic) or antagonize their action (anti-estrogenic, antiandrogenic). Environmental levels of EDCs have been proven to affect the development and functioning of the reproductive system, causing developmental and reproductive disorders, including infertility.

MXC is a xenoestrogenic organochlorine pesticide with moderate persistence in the environment, which has been proved to negatively affect spermatogenesis, disrupting the development of germ cells, and interfering with sperm function.

Spermatogonia are the first differentiated cell population of the germ line, representing the precursor cell population of the spermatogenic process. Thus, the disturbance of spermatogonia homeostasis can be a major trigger of male infertility. Additionally, and worryingly, their location outside the BTB increases their susceptibility to noxious stimuli, as is the case of EDCs. However, little is known about the disrupted molecular mechanisms behind the action of MXC, mostly in specific testicular cell populations like spermatogonia.

Following this rational, the present dissertation aims to characterize the impact of MXC on survival of spermatogonial cells, specifically analysing the (de)regulation of the apoptotic process and antioxidant defences. For this, a mouse-derived spermatogonial cell line (GC-1spg) was maintained in culture for 48 hours in the presence or absence of different MXC concentrations. After cell viability analysis, protein expression and activity of apoptotic process key-modulators and antioxidant enzymes were evaluated. In addition, the expression of ERs and AR and its regulation by MXC was assessed in this specific testicular cell type.

3. Materials and Methods

3.1. Cell line

GC-1spg, a mouse-derived spermatogonial cell line, which corresponds to a spermatogonia type B stage (ATCC, CRL-2053™) was kindly provided by Professor Sandra Rebelo, iBiMED, University of Aveiro.

Spermatogonia B lie at the basement membrane, being precursor cells of the spermatogenic process (18). Thus, any disturbance to their biological activity could have a profound impact on sperm quality and quantity. Also, their location outside the BTB makes them more susceptible to the influence of exogenous factors, as is the case of EDCs. Together, these premises underlie the choose of spermatogonia B as the present study model.

3.2. Cell culture and methoxychlor treatment

GC-1spg cell line was cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, 21068028, Thermo Fisher), supplemented with 10% fetal bovine serum (FBS, F7524, Sigma-Aldrich) and 1% penicillin-streptomycin-amphotericin B solution (A5955, Sigma-Aldrich), at 37 °C in an atmosphere of 5% CO_2.

For cell viability assays, approximately 7 500 cells/well were grown in a 96-well plate until reaching a confluence of 60-70%. Culture medium was then replaced by phenol red-free DMEM (31053028, Thermo Fisher) containing 5% charcoal-stripped FBS (F6765, Sigma-Aldrich). After maintenance for additional 24 hours in this culture medium, cells were exposed for 48 hours to the EDC MXC (36161, Sigma-Aldrich), in a concentration range of 5-100 μM (158, 159, 160, 161).

In order to perform the analysis of apoptotic pathway's and oxidative stress' key-players, approximately 500 000 GC-1spg cells were seeded in each T-flask (Orange Scientific), in the presence (10 μM and 25 μM) or absence of MXC for 48 hours. Concentrations were selected through the previous-mentioned cell viability assays. At the end of the experiment, GC-1spg cells were recovered and stored at -80 °C until protein isolation.

3.3. Cell viability assays

The viability of MXC-treated GC-1spg cells, was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric test used to assess cell viability as a function of redox potential. The principle behind

MTT assay relies on the fact that metabolically active cells convert the water-soluble MTT (yellow coloured) to an insoluble purple formazan. The formazan crystals are then dissolved using organic solvents and their concentration determined spectrophotometrically.

After MXC treatment, 50 µL of MTT (1 mg/mL, ab146345, Abcam) were added and cells were incubated in the dark for 4 hours at 37 °C. After incubation, the medium and MTT solution were carefully removed and 100 µL methanol were added to dissolve the formazan crystals. The formazan content was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The value of absorbance was directly proportional to the number of viable cells. All experiments were independently repeated at least three times.

3.4. Total Protein Extraction and Quantification

Total protein was isolated from MXC-treated GC-1spg and control groups using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail (Sigma-Aldrich) and 10% of phosphatases inhibitor (PhosSTOP, 78427, Thermo Fisher). Upon being kept on ice for 40 minutes and occasionally mixed, samples were centrifuged at 14 000 rpm for 20 minutes at 4 °C in a Hettich Mikro 200R centrifuge. Total protein (supernatant) was recovered to a fresh eppendorf tube in order to assess its concentration using the bicinchoninic acid (BCA) method with Pierce BCA Protein Assay (23225, Thermo Fisher). Briefly, in each well of a 96-well plate, 1 µL of protein sample was mixed with 80 µL of BCA work reagent and 19 µL of milli-Q water. In the blanks, 1 µL of RIPA buffer was added instead of 1 µL of protein sample. The absorbance was measured spectrophotometrically (xMark™ Spectrophotometer, Bio-Rad) at 562 nm. The standard curve for protein quantification was obtained using serial concentrations of bovine serum albumin. The proteins were stored at -80 °C.

3.5. Superoxide Dismutase Assay

SOD activity in control and MXC-treated GC-1spg cells was measured through a competitive inhibition assay (19160, Sigma-Aldrich) using a tetrazolium salt and xanthine oxidase, following the manufacturer's instructions. Upon reduction with superoxide anion, a water-soluble formazan dye is produced, which is linear with xanthine oxidase activity and inhibited by SOD. Briefly, 2,5 µL of total protein extracts were incubated with 100 µL tetrazolium salt and 2,5 µL xanthine oxidase (sample) or

2,5 µL of dilution buffer (sample blank). Two additional blanks were performed, the first with 2,5 µL H_2O, 100 µL tetrazolium salt and 2,5 µL xanthine oxidase, and the second with 2,5 µL of dilution buffer instead of the latter. The assay was monitored by measuring the absorbance at 450 nm, after a reaction time of 20 minutes at 37 °C using the xMark™ Microplate Spectrophotometer (Bio-Rad). Percentage of reaction inhibition, extrapolated by the decrease in absorbance values, indicated the SOD activity. Results were expressed as the activity ratio (percentage of inhibition) per µg of protein.

3.6. Glutathione peroxidase Assay

GPx activity in control and MXC-treated GC-1spg cells was determined using a commercial kit (703102, Cayman Chemical) according to the manufacturer's protocol. Briefly, GPx activity was measured by indirectly monitoring the glutathione reductase coupled reaction. Oxidized glutathione, produced in the reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. In a 96-well plate, 5 µL total protein extracts were incubated with 25 µL NADPH, 25 µL Co-substrate and 25 µL GPx assay buffer. In the blanks, 25 µL NADPH, 25 µL co-substrate and 30 µL GPx assay buffer were added. The absorbance was measured at 340 nm every minute and 5-time points were registered using the xMark™ Microplate Spectrophotometer (Bio-Rad). Under these conditions in which the GPx activity is rate limiting, the rate of decrease in absorbance is directly proportional to GPx activity in the sample. Results were expressed as U/L/µg of protein.

3.7. Caspase-3 like activity assay

Caspase-3 activity assay was performed as previously described (162, 163). Briefly, in a 96-well plate, 5 µL of total protein extracts from each experimental condition 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 (AcDEVD-p-nitro-aniline, pNA; Sigma-Aldrich). Blanks were performed without protein. The incubation was undertaken for 90 minutes at 37 °C. Upon caspase cleavage, pNA is released producing a yellow color, which is measured spectrophotometrically at 405 nm (xMark™ Microplate Spectrophotometer, Bio-Rad). The amount of generated pNA was

obtained by extrapolation with a standard curve of free pNA at different concentrations.

3.8. Western blot analysis

Protein extracts (25 μg) from each experimental group were mixed with a previously prepared loading buffer (1610747, Bio-Rad, 10% 2-Mercaptoethanol) and denatured at 100 °C. Proteins were resolved in a 12.5% gel by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-AR (1:250, sc-815, Santa Cruz Biotechnology), rabbit anti-ERα (1:1000, ab32063, Abcam), rabbit anti-ERß (1:1000, ab3576, Abcam), rabbit anti-Bax (1:1000, 2772, Cell Signalling Technology), rabbit anti-Bcl-2 (1:1000, ab194583, Abcam), rabbit anti-p53 (1:1000, sc-6243, Santa Cruz Biotechnology) and rabbit anti-caspase-8 (1:500, 4790, Cell Signalling Technology) primary antibodies. A mouse anti-β-actin antibody (1:10000, 5441, Sigma-Aldrich) was used as for protein loading control in all western blot analyses. Goat-anti-rabbit IgG-HRP (1:40000, sc-2004, Santa Cruz Biotechnology) or goat-anti-mouse IgG-HRP (1:40000, sc-2005, Santa Cruz Biotechnology) were used as secondary antibodies.

The membranes were incubated with enhanced chemiluminescence substrate (Bio-Rad) for 5 minutes 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 β-actin band density. Results are presented as fold-variation relatively to the control group (GC1-spg without any treatment).

3.9. Statistical Analysis

The statistical analysis of all data obtained was performed using 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 ± S.E.M.

4. Results

4.1. Methoxychlor treatment decreased the viability of GC-1spg in a dose-dependent manner

The viability of GC-1spg cells after 48 h of exposure to different MXC concentrations (5, 10, 25, 50 and 100 μM) was evaluated by the MTT assay. The results showed that the treatment with the higher concentrations of MXC reduced the viability of GC-1spg cells and, apparently, in a dose-dependent manner (Figure 6). Thus, concentrations in the range of 5 to 25 μM MXC showed no significant differences when compared to control (5 μM: 93,59 ± 6,64%; 10 μM: 90,35 ± 3,81%; 25 μM: 95,14 ± 3,06%; control group: 100,00 ± 2,77%; Figure 6). At 50 and 100 μM, MXC caused a significant decrease in cell viability (82,40 ± 3,85 and 63,86 ± 4,15% compared to 100,00 ± 2,77% in control, $p<0,05$ and $p<0,0001$, respectively; Figure 6).

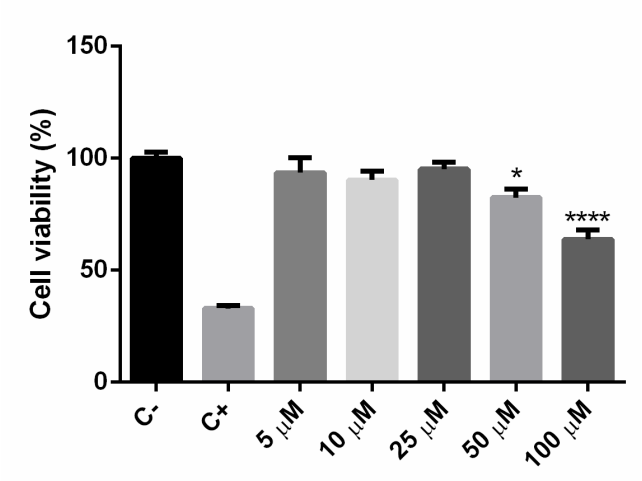

Figure 6. Percentage of viable GC-1spg cells after exposure to several concentrations of methoxychlor (MXC, 5, 10, 25, 50 and 100 μM) for 48 h, evaluated by the MTT assay. Results are expressed as % of control group. Data are presented as mean ± S.E.M. (n≥5, mean of three independent assays). Statistically significant differences relatively to control (C-) are indicated as * $p<0.05$ and **** $p<0.0001$.

After analysis and integration of the obtained results, the 10 μM and 25 μM concentrations of MXC were selected for the subsequent analyses, as they are the highest concentrations not affecting the viability of GC1-spg cells.

4.2. GC-1spg antioxidant defences are prompted upon exposure to methoxychlor

GPx is an antioxidant enzyme involved in the detoxification of ROS (68). GPx catalyses the reduction of various hydroperoxides (e.g., H_2O_2) to H_2O via oxidation of reduced GSH into its disulfide form (GSSH). In its turn, SOD is the key enzyme involved in the detoxification of superoxide radicals and may play a vital role in interindividual variation in superoxide radical turnover (69). SOD catalyses the conversion of $O2^{\cdot-}$ into molecular O_2 and H_2O_2, and thus being critical for protecting the cell against the toxic products of aerobic respiration (70,71).

In a general way, MXC augmented the activity of these antioxidant enzymes in GC-1spg cells. The activity of GPx was higher in GC-1spg cells treated with both 10 and 25 µM MXC when compared to untreated cells (0,06 ± 0,01 U/L/µg protein, p<0,01 and 0,11 ± 0,02 U/L/µg protein, p<0,01, respectively, vs. 0,03 ± 0,00 U/L/µg protein in control group; Figure 7A). Similarly, MXC induced a significant increase in the SOD activity in GC-1spg cells (86,43 ± 0,59 %/µg protein, p<0,01 and 83,35 ± 2,68 %/µg protein, p<0,01, respectively, vs. 43,61 ± 9,95 %/µg protein in untreated cells; Figure 7B).

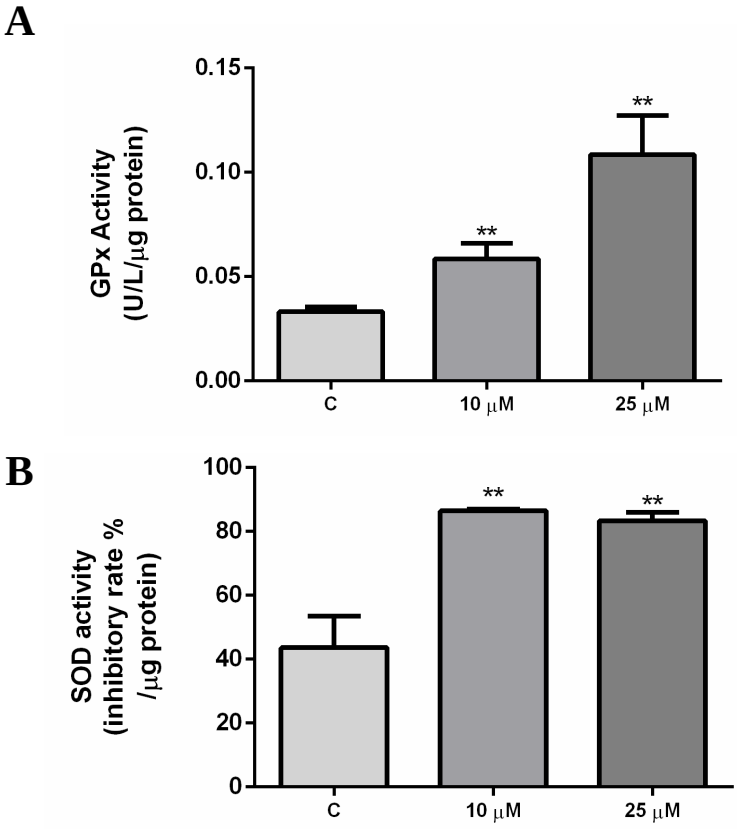

Figure 7. Glutathione peroxidase (GPx, A) and superoxide dismutase (SOD, B) activity in GC-1spg cells cultured for 48 h, in the presence or absence of 10 and 25 µM methoxychlor

(MXC). Data are presented as mean ± S.E.M. (n≥5). Statistically significant differences relatively to control (C) are indicated as **p<0,01.

4.3. GC-1spg apoptotic status after methoxychlor treatment

4.3.1. The enzymatic activity of caspase-3 was increased in methoxychlor-exposed GC-1spg

Caspase-3 is a major mediator of both apoptotic and necrotic cell death. This executor caspase cleaves and inactivates proteins crucial for the maintenance of cellular cytoskeleton, DNA repair, signal transduction and cell cycle control, being known as the endpoint of apoptosis (42). Both 10 and 25 μM MXC significantly increased the activity of caspase-3 when compared with GC-1spg without any treatment (1,37 ± 0,10-fold variation to control, p<0,05; 1,51 ± 0,09-fold variation to control, p<0,01, respectively; Figure 8).

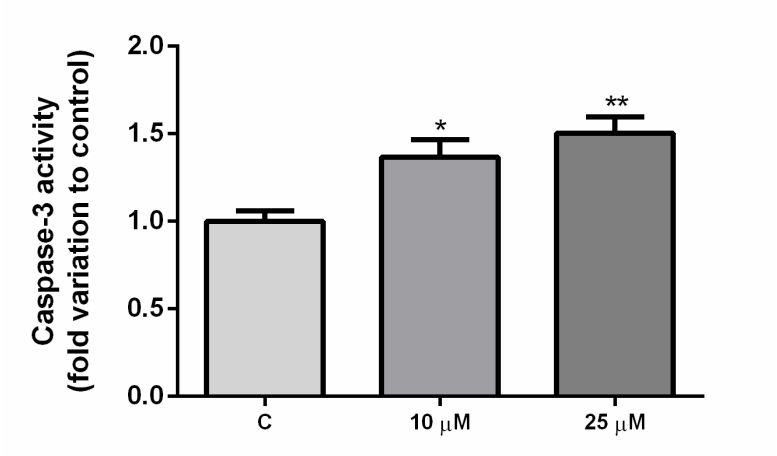

Figure 8. Caspase-3 activity in GC-1spg cells cultured for 48 h, in the presence or absence of 10 and 25 μM methoxychlor (MXC). Data are presented as mean ± S.E.M. (n≥5 in each group). Results are expressed as fold-variation comparatively to control in absence of MXC (C); Statistically significant differences relatively to control are indicated as *p<0,05 and **p<0,01.

4.3.2. Methoxychlor exposure modulated apoptosis-related protein expression levels in GC-1spg

Bcl-2 protein family members are the key regulators of intrinsic/mitochondrial pathway of apoptosis and the interaction and relative abundance between proapoptotic (Bax) and antiapoptotic (Bcl-2) members form a dynamic equilibrium that determines the threshold for apoptosis (72). The "point of no return" in this pathway is defined by mitochondrial outer membrane permeabilization (MOMP), which leads to the release of cytochrome c. Bcl-2 family proteins regulate MOMP and thereby determine the cellular commitment to apoptosis (73).

In MXC-exposed GC-1spg cells, no significant alterations were perceived on the expression of Bax when compared with the control group (non-treated cells) (1,08 ± 0,07-fold variation to control, Figure 9A). However, the expression of the anti-apoptotic protein Bcl-2 was significantly decreased (0,22 ± 0,09-fold variation to control, $p<0,01$, Figure 9B). After analysis of the expression of Bax and Bcl-2, the Bax/Bcl-2 protein expression ratio was calculated as indicator of a pro- or anti-apoptotic cell state. This ratio was significantly increased in MXC-stimulated cells when compared with untreated group (4,86 ± 0,73-fold variation to control, $p<0,01$, Figure 9C).

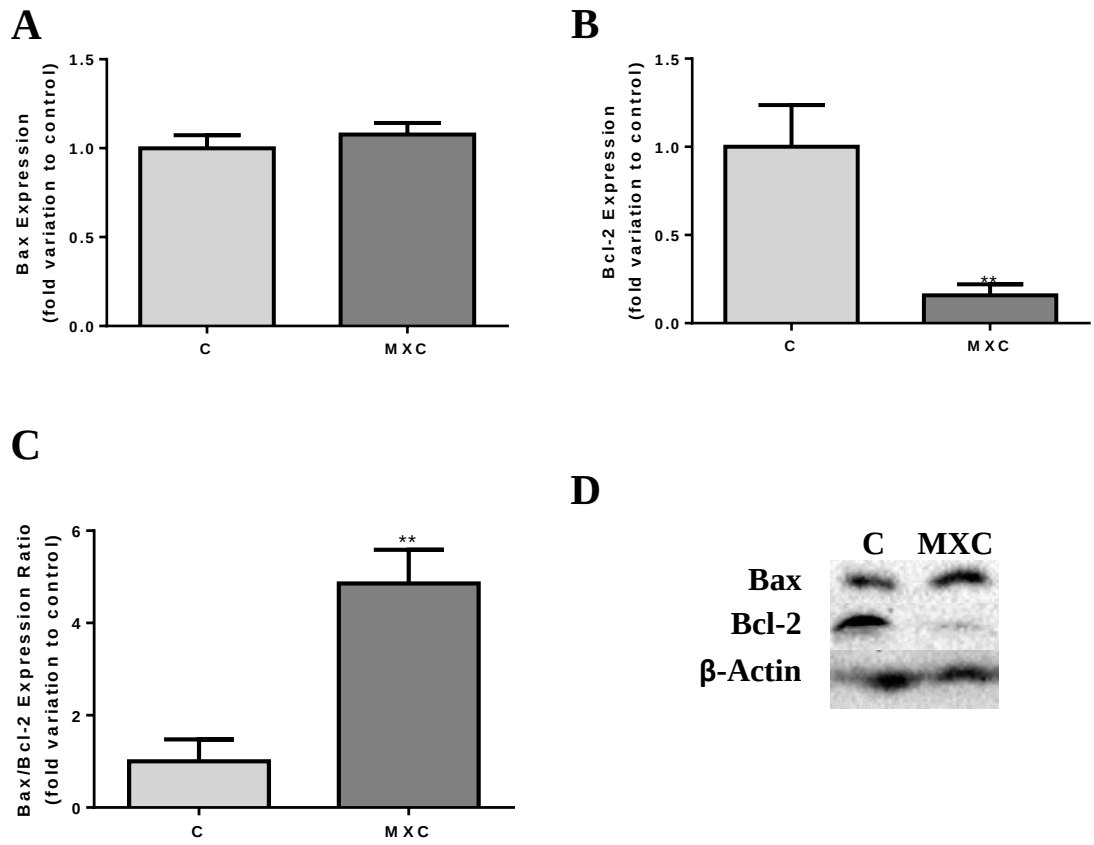

Figure 9. Protein expression of the Bcl-2 family proteins, Bax (A) and Bcl-2 (B), in GC-1spg cells cultured for 48 h, in the presence or absence of 25 µM methoxychlor (MXC). Bax/Bcl-2 protein ration and representative immunoblots are shown in panel C and D, respectively. Data are presented as mean ± S.E.M. ($n\geq5$). Results are expressed as fold-variation comparatively to control in absence of MXC (C); Statistically significant differences relatively to control are indicated as **$p<0,01$.

Caspase-8 has been classified as the main initiator caspase in death-receptor-mediated apoptosis, in other words, an initiator of extrinsic cell death (74). Upon stimulation of death receptors, such as TNFR1, caspase-8 forms a complex with FADD, receptor-interacting protein kinase 1 (RIPK1), and TNFR1-associated death domain protein (TRADD) and is activated (74). The activated caspase-8 induces apoptotic cell

death through the processing of effector caspases, such as caspase-3, -6, and -7 (74). Curiously, in GC-1spg treated cells, the expression of caspase-8 was significantly decreased when compared with non-treated group (0,30 ± 0,17-fold variation to control, p<0,01, Figure 10).

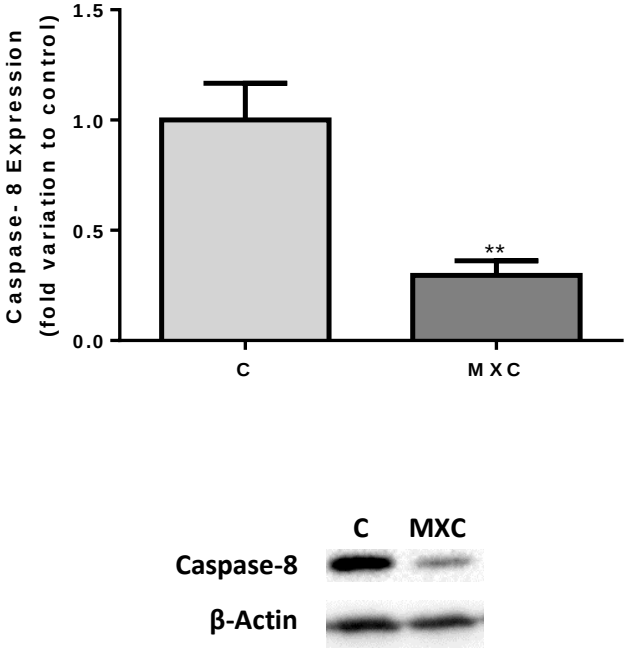

Figure 10. Protein expression of caspase-8 in GC-1spg cells cultured for 48 h, in the presence or absence of 25 μM methoxychlor (MXC). Representative immunoblots are shown. Data are presented as mean ± S.E.M. (n≥5). Results are expressed as fold-variation comparatively to control in absence of MXC (C); Statistically significant differences relatively to control are indicated as **p<0,01.

The protein p53 is known for its tumour suppressor function, but it is also a regulator of important processes for maintenance of homeostasis and response to stress. Its activity is generally antiproliferative and when the cell is damaged beyond repair or intensely stressed contributes to apoptosis (75). After MXC treatment, the expression of p53 was significantly decreased when compared with non-treated group (0,30 ± 0,06-fold variation to control, p<0,01, Figure 11).

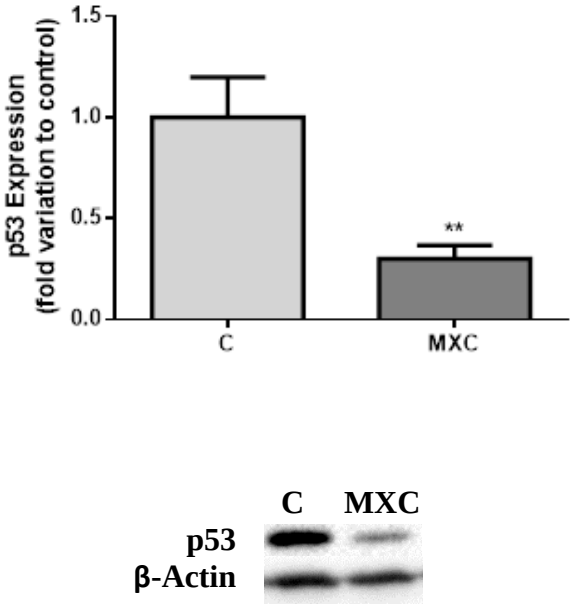

Figure 11. Protein expression of p53 in GC-1spg cells cultured for 48 h, in the presence or absence of 25 µM methoxychlor (MXC). Representative immunoblots are shown. Data are presented as mean ± S.E.M. (n=6). Results are expressed as fold-variation comparatively to control in absence of MXC (C); Statistically significant differences relatively to control are indicated as **p<0,01.

4.4. Estrogen and androgen receptors are expressed in GC-1spg cells, being downregulated by methoxychlor

The biological actions of estrogens are traditionally mediated by binding to one of two specific ERs, ERα or ERß, which belong to the NRs superfamily, a family of ligand-regulated transcription factors (76). The regulatory key-role of estrogens in the testis is corroborated by estrogen biosynthesis *in situ* and by the fact that the absence of ERs induces adverse effects on spermatogenesis (77). Androgens also exert their action through a NR, named AR, and its signalling in the testis is essential for spermatogenesis, being required for the maintenance of spermatogonial numbers (78). Thereby, the pertinence of studying the impact of MXC on these hormone receptors is prompted by its classification as an EDC with xenoestrogenic and anti-androgenic activity (79).

In the present dissertation, it was demonstrated for the first time that GC-1spg express ERß. In its turn, the expression of ERα and AR in GC-1spg was previously described by others (80–82). Upon exposure to MXC, the expression of all these receptors was significantly reduced (ERα: 0,41 ± 0,06-fold variation to control, p<0,001; ERß: 0,84 ± 0,09-fold variation to control, p<0,05; AR: 0,77 ± 0,04-fold variation to control, p<0,05; Figure 12).

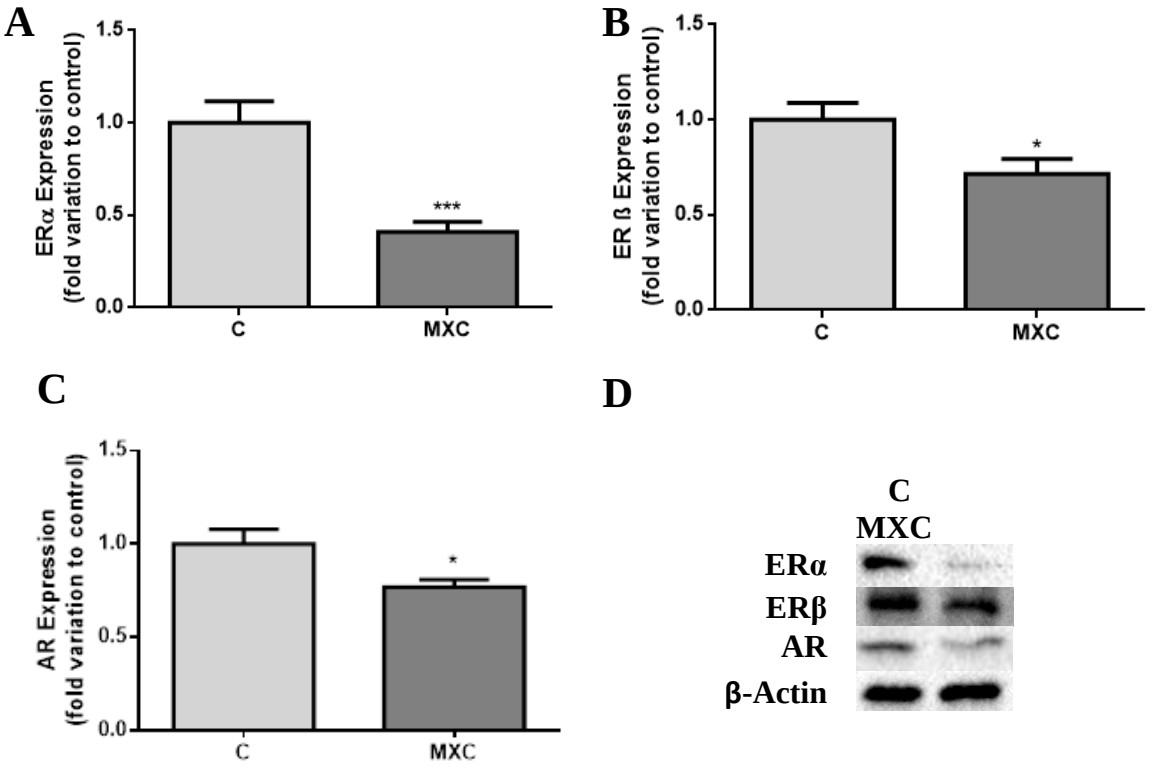

Figure 12. Protein expression of estrogen receptors (ERs), ERα (A) and ERß (B), and androgen receptor (AR) in GC-1spg cells cultured for 48 h, in the presence or absence of 25

µM methoxychlor (MXC). Representative immunoblots are shown in panel D. Data are presented as mean ± S.E.M. (n≥5). Results are expressed as fold-variation comparatively to control in absence of MXC (C); Statistically significant differences relatively to control are indicated as *p<0,05 and ***p<0,001.

5. Discussion

Infertility is a global public health problem, involving developed and developing countries (83). Concerning specifically male infertility, several factors can contribute to this condition such as hormonal imbalances, anatomical causes, sexually transmitted diseases, genetic factors, and environment and lifestyle (83). Indeed, environmental or occupational exposure to toxicants, like pesticides, is among the idiopathic risk factors (83). MXC is an insecticide and an EDC that is persistent in the environment (63). Significant amounts of MXC and deriving metabolites can be measured in human tissues, long after stopping its use (63). Nevertheless, there are no reports focused directly on assessing the molecular mechanism of MXC toxicity on spermatogonia survival. To start fulfilling this lack of knowledge, the present dissertation investigated the effect of MXC in specific mechanism of GC-1spg cells death.

Initially, the analysis of the results obtained in the MTT assay, suggested that GC-1spg cells showed decreased cell viability in response to MXC-treatment, probably in dose-dependent manner (Figure 6). At low concentrations, more precisely until 25 μM, there was no reduction in cell viability. After integration of the information on the effects of MXC on cell viability, the concentrations of 10 and 25 μM MXC were selected to analyse their influence in GC-1spg survival/death pathways, even before a reduction in cell viability is perceived.

The maintenance of spermatogenesis is controlled by gonadotropins and T, which effects are modulated by a complex network of locally produced factors, including hormones such as estrogens (84). Physiological effects of estrogens are mainly mediated by the classical nuclear ERα and ERß, which culminate in both genomic and rapid signalling events (84). Several EDCs have been shown to disrupt hormonal signaling in exposed wildlife, lab animals, and mammalian cell culture by binding to ERα and ERβ and affecting the expression of estrogen responsive genes (85). The results obtained in response to MXC exposure showed a decreased expression of ERα and ERß in GC-1spg cells (Figure 12). This observation was particularly intriguing, since this compound is extensively described as a xenoestrogen (86,87). However, over the last few years, substantial evidence has been supporting MXC as ER antagonist (88–90), with some studies even classifying MXC as an anti-estrogenic EDC (91–93). Thus, raising the question if MXC could be simply classified as xeno- or anti-estrogenic or if its endocrine disrupting effects could be dependent on the cells/tissue affected. E_2 signaling have been shown to be intrinsically related to cell survival and proliferation pathways, and in a concentration-dependent manner (94). Experimental

evidence shows that both ERα and ERβ regulate germ cell development, promoting SSCs' self-renewal (94). The mechanisms by which E_2 stimulate spermatogonia are complex, with many factors involved (94). It was hypothesized that E_2 could be involved in the regulation of mitosis of mouse spermatogonia (GC-1spg) through the activation of rapid EGFR/ERK/c-fos signaling, involving ERs (82). Therefore, since estrogenic signalling is believed to sustain spermatogenesis by promoting the proliferation of spermatogonia (72), the downregulation of ERs observed in the present work can be a pathway through which MXC affects GC-1spg proliferation, as seen on the MTT assays.

The classical genomic mechanism of androgenic signalling, involving hormones such as T, occurs when they diffuse into the cell and bind to AR (78). In the present dissertation, as in other studies (80,81), AR expression was observed in GC-1spg. Androgenic signalling in the testis is essential for normal spermatogenesis (78), supported by the higher intratesticular T levels relatively to those in the serum (up to 100 times more) (95). The activity of AR plays an important role during at least three steps of spermatogenesis: first, for progression through meiosis I, secondly, the transition from round to elongated spermatids, and finally during the terminal stages of spermiogenesis (96). Interestingly, when AR expression was inactivated in germ cells, testis development, every spermatogenic stages and sperm maturation were unaffected (97,98). These results support the idea that functional AR in germ cells is not essential for spermatogenesis and normal fertility of mice. However, the expression of AR in germ cells is still controversial and it is believed that the androgenic regulation is mediated by other cells (96). SCs are generally believed to be the primary mediators of the androgenic regulation of spermatogenesis because they provide physical and nutritional support to the developing germ cells (96). SCs selective AR knockout blocked spermatogenesis in meiosis since spermatocytes, round spermatids, and elongated spermatids were reduced to 64, 3 and 0%, respectively (99). The fact that only the germ line upon spermatocytes was affected enforces the hypothesis that the androgenic regulation of spermatogonia could be independent of SCs. The observed MXC-induced decrease in AR expression in GC-1spg cells, along with all this evidence, highlights the deleterious effect that MXC treatment could have on GC-1spg, affecting AR related pathways and mechanisms.

Besides its importance to maintain normal male sexual development and the spermatogenic process, T is needed for inhibition of germ cell apoptosis as when it is absent or in low levels, germ cells undergo apoptosis (100–102). However, the exact apoptotic pathway by which androgen withdrawal induces germ cell death remains elusive. Also, it has been largely reported that estrogens analogues have a profound

impact in testicular physiology by enhancing the apoptotic rate of germ cells (72). It is well-described that estrogens have influence on apoptosis and fluctuations in their levels or in the expression of their receptors can affect the process (72). Following this premise, concomitantly with the fact that in the present work a decrease in ERs and AR expression levels was shown in MXC-treated GC-1spg, it is liable to presume that MXC may modulate GC-1spg apoptotic pathways.

Apoptosis is tightly regulated by a family of proteases called caspases that are normally found in healthy cells as inactive precursors but become activated during apoptosis (103). Caspases play an important role on male infertility (104). Samples from infertile patients were characterized by high numbers of cells with active caspases, especially in cytoplasmic residues (105). On the other hand, germ cell apoptosis is an essential process to sustain male reproductive health. In mammalian testes, germ cells expand clonally through many rounds of mitoses before undergoing the differentiation steps that result in mature sperm (105). This clonal expansion is excessive and thus requires a mechanism such as apoptosis to match the number of germ cells with the supportive capacity of SCs (105). Therefore, apoptosis controls the overproduction of male gametes and restricts the normal proliferation levels during conditions unsuitable for sperm development, being a key regulator of spermatogenesis in normal and pathological conditions (10). The point of no return in the apoptotic cascade occurs with caspase-3 activation, an important and reliable apoptotic marker (40,44,104,106,107). Here, the effect of MXC on apoptosis of GC-1spg cells was evaluated by analysing the expression and activity of key regulators of apoptotic pathways. MXC had a pro-apoptotic effect on GC-1spg cells, which was demonstrated by the increase on the activity of the apoptosis effector caspase-3 (Figure 8). Although no alteration on the pro-apoptotic protein Bax expression levels was perceived (Figure 9A), MXC treatment increased Bax/Bcl-2 ratio (Figure 9C) by notoriously decreasing the expression of the anti-apoptotic protein Bcl-2 (Figure 9B). Curiously, the expression of caspase-8, which classically triggers the extrinsic apoptotic pathway in response to the activation of cell surface death receptors, was diminished in MXC-exposed GC-1spg (Figure 10). These results suggest that MXC induced apoptosis on GC-1spg and, most probably, through the activation of the intrinsic pathway. In the intrinsic pathway, exogenous stimuli (e.g., toxic chemicals, radiation and viral infection) directly generate intracellular signals which lead to biochemical changes within the cell (108,109). When a stimulus of this nature is present, it elicits disruption of the mitochondrial transmembrane that dissipates the membrane potential, rendering it more permeable (109,110) This also results in the formation of mitochondrial permeability transition pores on the outer membrane that channels pro-

apoptotic factors into the cytosol (110). Cytochrome c interacts with Apaf-1, resulting in the formation of apoptosome which cleaves procaspase-9 to yield active caspase-9 that, in turn, activates effector caspases (i.e., caspase-3) (43)(62). On the other side, the extrinsic pathway of apoptosis begins outside a cell, depending on the conditions in the extracellular environment or by cell-to-cell contact (45). This rationale corroborates the fact that MXC is activating apoptosis through the intrinsic pathway, as it is an EDC and exogenous toxicants can trigger this pathway. Furthermore, caspase-8 establishes the connection between the intrinsic and the extrinsic pathway, inducing Bid and cleaved Bid (cBID), activating the effector proteins Bax and BAK, which mediate MOMP (43). This could justify the fact that Bax expression was not altered in MXC-treated GC-1spg, due to insufficient caspase-8 levels to further stimulate Bax activation. Given their location, spermatogonia are more susceptible to exogenous stimulus which warrant the need to develop proper protection mechanisms and strategies, this could represent another justification for the decrease in caspase-8 levels. Cells that show increased resistance to stress are characterized by their requirement for higher levels of apoptotic stimuli for cell death to be induced (111). The ability to evade apoptosis has been shown to be caused by a range of different alterations including physiological changes such as the inactivation/downregulation of certain apoptotic molecules (111). A very efficient way to acquire resistance to FasL-induced apoptosis, is to downregulate death receptor surface expression (111). Maybe GC-1spg present decreased expression of the Fas receptor in an attempt to resist apoptosis induced by MXC. However, the expression of this receptor needs to be studied to elucidate about this hypothesis.

The tumour suppressor p53 has been shown to cause cell-cycle arrest and apoptosis in response to DNA damage (112). p53 mediates cell apoptosis by activating mitochondrial and death receptor–induced apoptotic pathways, both pathways resulting in the induction of caspase signalling, which then induces apoptosis. The mitochondrial pathway is mainly regulated by p53 effector Bcl-2 proteins (112). DNA damage or stress increase the levels of p53 protein, which in turn induces p21 transcription and leads to cell-cycle arrest at G1, allowing cells to survive until the damage has been repaired or the stress removed (112). Considering that MXC induced apoptosis in GC-1spg, an increased expression of p53 was expected. Nevertheless, MXC-exposed GC-1spg presented lower levels of p53 (Figure 11). This intriguing result also brings the pertinence of the previous-justified specific mechanisms of resistance to death in spermatogonia. In this context, a study refers that spermatogonia predominantly express testis-specific protein Y-linked 1 (TSPY1) (113). TSPY1 could decrease the p53 levels by inducing the degradation of ubiquitinated ubiquitin-specific peptidase 7 (USP7) (113). The observations reveal the significance of TSPY1 as a

suppressor of USP7-mediated p53 function in inhibiting p53-dependent cell proliferation arrest (113). These findings suggest an additional mechanism underlying the regulation of spermatogonial p53 function. While p53 plays a conserved role connecting the DNA damage response pathway to the core apoptotic machinery, there are also p53-independent mechanisms to induce cell death following chromosome damage (114). A study propose that these mechanisms act in parallel to the canonical DNA damage response pathway to eliminate cells with altered genomes following harmful exogenous stimuli, as ionizing radiation (114). Also, various differentiating tissues show considerable heterogeneity in stress-induced transcription from p53-dependent promoters (115). These differences in induction of p53 and cell-cycle arrest imply that signal processing and response within the DNA-damage-response pathway are regulated in a tissue-specific fashion (115). A study showed that embryonic stem cells were exquisitely sensitive to DNA damage through the activation of an apoptotic program that was effectively initiated in the absence of p53 (115). These results suggested a p53-independent, DNA-damage-induced response as the frontline strategy to insure genomic stability (115). In another study, apoptosis was induced by doxorubicin in a p53-null system with the involvement of mitochondrial membrane depolarization, cytochrome c release, caspase-3 activation and ROS generation (116). Interestingly, results suggested that ROS might act as the signal molecules for doxorubicin induced cell death and the process is still functional even in the absence of p53 (116).

When ROS production greatly exceeds the capacity of the endogenous cellular antioxidant defence system, OS occurs (117). OS affects spermatozoa in three main ways: membrane lipid peroxidation, DNA damage and induction of apoptosis (117). Antioxidant enzymes, which include GPx and SOD, play an important role in spermatogenesis and normal sperm function (118). SOD catalyses the dismutation of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide (118). GPx reduces hydrogen peroxide to water (71). The results obtained in this dissertation showed an increased activity of the enzymes SOD and GPx in GC-1spg cells treated with MXC (Figure 7). Some studies show that a significant increase in SOD and GPx activities might be meant to scavenge the overproduction of ROS under the OS induced by EDCs, functioning as a compensatory mechanism (119–122). This is easily explained as these enzymes are induced for the conversion of free radicals into less harmful or harmless metabolites with direct stimulatory effect of ROS (120). This evidence leads to believe that GC-1spg increase the activity of antioxidant enzymes in order to oppose the oxidative damage induced by MXC. Coherently, in several studies, MXC has been reported to elevate OS and consequently increase H_2O_2 production and

lipid peroxidation (123–127). A study even reports increased SOD activity after MXC exposure (121). SOD activity was also reported to be increased in fish exposed to other EDCs, such as paraquat, 2,3,7,8-Tetrachlorodibenzofuran or hexachlorobenzene (128). Another study showed that exposure to bisphenol A increased both SOD and GPx activity (127).

Altogether, our findings indicate that the expression and activity of key players in the apoptotic process were significantly altered at doses as low as 10 and 25 μM, suggesting that MXC may be effective even at low concentrations, as typically observed in other EDCs. The outcomes of this dissertation contributed to highlight the impact of environmental exposure to MXC in male fertility and to start to shed light to their mechanism of action.

6. Conclusions and Future Perspectives

Male infertility has increased over the years, becoming a global concern. Daily exposure to EDCs is one of the main causes, requiring greater awareness of its consequences. However, the molecular mechanism underlying EDCs actions leading to pathologies of the reproductive system are not completely characterized, specifically their effects in spermatogonial cells.

The present dissertation demonstrated that MXC-treatment led to increased apoptosis mediated probably by the activation of the intrinsic pathway in GC-1spg cells. In addition, MXC-treatment enhanced the activity of antioxidant defences. The results presented herein are the first report demonstrating the expression of ERß in GC-1spg and evidencing the impact of MXC modulating hormone receptors levels and the survival of these cells. The expression levels of the evaluated sex steroid receptors, AR, ERα and ERβ, were decreased upon MXC exposure. Although preliminary, these findings are extremely relevant, exploiting the mechanisms and pathways through which EDCs exert their well-described deleterious effects in male reproductive health. Therefore, shading light on putative targets for the development of novel male fertility preservation strategies and treatments.

In the near future, the full comprehension of MXC impact on the molecular mechanisms here investigated will be of utmost importance. With is envisaged, it would be interesting to confirm if the expression of Fas receptor is also reduced in GC-1spg, investigating their capability of resistance to death receptors induced-apoptosis. To complement these results, it will be opportune to study p53-independent mechanisms, because we believe these cells could have mechanisms to act in parallel to the DNA damage response pathway. Here, the activity of antioxidant enzymes was evaluated. However, and given the information present in the literature, a more direct way of investigating the potential of MXC as an OS inductor should be also performed, such as the measurement of ROS and lipid peroxidation levels.

Furthermore, the function of AR in spermatogonia needs to be explored, as here, it was hypothesized that in contrary to what is observed in other germ cells they could have their own androgenic regulation. Noteworthy, the xeno/anti-estrogenic dual behaviour of MXC needs to be unveiled. For this, the analysis of its impact on ERs expression levels must be compared in different cell types. The importance of these studies transcends the focus on MXC, broadening the horizons about the dimension and complexity of the EDCs' hormone (dys)regulation mechanisms.

Ultimately, assays comprising longer exposure times along with lower MXC-concentration must be performed in order to better mimicking the real context of EDCs

exposure. After solving the questions aroused, *in vivo* approaches could be developed allowing a more comprehensive view of the harmful effects of EDCs on male reproduction.

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