Protective potentialities of propolis towards spermatogonial oxidative damage

VERSÃO FINAL APÓS DEFESA

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Declaração de Integridade

Eu, Filipa Maia Duarte, que abaixo assino, estudante com o número de inscrição M12348 de Bioquímica da Faculdade de Ciências, declaro ter desenvolvido o presente trabalho e elaborado o presente texto em total consonância com o **Código de Integridades da Universidade da Beira Interior**.

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Universidade da Beira Interior, Covilhã 21 /02 /2024

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Resumo

A fertilidade masculina depende de uma espermatogénese bem-sucedida, assim como de uma série de fatores genéticos, ambientais e fisiológicos. Neste contexto, o dano oxidativo no tecido testicular afeta o sistema reprodutor, dando origem a cerca de 30 a 80% dos casos de infertilidade. Foi reportado que elevados níveis de espécies reativas de oxigénio (ROS) perturbam o desenvolvimento das células germinativas e interferem na função espermática. Vários antioxidantes naturais foram descritos com a função de prevenir ou reduzir esses mesmos danos. A própolis é uma mistura natural resinosa produzida pelas abelhas com inúmeras propriedades farmacológicas e biológicas, incluindo também a sua capacidade antioxidante. Desta forma, a própolis é uma boa candidata para proteger o sistema reprodutor masculino contra o stress oxidativo (OS). Além disso, e apesar de todas as evidências sobre o potencial protetor da própolis, não existem estudos diretamente focados no seu efeito em populações de células testiculares específicas. Seguindo este raciocínio, a presente dissertação teve como objetivo analisar o papel da própolis na proteção contra o dano oxidativo em espermatogónias. Após avaliação da composição fitoquímica e da atividade antioxidante da própolis, células espermatogoniais do tipo B (GC-1spg) foram tratadas com própolis (0.1-500 μg/mL; 12-48 horas), na presença ou na ausência de estímulo oxidante (tert-butil hidroperóxido, TBHP, 0.005-3.6 μg/mL, 12 horas). A citotoxicidade da própolis e do TBHP, misturados ou não, foi analisada pelo ensaio MTT e a proliferação por imunocitoquímica do Ki-67. A taxa de apoptose, os níveis de ROS e as defesas antioxidantes foram estudados através de métodos colorimétricos.

Os resultados obtidos para a própolis utilizada neste estudo, mostraram alto teor de compostos fenólicos totais e flavonóides, com atividade antioxidante moderada. A própolis (0.1 μg/mL) aumentou a viabilidade das células GC-1spg, contrabalançando o impacto da exposição ao TBHP (1.8 μg/mL). Além disso, independentemente da presença de TBHP, a própolis reduziu os níveis de ROS nas células GC-1spg. Quando na presença de TBHP, a própolis diminuiu a atividade da caspase-3 e atenuou a diminuição da taxa de proliferação induzida por TBHP, aumentando a atividade da glutationa peroxidase.

O presente trabalho destacou os benefícios dos compostos naturais para a fertilidade masculina, mais precisamente, a ação protetora da própolis contra o OS induzido por TBHP em espermatogónias (GC-1spg). Na verdade, as ações nocivas do TBHP foram reduzidas, ou mesmo neutralizadas, na presença da própolis. Estes resultados destacam o papel da própolis como agente protetor contra o OS, o que é crucial no contexto da espermatogénese e da fertilidade masculina.

Palavras-chave

antioxidante;espermatogónias;própolis;stress oxidativo;tert-butil hidroperóxido

Resumo alargado

A espermatogénese é um processo complexo que começa na puberdade e continua ao longo de toda a vida de um indivíduo, com o objetivo de formar espermatozoides funcionais. A fertilidade masculina depende de uma espermatogénese bem-sucedida, assim como de uma série de fatores genéticos, ambientais e fisiológicos. O nosso corpo está constantemente sob ataque de espécies reativas de oxigénio (ROS), o que confere alta reatividade e consequentemente danos a nível celular. Para combater esta oxidação são desenvolvidas defesas antioxidantes no organismo que, quando perturbadas ou em quantidade insuficiente, não permitem o restabelecimento da homeostase oxidativa, originando stress oxidativo (OS). O dano oxidativo no tecido testicular afeta o sistema reprodutor, dando origem a cerca de 30 a 80% dos casos de infertilidade. Foi reportado que baixos níveis de ROS são necessários no testículo para funções como a capacitação espermática ou a reação acrossomal, mas níveis elevados de ROS neutralizam os antioxidantes e causam OS, perturbando o desenvolvimento das células germinativas e interferindo na função espermática. Estas células espermáticas são incapazes por si só de reparar os danos causados pelo OS uma vez que sofrem falta de algumas enzimas citoplasmáticas essenciais. O ambiente testicular apresenta proteção antioxidante de modo a prevenir uma redução da produção de espermatozóides contendo defeitos morfológicos, a fragmentação de DNA e capturar as ROS. Este mecanismo de defesa inclui enzimas antioxidantes como a superóxido dismutase, a catalase e a glutationa peroxidase. No entanto, em condições que culminam em elevado OS, estas defesas não conseguem colmatar os danos oxidativos. Neste contexto, vários antioxidantes naturais como o alho, o gengibre e a própolis foram descritos com a função de prevenir ou reduzir esses mesmos danos. A própolis é uma mistura natural resinosa produzida pelas abelhas com inúmeras propriedades farmacológicas e biológicas, incluindo também a capacidade antioxidante através da remoção de radicais livres. Esta atividade antioxidante deve-se ao seu enriquecimento em polifenóis como os compostos fenólicos e flavonóides, com a grande maioria dos resultados demonstrando redução nos marcadores de OS. Recentemente, foi demonstrado que a própolis aumenta a produção espermática, assim como a mobilidade, contagem e qualidade dos espermatozóides. Deste modo, a própolis é uma boa candidata para proteger o sistema reprodutor masculino contra o OS. Apesar de todas as evidências sobre o potencial protetor da própolis no homem, não existem estudos diretamente focados no seu efeito em populações específicas de células testiculares. Seguindo este raciocínio, a presente dissertação teve como objetivo analisar o papel da própolis na proteção contra o dano oxidativo em espermatogónias. Após avaliação da composição fitoquímica e da atividade antioxidante da própolis, células

espermatogomiais do tipo B (GC-1spg) foram tratadas com própolis (0.1-500 μg/mL; 12-48 horas), na presença ou na ausência de estímulo oxidante (tert-butil hidroperóxido, TBHP, 0.005-3.6 μg/mL, 12 horas). A citotoxicidade do própolis e do TBHP, misturados ou não, foi analisada pelo ensaio MTT e a proliferação por imunocitoquímica do Ki-67. A taxa de apoptose, os níveis de ROS e as defesas antioxidantes foram estudados através de métodos colorimétricos.

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Abstract

Male fertility is dependent on successful spermatogenesis, as well as an array of genetic, environmental, and physiological factors. In this context, oxidative damage in testicular tissue affects the reproductive system, leading to 30 to 80% of infertile cases. High levels of reactive oxygen species (ROS) have been reported to disrupt the development of germ cells and to interfere with sperm function. Several natural antioxidants have been described to prevent or reduce this damage. Propolis is a natural resinous mixture produced by honeybees with plenty of pharmacologic and biological properties, including antioxidant capacity. Hence, propolis is a good candidate to protect the male reproductive system against oxidative stress (OS). Moreover, despite all the evidence about the protective potential of propolis, there are no reports directly focused on the effect of propolis on specific testicular cell populations. Following this rationale, the present dissertation aimed to analyse the role of propolis in protecting spermatogonial cells from oxidative damage. After evaluating the phytochemical composition and the antioxidant activity of propolis, type B spermatogonia cells (GC-1spg) were treated with propolis (0.1-500 μg/mL; 12-48 hours), in the presence or the absence of an oxidant stimulus (tert-butyl hydroperoxide, TBHP, 0.005-3.6 μg/mL, 12 hours). The cytotoxicity of both propolis and TBHP mingled or not, was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and proliferation by Ki-67 immunocytochemistry. The apoptosis rate, ROS levels, and antioxidant defences were studied through colorimetric methods.

The obtained results showed a high content of total phenolics and flavonoids in the propolis extract, and moderate antioxidant activity. Propolis (0.1 μg/mL) increased the viability of GC-1spg cells, counterbalancing the impact of TBHP exposure (1.8 μg/mL). Additionally, regardless of TBHP presence, propolis reduced ROS levels in GC-1spg cells. In the presence of TBHP, propolis decreased caspase-3 activity and attenuated the TBHP-induced decrease in proliferation rate, increasing glutathione peroxidase activity. The present work highlighted the benefits of natural compounds to male fertility, more precisely, the protective action of propolis against TBHP-induced OS in spermatogonial cells (GC-1spg). Indeed, the disruptive actions of TBHP were counteracted, or even neutralized in the presence of propolis. These findings highlight propolis role as a protective agent against OS, which is crucial in the context of spermatogenesis and male fertility.

Keywords

Antioxidant, oxidative stress, propolis, spermatogonial cells, tert-butyl hydroperoxide.

Table of Contents

List of Publications

Article

Duarte F[#], Feijó M[#], Luís Â, Socorro S, Maia CJ, Correia S. (2024) Propolis Protects GC-1spg Spermatogonial Cells against Tert-Butyl Hydroperoxide-Induced Oxidative Damage *International Journal of Molecular Sciences 25*, 614. [#]Contributed equally. (IF: 5.6, Q1)

Poster Communication

Duarte F, Feijó M, Luís Â, Socorro S, Maia CJ, Correia S. Unveiling the preventive role of propolis facing oxidative damage in spermatogonial cells. XVIII International CICS-UBI Symposium. 10-11 July 2023, Covilhã, Portugal

List of Figures

List of Tables

List of Abbreviations

AAI	Antioxidant activity index
A_{al}	$A_{aligned}$
A_{pr}	A_{paired}
AR	Androgen receptor
A_s	A_{single}
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid protein
BTB	Blood-testis barrier
CAT	Catalase
CO_2	Carbon dioxide
Cu/Zn-SOD	Copper-zinc superoxide dismutase
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E2	17β-estradiol
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GAE	Gallic acid equivalents
GC-1spg	Gonadal cell-1 spermatogonia
GnRH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
LC	Leydig cell
LH	Luteinizing hormone
MDA	Malondialdehyde
Mn-SOD	Manganese superoxide dismutase
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate

NADPH	Nicotinamide adenine dinucleotide phosphate
$O_2^{\cdot -}$	Superoxide anions
$^{\cdot}OH$	Hydroxyl
OS	Oxidative stress
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween-20
pNA	p-nitro-aniline
QE	Quercetin equivalents
RIPA	Radioimmunoprecipitation assay
$^{\cdot}ROO$	Proxyl
ROS	Reactive oxygen species
SC	Sertoli cell
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SeTs	Seminiferous tubules
SOD	Superoxide dismutase
SSC	Spermatogonial stem cells
T	Testosterone
TBHP	Tert-butyl hydroperoxide

I – Introduction

1. An insight into testicular anatomy and physiology

The male reproductive tract involves several structures which include the testes, efferent ducts, epididymis, deferent ducts, accessory sex glands, and penis [1,2]. Testes have an oval shape and a mean volume of 20 cm^3, the average width is 2.5 cm and the length is between 3.5 and 5.5 cm (Figure 1) [3]. They are suspended in the scrotum, a fibromuscular structure that is divided into two sacs, one for each testis. This is not only to protect the male gonad but also to maintain the testicular temperature approximately 2-3 °C below the body core temperature [2-4]. The testicular capsule is suspended by the spermatic cord [5]. Each testis is enclosed in a capsule that is composed of three layers: *tunica vaginalis*, *albuginea,* and *vasculosa*. The outer tunica, *vaginalis*, is a dense saclike extension of the peritoneum that results in direct communication between the peritoneal cavity and the scrotum [6]. The *tunica albuginea* is a dense white fibrous capsule composed of collagen fibres. This layer protects the innermost cover of the testicle, the *tunica vasculosa*, which contains the blood vessels [6-9]. Connective tissue septa divide the organ into 250 to 300 pyramidal lobules that hold the seminiferous tubules (SeTs) [7]. SeTs are typically long, highly coiled, and looped [6]. These are covered by *lamina propria*, which consists of a basal membrane, a layer of collagen, and the peritubular cells (myofibroblasts). These characteristics differentiate the human testicle from the majority of the other mammals, whose SeTs are surrounded only by two to four layers of myofibroblasts [10].

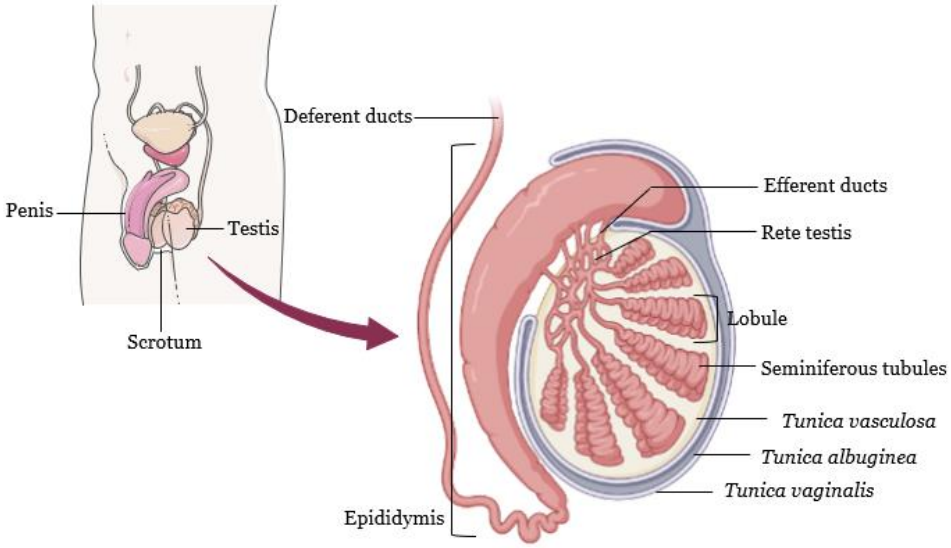

Figure 1: Schematic representation of the testis. Testes are enclosed in a capsule that is composed of three layers: tunica *vaginalis* (outer), *albuginea* (intermediate), and *vasculosa* (innermost) which results in the formation of pyramidal lobules containing the seminiferous tubules (SeTs). SeTs converge to the rete

testis which is connected to the efferent ducts and then to the epididymis. Figure was drawn by using pictures from BioRender and Servier Medical Art.

Testis accomplished two major functions: endocrine, producing sex steroid hormones, and exocrine, with the production of highly differentiated gametes, the spermatozoa [11]. Steroidogenesis relies on enzymatic reactions leading to the production of male steroid hormones, occurring in the interstitial compartment, a structure that represents 12 to 15% of the total testicular volume [10]. In this compartment, the most important cells are the Leydig cells (LCs), the source of testicular testosterone (T) [10]. Aside from LCs, the interstitial compartment also contains immune cells, fibroblasts, loose connective tissue, nerves and blood and lymph vessels [10]. Steroid hormones are essential to sustain spermatogenesis, the process through which gametes are produced and that occurs in the tubular compartment. This compartment, representing about 60 to 80% of the total testicular volume, contains the germ cells and two different types of somatic cells, the Sertoli cells (SCs) and the peritubular cells [6, 10]. SCs are lined in the lumen of the SeTs and can be considered as the supporting structure of the germinal epithelium[6, 7, 10]. The SCs have several distinct functions that facilitate the maturation of the germ cells. First, they provide physical support where the germ cells develop and migrate toward the lumen of the tubule[10]. Second, the SCs form the blood-testis barrier (BTB) with specialized tight junctions that exist between these cells [6, 7]. Third, SCs create the focused microenvironment essential for germ cell maturation [10]. These distinctive functions also include fluid secretion, phagocytosis, production of a variety of molecules and also create and maintain the permeability of the lumen tubules [7, 10]. Germ cells are immersed in a fluid of unique composition, the tubular fluid, that contains an elevated concentration of potassium ions and a low concentration of sodium ions. Other constituents are bicarbonate, magnesium and chloride ions, glucose, inositol, glycerophosphorylcholine, carnitine, amino acids, and several proteins [10, 12].

2. Male fertility from the genesis: the spermatogenic process

a) Spermatogenesis and the peculiarities of spermatogonia cell populations

Spermatogenesis is a complex process that occurs in the SeT, involving mitotic cell division, meiosis, and the spermiogenesis, to create functional sperm [1, 13-15]. This process differs from species to specie: in human, it takes about 64 days to complete, while in rats it is 48 days and in mice it only takes 35 days [16, 17].

Spermatogenesis (Figure 2) starts at puberty and continues throughout the entire life of the individual [18]. This process begins with spermatogonial stem cells (SSCs) that mitotically originate daughter stem cells or differentiate into spermatogonia [19]. Spermatogonia multiply by mitosis, producing two types of daughter cells: type A (without heterochromatin) and type B spermatogonia. Type A cells continue to multiply from puberty until death, while type B cells migrate closer to the tubule lumen and differentiate into slightly larger cells, the primary spermatocytes. These cells pass through the BTB and move through the lumen, where meiosis I occurs and forms two haploid secondary spermatocytes. Each of these undergoes meiosis II, dividing into two spermatids. Finally, spermatids undergo a series of nuclear and cytoplasmic changes, spermiogenesis, to develop into mature spermatozoa [6, 7]. The spermatozoa produced are released in the lumen of the SeT as immature gametes. Spermatozoa full fertilization capacity, progressive motility, and ability to fertilize, are acquired 1 or 2 weeks after the passage through the epididymis [20-22].

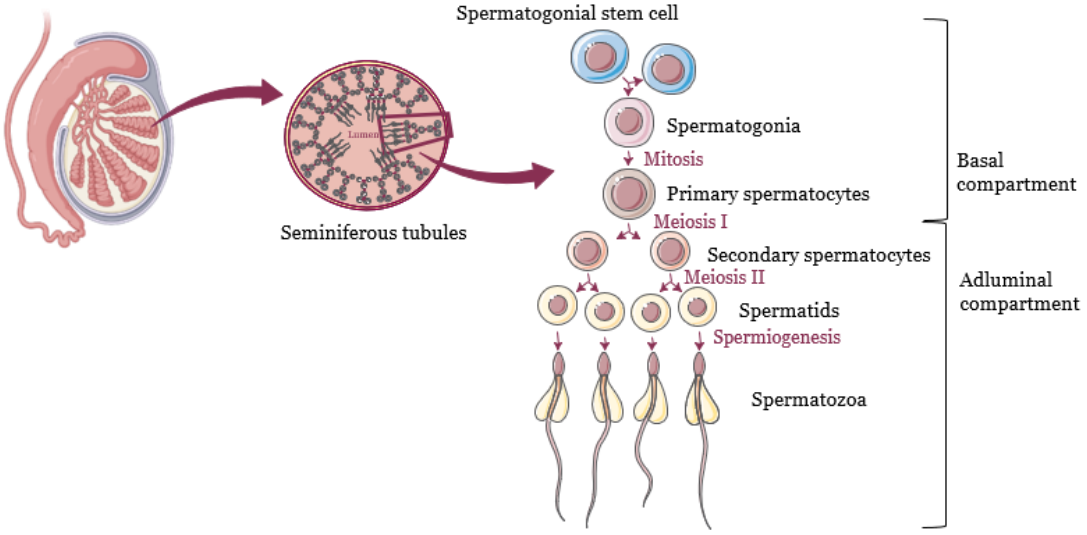

Figure 2: Schematic representation of the spermatogenic process. Spermatogenesis occurs in the seminiferous tubules. Spermatogonial stem cells differentiate into spermatogonia that multiplies by mitosis to form primary spermatocytes. Then, the first meiotic division occurs originating secondary spermatocytes. The secondary spermatocytes originate the spermatids by concluding the second meiotic division, which then originates the spermatozoa by spermiogenesis. Figure was drawn by using pictures from BioRender and Servier Medical Art.

In rodents testis, undifferentiated stem and progenitor spermatogonia are defined in part by their clonal arrangement in the seminiferous epithelium and part by molecular markers (ID4, GFRA1, PLZF, SALL4 and others, Figure 3) [23]. SSCs are the least differentiated spermatogonia (0.03% of total adult testis), called A_{single} spermatogonia (A_s), remaining as single cells on the basement membrane of the SeT [23, 24]. Mitotic division of A_s produces a pair of A_{paired} spermatogonia (A_{pr}) that will either complete cytokinesis to produce two new A_s (self-renewing division) or remain joined by an

intracellular bridge and generate $A_{aligned4}$, $A_{aligned8}$, and $A_{aligned16}$ (A_{al}), which will differentiate to type A1 spermatogonia and consequently will produce types A2, A3, A4, Intermediate and B spermatogonia [23-25]. These differentiated cells appear sequentially in time as they mature and acquire more heterochromatin and decrease their nuclear sizes. All of these cells are actively engaged in proliferation and their identification of specific spermatogonial cell types is performed according to the chromatin distribution in their nucleus [26]. Ultimately, 1 spermatogonia is capable of originating 4096 spermatozoa, which means that a small pool of stem cells in the rodent testis can produce 40 million sperm per gram of testis parenchyma each day [23, 25].

In contrast, in higher primates (human and nonhuman, like monkeys) stem cells are defined based on differences in nuclear morphology and staining intensity with hematoxylin (Figure 3) [23]. A_{dark} spermatogonia are relatively small, spherical, or slightly ovoid cells on the basement membrane of SeT having dark nuclei and rarely proliferate under normal circumstances, while A_{pale} spermatogonia are identified as relatively larger or oval cells on the basement membrane of the SeT contain pale nuclei and proliferate more frequently [23, 27]. Some authors propose that A_{dark} and A_{pale} are the same populations of cells that are simply at different stages of the cell cycle (A_{dark}: G0 versus A_{pale}: G1/S/G2/M), even though there is only evidence supporting this [23]. In nonhuman primates, A_{dark} and A_{pale} spermatogonia are present in equal numbers and comprise 4% of germ cells in the testis, with 1 spermatogonia originating 256 spermatozoa, by 8 transit amplifying divisions. On the other hand, in humans, A_{dark} and A_{pale} spermatogonia constitute 22% of germ cells, compensating for the occurrence of only 5 transit-amplifying divisions that give rise to 32 spermatozoa. Thus, the sperm output in monkeys is about 40 million sperm per gram of testicular parenchyma per day, while in humans the output is reduced to 4.4 million sperm per gram of testicular parenchyma per day [23].

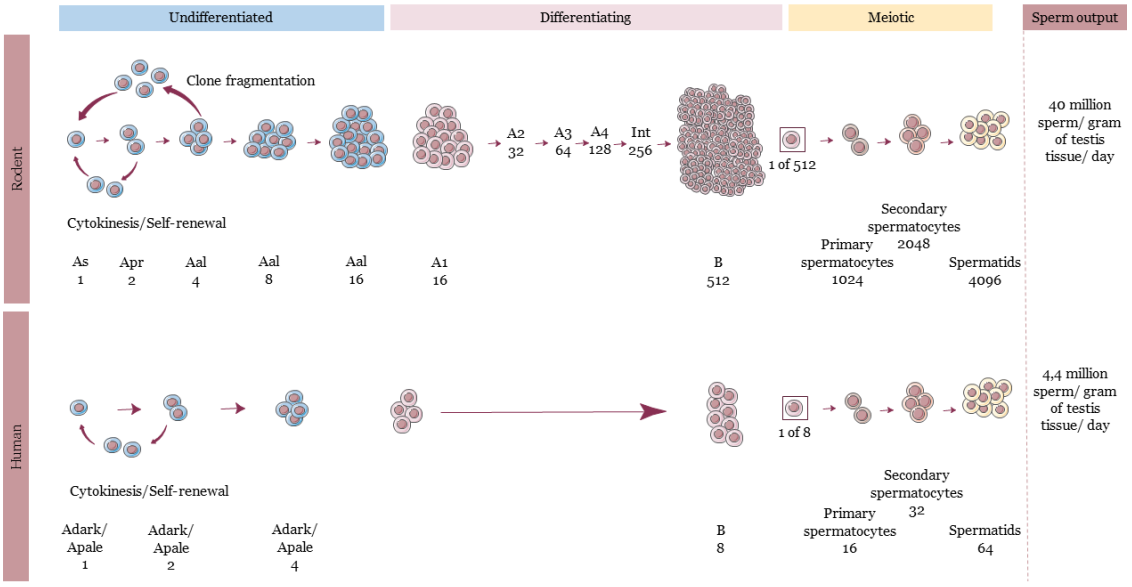

Figure 3: Clonal development in the spermatogenic lineages of rodents and humans. Undifferentiated spermatogonia are described as A_s (A_{single}), A_{pr} (A_{paired}) or, A_{al} ($A_{aligned}$) in rodents, and A_{dark} or A_{pale} in humans. During spermatogenic development, A_s and A_{dark} and/or A_{pale} undergo one or more mitotic divisions to give rise to cells of larger clones of interconnected cell sizes through transit-amplifying mitotic divisions. A) Clonal development in rodents features 3–4 transit-amplifying divisions in the pool of undifferentiated A_s, $A_{pr,}$ and A_{al} spermatogonia followed by 6 amplifying divisions in the pool of differentiated spermatogonia (A1–A4, Intermediate, B), which give rise to primary spermatocytes. Two additional meiotic divisions produce round spermatids that undergo spermiogenesis to produce sperm. B) Clonal development of spermatogonia in humans features 0, 1, or 2 transit-amplifying divisions in the pool of undifferentiated A_{dark}/A_{pale} spermatogonia followed by a single transit-amplifying division in differentiated B spermatogonia that give rise to primary spermatocytes. Figure was drawn by using pictures from BioRender and Servier Medical Art.

b) Hormonal regulation of spermatogenesis

Male fertility is dependent upon successful spermatogenesis, a multi-step process that depends on hormonal messengers acting through endocrine, paracrine, and autocrine pathways [28, 29].

The endocrine control of spermatogenesis is commanded by neuroendocrine activity along the hypothalamic-pituitary-testicular axis, where the decapeptide gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner by the hypothalamus to the anterior pituitary [30]. This organ secretes two glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), that act directly on the testis to stimulate somatic cell function in support of spermatogenesis [29-31]. LH and FSH act on LC and SCs, respectively, mediating their actions via specific transmembrane receptors, LH-R and FSH-R [30, 31]. LH`s primary function is to stimulate LCs to produce T, by the increased expression of key steroidogenic enzyme genes as well as transcription factors [31]. FSH activates several signalling pathways in SCs to support germ cell development [31] and, combined with T stimulates SCs to produce factors required for the development of germ cells, such as growth factors and nutrients [32 33]. SCs also secrete glycoproteins that

facilitate the transport of ions and hormones or provide bioprotective functions (transferrin and androgen-binding protein), proteases, and proteases inhibitors [34].

T is present in high levels in men's testis (340 to 2000 nM) when compared to the serum (8 to 35 nM), and is required for four critical processes during spermatogenesis: maintenance of the BTB, meiosis, SCs-spermatid adhesion, and sperm release [28, 35, 36]. This hormone acts as a paracrine factor, being the major androgen in the testis that regulates spermatogenesis [28]. Androgen effects are mediated by the androgen receptor (AR) which, when interacting with T, regulates LCs through autocrine feedback and in their absence, spermatogenesis does not proceed [28, 29, 35]. Indeed, the hypothalamic-pituitary-gonadal axis is controlled by negative feedback mechanisms. T can inhibit LH secretion in two ways: by acting on the hypothalamus directly and decreasing the GnRH release, which results in a decrease in the secretion of gonadotropins, or by acting directly on the anterior pituitary gland to decrease the LH response. Another hormone responsible for the regulation of this mechanism is inhibin, which acts in the anterior pituitary gland, by decreasing FSH release (Figure 4) [2].

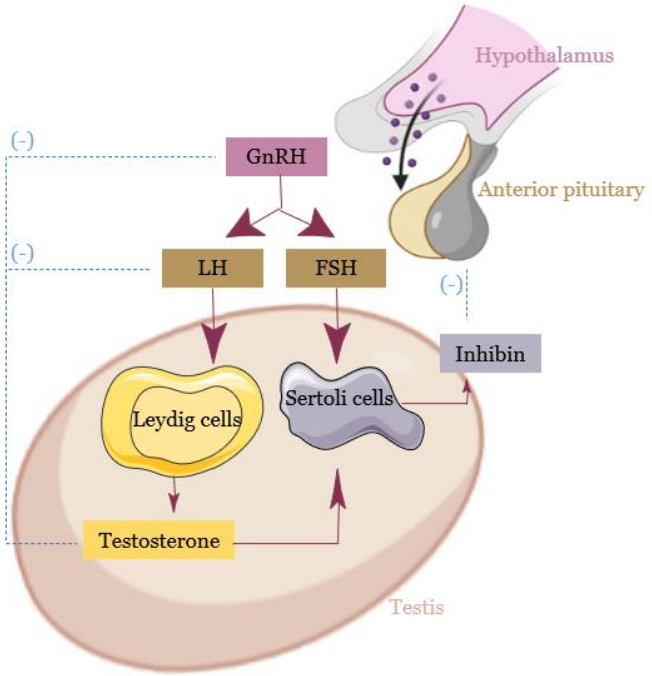

Figure 4: Schematic representation of hormonal regulation of the spermatogenesis. *The* hypothalamus releases decapeptide gonadotropin-releasing hormone (GnRH) to the anterior pituitary. This gland secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that act directly on the testis. LH stimulates Leydig cells and FSH stimulates the activity of Sertoli cells, inducing the production of testosterone and inhibin, respectively. Testosterone induces a negative feedback in the pituitary gland and hypothalamus, decreasing the levels of GnRH and LH, and inhibin in the anterior pituitary decreasing FSH. Figure was drawn by using pictures from BioRender and Servier Medical Art.

In the last decades, the role of estrogens in the male reproductive system has been explored. Estrogens can be synthesized by germ cells, LCs, and SCs, producing a relatively high concentration in rete testis fluid [37-39]. Germ cells express the aromatase cytochrome P450 enzyme, the enzyme responsible for aromatization of testosterone to

17β-estradiol (E_2) [37, 38]. While a major source of estrogens in the immature testes is the SCs, LCs produce most of the estrogens in the adult testes [40]. In men, this hormone is essential for modulating libido, erectile function, and spermatogenesis, as well as regulating chromatin remodelling of spermatids, loss of acrosome and increased motility of spermatozoa ejaculated sperm [40-43]. Noteworthy, E_2 also regulates fluid absorption in the efferent ducts and rete testis, by maintaining the appropriate osmolality and sperm concentration [43].

In addition to the hormonal regulation of spermatogenesis, growth and differentiation factors, like vitamin A and interleukins, as well as intracellular factors also have an important role. Several factors and/or pathologies can affect the spermatogenic process, such as obesity, diabetes, genetic factors, and environmental chemicals [44, 45]. Herein, most of them result in oxidative status imbalance.

3. Oxidative stress in male (in)fertility

Except for some anaerobic and aerotolerant species, all organisms require oxygen for efficient production of energy in the form of ATP, through oxidative phosphorylation [46]. The reactivity of this gas allows the evolution of complex multicellular organisms but also renders it liable to attack any biological molecule, like proteins, lipids, or DNA [46, 47]. Therefore, our body is under constant oxidative attack from reactive oxygen species (ROS) [47]. ROS are oxygen-derived free radicals containing one or more unpaired electrons, and it is this incomplete electron shell that confers their high reactivity. Free radicals can be generated from many elements, but in biological systems, it is those involving oxygen and nitrogen that are the most important [47]. ROS (Figure 5) includes highly reactive free radicals including hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), and proxyl ($^{\cdot}ROO$) [48-50]. The antioxidant defence is, in part, capable of delaying or inhibiting the oxidation of a substrate, involving both enzymatic and non-enzymatic processes, nevertheless, this balance can be perturbed, leading to oxidative stress (OS) [47, 51]. The term “oxidative stress” can be defined as an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage [51-53]. OS plays a central role in the pathophysiology of many different disorders, including male infertility [47, 51, 54].

Males are responsible for 20% to 30% of infertility cases, with 7% of all men worldwide diagnosed as infertile [54, 55]. During recent years, an increasing percentage of male infertility has been attributed to an array of genetic, environmental, and physiological factors [56, 57]. At the cellular level, sperm damage by ROS is the main cause of infertility in 30 to 80% of infertile men [49, 54, 57]. Low levels of ROS are necessary for sperm capacitation,

hyperactivation, acrosomal reaction, and spermatozoon-oocyte fusion [49, 54, 55]. At the same time, high levels of ROS neutralize antioxidants in the seminal plasma and cause OS and, consequently, DNA damage in the nucleus and mitochondria [54, 55]. Sperm cells have cytoplasmic membranes rich in unsaturated fatty acids, therefore they are faced with lipid peroxidation under the influence of ROS [49]. Moreover, these cells are incapable of repairing damage by OS because they suffer from a lack of essential cytoplasmic enzymes [49]. Furthermore, lower motility and death of sperm occur due to the loss of ATP caused by lipid peroxidation followed by axonemal injury [49].

To neutralize the effects of ROS, the testis exhibits antioxidant protection against the production of abnormal spermatozoa, prevents DNA fragmentation, scavenges ROS produced by leukocytes, reduces cryodamage to spermatozoa, improves semen quality, blocks premature sperm maturation and stimulates spermatozoa [58]. The antioxidant defence mechanism includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) [58, 59]. SOD acts as an endogenous cellular defence system that degrades superoxide into oxygen and hydrogen peroxide, with the last being further detoxified by GPx or CAT [60]. There are two types of SOD, a mitochondrial type, manganese superoxide dismutase (Mn-SOD), and a cytoplasmic type, copper-zinc superoxide dismutase (Cu/Zn-SOD). Both SOD act as antioxidant enzymes, and the last one is expressed predominantly in spermatogonia [61]. Moreover, testis can exhibit non-enzymatic antioxidants such as ascorbate, urate, vitamins A and E, albumin, pyruvate, ubiquitol, taurine, reduced glutathione (GSH), carotenoids and other antioxidants [50, 58, 59, 62].

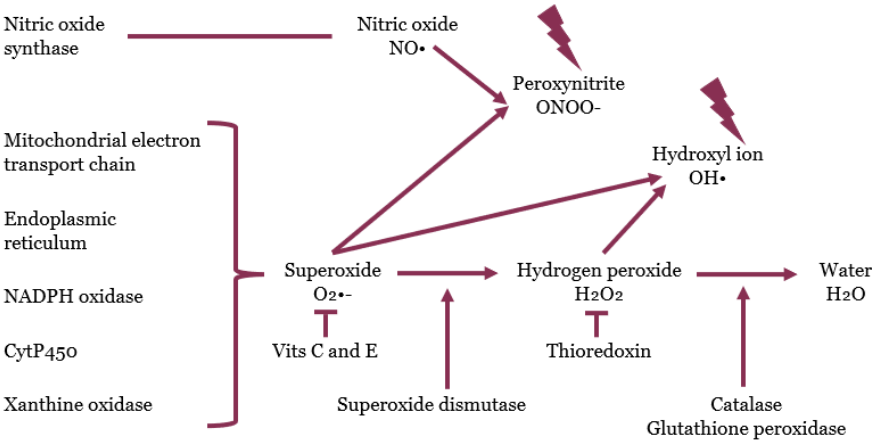

Figure 5: Reactive oxygen species (ROS), their potential origins, and detoxification pathways. The oxygen free radical superoxide anion ($O_2^{\cdot-}$) derivates from different sources such as mitochondria and endoplasmic reticulum. Superoxide is detoxified by the superoxide dismutase enzyme, which convert it to hydrogen peroxide (H_2O_2). Hydrogen peroxide is then detoxified to water (H_2O) by the enzymes catalase and glutathione peroxidase. Excessive generation of superoxide can also lead to interactions with nitric oxide ($NO^{\cdot}$) to form a powerful pro-oxidant peroxynitrite ($ONOO^-$, adapted from [47]).

4. Natural products as male fertility guardians: propolis

a) Composition and biological properties

Propolis, or bee glue is a natural substance synthetised by honeybees from numerous plant resinous secretions, such as blossoms, gums and plants, and also from leaf buds of different plant species like pines, palms, alder, poplar, conifer, and then mixed with salivary and enzymatic secretions [63-68]. Each colony is estimated to produce 150 to 200 g of propolis per year [65]. The area and the plant source can vary the colour of propolis, as well as its composition [65]. Due to its waxy nature and mechanical properties, propolis is used by honeybees as cement to keep moisture and temperature stable in the hive all year around and to seal cracks or open spaces [63]. At elevated temperatures, propolis is soft, pliable, and very sticky, melting at 60 to 70 ºC [63, 65, 68]. However, when cooled it becomes hard and brittle [63].

Chemical propolis composition is not well-defined and varies considerably from region to region along with vegetation, from season to season, and from hive to hive, and as a result of its great variability, over than 300 different chemical compounds have been identified in propolis [64, 65, 68, 69]. Propolis is typically composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances [64, 65, 70]. Nevertheless, the main chemical groups present in propolis resin comprise phenolic acids or their esters, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β-steroids [64, 65, 70]. Flavonoids are the main compounds of propolis as they are responsible for its pharmacological activity [71]. Among them, it could be found flavones, and terpenoids, contributing not only to the odour, but also for the biological properties of this natural substance, amino acids, vitamins, and minerals [63, 64, 71].

The use of different solvents for molecule extraction, for example, ethanol, methanol, acetone, or chloroform, changes the activity of the main biologically active constituents in propolis [63, 65, 68, 72].

Aqueous extract of propolis is known to modulate macrophage antitumoral activity and increase the production of lymphocyte activation factors, and ethanolic extract of bee glue enhances natural killer cells' activity against lymphoma in mice [63, 73, 74]. The ethanolic extract also has shown significant effects against *S. aureus*, *E. coli,* and *Enterococcus sp* [63, 75, 76].

The use of propolis in traditional medicine has been refined over time [63, 65, 74]. It goes back to the Greeks and Romans where it was used as a skin and oral disinfectant [63, 65, 74]. In Ancient Egypt was commonly used in the mummification process and in Persia it was described in the treatment of eczema, myalgia, and rheumatism [63, 65, 74]. Later, during World War II propolis was used for healing wounds and tissue regeneration [63, 65, 74]. Nowadays, propolis is used as an antibacterial and antifungal, anti-inflammatory,

antiviral, antioxidant, anaesthetic, antitumoral, antiproliferative, antimutagenic, and antihepatotoxic in addition to being used for cytotoxic activity and in neurodegenerative and depressive diseases [63-65]. It is found commercially in the form of dentifrices, mouth rinses, antiseptic mixtures, cough syrups, soap, creams, gels, shampoos, lotions as well as candies, powder, cakes, wine, chewing gums, and chocolate bars, and is also used for the preservation of meat [65, 72, 77-79].

To generally healthy humans, a safe dose of propolis has been reported to be 70 mg/day [71, 80].

The antioxidant activity of propolis is due to its enrichment of polyphenols such as phenolic acids and flavonoids, with the vast majority of outcomes demonstrating a reduction in OS markers [63, 71, 81]. These components of propolis could enter the skin and protect the epidermis and dermis from free radicals, produced due to radiations or before the maturation of dermal cells aging [65]. The chemical structure of the constituent polyphenols allows propolis to remove free radicals, which are the primary cause of lipids, nucleic acids, and proteins oxidation, inhibit the formation of ROS, chelating metal ions and exerting synergistic roles with other antioxidants [64, 65, 71, 82]. The antioxidant activity of propolis has also been connected to its protective effect against heavy metal toxicity, cardiovascular anomalies, insufficient liver function and brain pathologies [65, 83]. Portuguese propolis is rich in phenols and has an important capacity for scavenging free radicals, as well as preventing lipid peroxidation [63, 65]. The strong antioxidant activity of propolis highlights this natural product as a potential protective factor against ROS impairment of male infertility.

b) Propolis action: a focus on male reproduction

Many genetic, environmental, and physiological factors have been implicated in defective sperm function, the most common cause of male infertility [57, 84]. Sperm damage by ROS has gained considerable attention for its role in inducing poor sperm function and infertility [57, 84]. Products that can offer spermatozoa protection are of great importance [84]. In this context, natural products, like garlic, ginger, grapes, and propolis, are being studied as protective agents and eventually as new supplementary therapeutic options for the treatment of male sexual disorders [84-87]. Specifically, propolis has the ability to scavenge free radicals, and as a result of that, has been studied as a protective factor against different OS inducers in the testis [63, 85].

Administration of propolis in rats and rabbits shows an improvement in body and reproductive organ weights, an increase in sperm production, motility, count, and quality, and a reduction in abnormal and dead sperm numbers [84, 86-89]. In co-administration with OS-inducers, like aluminium, cadmium, and copper, propolis

treatment decreases the number of apoptotic cells, increases CAT, GSH, SOD, testosterone, and GST levels, at the same time decreases malondialdehyde (MDA) levels [84, 85, 90]. In men, propolis administration demonstrates a reduction in the free radical-induced lipid peroxidation, as well as an increase in SOD activity [86]. Besides that, it also shows the capacity to protect the DNA in spermatozoa and the sperm membrane from oxidative damage [84, 86]. Moreover, like in rats, in men, the reproductive organs' weight is also restored and there is an increase in sperm production, motility, and concentration, as well as a reduction in abnormal and dead sperm [91].

Despite all the evidence associating the protective potential of propolis for male infertility, little is known about the effect of this natural product, on specific testicular cell populations.

II – Aim of Dissertation

Male fertility is dependent upon successful spermatogenesis. In this context, sperm damage by ROS contributes to 30 to 80% of infertility cases. Low levels of ROS are necessary for sperm development, with high levels of ROS disrupting the development of germ cells and interfering with sperm function. Herein, several compounds with antioxidant potential have been studied to prevent or reduce this damage. Propolis, a natural substance synthetised by honeybees, has the ability to remove free radicals, being a good candidate to protect the male reproductive tract against OS. Moreover, despite all the evidence about the protective potential of propolis, there are no reports directly focused on the effect of this natural product on specific testicular cell populations.

Following this rationale, the present work aimed to analyse the protective potentialities of propolis toward spermatogonial oxidative damage. For this purpose, a mouse-derived spermatogonial cell line (GC-1spg) was treated with propolis, followed by exposure to a well-known OS inducer, the TBHP, in the presence/absence of propolis. The following parameters were evaluated:

- The phytochemical composition and the antioxidant activity of propolis;
- The cytotoxicity of both propolis and TBHP;
- GC-1spg cell viability, apoptosis, proliferation, ROS levels, and antioxidant defences to analyse the protective potential of propolis against the TBHP damaging actions.

III – Materials and Methods

1. Propolis characterization

Commercial Propolis (Biprol®, *Myroxylon peruiferum* and *Matriacaria chamomilla*) was purchased in the form of a propolis ethanolic extract in a local store of natural products.

a) Total phenolic compound determination

The total phenolic compounds were determined by Folin-Ciocalteu's colorimetric method (Figure 6) [92]. Propolis was first diluted in methanol while gallic acid was diluted in water, and both were mixed with 0.2 N Folin-Ciocalteu's reagent. The mixture was then incubated for 5 min, followed by the addition of aqueous Na_2CO_3 (75 g/L). After a 90-min incubation at 30 °C, the total phenols were determined spectrophotometrically at 765 nm (Helios–Omega, Thermo Scientific, USA). The standard curve was constructed using standard solutions of gallic acid in methanol and the total phenolic values were expressed as gallic acid equivalents (mg GAE/g of extract).

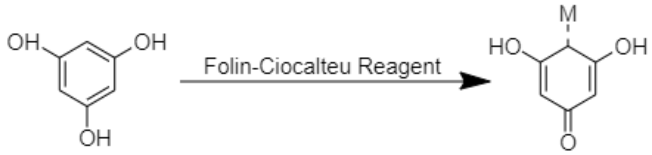

Figure 6: Schematic representation of the reduction of the Folin-Ciocalteu reagent. The reaction involves the oxidation of phenols in an alkaline solution by the yellow molybdotungstophosphoric acid heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue [92].

b) Flavonoid content determination

The flavonoid content was determined by the aluminium chloride colorimetric method (Figure 7) [92]. After dilution in methanol, propolis was mixed with 10% aluminium chloride, and 1 M potassium acetate. This solution was incubated at room temperature for 30 min and the absorbance of the reaction mixture was measured at 415 nm (Helios–Omega, Thermo Scientific). The calibration curve was constructed by preparing quercetin solutions in methanol. Total flavonoid values were expressed as quercetin equivalents (mg QE/g of extract).

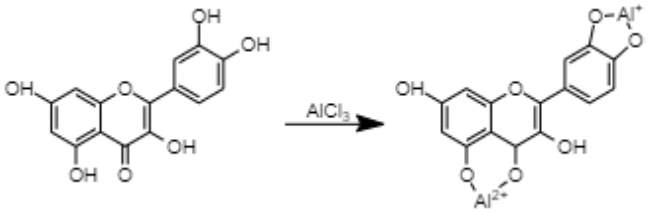

Figure 7: Schematic representation of the reaction of flavonoid-aluminium chloride complex formation. The reaction is based on the nitration of an aromatic ring bearing a catechol group with its positions substituted after the addition of Al(III), forming a yellow solution of complex [93].

c) Evaluation of antioxidant activity by DPPH scavenging assay

The antioxidant activity of propolis was determined by the radical scavenging activity method using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Figure 8). Aliquots of methanolic solutions of the propolis at different concentrations were added to a DPPH methanolic solution. After a 90 min incubation at room temperature in the dark, the absorbance was measured at 517 nm (Helios–Omega, Thermo Scientific). The radical scavenging activity was calculated by $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$. The half maximal inhibitory concentration (IC_{50}) was calculated using a calibration curve, and the Antioxidant Activity Index (AAI) was calculated by AAI = final concentration of DPPH in the control sample/IC_{50} [92].

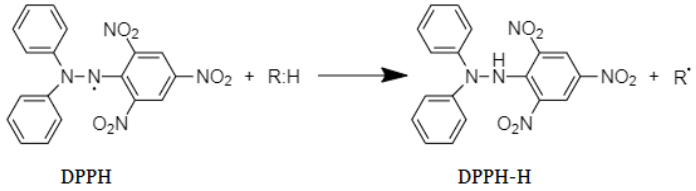

Figure 8: Schematic representation of the DPPH scavenging reaction mechanism. This reaction is based on an electron-transfer that produces a violet solution in methanol [94].

2. Cell culture

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

GC-1spg cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, D0822, Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS, P211107, PAN-Biotech, Germany) and 1% penicillin-streptomycin-amphotericin B solution (A5955, Sigma-Aldrich), at 37 °C in an atmosphere of 5% CO_2. Culture medium was replaced every 2-3 days and cells were sub-cultivated each time they reach a confluence of 80-90%. When performing assays, the culture medium was replaced by phenol red-free DMEM (D1145, Sigma-Aldrich) containing 10% FBS (P211107, PAN-Biotech) and adjusting the concentration of L-glutamine (3.97 mM, BP379, Thermo Fisher, USA), not present in this formulation.

3. Cell viability assays

For cell viability assays, approximately 2 500 cells/well were grown in a 96-well plate (734-0023, VWR International, USA) for 24 h. To assess the cytotoxicity of TBHP (458139, Sigma-Aldrich), GC-1spg cells were exposed for 12 h to different concentrations (0.005, 0.009, 0.018, 0.045, 0.09, 0.9, 1.8 and 3.6 µg/mL) of this OS inducer. In the case of propolis, cells were treated during 12, 24 and 48 h to a 0.1, 1, 10, 100, 250 or 500 µg/mL concentration. When simultaneously assaying propolis and TBHP, cells were first exposed to propolis (0.1 and 1 µg/mL) during 24 h, followed by a 12 h exposure to either TBHP only (0.9 and 1.8 µg/mL) or propolis and TBHP (0.1/1 and 1.8 µg/mL, respectively). Ethanol (0.61%) was used as vehicle, corresponding to the percent of ethanol in the most concentrated solution of propolis.

The viability of GC-1spg cells, was then 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 TBHP/propolis treatment, MTT (1 mg/mL, ab146345, Abcam, UK) was added and cells were incubated in the dark for 3 hours at 37 °C. After incubation, the medium and MTT solution were carefully removed, and dimethyl sulfoxide (DMSO, 276855, Sigma-Aldrich) was added to dissolve the formazan crystals. The formazan content was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, USA). The value of absorbance was directly proportional to the number of viable cells. All experiments were independently repeated at least two times.

4. Propolis and TBHP treatments

To perform subsequent analyses, approximately 200 000 GC-1spg cells were seeded in each 25 cm^3 tissue culture flask (70025, SPL Life Sciences, USA), except for the ROS levels determination assay, where approximately 2 500 cells were seeded per well in a 96-wellplate. After cell adhesion, 0.1 µg/mL propolis was added to GC-1spg cells during 24 h. Then, the culture supernatant was replaced by fresh medium containing 0.1 µg/mL propolis or 1.8 µg/mL TBHP only or 0.1 µg/mL propolis and 1.8 µg/mL TBHP, and cells were stimulated for additional 12 h. In the control group, culture media was replaced at the same time-points as the other study conditions. At the end of the experiment, culture media and GC-1spg cells were recovered and stored at -80 °C.

5. ROS level determination

ROS levels were measured using the probe dihydroethidium (DHE) as described by Georgiou *et al* [95]. Briefly, DHE uses fluorescence for detecting ROS generation and is specific for superoxide and hydrogen peroxide [96]. At the end of the exposure time (24 h + 12 h), cells were cultured with 0.5 µM DHE, for 20 min, at 37 °C. After washing cells twice with phosphate buffered saline, the emitted fluorescence was read in a spectrofluorometer (SpectraMax M5e; Molecular Devices, USA) at 510 nm (excitation)/580 nm (emission). Cell nuclei were stained with Hoechst 33342 (5 µg/mL, 1910197; Thermo Fisher) for cell number normalization, and fluorescence read at 352 nm (excitation)/454 nm (emission).

6. Total protein extraction and quantification

Total proteins were isolated from control, propolis, TBHP and propolis + TBHP-treated GC-1spg cells, using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA) supplemented with 1% protease-inhibitors cocktail (A7779, PanReac AppliChem, Darmstadt, Germany) and 10% phosphatase inhibitors cocktail (4906845001, Roche, Basel, Switzerland). After being kept on ice for 30 min and homogenized, cells were centrifuged at 14000 rpm, 20 min, 4 ºC, in a Hettich Mikro 200R centrifuge, and the supernatant containing proteins was collected. Total protein concentration was determined through the Bicinchoninic Acid Protein (BCA) method, using Bovine Serum Albumin as standard. The proteins were stored at -80 ºC.

7. Evaluation of antioxidant enzyme activity

To analyse the antioxidant status of GC-1spg cells, the activity of the antioxidant enzymes GPx and SOD was evaluated.

GPx activity was determined using a commercial kit (703102, Cayman Chemical, USA) according to the manufacturer's protocol. 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 nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm [97]. In a 96-well plate, 5 µL total protein extracts were incubated with NADPH, co-substrate and GPx assay buffer. In the blanks, sample was replaced by GPx assay buffer. The absorbance was measured at 340 nm every minute and 7-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. SOD activity 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 [98]. Briefly, 10 µg of total protein extracts were incubated with tetrazolium salt and xanthine oxidase (sample) or dilution buffer (sample blank). Two additional blanks were performed, the first with H_2O, tetrazolium salt and xanthine oxidase, and the second with dilution buffer instead of the latter. The assay was monitored by measuring the absorbance at 450 nm, after a reaction time of 60 min 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).

8. Caspase-3 like activity assay

Protein samples of each experimental condition were used to determine caspase-3 like activity. In a 96-well plate, 5 µL total protein extracts were incubated with 85 µL of assay buffer (20mM HEPES pH 7.4; 2 mM EDTA; 0.1% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate, CHAPS; 5 mM dithiothreitol DTT) and with 2 mM of caspase-3 substrate (AcDEVD-pNA; Sigma-Aldrich). Blanks were performed without protein. The incubation was undertaken overnight at 37 °C and the absorbance values were read at 405 nm using the xMark™ Microplate Spectrophotometer (Bio-Rad). Upon caspase cleavage, pNA is released producing a yellow colour, which is measured spectrophotometrically at 405 nm. The amount of generated pNA was obtained by extrapolation with a standard curve of free pNA at different concentrations. Results were expressed as caspase-3 activity/µg protein.

9. Ki-67 fluorescent immunohistochemistry

GC-1spg cells were fixed with 4% PFA for 10 min and permeabilized with 1% Triton X-100 for 5 min at room temperature. Unspecific staining was blocked by incubation with PBS containing 0.1% (w/v) Tween-20 (PBST) and 20% FBS for 1 h. Then, cells were incubated with rabbit anti-Ki-67 (1:50, ab16667; Abcam) primary antibody for 1 h at room temperature. Alexa Fluor 546 goat anti-rabbit IgG was used as secondary antibody (1:1000, 1813035; Invitrogen, USA). Specificity of the staining was assessed by the omission of primary antibody. Cell nuclei were stained with Hoechst 33342 (5 µg/mL, 1910197) for 10 min. Lamellae were washed with PBST and mounted with Dako

fluorescent mounting medium. Images were acquired using the Axio Imager A1 microscope (Carl Zeiss, Germany) and assessed with AxioVision 4.8 software (Carl Zeiss).

10. Statistical Analysis

Statistical analysis of all the obtained data was performed with the GraphPad Prism v6.01 software (GraphPad Software, San Diego, California, USA). Statistically significant differences between the tested groups were obtained by one-way analysis of variance (ANOVA), followed by Tukey's test. 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 ± standard error of the mean (S.E.M).

IV – Results

1. Propolis extract characterization

Phenolic and flavonoid compounds were reported to protect the reproductive system against toxic substances, improving normal spermatozoa morphology, sperm motility, and testosterone levels [85, 88]. Therefore, the content of phenolics and flavonoids from the propolis used in this research was analysed.

Total phenolics and flavonoids were in a proportion of 168.70 ± 0.08 mg GAE/g propolis and 36.90 ± 0.19 mg QE/g propolis, respectively, which is equivalent to 16.87% total phenolics and 3.69% flavonoids in the extract (Table 1).

Table 1: Phytochemical composition of propolis.

Total phenolics (mg GAE/g propolis)	Total phenolics (%)	Flavonoids (mg QE/g propolis)	Flavonoids (%)
168.70 ± 0.08*	16.87	36.90 ± 0.19*	3.69

*Data are presented as mean ± S.E.M.

Since oxidative damage adversely affects reproductive tissues, the antioxidant activity of propolis was assessed to evaluate its potential in preventing or reducing OS [85, 88]. This extract presented moderate antioxidant activity, as its antioxidant activity index (AAI) values were between 0.5 and 1.0, and the IC_{50} was 63.32 ± 13.04 µg/mL (Table 2) [92].

Table 2: Antioxidant properties of propolis measured by DPPH method.

IC_{50} (µg/mL)	Antioxidant activity index (AAI)	Antioxidant activity classification
63.32 ± 13.04*	0.77 ± 0.13*	Moderate

*Data are presented as mean ± S.E.M.

2. Cytotoxicity assessment of propolis in GC-1spg cells

The viability of GC-1spg cells was measured by the MTT assay after exposure to 0.1, 1, 10, 100, 250, or 500 µg/mL propolis during 12, 24, and 48 h. Ethanol (0.61%) was used as a vehicle, corresponding to the amount of solvent at the higher concentration of propolis. At this concentration, ethanol did not affect the viability of GC-1spg cells, as no differences were noticed between vehicle and control groups at any time (12 h: 100.00 ± 0.93% in control vs. 92.61 ± 6.21% in vehicle; 24 h: 100.00 ± 1.26% in control vs. 105.20

± 2.05% in vehicle; 48 h: 100.00 ± 0.73% in control vs. 103.80 ± 1.88% in vehicle; Figure 9).

The results from 12 h showed no significant differences in cell viability at lower concentrations of propolis (control: 100.00 ± 0.93%; 0.1 µg/mL: 101.40 ± 2.82%; 1 µg/mL: 97.28 ± 1.86%; 10 µg/mL: 100.00 ± 1.65%; Figure 9). At higher concentrations, propolis caused a substantial decrease in the viability of GC-1spg cells (control: 100.00 ± 0.93% vs. 100 µg/mL: 81.56 ± 2.54%, $p<0.0001$; 250 µg/mL: 25.69 ± 1.53%, $p<0.0001$; and 500 µg/mL: 24.53 ± 0.79%, $p<0.0001$; Figure 9). Between 10 and 100 µg/mL, and 100 and 250 µg/mL, the viability decreased significantly (18.46%, $p<0.0001$ and 55.87%, $p<0.0001$, respectively).

After 24 h the results were similar, with no impact in cell viability at lower concentrations (control: 100.00 ± 1.26%; 0.1 µg/mL: 96.15 ± 1.69%; 1 µg/mL: 101.50 ± 1.88%; 10 µg/mL: 104.10 ± 1.73%; Figure 9) and reduced GC-1spg viability when treated with 100, 250 and 500 µg/mL propolis (control: 100.00 ± 1.26% vs. 100 µg/mL: 49.06 ± 2.01%, $p<0.0001$; 250 µg/mL: 12.98 ± 0.32%, $p<0.0001$; and 500 µg/mL: 19.77 ± 0.62%, $p<0.0001$; Figure 9). Between 10 and 100 µg/mL, and 100 and 250 µg/mL, the viability also decreased significantly (55.02%, $p<0.0001$ and 36.09%, $p<0.0001$, respectively).

When exposed during 48 h to propolis, a significant increase in the viability of GC-1spg cells was noticed at 0.1 µg/mL concentration (control: 100.00 ± 0.73% vs. 0.1 µg/mL: 108.20 ± 0.70%, $p<0.01$; Figure 9). The 1 and 10 µg/mL concentrations showed no significant differences (control: 100.00 ± 0.73%; 1 µg/mL: 103.20 ± 1.22%; 10 µg/mL: 103.00 ± 1.15%; Figure 9). At 100, 250 and 500 µg/mL, propolis caused a substantial decrease in cell viability (control: 100.00 ± 0.73% vs. 100 µg/mL: 18.34 ± 0.47%, $p<0.0001$; 250 µg/mL: 7.84 ± 0.09%, $p<0.0001$; and 500 µg/mL: 12.25 ± 0.13%, $p<0.0001$; Figure 9). The viability of GC-1spg cells was significantly decreased between 0.1 and 10 µg/mL, 10 and 100 µg/mL, and 100 and 250 µg/mL (4.97%, $p<0.01$; 84.61%, $p<0.0001$, and 10.51%, $p<0.001$, decrease in cell viability, respectively; Figure 9).

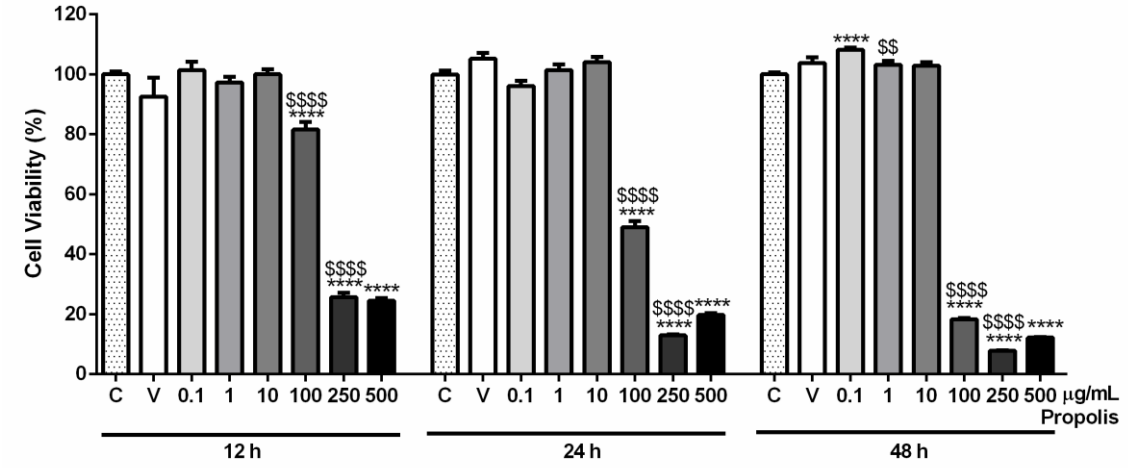

Figure 9: Cell Viability of GC-1spg cells in the presence of different concentrations of propolis extract (0.1, 1, 10, 100, 250, and 500 µg/mL) for 12 h, 24 h, and 48 h, evaluated by MTT assay. Results are expressed as % of the control group (C). Ethanol was used as a vehicle control in a concentration of 0.61% (V). Data are presented as mean ± S.E.M. (6 replicates/group, mean of two independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference between consecutive concentrations (0.1 vs. 1, 10 vs. 100, and 100 vs. 250 µg/mL propolis, ****p<0.0001; $^{\$\$}p$<0.01; $^{\$\$\$\$}p$<0.0001).

After analysis and integration of the obtained results, the 0.1 µg/mL and 1 µg/mL concentrations of propolis and the time-point of 24 h, were selected for the subsequent analyses, as the viability of GC-1spg cells was not affected.

3. Propolis attenuated the impact of the OS inducer tert-butyl hydroperoxide (TBHP) in GC-1spg viability

As the main aim of this study was to demonstrate the antioxidant potential of propolis, TBHP was used as an OS inducer, taking on additional importance considering the environmental perspective and their association with male reproduction damage [99, 100]. TBHP is widely used as a starting material or as a reactive ingredient product in the chemical industry and can be released into water, soil, and air during its production resulting in significant physical hazards [99, 100].

The viability of GC-1spg cells after 12 h of exposure to different concentrations of TBHP (0.005, 0.009, 0.018, 0.045, 0.09, 0.9, 1.8 and 3.6 µg/mL) were evaluated by MTT assay. Concentrations in the range of 0.005 to 0.09 µg/mL showed no significant differences when compared to control (control: 100.00 ± 1.43%; 0.005 µg/mL: 98.99 ± 4.79%; 0.009 µg/mL: 104.30 ± 3.32%; 0.018 µg/mL: 90.87 ± 2.83%; 0.045 µg/mL: 98.19 ± 3.47%; 0.09 µg/mL: 90.61 ± 2.70%; Figure 10). Concentrations above 0.09 µg/mL TBHP reduced the viability of these cells (control: 100.00 ± 1.43% vs. 0.9 µg/mL: 84.41 ± 2.64%, p<0.01; 1.8 µg/mL: 61.71 ± 2.22%, p<0.0001; and 3.6 µg/mL: 20.77 ± 3.88%, p<0.0001; Figure 10). The effect was dose-dependent, as significant differences were

observed between 0.9 and 1.8 µg/mL TBHP (22.71%, $p<0.0001$) and between 1.8 and 3.6 µg/mL TBHP (40.94%, $p<0.0001$).

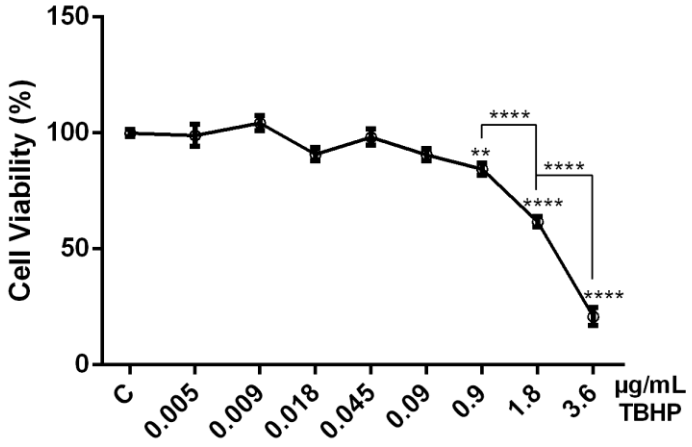

Figure 10: Cell Viability of GC-1spg cells in the presence of different concentrations of tert-butyl hydroperoxide (TBHP, 0.005, 0.009, 0.018, 0.045, 0.09, 0.9, 1.8 and 3.6 µg/mL) for 12 h, evaluated by MTT assay. Results are expressed as % of control group. Data are presented as mean ± S.E.M. (6 replicates/group, mean of two independent assays). (*) Statistically significant difference when compared between groups, comparisons relatively to control are above each error bar (without bounding-line, **$p<0.01$; ****$p<0.0001$).

After analysis and integration of the obtained results, the 0.9 µg/mL and 1.8 µg/mL concentrations of TBHP were selected for the subsequent analyses, as the accentuated decrease in cell viability after exposure to 3.6 µg/mL TBHP (only ≈ 21% viable cells) may difficult the study of the molecular mechanisms in the genesis of GC-1spg cell damage by TBHP.

To study the protective potential of propolis against TBHP cytotoxicity in GC-1spg cells, a 24h-pre-treatment with propolis was followed by exposure for 12 h to TBHP in the absence (Figure 11) or in the presence of propolis (Figure 12). The evaluation of TBHP impact with or without the simultaneous presence of propolis was essential to allow a more precise and phased choice of concentrations for both propolis and TBHP stimuli.

In the exposure in the absence of propolis after the 24h-pre-treatment and as previously seen, both TBHP selected concentrations significantly decreased cell viability (control: 100.00 ± 2.02 vs. 0.9 µg/mL TBHP 84.41 ± 2.64%, $p<0.01$; and 1.8 µg/mL TBHP: 61.71 ± 2.22%, $p<0.0001$; Figure 11). Propolis pre-treatment was not able to modulate the effect of 0.9 µg/mL TBHP in GC-1spg cells viability (without propolis: 84.41 ± 2.64%, $p<0.01$; 0.1 µg/mL propolis: 81.74 ± 2.51%, $p<0.0001$; 1 µg/mL propolis: 90.16 ± 2.63%; Figure 11). Curiously, when the cells were exposed to 1.8 µg/mL TBHP, propolis attenuated TBHP impact (without propolis: 61.71 ± 2.22%, p<0.0001 vs. 0.1 µg/mL propolis: 77.96 ± 3.48%, $p<0.01$; and 1 µg/mL propolis: 73.53 ± 4.45%, $p<0.05$). However, it was not sufficient to equalize control levels (control: 100.00 ± 2.02% vs. 0.1

μg/mL propolis: 77.96 ± 3.48%, p<0.0001; and 1 μg/mL propolis: 73.53 ± 4.45%, p<0.0001; Figure 11).

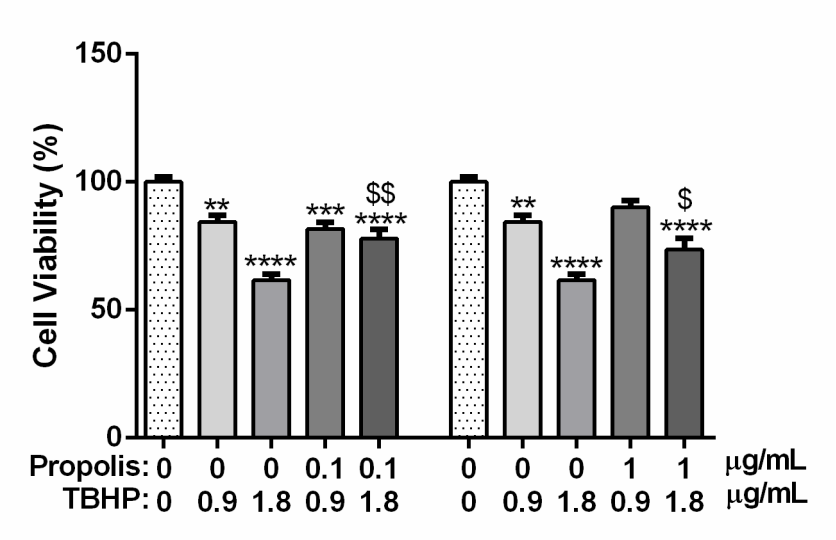

Figure 11: Cell Viability of GC-1spg cells in the presence of different concentrations of propolis extract (0.1 and 1 μg/mL) for 24 h, and tert-butyl hydroperoxide (TBHP, 0.9 and 1.8 μg/mL) for 12 h, evaluated by MTT assay. Results are expressed as % of control group. Data are presented as mean ± S.E.M. (6 replicates/group, mean of two independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference when compared to TBHP respective group. (**p<0.01; ***p<0.001; ****p<0.0001; $^{\$}p$<0.05; $^{\$\$}p$<0.01).

After analysis and integration of the obtained results, the 1.8 μg/mL concentration of TBHP was selected. Here, the viability of cells exposed to 0.1 and 1 μg/mL propolis during 36 h (summatory of 24 h pre-TBHP stimulus and 12 h TBHP stimulus) was evaluated. Despite 1 μg/mL propolis had no effect (control: 100.00 ± 2.06%; 1 μg/mL propolis: 101.70 ± 5.62%; Figure 12), the lowest concentration increased the viability of GC-1spg cells (control: 100.00 ± 2.06% vs. 0.1 μg/mL propolis: 125.70 ± 3.55%, p<0.0001; Figure 12). After exposure to 1.8 μg/mL TBHP, GC-1spg viability was significantly decreased (control: 100.00 ± 2.06% vs. 1.8 μg/mL TBHP: 79.04 ± 1.76%, p<0.01; Figure 12). This time, propolis attenuated TBHP effect at any of the selected concentrations (without propolis: 79.04 ± 1.76% vs. 0.1 μg/mL propolis: 104.20 ± 3.18%, p<0.001; and 1 μg/mL propolis: 102.50 ± 5.02%, p<0.01; Figure 12), and even equalized control levels (control: 100.00 ± 2.06%; 0.1 μg/mL propolis: 104.20 ± 3.18%; 1 μg/mL propolis: 102.50 ± 5.02%; Figure 12).

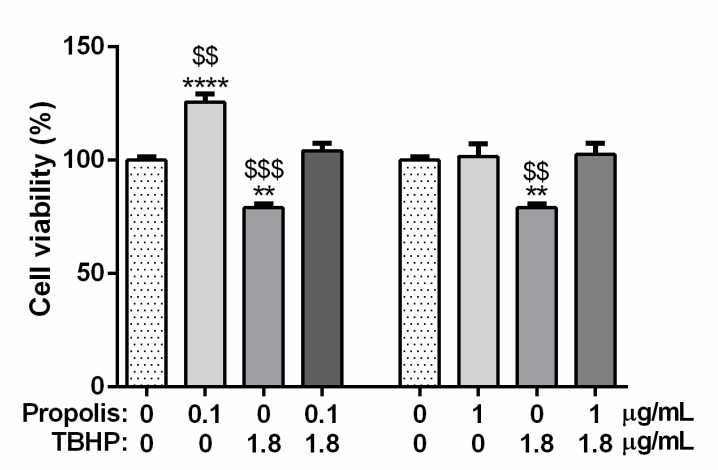

Figure 12: Cell Viability of GC-1spg cells in the presence of different concentrations of propolis extract (propolis, 0.1 and 1 µg/mL) alone for 36 h, and after a propolis pre-treatment for 24, followed by tert-butyl hydroperoxide (1.8 µg/mL TBHP, with or without propolis) for 12 h, evaluated by MTT assay. Results are expressed as % of control group. Data are presented as mean ± S.E.M. (6 replicates/group, mean of four independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference when compared to TBHP plus propolis respective group. (**p<0.01; ****p<0.0001; $^{\$\$}p$<0.01; $^{\$\$\$}p$<0.001).

Considering the obtained results, the 0.1 µg/mL concentration of propolis was selected for the subsequent analyses, as both propolis concentrations reversed TBHP effect and 0.1 µg/mL propolis was also able to increase cell viability in the absence of TBHP. Representative micrographs of GC-1spg cells from each study condition (control, 1.8 µg/mL TBHP, 0.1 µg/mL propolis, and both) are shown in Figure 13.

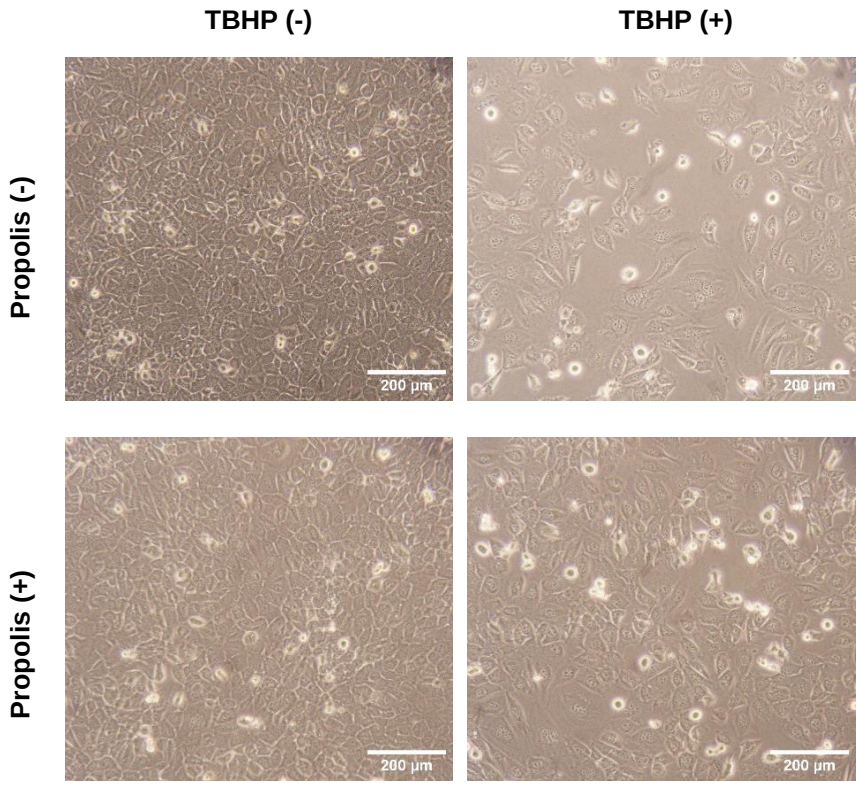

Figure 13: Representative images of GC-1spg cells from each study condition. Cells were treated with 0.1 µg/mL propolis for 24 h, and tert-butyl hydroperoxide (1.8 µg/mL TBHP, with or without 0.1 µg/mL propolis) for the subsequent 12 h. Micrographs were taken by Olympus CKX41, with an ampliation of 100x. The scale bar represents 200 µm.

4. Propolis decreased caspase-3 activity in TBHP-exposed cells

Caspase-3 has been recognized as an endpoint of apoptosis, activated in apoptotic cells by both extrinsic (death ligands) and intrinsic (mitochondrial) pathways [101]. Despite not presenting significant differences, propolis slightly diminished the activity of caspase-3, promoting a significant decrease when cells were also exposed to TBHP (TBHP: 0.93 ± 0.09–fold variation to control; propolis: 0.78 ± 0.05–fold variation to control; TBHP + propolis: 0.54 ± 0.03–fold variation to control, $p<0.05$; Figure 14).

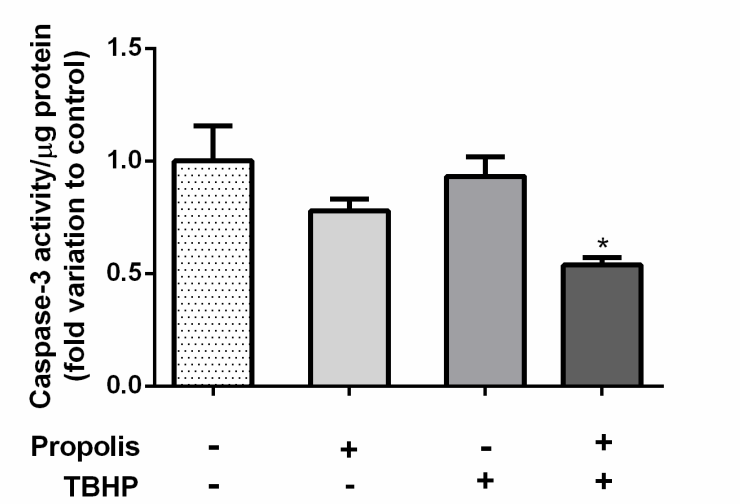

Figure 14: Caspase-3 activity in GC-1spg cells cultured for 24 h in the presence of propolis (0.1 μg/mL) and 12 h in the presence of tert-butyl hydroperoxide (TBHP, 1.8 μg/mL with or without 0.1 μg/mL propolis). Results are expressed as fold variation to the control group. Data are presented as mean ± S.E.M. (5 replicates/group, mean of two independent assays). (*) Statistically significant difference when compared to control (*p<0.05).

5. Propolis attenuated the decrease in the number of proliferative GC-1spg in response to the OS inducer TBHP

The cell proliferation index was determined by the number of Ki67-positive cells relative to the total cell number, as this protein is detected in the nucleus of proliferating cells in all active phases of the cell division cycle [102]. There were no significant differences in the number of ki67-positive cells in the presence of propolis, even upon TBHP exposure (propolis: 1.20 ± 0.11–fold variation to control; TBHP + propolis: 0.72 ± 0.24–fold variation to control; Figure 15) despite TBHP-treated cells showed a significantly decreased proliferation index when compared to control (TBHP: 0.11 ± 0.11–fold variation to control, p<0.001; Figure 15). Therefore, the proliferation index increased in 0.61-fold variation to control, p<0.05, when comparing TBHP exposure in the absence or in the presence of propolis.

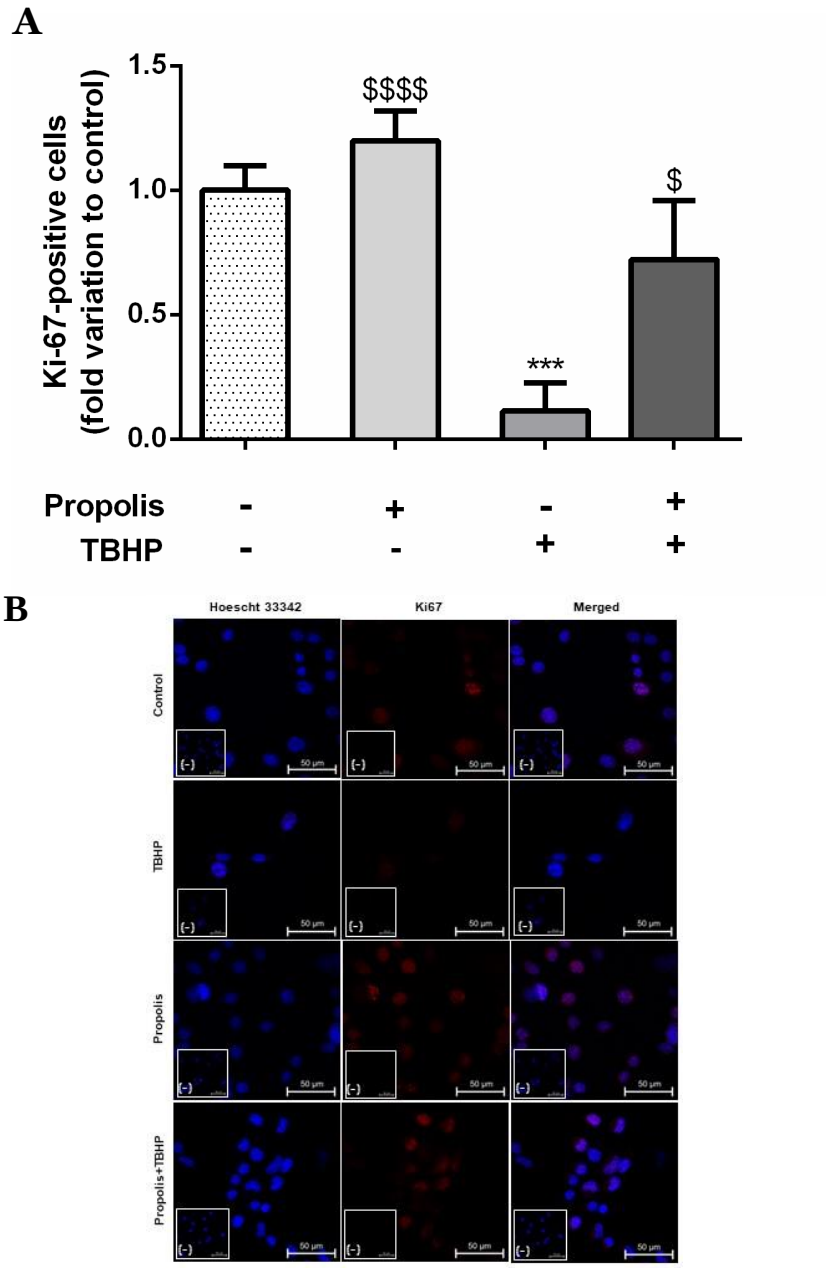

Figure 15: Proliferative activity of GC-1spg cells cultured for 24 h in presence of propolis (0.1 µg/mL) and 12 h in the presence of tert-butyl hydroperoxide (1.8 µg/mL TBHP, with or without 0.1 µg/mL propolis). Proliferative index determined by Ki-67 positive cells normalized with Hoechst-stained cells (A) Results are expressed as fold variation to control group. Data are presented as mean ± S.E.M. (6 replicates/ group, mean of two independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference when compared to TBHP group. (***$p<0.001$; $^{\$}p<0.05$; $^{\$\$\$\$}p<0.0001$). ***Representative confocal microscopy images of proliferative activity (B).*** Images were taken by Zeiss LMS 710 laser scanning confocal microscope, with an ampliation of 400x. The scale bars represent 50 µm. Ki67-positive cells are stained as red and the nuclei (Hoechst) as blue. Negative controls were assessed by the omission of primary antibody and provided as insert panels (-).

6. Propolis reduced reactive oxygen species in GC-1spg cells, even in the presence of the OS inducer TBHP

DHE acts as a fluorescent probe for the detection of ROS generation and is specific for superoxide and hydrogen peroxide [95]. Here, DHE results were normalized with Hoechst fluorescence and expressed as % relatively to the control group. TBHP-treated cells showed a significant increase in DHE fluorescence when compared to the control group (control: 100.00 ± 4.78% vs. TBHP: 128.30 ± 4.54%, $p<0.05$; Figure 16). Noteworthy, the intensity of fluorescence was significantly decreased in the propolis study conditions, regardless of TBHP exposure (control: 100.00 ± 4.78% vs. propolis: 70.85 ± 5.99%, $p<0.05$; and TBHP + propolis: 55.04 ± 8.97%, $p<0.0001$ Figure 16). This resulted in a decrease in DHE fluorescence of 73.24%, $p<0.0001$ when comparing TBHP exposure with or without propolis.

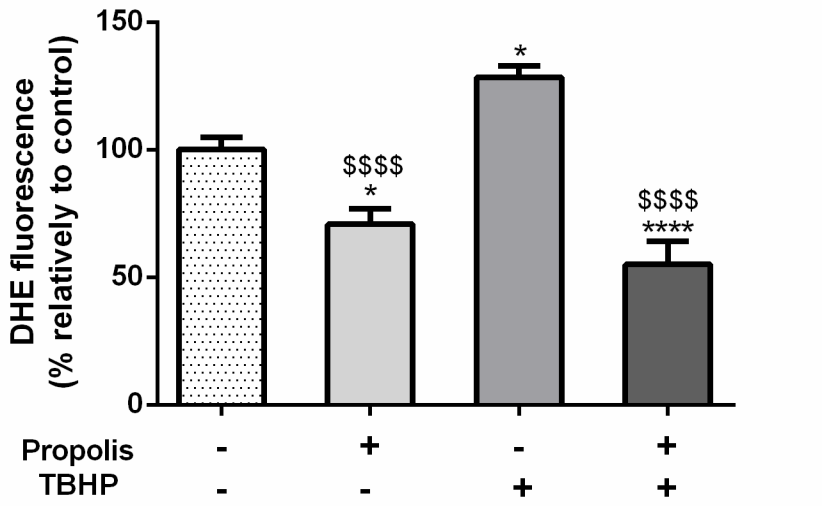

Figure 16: Dihydroethidium (DHE) fluorescence normalized with Hoechst in GC-1spg cells cultured for 24 h in the presence of propolis (0.1 µg/mL) and 12 h in the presence of tert-butyl hydroperoxide (1.8 µg/mL TBHP, with or without 0.1 µg/mL propolis). Results are expressed as % of control group. Data are presented as mean ± S.E.M. (6 replicates/ group, mean of two independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference when compared to TBHP group. (*$p<0.05$; ****$p<0.0001$; $$$$$p<0.0001$).

7. The antioxidant potential of propolis was evidenced by the accentuated rise of GPx activity in GC-1spg exposed to TBHP

GPx is an important intracellular enzyme that breaks down H_2O_2 to H_2O [103]. In the presence of TBHP, it was noticed a decrease in the activity of GPx, despite not being significantly different when compared to the control group (control: 0.05 ± 0.01 U/L/µg protein; TBHP: 0.04 ± 0.01 U/L/µg protein; Figure 17A). The antioxidant activity of GPx

was only significantly increased, when GC-1spg cells were treated with propolis and TBHP (control: 0.05 ± 0.01 U/L/µg protein vs. TBHP + propolis: 0.14 ± 0.03 U/L/µg protein, $p<0.05$; propolis: 0.06 ± 0.01 U/L/µg protein; Figure 17A), being significantly increased when compared to TBHP or propolis groups (increase of 0.10 U/L/µg protein, $p<0.01$ and 0.08 U/L/µg protein, $p<0.05$, respectively; Figure 17A).

SOD catalyses the dismutation of $O_2^{\cdot -}$ into molecular O_2 and H_2O_2, consequently rendering the potentially harmful superoxide anion less hazardous [103]. Here, no significant differences in any experimental group were observed concerning SOD activity (control: 25.28 ± 1.55%; TBHP: 25.85 ± 0.28%; propolis: 23.03 ± 0.54%; TBHP + propolis: 25.28 ± 1.25%; Figure 17B).

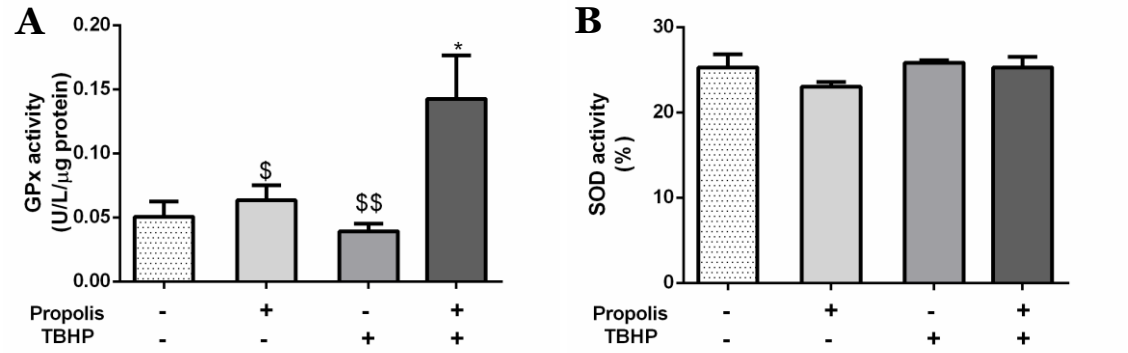

Figure 17: Glutathione peroxidase (GPx, A) and superoxide dismutase (SOD, B) activity in GC-1spg cells cultured for 24 h in the presence of propolis (0.1 µg/mL) and 12 h in the presence of tert-butyl hydroperoxide (1.8 µg/mL TBHP, with or without 0.1 µg/mL propolis). Data are presented as mean ± S.E.M. (5 replicates/ group, mean of two independent assays). (*) Statistically significant difference when compared to control; ($) Statistically significant difference when compared to TBHP plus propolis group. (*$p<0.05$; $^{\$}p<0.05$; $^{\$\$}p<0.01$).

V – Discussion

In the present dissertation, the ability of propolis extract to protect against oxidative damage induced by TBHP in spermatogonial cells was studied.

Firstly, the phytochemical composition and antioxidant properties of the commercial propolis extract used in this dissertation were characterized. The propolis extract was constituted by 16.87% total phenolic compounds, from which 3.69% were flavonoids. The IC_{50} value, obtained through the DPPH assay, was 63.32 ± 13.04 μg/mL and, as the value is inversely proportional to the antioxidant activity, the results demonstrated a moderate antioxidant activity with an AAI of 0.77 ± 0.13. The chemical composition of propolis is strongly dependent on the plant sources available around the hive, the geographical and climatic conditions, as well as the surrounding flora [104-106]. Propolis from Europe and North America show similar phenolic composition, with the main compounds being flavonoids, phenolic acids, and their esters [104]. Moreover, terpenes were the major components in propolis from Mediterranean Sea areas, and tropical areas, particularly in Brazil, propolis presented a composition rich in prenylated phenylpropanoids and caffeoylquinic acids [104]. Flavonoids, as one of the most diverse and widespread groups of natural compounds, are probably the most important phenolic compounds [107]. These compounds possess a broad spectrum of biological and chemical activities, including radical scavenging properties [107]. Many studies have determined the total phenolic and flavonoid content in propolis. In areas rich in these compounds, like Europe, the total phenolics range from 200–340 mg GAE/g in Spain to 115–210 mg GAE/g in Turkey. In the south hemisphere, the values decrease to 127-142 mg GAE/g in Brazil [108]. The flavonoid content follows the same tendency with values from 72–161 mg QE/g in Spain to 13–379 mg QE/g in Turkey, while in Brazil the flavonoid value decreased to 33-53 mg QE/g [108]. The results obtained in this dissertation presented a total phenolic of 168.7 ± 0.08 mg GAE/g and a flavonoid content of 36.9 ± 0.08 mg QE/g being in the same range as in areas considered rich in these compounds. The antioxidant activity is basically due to the presence of phenolic compounds and flavonoids, although the exact mechanism of action is unknown [107, 109]. Among the mechanisms proposed are free radical sequestration, hydrogen donation, metallic ion chelation, or the capability to act as a substrate for radicals such as superoxide and hydroxyl [109]. As mentioned, propolis is known to contain large amounts of polyphenols and flavonoids, which are strongly related to its ROS-scavenging properties [110]. This free radical scavenging capacity, as described for the chemical composition, also change with the geographic location and with the season of the year [111-113]. For example, a study showed that propolis collected in autumn, winter, and spring presented similar antioxidant potentials (IC_{50}: 58.8 ± 6.7;

65.7 ± 12.2 and 67.0 ± 7.5 μg/mL, respectively), while propolis collected in summer showed a less antioxidant capacity among the seasonal samples (IC_{50}: 98.7 ± 2.5 μg/mL). In general, DPPH free radical-scavenging activity ranged from about 20 to 190 μg/mL [111, 114]. The free radical scavenging activity analysed in this dissertation is within this range, once the results showed an IC_{50} of 63.32 ± 13.04 μg/mL. In sum, despite the exact geographical and climatic conditions, as well as the surrounding flora of the commercial propolis studied in this dissertation were not provided, the present results demonstrated that it's phytochemical properties and antioxidant potential are similar to the ones found in the literature.

To evaluate the potential protective role of propolis in spermatogonia cells, the viability of GC-1spg cells treated with propolis was analysed. In the 3 timepoints chosen (12, 24 and 48 h), at higher concentrations (100, 250 and 500 μg/mL), propolis caused a substantial decrease in cell viability. At low concentrations (0.1 and 1 μg/mL, 24 h), the viability of GC-1spg was not affected, being these concentrations and time-point selected for subsequent analyses. When exposed to the well-known OS inducer TBHP, the viability of GC-1spg cells decreased in a concentration-dependent manner. After analysis and integration of the obtained results, the concentrations of 0.9 and 1.8 μg/mL TBHP (equivalent to 10 and 20 μM, respectively) were selected. TBHP cytotoxic assays in murine hepatocytes demonstrated a 40%-cell viability reduction at a concentration of 250 μM and a total loss of viability with 500 μM TBHP [115, 116]. In the human normal liver cell line QZG, the viability decreased markedly in cells treated with 200 μM-8 mM TBHP, and concentrations higher than 64 μM TBHP reduced human prostate epithelial cells viability by 98% [117, 118]. In general, the concentration range of TBHP in the above-mentioned studies was higher than the one from the present work. Indeed, this observation enforces the sensitivity of GC-1spg to noxious stimuli and, in particular, to the disruptive effects of TBHP, prompting the emergence of protective approaches.

When treated for 24 h with propolis and 12 h with TBHP without/with propolis, propolis attenuated the TBHP effect. The 0.1 μg/mL concentration of propolis was selected for the subsequent analyses, as it could reverse the effect of TBHP and also increase the cell viability of GC-1spg in the absence of TBHP. Indeed, propolis has been reported to control cell growth and viability, presenting selectivity between normal and cancer cell lines, due to its different types of compounds [119]. On the one hand, propolis has an inhibitory effect against several cancer cells through an increase in apoptosis. On the other hand, the flavonoids present in this resin are powerful antioxidants and capable of scavenging free radicals and thereby protecting normal cells [119]. In fact, previous studies in Caco-2 cells (a cell line from colon carcinoma) showed that 1 mg/mL propolis decreased cell viability by around 25% [120, 121], while studies in normal cells, such as human

lymphocyte, rat kidney, liver and spleen, demonstrated that the same concentration of propolis (1 mg/mL) stimulated cell growth and increased final cell numbers (148% in spleen cells and 118% in liver cells) [119]. In the present work, a concentration as low as 0.1 µg/mL propolis preserved GC-1spg cell viability upon the noxious stimuli of TBHP. Corroborating, the protective potentialities of propolis have been studied against other damaging factors, such as anticancer drugs [91]. A study with male rats demonstrated that the sperm viability after a 4-week treatment with the anticancer drug paclitaxel decreased to 23% and when in the presence of propolis this viability was reestablished to 69%[91]. Similar results were observed in rats, where propolis minimized the reproductive toxicity of chlorpyrifos in sperm [122].

ROS are normally produced during metabolic events of living cells, particularly by mitochondrial electron-transport chain [88, 123]. However, high levels of ROS result from the dysregulated balance between their production and elimination, inducing OS, as well as many diseases such as cardiovascular diseases, diabetes mellitus, and cancer [123, 124]. As expected, after exposure to the well-known OS inducer TBHP, ROS were significantly increased in GC-1spg cells. This overproduction of ROS following TBHP exposure agrees with several studies on mice testis and sperm [99, 100, 124, 125]. Relatively to propolis, our results demonstrated a significant decrease in ROS once again corroborating its antioxidant potential. According to the literature, the main antioxidant mechanisms of propolis are associated with the capacity of polyphenols to scavenge ROS and decrease xanthine oxidase activity, thus interrupting the cascade of reactions leading to the peroxidation of lipids [126-128]. These findings were also demonstrated by studies made in diabetic rats, human keratinocyte cells, and sperm where they empathized that the antioxidant capacity of propolis was due to its phenolic compounds [129-131]. Phenolic acids are compounds built of a benzene ring, carboxyl and hydroxyl groups and their antioxidative activity depends on the number of hydroxyl groups in their molecules and on the steric effects [126]. Thereby, the position of hydroxyl groups, as well as the type of substitution on the aromatic ring, influence the antioxidative activity of these compounds [126].

To neutralize OS effects, testis exhibit antioxidant defence mechanisms that includes antioxidant enzymes such as SOD, CAT and GPx [58, 59]. SOD degrades superoxide into oxygen and hydrogen peroxide, with the last being further detoxified by GPx or CAT [60]. In this dissertation, propolis increased GPx activity in GC-1spg cells, regardless of TBHP exposure. However, GPx levels appeared to be unaffected by propolis or TBHP alone, and neither propolis nor TBHP altered SOD activity. The main biological role of the GPx enzyme is to protect the organism from oxidative damage, by catalysing the conversion of H_2O_2 to H_2O [124]. Despite of constant GPx activity, ROS levels were elevated by TBHP

treatment, raising the possibility that the detoxification of H_2O_2 was not being catalysed as demanded, resulting in increased ROS that leads to OS [124]. In literature, studies are controversial once on one hand TBHP induces a decrease in SOD and GPx activity in mice hepatocytes and on the other hand causes an increase in GPx level in mice testis [100, 124, 132]. Other previous studies demonstrated that TBHP-treated rats presented a significant decrease in SOD activity in testis while other studies in hepatocytes demonstrated an increase in its activity [99]. The discrepancies may be due to different exposure conditions, as well as, to tissue/cell-specific features, which may lead to different results [99]. Here, propolis only increased GPx activity in the presence of TBHP. In other studies performed in mice erythrocytes and rat testis, propolis increased GPx activity only at the highest dose, and the authors suggested that it was likely due to the pro-oxidant effect of that concentration of propolis [133,134]. These results led to the hypothesis that besides the dependence on the type of cell/tissue studied, the disparities in GPx activity can be concentration/dose-dependent. In other words, despite low concentrations of propolis alone did not affect GPx activity, when the concentration is higher it could by itself raise GPx activity, even in the absence of a damaging stimulus. Some studies evidence a significant increase in SOD activity after propolis treatment in erythrocytes, testis, or sperm. [80, 85, 90]. However, the results here obtained did not show any alterations in this detoxicant enzyme. According to a study in rats, an SOD defence system against OS is developed in late-stage spermatocytes at 4 weeks after birth [61], which may be the explanation for why SOD did not affect GC-1spg cells, once they correspond to a spermatogonia B stage.

It has been reported that ROS initiates a cascade of reactions that ultimately trigger apoptosis [135]. The proper regulation of the caspase cascade plays an important role on male fertility, sperm differentiation and testicular maturity [136, 137]. Hence, the effect of propolis on the apoptosis of GC-1spg cells was evaluated by analysing the activity of the endpoint regulator of the apoptotic pathway, caspase-3 [101]. Propolis alone did not affect the activity of caspase-3 on GC-1spg cells, but when in the presence of TBHP, the activity of this executor caspase was significantly decreased. Once again, propolis effect, anti-apoptotic in this case, was only observed in the presence of a noxious stimulus. TBHP itself did not show any effect in caspase-3 activity, demonstrating that the reduction in GC-1spg cell viability could not be attributed to programmed cell death [135]. In previous studies, propolis was able to attenuate the effect of damaging stimuli-induced apoptosis in germ cells, as well as in rat sperm [89, 138, 139]. When exposed to cadmium, the number of apoptotic cells in testicular tissue increased in rats, but when pretreated with propolis the number of apoptotic cells was similar to control group [89]. Similar results were obtained in rats exposed to doxorubicin and in mice exposed to mitomycin C [138, 139].

Khayyal *et al* attributed this anti-apoptotic effect of propolis to its content of aromatic acids, including phenolic and caffeic acids [140].

Studies have demonstrated that OS decreases the proliferative capacity of cells [141]. During the normal spermatogenic cycle, there is active proliferation of A_s, A_{pr} and A_{al} spermatogonia in mice, and little or no proliferation in the remaining stages [142]. However, when the numbers of A4, Intermediate, and B spermatogonia are low, the proliferation period is extended by a feedback mechanism. This way, any stimuli affecting spermatogonia proliferative capability could be detrimental to the spermatogenic process. In the present work, a significant decrease in the proliferation of GC-1spg cells when exposed to the OS inducer TBHP was observed. Again, propolis reestablished the proliferative capability of GC-1spg cells. This observation is in line with the study from Najafi *et al*, where, after being exposed to propolis, human lymphocytes, rat kidney, liver and rat spleen cells showed a faster rate of cell proliferation and increased cell number [119].

VI – Conclusions and Future Perspectives

The present dissertation highlighted the benefits of natural compounds to male fertility, more precisely the protective potential of propolis against TBHP-induced OS in spermatogonial cells (GC-1spg). Herein, the disruptive actions of TBHP were counteracted, or even neutralized in the presence of propolis. Concretely, propolis stimulated GC-1spg cell survival/proliferation, increasing antiapoptotic and antioxidant enzymatic activities when cells were exposed to TBHP while reducing ROS levels. Although preliminary, these findings highlight the propolis role as a protective agent against OS, which is crucial in the context of spermatogenesis and male fertility.

In the future, full comprehension of the molecular mechanisms responsible for propolis action on GC-1spg cells and other specific testicular cell populations will be of utmost importance. For this purpose, the expression and/or activity levels of the molecules involved in the studied pathways, specifically apoptosis, and proliferation, should be evaluated, allowing the identification of specific molecular targets for the development of directed preventive and/or therapeutic approaches. Complementing our results regarding ROS elevation, the lipid peroxidation and protein carbonyl levels would be interesting to investigate. Also, non-enzymatic antioxidants present in testis, like ascorbate, retinoids, and carotenoids, should be analysed in the future.

In this work, an aim-focused characterization of propolis was performed, as the extract is already commercialized. However, especially in the case of crude extracts, the panoply of propolis' potentialities (antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, anaesthetic, antimutagenic), as well as the influence of geographic and climatic conditions in its constituents, require a deeper analysis with a full characterization (e.g., liquid chromatography-mass spectrometry) in other to better explore the overall benefits of this resin.

Ultimately, translation to *in vivo* studies could also provide valuable insights on the effect of propolis against TBHP-induced OS in testis and, consequently, on the development of more efficient fertility-preserving approaches.

In sum, the results obtained in this dissertation brought new research venues to further explore the potentialities of natural compounds as guardians of male fertility.

VII – References

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