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The protective effect of regucalcin against radiation-induced testicular damage

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Resumo

O cancro testicular é a malignidade masculina mais frequente nos jovens, e a radioterapia é normalmente usada no seu tratamento. No entanto, a exposição à radiação tem vários efeitos secundários na fertilidade masculina, tornando-se necessária a identificação de estratégias efectivas para a proteger do dano testicular provocado pela radioterapia. A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que se encontra amplamente expressa no tracto reprodutor masculino. Vários estudos demonstraram a capacidade supressora da RGN na morte celular de diferentes tipos de células. Anteriormente, o nosso grupo de investigação mostrou que a sobre-expressão de RGN teve efeitos benéficos na espermatogénese por suprimir a apoptose induzida quimicamente. Para além disso, a RGN é regulada positivamente em linhas celulares radiorresistentes, sugerindo que esta pode proteger de danos causados pela radiação. O presente trabalho visa avaliar se a RGN desempenha um papel benéfico na recuperação da espermatogénese após radioterapia. Ratos, quer transgénicos que sobre-expressam a RGN (Tg-RGN) quer os seus homólogos selvagens (Wt), foram expostos a raios-X. Às dez semanas de recuperação após a radioterapia, o estado testicular e os parâmetros espermáticos foram avaliados. A expressão da RGN, bem como de vários reguladores do ciclo celular e da apoptose foi também avaliada. Para além disso, a actividade enzimática da caspase-3 foi determinada. Às dez semanas de recuperação após a radioterapia, tanto o estado testicular como os parâmetros espermáticos parecem ter sido menos afectados pelos raios-X nos Tg-RGN. Verificou-se ainda uma diminuição da expressão de p53 e de p21, o que pode indicar a reiniciação da espermatogénese. Para além disso, a reduzida actividade da caspase-3 detectada nos Tg-RGN está de acordo com os baixos níveis de caspase-8 e com o elevado rácio Bcl-2/Bax, sugerindo que os Tg-RGN são mais resistentes à apoptose testicular em resposta à radiação. A expressão de RGN aumentou significativamente nos ratos Wt, suportando o seu envolvimento na resposta anti-apoptótica. De forma geral, estes resultados indicam que a sobre-expressão da RGN desempenhou um papel protector relativamente ao dano testicular induzido pela radiação.

Palavras-chave

Testículo, Epidídimo, Espermatogénese, Espermatozóides, Radioterapia, Preservação da fertilidade, Regucalcina, Dano testicular, Apoptose, Oncofertilidade masculina.

Resumo Alargado

O cancro testicular é a malignidade masculina mais frequente nos jovens, apresentando mais de 10 novos casos por 100.000 homens por ano na Europa, e uma mortalidade de 0.3 casos por cada 100.000 homens por ano. A radioterapia é normalmente usada no tratamento do cancro testicular, mas a exposição à radiação tem vários efeitos secundários na fertilidade masculina porque os testículos são dos órgãos mais sensíveis à radiação devido à elevada taxa de divisão das células germinativas. Considerando a idade de incidência do cancro testicular e as suas elevadas taxas de sobrevivência, a maioria dos doentes ambiciona ainda ter filhos no futuro. Para além disso, os problemas reprodutivos são considerados pelos doentes uma das consequências mais frequentes e angustiantes do tratamento oncológico, tornando-se necessária a identificação de estratégias efectivas para os proteger do dano testicular provocado pela radioterapia. A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que se encontra amplamente expressa no tracto reprodutor masculino quer em rato quer em humano, incluindo nas vesículas seminais, epidídimos, e testículos. Vários estudos demonstraram a capacidade da RGN como supressora da morte celular em diferentes tipos de células. Anteriormente, o nosso grupo de investigação mostrou que a sobre-expressão de RGN teve efeitos benéficos na espermatogénese, nomeadamente, na supressão da apoptose induzida por fármacos como a tapsigargina ou actinomicina D. Para além disso, a RGN é regulada positivamente em linhas celulares radiorresistentes, sugerindo que esta proteína pode proteger de danos causados pela radiação. O presente trabalho visa avaliar se a RGN desempenha um papel benéfico na recuperação da espermatogénese após radioterapia.

Com esse intuito, ratos com três meses, quer transgénicos que sobre-expressam a RGN (Tg-RGN) quer os seus homólogos selvagens (Wt), foram submetidos a uma dose única de 6 Gray (Gy) de raios-X. Às dez semanas de recuperação da radioterapia, o estado testicular e os parâmetros espermáticos da cauda do epidídimo foram analisados. Os animais e os seus testículos foram pesados, permitindo o cálculo do índice gonado-somático (GI). Os testículos foram processados histologicamente de modo a determinar o índice de diferenciação tubular (TDI), ou usados para análise da expressão de genes. A expressão da RGN, bem como de vários reguladores do ciclo celular e da apoptose foi avaliada por Western Blot (WB). Além disso, também a medição colorimétrica da actividade enzimática da caspase-3 foi incluída como um ponto final da apoptose.

Às dez semanas de recuperação da radioterapia, o GI estava diminuído tanto nos animais Tg-RGN como nos Wt, mas esta diminuição parece ser menor no Tg-RGN. Em resposta aos raios-X, também foi observada uma diminuição no TDI. Também a contagem, a motilidade, e a viabilidade dos espermatozóides parecem ter sido menos lesadas nos Tg-RGN. Quanto à morfologia dos espermatozóides, a percentagem de espermatozóides normais nos Tg-RGN parece ter sido menos afectada pela radiação, incluindo uma reduzida variação na

percentagem de defeitos de pescoço e de cauda. Embora a diminuição da expressão de p53 e de p21 verificada às dez semanas de recuperação após a radioterapia seja provavelmente independente dos níveis de expressão de RGN, estes resultados sugerem a activação do ciclo celular e a reiniciação da espermatogénese. Considerando o reconhecido aumento da apoptose em resposta à radiação, a expressão do receptor de Fas (FasR) e do seu ligando (FasL) diminuiu inesperadamente. Contrariamente ao observado nos ratos Wt, a expressão de caspase-8 aumentou apenas moderadamente nos Tg-RGN, sugerindo uma menor susceptibilidade à apoptose. Em concordância, após a radioterapia, o rácio da expressão de proteína Bcl-2 (anti-apoptótica)/Bax (pró-apoptótica) foi significativamente mais alto nos ratos Tg-RGN, o que sugere também uma menor taxa de apoptose. Para além disso, ao contrário dos Wt, não foi observada diferença significativa na actividade da caspase-3 nos Tg-RGN, sugerindo menores níveis de apoptose testicular também nestes animais. Curiosamente, verificou-se ainda que a exposição à radiação aumentou significativamente a expressão de RGN nos ratos Wt, o que suporta o envolvimento desta proteína na resposta anti-apoptótica.

De forma geral, os resultados obtidos indicam que a sobre-expressão da RGN desempenhou um papel protector relativamente ao dano testicular induzido pela radiação, provavelmente por suprimir a apoptose das células testiculares. Apesar de serem necessários mais estudos, estes resultados despertam a curiosidade acerca da manipulação dos níveis de RGN nos testículos, no sentido de poder ajudar a preservar a fertilidade de doentes do sexo masculino submetidos a tratamentos oncológicos.

Abstract

Testicular cancer is the most common malignancy among young men, and radiation therapy is a generally used as treatment. However, the exposure to radiation has several adverse effects on male fertility. Considering the high survival rates and the age of incidence of this malignancy, it is mandatory to identify effective strategies protecting against radiation-induced testicular damage. Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that is broadly expressed in the male reproductive tract. Several studies have demonstrated RGN ability suppressing cell death in different cell types. Previously, our research group showed that RGN overexpression had beneficial effects on spermatogenesis by suppressing chemical-induced apoptosis. Moreover, RGN is upregulated in radioresistant cell lines, suggesting that it also can protect from radiation damage. The present work aimed to evaluate whether RGN may play a role in spermatogenesis recovery after radiation treatment. For this purpose, transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts were exposed to X-rays. At ten weeks of recovery after irradiation, the testicular status and the epididymal sperm parameters were evaluated. The expression of RGN and several cell cycle and apoptosis regulators was also evaluated. In addition, the enzymatic activity of caspase-3 was measured. Upon radiation treatment and ten weeks of recovery, both the testicular status and sperm parameters seem to have been less affected by X-rays in Tg-RGN. We also found a diminished expression of p53 and p21, which may indicate the reinitiating of spermatogenesis. Moreover, the reduced activity of caspase-3 detected in Tg-RGN is in accordance with low levels of caspase-8 and increased Bcl-2/Bax ratio, suggesting that these animals are more resistant to testicular apoptosis in response to radiation. RGN expression was significantly enhanced in Wt rats after irradiation, supporting its involvement in the anti-apoptotic response. Altogether, the present findings point out a protective role for RGN overexpression against radiation-induced testicular damage.

Keywords

Testis, Epididymis, Spermatogenesis, Sperm, Radiation therapy, Fertility preservation, Regucalcin, Testicular damage, Apoptosis, Male oncofertility.

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List of Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
Ac-DEVD-pNA	Acetyl-Asp-Glu-Val-Asp p-nitroanilide
ATP	Adenosine Triphosphate
ABP	Androgen-binding protein
ART	Assisted reproductive techniques
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
DTT	Dithiothreitol
FasL	Fas ligand
FasR	Fas receptor
FSH	Follicle-stimulating hormone
GI	Gonadosomatic index
GnRH	Gonadotropin releasing hormone
Gy	Gray
HBSSf	Filtered Hank´s buffered salt solution
H&E	Hematoxylin and eosin
LH	Luteinizing hormone
NIH	National Institutes of Health
NOS	Nitric oxide synthase
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
pNA	p-nitro-aniline
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RGN	Regucalcin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STP	Steroidogenesis stimulating protein
SOD	Superoxide dismutase
T	Testosterone
Tg-RGN	Transgenic rats overexpressing RGN
TDI	Tubular differentiation index
WB	Western Blot
Wt	Wild-type

I. Introduction

1. Brief overview of the testicular, epididymal and sperm structure

Testes, the key organs of the male reproductive tract, are whitish and ovoid paired structures suspended outside of the abdomen in the scrotum, which is internally distributed into two sacs, one for each testis (Setchell et al. 1994).

In mammals, the central functions of the testes are: 1) steroid synthesis and secretion; and 2) spermatozoa production (Cooke and Saunders 2002). The structural organization of testes determines the physical division of their dual function. Steroidogenesis takes place in the interstitium (vascularized region) whereas spermatogenesis occurs within the seminiferous tubules (avascular compartment), highly convoluted structures that are the functional units of the testis (Figure I-1). The testicular functions are coordinated by the communication between hormone and gamete-producing compartments (Schlatt et al. 1997). The interstitial area is coated by a tough fibrous membrane called tunica albuginea (Figure I-1) (Saladin 2003). There is also an outer tissue layer, named tunica vaginalis, a thin serous sac derived from the peritoneum during the descent of the testes, which covers both anterior and lateral surfaces of the testes but not their posterior surfaces (Setchell et al. 1994; Kent 2001). Fibrous internal septa, extending from the tunica albuginea, divide the testis in 250 to 300 wedge-shaped testicular lobules, each one enclosing 1 to 3 loop-shaped seminiferous tubules (Figure I-1) (Kent 2001; Rabbani et al. 2010).

The interior of each seminiferous tubule (Figure I-1) is limited by a basal membrane, composed by germ cells that form numerous concentric layers penetrated by a single type of somatic cell, the Sertoli cell (de Rooij and Mizrak 2008). Externally, the seminiferous tubules are surrounded by mesenchymal cells, including the peritubular myoid cells whose contractile elements produce peristaltic waves along the tubules (Gaytan et al. 1994a; Gaytan et al. 1994b). The seminiferous tubules are connected with the *rete testis* by means of the *tubulus rectus*, which in turn are linked to the efferent ductules (Figure I-1) (Rabbani et al. 2010). The seminiferous tubules, which contain germ cells in different stages of development and Sertoli cells, represent about 80% of the testicular mass (Sharpe 1984; Colborn et al. 1993; Sikka and Wang 2008).

The interstitium contains blood and lymphatic vessels and various cell types, including fibroblasts, leukocytes, macrophages and endocrine cells, the Leydig (interstitial) cells (Sharpe 1984; Colborn et al. 1993). The major source of the testosterone (T) are the Leydig cells (Haider 2004), which play an important role in downstream masculinization events, descent of the human testes into the scrotum before birth and initiation and maintenance of spermatogenesis (Akingbemi 2005; Sikka and Wang 2008).

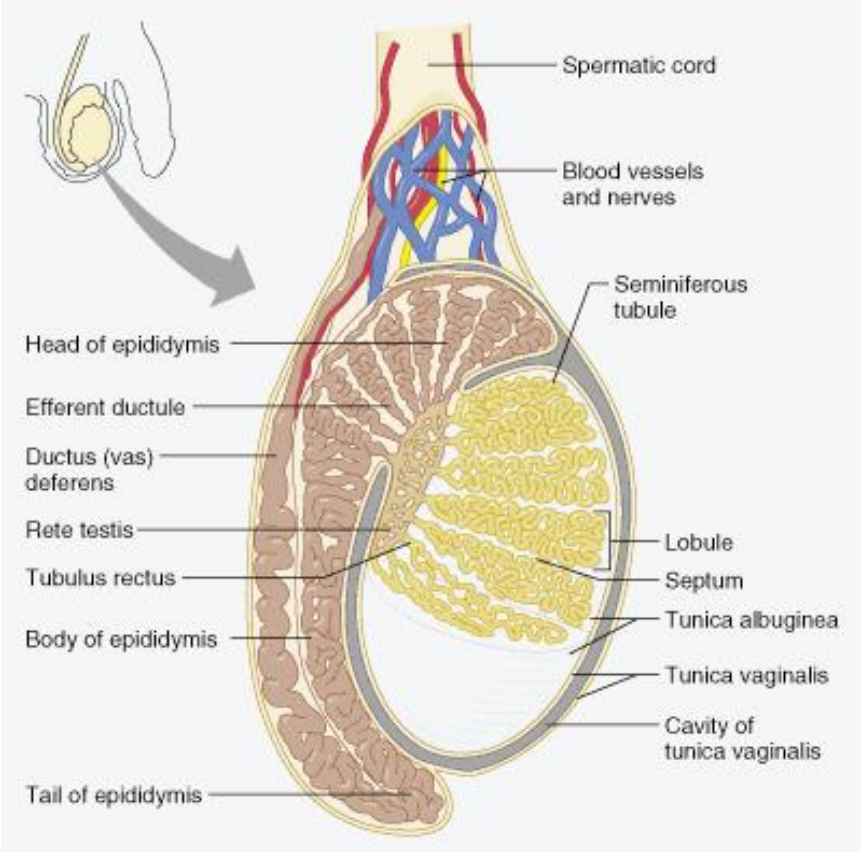

Figure I-1. Schematic representation of the mammalian testis and its relationship with the epididymis. The testis is encased by two tissue layers, from the inside to the outside, tunica albuginea and tunica vaginalis. Various septa extending from the tunica albuginea divide the testis in lobules, where the seminiferous tubules are located. The seminiferous tubules converge to the *rete testis* that is connected to the efferent ductules. The head of the epididymis receive testicular secretions by several efferent ductules (Marieb 2001).

The efferent ductules are mainly involved in fluid homeostasis and reabsorb more than 95% of the luminal fluid released from the seminiferous epithelium (Clulow et al. 1994). Moreover, these ductules carry spermatozoa from the *rete testis* and concentrate it before sperm maturation in the epididymis (Lee et al. 2009). The epididymis is a highly compartmentalized organ that is usually divided in three distinct regions (Figure I-1 and Figure I-2), the *caput* (head), *corpus* (body) and *cauda* (tail), which cooperate with different functions to reach the ultimate goal, sperm maturation and its fertilizing capacity (Robaire et al. 2006).

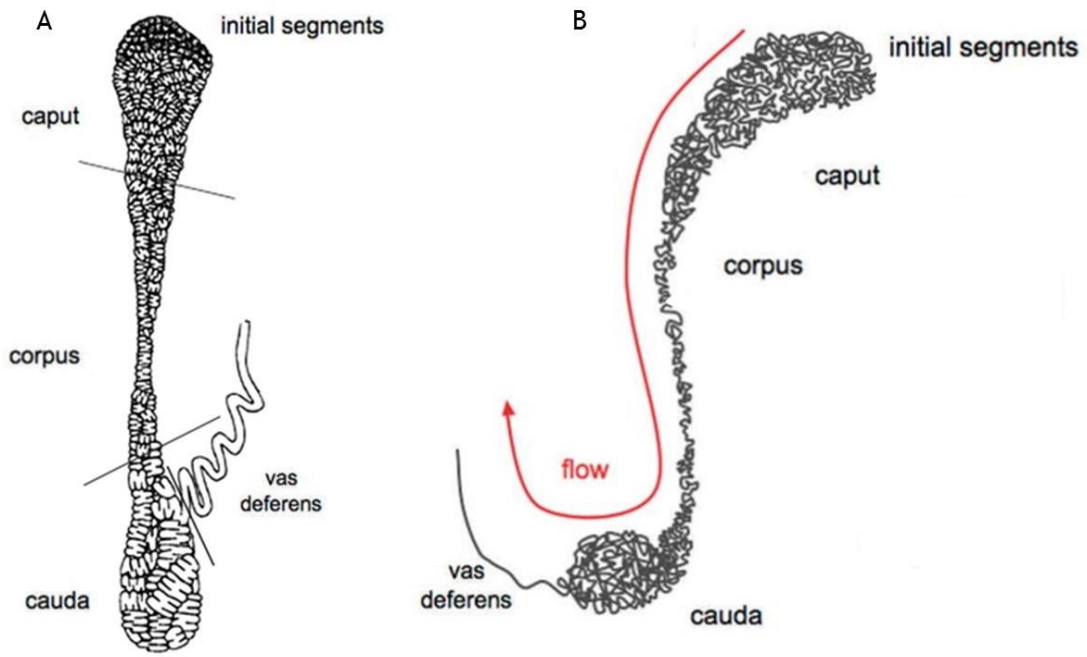

Figure I-2. Schematic organization of the rat epididymis. (A) Frontal view showing the three regions of the epididymis – *caput*, *corpus*, and *cauda* – as well as the initial segments. Oblique lines indicate sites where different regions were segmented. (B) Sagittal view evidencing the transit flow through the epididymis (adapted from (Guo et al. 2007) and (Shum et al. 2011)).

The sperm cell (Figure I-3) contains a haploid nucleus, with the acrosomal vesicle lying in front of it. The acrosomal vesicle, or acrosome, is derived from the Golgi apparatus and contains enzymes that digest proteins and complex sugars. These stored enzymes are used to lyse the outer coverings of the oocyte. Together, the acrosome and nucleus constitute the head of the sperm (Figure I-3) (Gilbert 2000). The flagellum of the mammalian spermatozoon consists of four distinct segments: the connecting piece (neck), the middle piece, the principal piece, and the end piece (Figure I-3) (Eddy 2006). The major motor portion of the flagellum is the axoneme, which is constituted by microtubules and responsible for sperm motility (Gilbert 2000; Rabbani et al. 2010). The flagellum is surrounded in turn by outer dense fibers extending from the neck into the principal piece of spermatozoa. The midpiece contains the mitochondrial sheath, a tightly wrapped helix of mitochondria surrounding the outer dense fibers and axoneme (Rabbani et al. 2010). The end piece is the thinnest portion of the sperm (Saladin 2003).

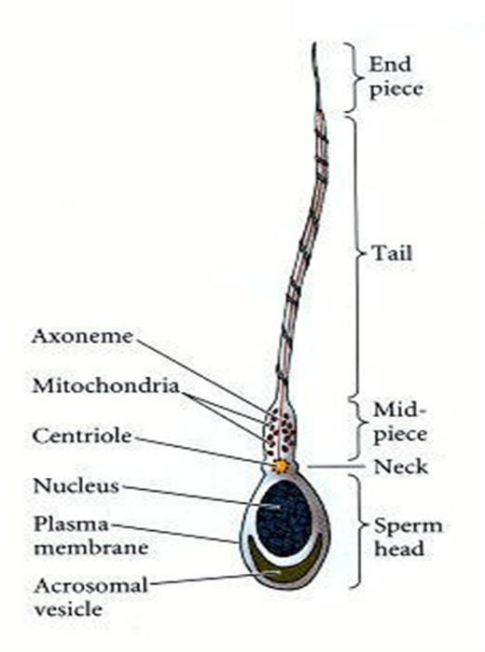

Figure I-3. Schematic representation of the structure of mammalian spermatozoa (adapted from (Gilbert 2000)).

There are some differences in the size and shape of the sperm head, in the length and relative amount of the different components of the flagellum among species (Rabbani et al. 2010). The head of human spermatozoa has a spatulate shaped whereas the rat sperm head is falciform-shaped (Figure I-4) (Eddy 2006).

Figure I-4. Schematic representation comparing the human (A) and rat (B) spermatozoa (adapted from (Frandson et al. 2009)).

2. Testicular and epididymal physiology

2.1 Spermatogenesis

Mammalian spermatogenesis is a complex biological process involving cell division and maturation of spermatogonial stem cells that culminates with the production of male gametes, the spermatozoa. It is a continuous and highly regulated process occurring in the seminiferous tubules within the testis (Hess and de Franca 2008). Sperm cells develop from the primordial germ cells and move towards the lumen of seminiferous tubule as they undergo a series of mitoses followed by the first and second meiotic division (Figure I-5) (Nussbaum et al. 2007; Sharma and Agarwal 2011).

Spermatogonial stem cells are localized at the basal membrane of the seminiferous tubules as single cells and upon division originate daughter cells, the spermatogonia (de Rooij and Mizrak 2008). The somatic Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact (Figure I-5) (Taylor et al. 2004). The cytoplasm of Sertoli cells extends as thin arms around the germ cells, spanning the thickness of the seminiferous epithelium. These cells supervise the several steps of spermatogenesis by providing structural and nutritional support to the germ cells (Griswold 1998; Taylor et al. 2004). The presence of tight junctions between neighbouring Sertoli cell forms the blood-testis barrier, which divides the seminiferous tubule in basal and adluminal compartments (Figure I-5) (Griswold 1998). The somatic Leydig cells are located in the interstitial space between seminiferous tubules and play a key role in the regulation of spermatogenic process (Haider 2004).

The expression of a large number of genes is developmentally regulated during spermatogenesis (Lambard et al. 2004), with both transcriptional and translational control mechanisms being responsible for temporal and stage-specific expression pattern (Angelopoulou et al. 2007). Each spermatogenic cycle in the seminiferous tubules comprises three main phases: mitosis, meiosis, and the final stage of cell differentiation, spermiogenesis (Hess and de Franca 2008). Spermatogenesis (Figure I-5) begins with the proliferation of spermatogonia and, after a species-specific fixed number of mitotic divisions, spermatogonia differentiate into primary spermatocytes (Clermont 1972). These proceed to the first division of meiosis resulting in secondary spermatocytes, which undergo the second meiotic division and become haploid spermatids. The cellular restructure in the spermiogenesis transforms round-spermatids in elongated-spermatids, and then, elongated-spermatids into spermatozoa, which are finally released into the lumen of the seminiferous tubule (Hess and de Franca 2008).

The spermatogenesis process in rat is similar to another mammalian species, including primates (Ross et al. 1995). The total duration of spermatogenesis is about 50 days in rat and 64 days in man. The additional required period for maturation in epididymis is about a week for rat and 8 to 17 days for man (Adler 1996).

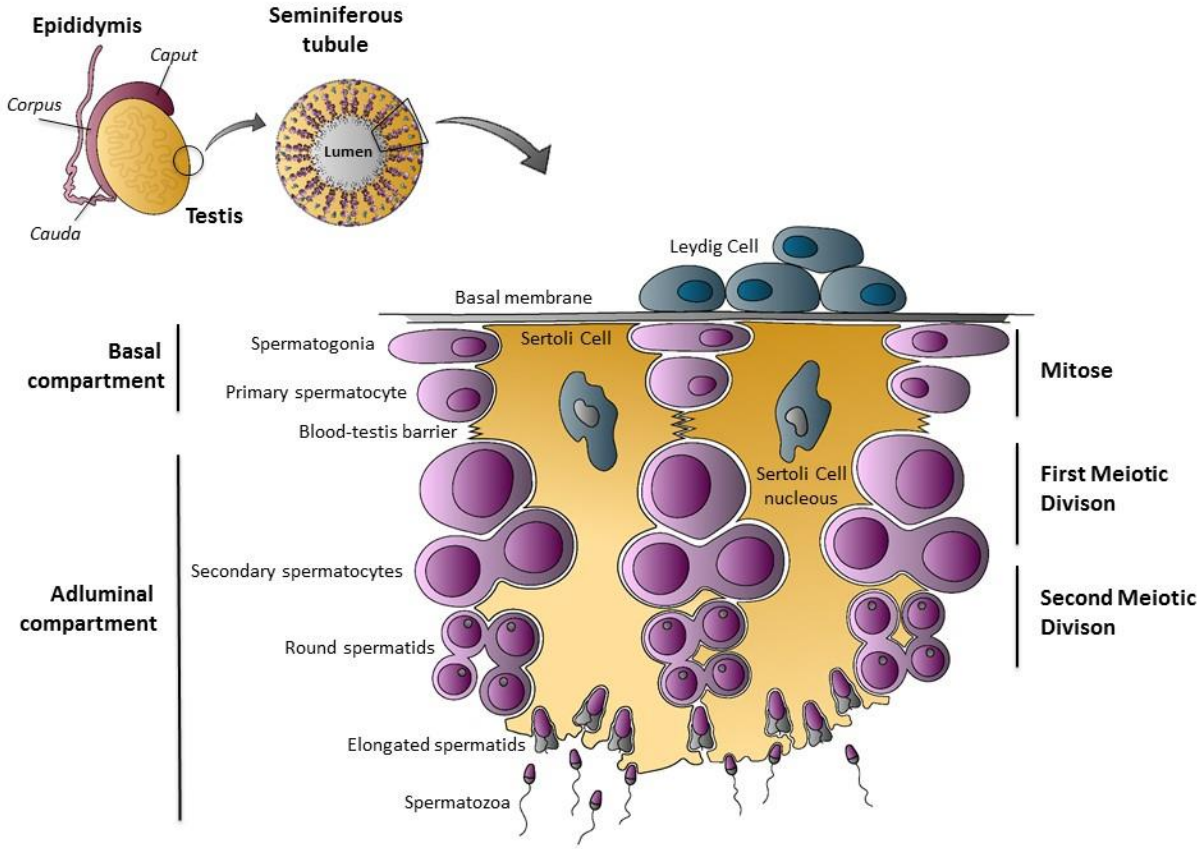

Figure I-5. Schematic representation of the testicular histology and mammalian spermatogenesis. The anatomic relationship between testis and epididymis, as well as the distinct functional regions of the epididymis are also shown (adapted from (Correia 2014)).

2.2 Control of spermatogenesis

The accomplishment of a successful spermatogenesis is dependent on various hormonal factors, which exert their actions via endocrine, paracrine, juxtacrine and autocrine signaling mechanisms. The central player in the hormonal control of spermatogenesis is the hypothalamic-pituitary-gonadal axis (Holdcraft and Braun 2004).

Generally, the hypothalamus releases gonadotropin releasing hormone (GnRH), which acts on the pituitary inducing the release of gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In the testis, LH acts on Leydig cells stimulating the synthesis of T, while FSH acts on Sertoli cells inducing the production of several growth factors and other stimulatory factors required for spermatogenesis (Walker and Cheng 2005; Walker 2009), which includes the androgen-binding protein (ABP) and the steroidogenesis stimulating protein (STP) (Jones and Lopez 2006) (Figure I-6).

Androgens and ABP, produced by Sertoli cells in the seminiferous tubules, are released along with sperm and transported from testis to epididymis (Norris and Carr 2013). ABP has a high

affinity for androgens and binds specifically 5α-dihydrotestosterone and T (Courot 1980). Through its binding activity, ABP may play a role in spermatogenesis and epididymal sperm maturation by enhancing the local concentration of androgens around the germinal cells and the male gametes (Courot 1980). STP enhances T secretion by Leydig cells (Figure I-6) (Jones and Lopez 2006).

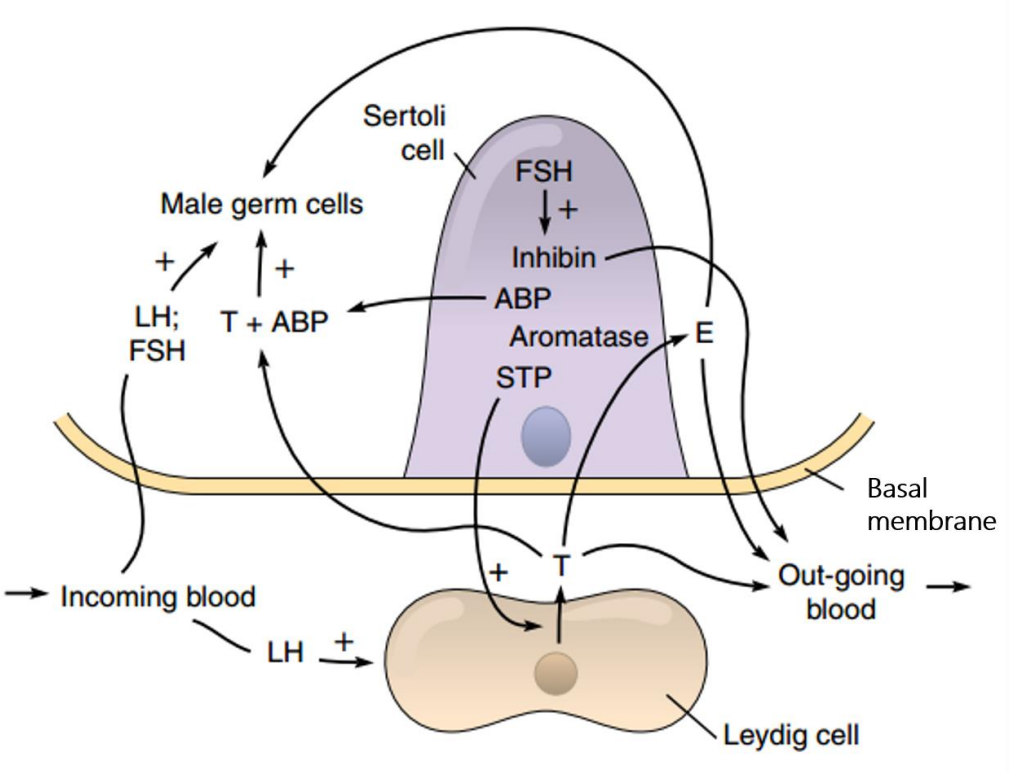

Figure I-6. The endocrinology of the testes. Leydig cells secrete testosterone (T) in response to the circulating luteinizing hormone (LH). Then, T diffuses into the seminiferous tubules and in Sertoli cells can be converted to estrogens (E) by the aromatase enzyme. T is also present in the germ cell region, where may be bound to the androgen-binding protein (ABP) or may remain in free-form to stimulate the spermatogenesis. Follicle stimulating hormone (FSH) also acts on Sertoli cells to induce the production of inhibin, ABP, aromatase, and steroidogenesis stimulating protein (STP). The STP leaves the tubule and helps the LH to increase the production of T. Finally, inhibin, E, and T enter the outgoing blood and exert a negative feedback on gonadotropin secretion. Note that E produced by Sertoli cells can stimulate male germ cells directly (adapted from (Jones and Lopez 2006)).

Moreover, T diffuses into the seminiferous tubule where together with FSH exerts stimulatory effects on the activity of Sertoli cells, which is determinant for germ cells survival, maturation and sperm production (Erkkila et al. 1997; Walker and Cheng 2005; Walker 2009). Besides, T regulates the spermatogenic process by a negative feedback mechanism on the hypothalamus and pituitary inhibiting, respectively, the release of GnRH and LH (Holdcraft and Braun 2004). Other negative feedback regulatory mechanism is driven by inhibin, which is a member of the transforming growth factor ß superfamily, produced by Sertoli cells in response to FSH (Figure I-6). Inhibin represses the production and release of FSH by the pituitary (Pierik et al. 2003; Bilezikjian et al. 2004) controlling the output of spermatogenesis. Even though androgens and FSH are considered the main regulators of spermatogenesis, in the last years, estrogens have also been recognized as important

modulators of spermatogenesis and male fertility (O'Donnell et al. 2001; Carreau and Hess 2010). The rodent and human testis express nuclear and membrane estrogen receptors, and also actively synthesize estrogens, including 17β-estradiol, which is the most potent of estrogens (Carreau and Hess 2010). The synthesis of estrogens occurs through the aromatization of androgenic precursors by the cytochrome-c P450 aromatase enzyme (Carreau et al. 2003; Carreau and Hess 2010). Estrogens can have a direct action on Leydig cells down-regulating the expression of steroidogenic enzymes involved in T biosynthesis (Sakaue et al. 2002). Since germ cells express estrogens receptors, estrogens produced by Sertoli cells are able to stimulate them directly (Correia et al. 2015). Estrogens also can act on the hypothalamus or pituitary exerting a negative feedback, suppressing the production of GnRH and LH, and consequently, decreasing the T levels (Hossaini et al. 2003; Chimento et al. 2014). Figure I-6 summarizes the hormonal modulation of spermatogenesis.

The output of spermatogenesis and the number of spermatozoa produced is regulated by an interaction between proliferation, differentiation and cell death (Pastor et al. 2011). Testicular germ cell apoptosis happens normally and continuously throughout the life (Wang et al. 2010). High rates of apoptosis, or programmed cell death, have been associated with the first waves of spermatogenesis (Aitken et al. 2011), and the germ cells that do not achieve the full maturity are more susceptible to die in response to numerous factors (Shaha et al. 2010). In fact, the quality control of spermatozoa is one of the most important aspects in spermatogenesis, and apoptosis is the best known quality control mechanism in testis (Shukla et al. 2012). Apoptosis occurs at the same time that spermatogonia undergo mitotic divisions and spermatocytes proceed through meiosis. Thus, apoptosis in the germ cell serves as a checkpoint to eliminate abnormal cells, as well as to provide an optimal germ/Sertoli cell ratio (Allan et al. 1992; Bartke 1995; Sinha Hikim et al. 2003). Spontaneous apoptosis provokes the loss of germ cells in the testis both in normal and pathological conditions. In the first case, it is estimated that up to 75% of potential spermatozoa degenerate in the testes of adult mammals (Huckins 1978). Regarding the pathological condition, the range of stimuli that trigger apoptosis is extraordinarily broad, including various forms of electromagnetic radiation, chemotherapeutic agents, environmental toxicants, heavy metals, heat exposure, growth factor depletion or hormonal alterations (Pastor et al. 2011; Aitken and Baker 2013). As for somatic cells, essentially two distinct pathways exist for the initiation of apoptosis of male germ cells: extrinsic or receptor-linked apoptosis and intrinsic or mitochondria-mediated apoptosis, which are summarized in Figure I-7 (Shukla et al. 2012).

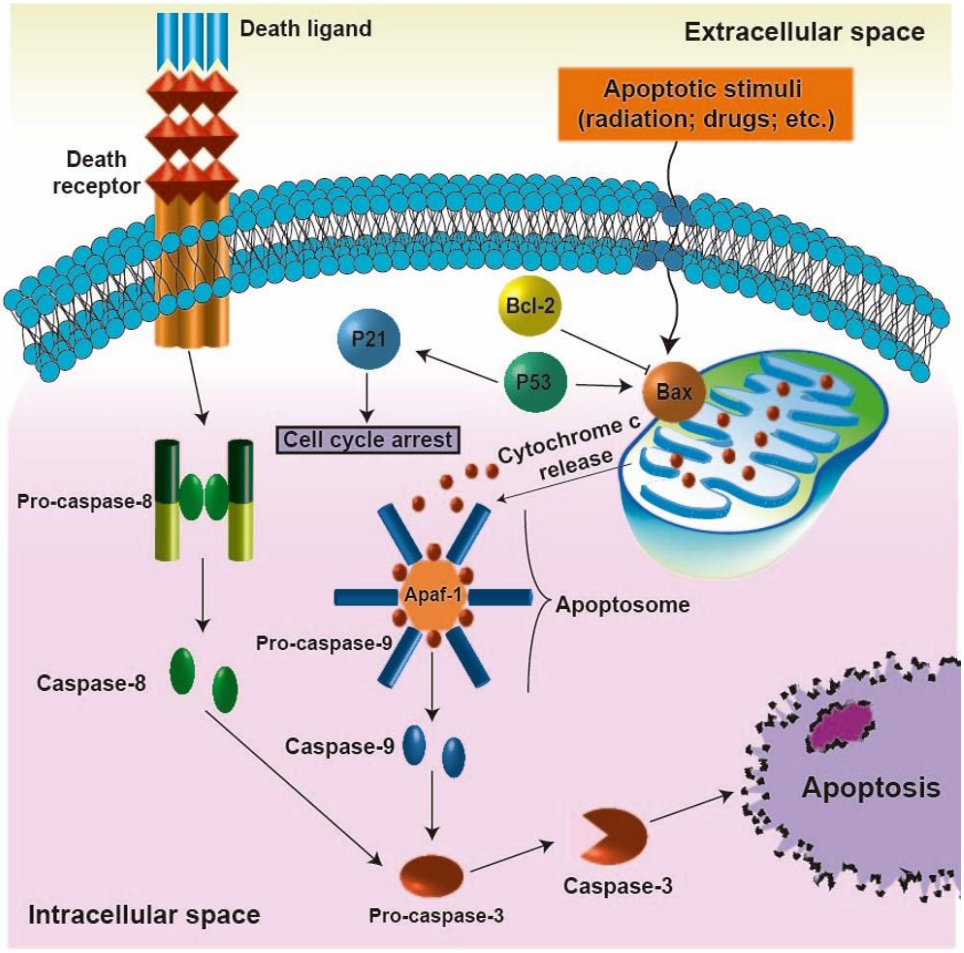

Figure I-7. Extrinsic and intrinsic pathways of apoptosis. Extracellular ligand binding (FasL or tumor necrosis factor, TNF) to death receptors (FasR and TNF receptor, TNFR) triggers the receptor-mediated (extrinsic) pathway resulting in the direct activation of initiator caspase-8. The mitochondrial (intrinsic) pathway is initiated in response to apoptotic stimuli (radiation, drugs, etc.) leading to the activation of proapoptotic members of the Bcl-2 protein family, such as the Bax. The Bax protein is translocated to the mitochondria allowing the permeabilization of mitochondrial membrane with consequent release of cytochrome c, which in turn, together with apoptotic protease activating factor 1 (Apaf-1), forms the apoptosome and activates caspase-9. Extrinsic and intrinsic pathways converge at the activation of the effector caspase-3. The transcription factor p53 is able to regulate downstream genes important in cell cycle arrest and apoptosis, including p21, Bax and Bcl-2. The cyclin-dependent kinase inhibitor p21 arrests the cell cycle at the G1 phase. Activation and inhibition are indicated by arrows and bar-headed arrows, respectively (adapted from (Correia et al. 2015).

2.3 Sperm maturation

Spermatozoa leaving the testis are non-functional gametes and it is only during the passage through the long convoluted tubule of the epididymis that they undergo a maturation process (Cornwall 2009). The four main functions of the epididymis comprise transport of spermatozoa, acquirement of the ability to move progressively and to capacitate, eventually gaining the ability to fertilize, and the creation of a specialized luminal microenvironment which allows the maturation process through the absorptive and secretory activities of the epididymis epithelial cells (Robaire et al. 2006; Guyonnet et al. 2011).

The maturation process is androgen-dependent and conducts several biochemical and functional changes in spermatozoa (Vreeburg et al. 1992). Estrogens are also involved in sperm maturation by the regulation of fluid absorption in the efferent ducts and *rete testis*, which is a fundamental event for maintenance of the adequate osmolality in the epididymis and sperm concentration (Correia et al. 2015). The *caput* and *corpus* regions perform early and late sperm maturation events, respectively, while the *cauda* stores the functionally mature spermatozoa (Robaire et al. 2006). This regional compartmentalization is characteristically evident both in the number and quantity of proteins secreted, with the *caput* as the most active, while the *corpus* and *cauda* possess a lower secretory activity (Dacheux et al. 2009).

The epididymal lumen is rich in inorganic ions and organic molecules that create the appropriate ionic, oxidative and pH environment for sperm maturation throughout epididymis transit (Cornwall 2009). The levels of calcium (Ca^{2+}) in the epididymal fluid are quite low in comparison with other ions, namely sodium, potassium, chloride, ammonium, and magnesium (Wales et al. 1966). Although the exact role of each component of the epididymal fluid needs to be deciphered, acidification has been shown to be essential for the alterations on sperm surface proteins required for sperm maturation and storage (Pholpramool et al. 2011). The acidification of epididymal fluid and water transport along epididymis are the critical events that ensure an appropriate environment. Acidification is implicated in sperm maturation and maintenance of its quiescent state during storage (Pholpramool et al. 2011) whereas water movement across the epididymis epithelium contributes to sperm concentration. This fact is crucial for proper sperm function, because sperm ability to reach maturation is enhanced by sperm concentration in the epididymal duct, achieved by water removal from the luminal fluid (Da Silva et al. 2006). In order to conserve energy and maintain structural integrity, sperm motility needs to be suppressed until it is required (Jones 1999). Some of the epididymal proteins contribute to the stabilization of the sperm plasma membrane preventing the occurrence of premature capacitation, whereas others proteins have been implicated in the acquisition of the sperm ability to bind and recognize the oocyte (Lefebvre et al. 2009; Cohen et al. 2011; Joseph et al. 2011). Physiological amounts of reactive oxygen species (ROS) are also involved in the regulation of some sperm functions, such as playing positive effects on maturation (Aitken and Baker 2004), capacitation (O'Flaherty et al. 2006), acrosome reaction (de Lamirande and O'Flaherty 2008), and sperm-oocyte fusion (Riffo and Parraga 1996), supporting the importance of preserving seminal ROS at low controlled levels through the delicate balance between ROS production and removal (Tremellen 2008).

In mammals, the normal duration of the transit through the epididymis *cauda* is in the range of 3 to 10 days, but spermatozoa can be stored in this segment for periods extending beyond 30 days (Rabbani et al. 2010).

3. Testicular damage and male (in)fertility

3.1 Testicular cancer

Testicular cancer is the most important malignancy in the young male, accouting >10 new cases per 100.000 males per year in Europe and a mortality of 0.3 cases per 100.000 males per year (McGlynn et al. 2003; Jemal et al. 2011). In fact, testicular germ cell tumors are the most common malignancy in males between 15 and 34 years old and also the most frequent cause of death from solid tumors (Chieffi and Chieffi 2013). Fortunately, the survival from cancer has been improved over the past few decades due to the advances in diagnostic tools, treatments and therapeutic modalities. As a result of early detection and successful adjuvant treatments, young cancer patients are living longer and subsequently the strategy of management has changed from cure with any cost to one in which quality of life has become increasingly important (Vassilakopoulou et al. 2015).

3.2 Cancer treatment

Depending on the type and stage of the testicular cancer and other factors, treatment options can include surgery, chemo- and/or radiotherapy (Albers et al. 2005; Brydoy et al. 2007).
The testes are very sensitive organs and, thus, highly affected by exogenous damaging factors, such as chemo- and radiotherapy treatments (Trost and Brannigan 2012b). For this reason, the treatment of oncological diseases usually results in temporary or permanent arrest of spermatogenesis (Stahl et al. 2006), as well as in disrupted sex hormone production (Brauner et al. 1983). Taking into account that the majority of young patients wish to be parents, the reproductive problems are considered one of the most common and distressing consequences of cancer treatment (Schover et al. 2014).
Focusing on radiotherapy, the extent of testicular injury is directly related with the dose of radiation delivered as well as the underlying cell type (Zhang et al. 2007). The germinal epithelium is more sensitive than the Leydig cells as a result of its high mitotic rate (Osterberg et al. 2014). Radiation therapy negatively affects spermatogenesis either transiently or permanently, by directly inducing DNA damage (Lushbaugh and Casarett 1976; Apperley and Reddy 1995). Besides the dose, several variables can affect the deleterious effect of radiation on gonadal function, such as source of radiation, gonadal shielding, scatter radiation, and individual susceptibility (Colpi et al. 2004; Trottmann et al. 2007). Seminiferous tubules are particularly sensitive to radiation because energies as low as 0.1 gray (Gy) results in temporary arrest of spermatogenesis. Increasing doses have been shown to cause azoospermia at 0.65 Gy, and doses >0.65 Gy but <1 Gy, 2–3 Gy, and 4–6 Gy, result in azoospermia lasting 9–18 months, 30 months, and 5 years to permanent, respectively (Trost and Brannigan 2012a). Leydig cells are only affected when doses reach >15 Gy (Colpi et al. 2004). Also, the radiation delivered directly to the testes for treatment of testicular

leukemia, or as part of the total body irradiation prior to bone marrow transplant, involves doses that result in permanent sterility in most men (Leonard et al. 2004).
Although with some controversy, it is accepted that malignancy itself is associated with male infertility since numerous biological processes are affected in parallel (Petersen et al. 1999). Indeed, azoospermia is present in ≈ 3% to 18% of men at the moment of cancer diagnosis (Tournaye et al. 2004). In addition, it was shown that spermatogenesis is affected in oncological patients with lymphoma and leukemia without testicular pathology, even before the onset of gonadotoxic therapies (Rueffer et al. 2001; Howell and Shalet 2005; Hotaling et al. 2013; Katz et al. 2013; Bujan et al. 2014). Also, altered sperm production in patients with testicular cancer before orchiectomy has been detected (Rives et al. 2012).

4. Preservation of male fertility and spermatogenesis recovery after testicular damage

4.1 Cryopreservation

Sperm cryopreservation preceding cancer treatments remains the only established method for fertility preservation in adult males (Hotaling et al. 2013), whereas in the pre-pubertal male the cryopreservation of testicular tissue is the adopted option for preserving fertility (Pennings and Mertes 2012). Other procedures are currently at experimental phase but are not devoid of ethical concerns (Pennings and Mertes 2012). Besides that, one of the major problems is related to the ability to avoid the oxidative damage that sperm cells and seminal plasma normally retain (Tremellen 2008). During the freeze–thawing practice, the antioxidant defenses could be insufficient (Bucak et al. 2010) to counteract the damage of that cryopreservation induces to the spermatozoa, which includes significantly decreased motility, viability, morphology, chromatin integrity, mitochondrial potential, *in vivo* fertilizing capacity, deterioration of acrosomal and plasma membrane integrity, and DNA damage (Bucak et al. 2010; Degl'Innocenti et al. 2013; Sharma et al. 2015). Furthermore, the use of cryopreserved sperm or tissue in assisted reproductive techniques (ART) has an economic obstacle due to the high costs associated (Linkeviciute et al. 2014).
The present challenge is to improve ART with cheaper and simpler alternatives with good acceptance by patients, and to develop effective strategies that would restrain the undesirable secondary effects of oncological treatments (Ko and Sabanegh 2014).

4.2 Hormonal and non-hormonal factors

There are several endogenous agents, hormonal and non-hormonal, which have been described to have protective or advantageous properties on the recovery of male reproductive function after testicular injury and cancer, as summarized in Tables I-1 and I-2. The knowledge reviewed in Tables I-1 and I-2 identify promising factors, which might be able

to mitigate the male fertility problems arising either from oncological treatments or other gonadal damage, opening new possibilities to ameliorate the recovery of spermatogenesis or to preserve fertility. Furthermore, the perspective of the endogenous molecules that could act as cryoprotectants in order to improve the quality of cryopreserved semen samples was also included.

Table I-1. Hormonal protective endogenous factors in male reproductive function.

Issue	Factor(s)	References
Antioxidant properties	IGF-1	(Selvaraju et al. 2010)
Chemical and/or radiation induced damage	↓↓ by GnRH agonists/analogues ↓↓ by T ↓↓ by E_2 ↓↓ by GH	(Glode et al. 1981; Meistrich and Kangasniemi 1997; Meistrich et al. 2001; Udagawa et al. 2001) (Masala et al. 1997; Aminsharifi et al. 2010) (Shetty et al. 2004; Porter et al. 2009) (Satoh et al. 2002; Nouri et al. 2009)
Enzymes involved in T biosynthesis	↓ by Estrogens	(Sakaue et al. 2002)
FSH	↓↓↓ by T ↓↓ by Estrogens	(Moss et al. 2013) (Hossaini et al. 2003)
HPG axis	↓↓↓ by T	(Moss et al. 2013)
LH	↓↓↓ by T ↓↓ by Estrogens	(Moss et al. 2013) (Hossaini et al. 2003)
Lipid peroxidation	↓↓ by IGF-1	(Selvaraju et al. 2009)
Modulation of SCF gene expression	E_2	(Correia et al. 2014b)
Seminiferous epithelial depth & tubular diameter	↑↑ by GH	(Nouri et al. 2009)
Spermatogenesis	↑↑↑ by GnRH agonists or antagonists ↓↓↓ by T ↑↑↑ by T ↑↑↑ by E_2 ↑↑ by IGF-1	(Meistrich and Shetty 2003) (Moss et al. 2013) (Masala et al. 1997; Aminsharifi et al. 2010) (Ebling et al. 2000; Bilinska et al. 2003; Shetty et al. 2004; Porter et al. 2009) (Glander et al. 1996; Selvaraju et al. 2010)
Sperm cryopreservation induced damage	↓↓ by IGF-1	(Selvaraju et al. 2010)
Sperm maturation	↑↑↑ by GH ↑↑↑ by IGF-1	(Moss et al. 2013) (Glander et al. 1996; Selvaraju et al. 2010)
Sperm membrane integrity	↑↑ by IGF-1	(Selvaraju et al. 2009)
Sperm motility	↑↑ by GH ↑↑ by IGF-1	(Radicioni et al. 1994; Ovesen et al. 1996; Nouri et al. 2009; Moss et al. 2013) (Selvaraju et al. 2009; Selvaraju et al. 2010)
Sperm production	↓↓ by T ↑↑ by GH ↑↑ by IGF-1	(Moss et al. 2013) (Radicioni et al. 1994; Moss et al. 2013) (Glander et al. 1996)
Sperm viability	↑↑ by GH	(Nouri et al. 2009)
T	↓↓↓ by GnRH agonists or antagonists ↓↓ by T ↓↓ by Estrogens ↑↑ by GH	(Meistrich and Shetty 2003) (Moss et al. 2013) (Hossaini et al. 2003) (Nouri et al. 2009)
Testis maturation	↑↑↑ by GH	(Rogol et al. 2002)

↑, up-regulated; ↓, down-regulated; ↑↑, increased; ↓↓, reduced; ↑↑↑, induced/stimulated; ↓↓↓, suppressed; Testosterone (T), follicle-stimulating hormone (FSH), hypothalamic-pituitary-gonadal (HPG), luteinizing hormone (LH), stem cell factor (SCF), gonadotropin releasing hormone (GnRH), 17β-estradiol (E_2), growth hormone (GH), insulin-like growth factor 1 (IGF-1).

Table I-2. Non-hormonal protective endogenous factors in male reproductive function.

Issue	Factor(s)	References
Acrosome reaction	↑↑↑ by NO	(Roessner et al. 2010)
Anti-apoptotic effect in testis	↑↑ by NO ↑↑ by Ghrelin Involvement of HGF ↑↑ by G-CSF ↑↑ by RGN	(Roessner et al. 2010) (Zhu et al. 2013) (Catizone et al. 2006) (Kim et al. 2011) (Correia et al. 2014a)
Antioxidant properties	Arginine Metallothioneins Ghrelin RGN	(Patel et al. 1998; Appleton 2002; Senbel et al. 2014) (Sato and Kondoh 2002) (Kheradmand et al. 2012) (Correia et al. 2013)
Chemical and/or radiation induced damage	↓↓ by Metallothioneins ↓↓ by Ghrelin ↓↓ by LGF ↓↓ by IL-1 & IL-6 ↓↓ by IL-6 ↓↓ by G-CSF ↓↓ by RGN	(Kheradmand et al. 2013; Maremanda et al. 2014) (Zhu et al. 2013; Garcia et al. 2015; Whirledge et al. 2015) (Perez-Crespo et al. 2011; Lobo et al. 2015) (Gerard et al. 1992; Syed et al. 1993; Legué et al. 2001) (Guitton et al. 1999) (Kim et al. 2011) (Correia et al. 2014a)
Epididymis weight	↑↑ by LGF	(Perez-Crespo et al. 2011)
Lipid peroxidation in sperm	↓↓ by Arginine	(Srivastava et al. 2006; Siddique and Atreja 2013)
Modulation of SCF gene expression	Ghrelin	(Barreiro et al. 2004)
Seminiferous epithelial depth & tubular diameter	↑↑ by G-CSF	(Kim et al. 2011)
Spermatogenesis	↑↑ by Arginine ↑↑↑ by LGF ↑↑↑ by IL-1 ↑↑ by G-CSF	(Aydin et al. 1995; Patel et al. 1998) (Perez-Crespo et al. 2011; Lobo et al. 2015) (Legué et al. 2001) (Kim et al. 2011)
Sperm abnormalities	↓↓ by G-CSF ↓↓ by RGN	(Kim et al. 2011) (Correia et al. 2013)
Sperm capacitation	↑↑↑ by NO	(Roessner et al. 2010)
Sperm cryopreservation induced damage	↓↓ by Arginine	(Siddique and Atreja 2013)
Sperm glycolysis rate	↑↑ by Arginine	(Patel et al. 1998; Srivastava et al. 2006)
Sperm maturation	↑↑ by RGN	(Correia et al. 2013)
Sperm membrane integrity	↑↑ by Arginine ↑↑ by Ghrelin	(Siddique and Atreja 2013) (Kheradmand et al. 2009)
Sperm motility	↑↑ by Arginine ↑↑ by NO ↑↑ by HGF ↑↑ by LGF	(Keller and Polakoski 1975; Scibona et al. 1994; Aydin et al. 1995; Rosselli et al. 1995; Patel et al. 1998; Appleton 2002; Morales et al. 2003; Srivastava et al. 2006; Imhof et al. 2012; Siddique and Atreja 2013) (Imhof et al. 2012) (Catizone et al. 2006) (Perez-Crespo et al. 2011)
Sperm production	↑↑ by Arginine ↑↑ by LGF ↑↑ by G-CSF	(Keller and Polakoski 1975; Aydin et al. 1995; Appleton 2002; Srivastava et al. 2006; Imhof et al. 2012) (Perez-Crespo et al. 2011) (Kim et al. 2011)
Sperm viability	↑↑ by Arginine ↑↑ by NO ↑↑ by RGN	(Rosselli et al. 1995; Siddique and Atreja 2013) (Imhof et al. 2012) (Correia et al. 2013)
Synthesis of VEGF and its receptors in testis	↑↑↑ by LGF	(Martin-Hidalgo et al. 2007)
T	↑↑ by LGF	(Lobo et al. 2015)
Testis maturation	↑↑↑ by HGF	(Catizone et al. 2006)
Testis weight	↑↑ by LGF ↑↑ by G-CSF	(Perez-Crespo et al. 2011) (Kim et al. 2011)

↑↑, increased; ↓↓, reduced; ↑↑↑, induced/stimulated; ↓↓↓, suppressed; Stem cell factor (SCF), vascular endothelial growth factor (VEGF), testosterone (T), nitric oxide (NO), hepatocyte growth factor (HGF), granulocyte colony stimulating factor (G-CSF), regucalcin (RGN), liver growth factor (LGF), interleukin 1 (IL-1), interleukin 6 (IL-6).

5. Regucalcin protein as a protective molecule in reproductive function

The differentiation and survival of spermatogenic cells has been indicated to implicate adjustments in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Berrios et al. 1998; Reyes et al. 2002; Mishra et al. 2006; Reyes et al. 2010; Sanchez-Cardenas et al. 2012). Germ cells produce Ca^{2+} currents that increase their density during the development from spermatogonia to early spermatids (Hagiwara and Kawa 1984). Thus, a tight control of Ca^{2+} homeostasis is a critical factor for spermatogenesis, including also for Sertoli cell function (Gorczynska and Handelsman 1995; Gorczynska-Fjalling 2004), for the maintenance of Sertoli cells tight junctions and integrity of the blood-testis barrier (Grima et al. 1998), and possibly for the Leydig cell steroidogenesis (Manna et al. 1999).

Overall, Ca^{2+} is required for sperm motility, capacitation, acrosome reaction, and, thus, acquisition of fertilization competence (Thomas and Meizel 1988; Sorensen et al. 1999; Breitbart 2002). One of the agents involved in the control of Ca^{2+} homeostasis is the regucalcin (RGN), also known as senescence marker protein 30 (Yamaguchi and Yamamoto 1978; Yamaguchi 2005). RGN has a molecular weight of 33 kDa and plays an important role in intracellular Ca^{2+} homeostasis by regulating the activity of Ca^{2+} pumps localized on cell membrane and endoplasmic reticulum of numerous cell types (Figure I-8) (Fujita et al. 1998; Tsurusaki and Yamaguchi 2000; Yamaguchi 2005).

Several studies have demonstrated that RGN is expressed in numerous tissues, such as liver (Shimokawa and Yamaguchi 1992; Ishigami et al. 2015), kidney (Yamaguchi and Kurota 1995; Zubiri et al. 2015), brain (Yamaguchi et al. 2000; Yamaguchi et al. 2008b), heart (Yamaguchi and Nakajima 2002; Akhter et al. 2006), bone (Yamaguchi et al. 2002a; Kagami et al. 2013), lung (Mori et al. 2004), submandibular gland (Ishii et al. 2005), ovary (Fayad et al. 2004; Kagami et al. 2013), breast (Marques et al. 2015a; Marques et al. 2015b), prostate (Maia et al. 2008; Maia et al. 2009; Vaz et al. 2015) and testis (Laurentino et al. 2011).

RGN expression is regulated by several hormones, including thyroid and parathyroid hormones (Yamaguchi et al. 2008a), aldosterone, insulin, calcitonin, and sex steroid hormones (Maia et al. 2008; Maia et al. 2009; Laurentino et al. 2011; Marques et al. 2014; Vaz et al. 2014).

This Ca^{2+}-binding protein also regulates several Ca^{2+}-dependent enzymes (Figure I-8), such as protein kinases, tyrosine kinases, phosphatases, phosphodiesterase, nitric oxide synthase (NOS) and proteases (reviewed by (Marques et al. 2014)). It has been proposed that RGN play an important role as a suppressor protein in the differentiation and proliferation of regenerating liver cells (Yamaguchi 2000). In addition, other studies support the role of RGN as a suppressor of cell proliferation in cancer tissues or cells and also in non-tumor tissues (Misawa et al. 2001; Vaz et al. 2014; Yamaguchi and Murata 2015). Recently, it was demonstrated that RGN overexpression diminished the incidence and aggressiveness of mammary gland tumors in rats treated with the carcinogen 7,12-dimethylbenz[α]anthracene,

which suggests a protective role of RGN against the onset and development of tumors (Marques et al. 2015b). In fact, RGN regulates the expression of oncogenes, tumor suppressor genes and cell cycle regulators inhibiting cell proliferation (Vaz et al. 2014; Yamaguchi and Murata 2015). Moreover, RGN may translocates to the nucleus modulating several nuclear functions (Yamaguchi and Sakurai 1991), such the inhibition of DNA and RNA synthesis (Yamaguchi and Kanayama 1996; Yamaguchi and Ueoka 1997).

Regarding the male reproductive tract, RGN is expressed in several tissues, including the testis, epididymis, seminal vesicles and prostate (Maia et al. 2008; Laurentino et al. 2011; Vaz et al. 2014). At testicular level, RGN is expressed in Leydig and Sertoli cells, as well as in all germ line both in human and rat testis (Laurentino et al. 2011). This Ca^{2+}-binding protein is largely recognized as an androgen-target gene (Maia et al. 2009; Laurentino et al. 2011; Vaz et al. 2014), and it has been pointed out that its increasing concentration might be a mechanism by which androgenic stimulation sustain germ cell survival and spermatogenesis (Laurentino et al. 2011).

Recently, several studies have demonstrated the role of RGN in regulating the expression of cell cycle and apoptosis regulators (Nakagawa and Yamaguchi 2005; Correia et al. 2014a; Vaz et al. 2014; Marques et al. 2015b). RGN inhibits the increase of $[Ca^{2+}]_i$ (Correia et al. 2013), inhibits caspase 8 activity (Ishigami et al. 2002), improves Akt pathway activity and increases the expression of anti-apoptotic agents Akt-1 and Bcl-2, providing a resistance to apoptosis (Yamaguchi 2013). Considering that the success of spermatogenesis depends on the tight balance between germ cell survival and death, a role for RGN in male spermatogenesis and fertility has been suggested (Laurentino et al. 2012). In order to investigate the protective function of RGN in testicular apoptosis, the effect of apoptosis-inducers such as thapsigargin and actinomycin D, was determined in the seminiferous tubules of transgenic animals overexpressing RGN (Tg-RGN) (Correia et al. 2014a). Concomitantly with RGN overexpression, it was detected reduced activity of caspase-3 and increased expression of anti-apoptotic Bcl-2 protein in the seminiferous tubules of Tg-RGN (Correia et al. 2014a). In addition, the mRNA expression of p53 and p21 was significantly diminished in Tg-RGN treated with thapsigargin or actinomycin D (Correia et al. 2014a). Altogether, these results suggest that RGN may act as a germ cell survival factor protecting the cells from noxious stimuli (Figure I-8) (Correia et al. 2014a). The importance of RGN for the spermatogenic output was also established by the fact that infertile men with abnormal spermatogenesis phenotypes exhibited different expression patterns of RGN at testicular level (Laurentino et al. 2012). Furthermore, significant alterations in the epididymal epithelium and sperm parameters between Tg-RGN and their wild-type (Wt) counterparts were found (Correia et al. 2013). Tg-RGN rats have lower sperm counts and reduced sperm motility, which may be associated to the lower Ca^{2+} influx in epididymis (Correia et al. 2013). However, this result is counterbalanced by greater sperm viability, higher percentage of normal sperm morphology, and a reduced incidence of tail defects, suggesting the involvement of RGN in sperm maturation (Correia et al. 2013). The beneficial effects of RGN on sperm parameters seems to

be explained by the lower levels of oxidative stress found in biological models with RGN overexpression (Handa et al. 2009; Correia et al. 2013). Indeed, the antioxidant activity of RGN has been described (Son et al. 2006; Correia et al. 2013; Marques et al. 2014). RGN increases the activity of superoxide dismutase (SOD) enzyme and diminishes NOS levels, reducing the generation of ROS (Figure I-8) (Ma and Yamaguchi 2002; Ma and Yamaguchi 2003; Yamaguchi et al. 2003; Fukaya and Yamaguchi 2004; Ichikawa and Yamaguchi 2004; Yamaguchi et al. 2005; Handa et al. 2009). The cytoprotective and antioxidant properties of RGN were also highlighted by studies in the RGN-knockout mice, which showed higher susceptibility to oxidative stress induced by exposure to cigarette (Sato et al. 2006), and displayed increased levels of anion superoxide in the brain (Son et al. 2006; Kondo et al. 2008; Sato et al. 2008). Also, RGN overexpression in a mouse carcinoma cell line was able to increase cell viability under oxidative damage induced by tert-butyl hydroperoxide (Son et al. 2008). Hence, the available studies evidenced the protective effect of RGN against oxidative stress, as well as its importance in order to keep ROS at physiological concentrations.

Altogether, the existing information indicates that RGN plays a role in the modulation of sperm production and maturation.

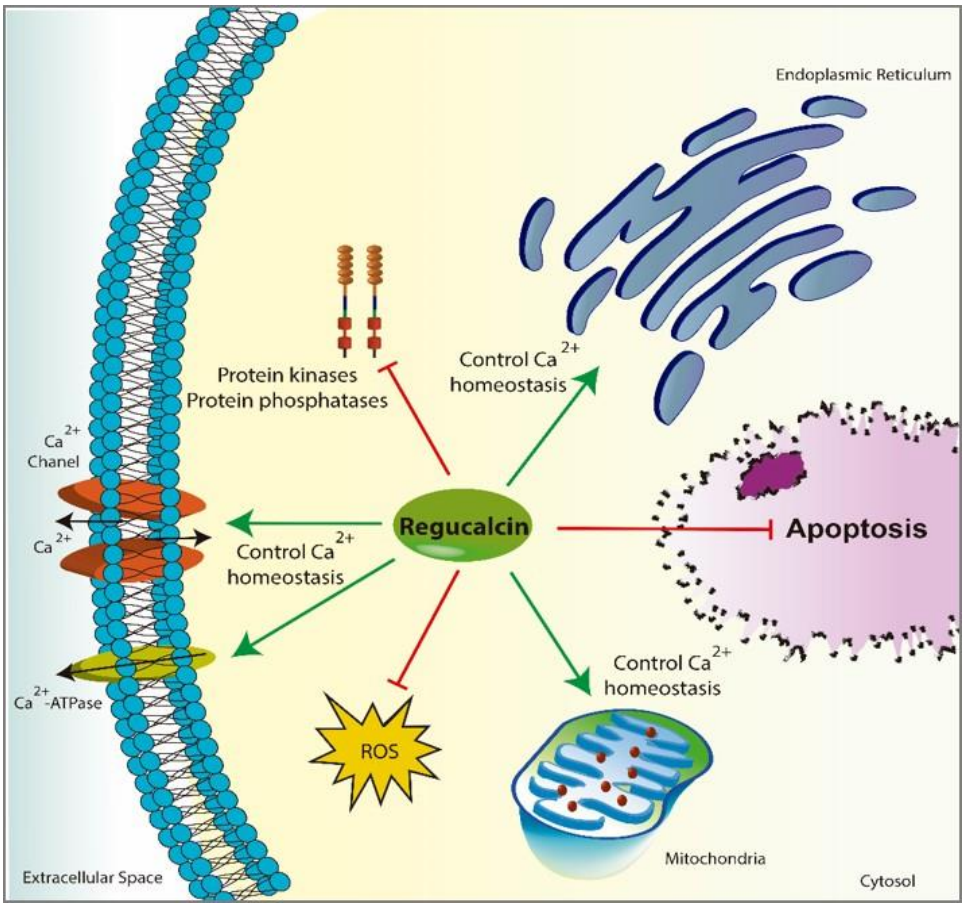

Figure I-8. The role of regucalcin (RGN) in cell biology. RGN regulates the activity of several Ca^{2+}-dependent enzymes and the concentration of intracellular Ca^{2+} by modulating the activity of Ca^{2+}-channels, and Ca^{2+}-ATPase in the plasma membrane, mitochondria and endoplasmic reticulum. It has suppressive effects on the activity of protein kinases and phosphatases. Another feature of RGN protein is its antioxidant activity by reducing the production of reactive oxygen species (ROS). Moreover, RGN is able to suppress apoptosis induced by noxious stimuli (Cardoso 2014).

II. Aim of the thesis

Cancer therapies and the oncological condition itself have adverse effects on male fertility, which implicate permanent or transitory impairment of reproductive function. In the case of radiation therapy, the extent of testicular injury is directly related to the dose of radiation delivered but, due to the sensitivity of seminiferous tubules, in general, it induces temporary or permanent azoospermia. Actually, the development of effective strategies that would restrain the undesirable secondary effects of oncological treatments is clearly warranted.

The Ca^{2+}-binding protein RGN has been indicated as a protein that suppresses apoptosis, and has cytoprotective and antioxidant effects, protecting cells against noxious stimuli. Moreover, RGN overexpression was associated with increased sperm viability and lower incidence of tail defects. This led us to hypothesize that RGN may have a protective role to counteract the effects of radiation on spermatogenesis. The present work aims to study the spermatogenesis recovery after radiation treatment in Tg-RGN comparatively with their Wt counterparts. For this purpose, a single dose of 6 Gy was delivered to the testes of Tg-RGN and Wt rats, and after a 10-week recovery period, the reproductive function of both groups was evaluated by determination of:

1. Gonadosomatic index (GI);
2. Tubular differentiation index (TDI);
3. Epididymal sperm parameters;
4. Expression of RGN and several cell cycle and apoptosis modulators.

III. Material and Methods

1. Animals

Three-months old Wt and Tg-RGN Sprague Dawley (*Rattus norvegicus*) rats were obtained, respectively, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan). Sprague Dawley Tg-RGN rats were originally generated by Yamaguchi M by means of oocyte transgene pronuclear injection (Yamaguchi et al. 2002b). Animals were handled in compliance with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive number 2010/63/EU. Rats were housed under a 12 h light:12 h darkness cycle, with food and water available ad libitum during the course of the experiment. Wt and Tg-RGN rats were randomly divided into control group receiving no treatment and irradiated group ($n \geq 5$ in each group).

2. Radiation Treatment

In vivo irradiation studies were performed in the Radiation Oncology Department, Coimbra University Hospital Centre, in a linear accelerator Varian Clinac 600 C (Varian Medical Systems), using a 4MV photon beam.

To perform *in vivo* irradiation, an immobilization device was first built in order to keep the rat in a lateral decubitus position. For radiotherapy planning a rat was anesthetized using intramuscular ketamine. Then a computerized tomography (CT) was acquired, keeping the animal in treatment position.

After CT acquisition, target volume (testes) delineation and three-dimensional (3D) computerized planning were performed using *Eclipse*™ Planning System (Varian Medical Systems, EUA). The goal was to have a homogeneous dose coverage of our target volume, with the dose within our volume ranging from 95% to 107% of the prescribed dose (6 Gy). In order to obtain the desired dose in our planning volume we used a pair of parallel opposed fields. Table, gantry and collimator positions were determined after 3D planning, as well as field size and monitor units for each field. Figure III-1 illustrates the 3D planning of the irradiation performed.

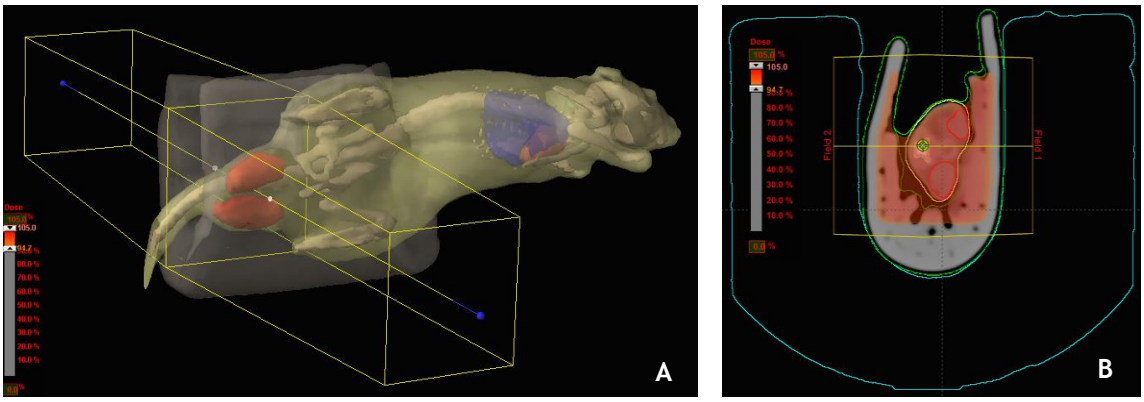

Figure III-1. Planning representation for testicular irradiation. (A) 3D representation of animal positioning and volume delineation (testes represented in red), as well as the irradiation fields. (B) CT axial slice with field and dose representation.

For irradiation the animals were anesthetized as previously described. Animal positioning and irradiation was performed according to planned. Wt and Tg-RGN rats were submitted to a single dose of 6 Gy of X-rays at a dose rate of 1 Gy/min.

3. Tissue Collection

Ten weeks after radiation treatment, rats were anesthetized by means of intraperitoneal injection with 100 µL of a mixture containing Ketamine and Xylazine (2:1) per 100 g of animal weight, and euthanized by cervical dislocation.

Gonads and body mass were measured for the calculation of GI. Testes were immediately frozen on liquid nitrogen and stored at -80ºC until total protein extraction or fixed in 4% paraformaldehyde (PFA) for histological processing. Epididymides from Wt and Tg-RGN animals were immediately removed and dissected free from fat. *Cauda* epididymis from each animal was used for the determination of sperm parameters.

4. Epididymal Sperm Count and Motility

Each epididymal *cauda* was minced with a sterilized scissor in 3 mL of filtered Hank´s buffered salt solution (HBSSf; Sigma-Aldrich) at 37°C in order to allow sperm dispersion in HBSSf, and the suspension was incubated for 5 minutes at 37ºC. Sperm motility was determined by placing a drop of 100 µL of the sperm suspension in a 37°C pre-warmed slide and covered with a cover slip. At least 10 random fields were assessed for each semen sample using an optical microscope (1000x magnification) with closed diaphragm (Primo Star, Zeiss) and the percentage of motile sperm was calculated. An aliquot of sperm suspension was diluted with HBSSf, and introduced into a Neubauer´s counting chamber (Tiefe Depth Profondeur, Optik Labor, Switzerland) for sperm counting under an optical microscope (1000x

magnification). Subsequently, sperm suspensions were also used to analyze sperm viability and morphology.

5. Epididymal Sperm Viability and Morphology Analysis

Sperm viability was assessed by using the one-step eosin-nigrosin staining technique. A sample of 5 µL of sperm suspension was mixed with 10 µL of a mixture of eosin and nigrosin stain (0,6% eosin; 5% nigrosin; 3% sodium citrate; pH of the stain was adjusted to 7.4 by adding a few drops of 0.1 M NaH_2PO_4) and placed on a pre-warmed slide. The head of viable sperm remains white head whereas non-viable sperm absorbs eosin due to the lost integrity of the cell membrane, which causes an increase in membrane permeability.

Sperm morphology was evaluated using the Kwik™-Diff stain kit (Thermo Scientific™). The Diff-Quik kit is composed by 3 sequential solutions: a fixative (methanol), an anionic/acidic dye (eosin) that stains positively charged/basic proteins (red), and a cationic dye (methylene blue and derivatives) that stains nuclei and negatively charged molecules (blue) (Mota and Ramalho-Santos 2006). First, the smear were done using 5 µL of sperm suspension mixed with 10 µL of HBSSf, dragged with a cover slip and allowed to air dry at 37ºC. Then, the dried slide was immersed in each solution of the staining kit for at least 1 min and dipped rapidly in water, and air dried. Afterward, the sperm was classified as normal or abnormal, and the abnormalities were divided in head, neck/midpiece or tail defects. Some abnormalities were exclusive but some sperm showed more than one type of defect. In such case, if one of the defects was on the head, it accounted exclusively for the number of head defects.

In order to determine the sperm viability and morphology, a total of 100 sperm cells in each semen sample were analyzed in random fields under a light microscope (Primo Star, Zeiss) with oil immersion (1000x magnification).

6. Testicular Histological Analysis

After histological processing, testicular sections were stained with hematoxylin and eosin (H&E) in order to determine the TDI, which is the percentage of seminiferous tubules showing germ cell differentiation, and thus, evidence of spermatogenesis recovery.

A minimum of 70 seminiferous tubules in one section of each animal were scored using an optical microscope (400x magnification; Zeiss) to evaluate the recovery of spermatogenesis at ten weeks after radiation treatment. A tubule was scored as differentiating if it contained cells in at least 3 different stages of germ cell differentiation: spermatogonia, spermatocyte and spermatid (round or elongated), or later stage. The same protocol was used both in irradiated and control groups of Tg-RGN and Wt animals.

7. Total Protein Extraction and Quantification

Testicular tissues were homogenized in 3 µL of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% nonidet-inhibitor and 10% phenylmethylsulfonyl fluoride (PMSF)) per mg of tissue by pipetting up and down. The samples were allowed to stand on ice 1 hour and occasionally mixed. Then, samples were centrifuged at 14000 rpm for 20 minutes at 4ºC, and supernatant containing total proteins was collected to fresh tubes. Total protein concentration was determined through the Bradford method with Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA), and using Bovine Serum Albumin (BSA) as a standard curve.

8. Western Blot

Total proteins (50 or 75 µg) were denatured at 100 ºC and resolved on 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) at 750 mA for 1h30min at 4ºC. Membranes were incubated overnight at 4ºC with rabbit anti-p53 (1:500, FL-393: sc-6243; Santa Cruz Biotechnology), rabbit anti-p21 (1:500, C-19: sc-397; Santa Cruz Biotechnology), rabbit anti-FasL (1:500, C-178: sc-6237; Santa Cruz Biotechnology), rabbit anti-FasR (1:500, A-20: sc-1023; Santa Cruz Biotechnology), rabbit anti-Bax (1:1000, no. 2772; Cell Signaling Technology), rabbit anti-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology), mouse anti-caspase-8 (1:500, D-8: sc-5263; Santa Cruz Biotechnology), or mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom). A mouse anti-α-tubulin monoclonal antibody (1:5000, T9026, Sigma–Aldrich) was used for protein loading control in all Western Blot (WB) 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. Membranes were developed with ECL substrate (Bio-Rad) for 5 minutes and scanned with the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective α-tubulin band density.

9. Caspase-3 Activity Assay

Caspase-3 activity assay was performed as previously described (Alves et al. 2011). Briefly, 25 µg of total protein extracted from testis were incubated with reaction buffer (25 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1 -propanesulfonate, 10% sucrose, and 10 mM dithiothreitoln (DTT), pH 7.5) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 hours at 37ºC in a 96-well plate. Upon caspase cleavage, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation of the standard curve of free pNA.

10. Statistical Analysis

Statistical significance of differences between experimental groups was evaluated by unpaired t-test, using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). All experimental data are shown as mean ± SEM.

IV. Results

1. Spermatogenic status at ten weeks of recovery after radiation treatment shows less injury in Tg-RGN

The TDI is a useful tool to analyze the spermatogenesis status because it is based on the presence of successive germ cell differentiation stages within the seminiferous tubules, which allowed the classification of tubules in differentiating (Figure IV-1A) and non-differentiating (Figure IV-1B).

The GI is an indicator of the testis condition because gonad weight and size are generally controlled by the secretory activity of Sertoli cells as well as by the sperm production in the seminiferous tubules (Biswas et al. 2001). At ten weeks of recovery after radiation treatment, the GI was found to be significantly decreased in both Wt and Tg-RGN animals comparatively with the respective controls ($p<0.001$) (Figure IV-1C). However, the GI showed a 0.39 ± 0.02 fold variation relatively to control in the Wt irradiated rats, whereas in Tg-RGN irradiated rats the GI displayed 0.51 ± 0.08 fold variation relatively to the control. In spite of the prominent diminution of the GI in both irradiated groups, this was much lower in Tg-RGN, 48.53 % vs. 61.41 % in Wt animals.

Likewise and also with a recovery time of ten weeks, the TDI was significantly reduced both in Wt (0.26 ± 0.05 fold variation) and Tg-RGN animals (0.29 ± 0.10 fold variation) in comparison with respective controls ($p<0.001$) (Figure IV-1D). In spite of the marked diminution of TDI in both irradiated groups, such diminution was slightly lower in Tg-RGN since it was approximately 71.49 % vs. 74.01 % in Wt.

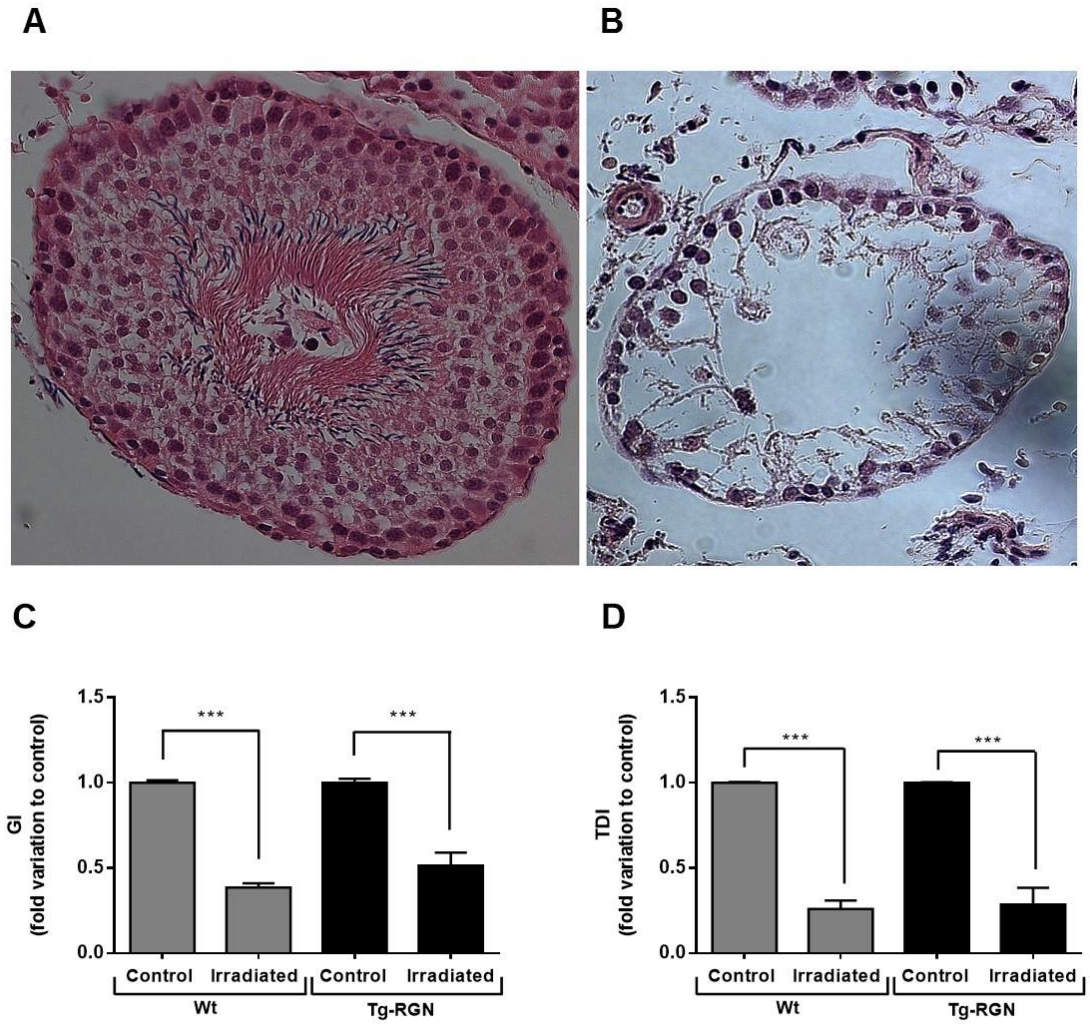

Figure IV-1. Representative photomicrographs of a differentiating (A) and non-differentiating (B) seminiferous tubule stained with H&E (400x magnification; Zeiss), and the effect of radiation treatment in GI (C) and TDI (D) both in Wt and Tg-RGN animals. Results are expressed as fold variation relatively to respective control group. Error bars indicate mean ± S.E.M. (n≥5 in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

2. Epididymal sperm parameters of Tg-RGN were less damaged by radiation treatment

2.1 Epididymal sperm count

At the control situation, without irradiation, the epididymal sperm counts were significantly lower in Tg-RGN rats when compared to their Wt littermates ($2.4 \times 10^8 \pm 1.2 \times 10^7$ vs. $1.7 \times 10^8 \pm 8.7 \times 10^6$, $p<0.001$) (Figure IV-2).

At ten weeks of recovery after radiation treatment, sperm counts were found to be significantly decreased both in Wt and Tg-RGN animals comparatively with the respective control ($p<0.001$) (Figure IV-2). In Wt irradiated rats, the number of sperm cells/mL decreased to $3.2 \times 10^7 \pm 2.4 \times 10^6$, and in Tg-RGN irradiated rats decreased to $3.1 \times 10^7 \pm 1.2 \times 10^7$ cells/mL. However, the % of reduction in sperm count triggered by radiation treatment was approximately 87% in Wt, whereas in Tg-RGN it was 82%, suggesting that the X-rays have less impact in Tg-RGN.

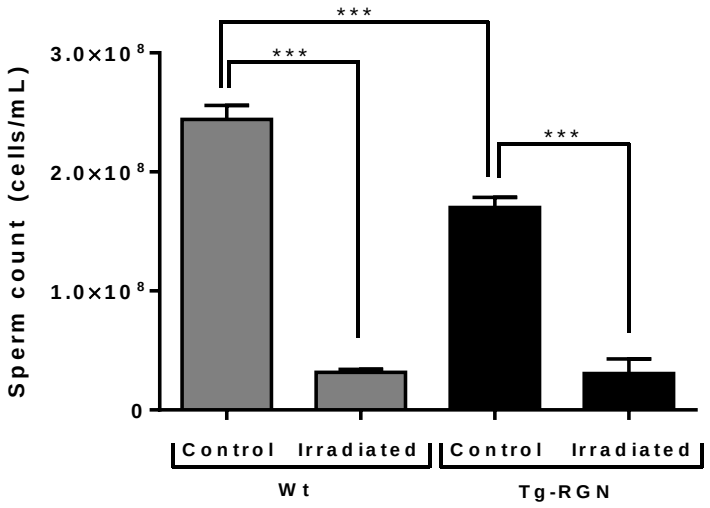

Figure IV-2. Effect of radiation treatment on sperm counts in Wt and Tg-RGN animals. Results are expressed as cells/mL. Error bars indicate mean ± S.E.M. ($n \geq 5$ in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

2.2 Epididymal sperm motility and viability

Spermatozoa leave the testis as non-functional gametes and it is only during the passage through the epididymis that they acquire the ability to move progressively, and eventually to fertilize (Cornwall 2009). The *caput* and *corpus* regions are responsible for the early and late sperm maturation, respectively. The epididymal *cauda* stores the functionally mature

spermatozoa (Robaire et al. 2006), and, for that reason, epididymal *cauda* sperm motility is an important parameter to evaluate the quality of semen (Zhou et al. 2015).

Since sperm cells are susceptible to changes in pH of the female cervical mucus, it may adversely affect the sperm viability (WHO 2010). Thus, the initial content of viable (Figure IV-3A) and non-viable (Figure IV-3B) sperm is crucial for semen quality assessment. Therefore, any factor that decreases the number of epididymal viable sperm will reduces the probability of fertilization.

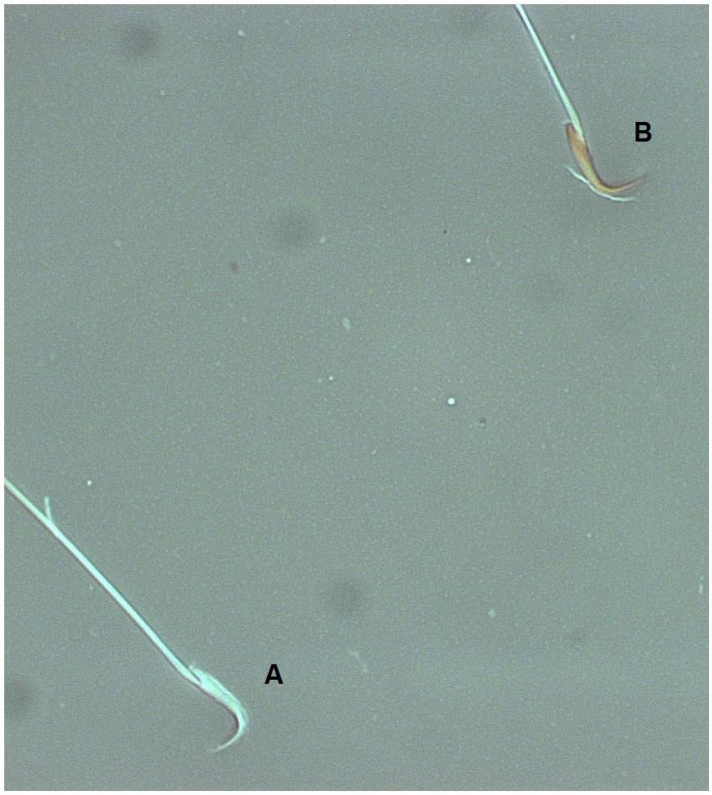

Figure IV-3. Representative photomicrographs of viable (A) and non-viable (B) rat sperm stained with Eosin-Nigrosin technique (1000x magnification; Zeiss).

Comparing the control groups, the percentage of epididymal motile sperm (Figure IV-4A) was significantly lower in Tg-RGN rats when compared to their Wt littermates (44.0 % ± 1.0 vs. 72.6 % ± 1.3, $p<0.001$). As excepted, at ten weeks of recovery after radiation treatment, the percentage of epididymal motile sperm displayed a statistical significant decline both in Wt and Tg-RGN comparatively to the respective control ($p<0.001$) (Figure IV-4A). Drastically, no motile sperm was found in Wt irradiated animals. On the other hand, Tg-RGN irradiated rats showed 6.8 % ± 4.3 of motile sperm.

Concerning the epididymal viable sperm, it was found a significant reduction both in Wt and Tg-RGN animals after radiation treatment in comparison with the respective control (Figure IV-4B). In Tg-RGN irradiated, 2.7 % ± 1.9 ($p<0.01$) of viable sperm was found, whereas in Wt irradiated no viable sperm could be observed ($p<0.001$).

Overall, the results of sperm motility and viability revealed a similar pattern, i.e., no motile or viable sperm were observed in Wt irradiates rats, in contrast with the Tg-RGN irradiated where it was possible to found viable or motile sperm.

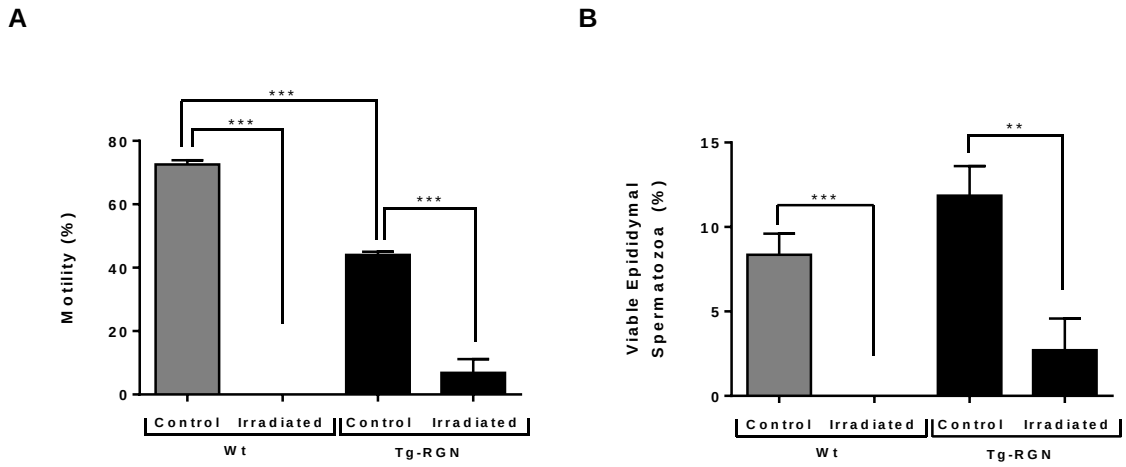

Figure IV-4. Effect of radiation treatment on epididymal sperm motility (A) and viability (B) in Wt and Tg-RGN animals. Error bars indicate mean ± S.E.M. (n≥5 in each group). * p<0.05; ** p<0.01; *** p<0.001 when compared with the respective control group.

2.3 Epididymal sperm morphology

Sperm morphology is another parameter evaluated in semen analysis. The shape of the head, midpiece and tail is commonly used to examine the sperm morphology. The sperm morphological abnormalities are a reflection of negative stress factors working on the organism (Menkveld et al. 2011). The presence of abnormal sperm is a decisive parameter to analyze the fertility potential of the individuals.

The sperm was classified as normal (Figure IV-5A) or abnormal, and the abnormalities were divided in head (Figure IV-5B), neck/midpiece (Figure IV-5C) or tail defects (Figure IV-5D). When more than one abnormality was detected in a single sperm cell, only the most important was considered, i.e., the head defect.

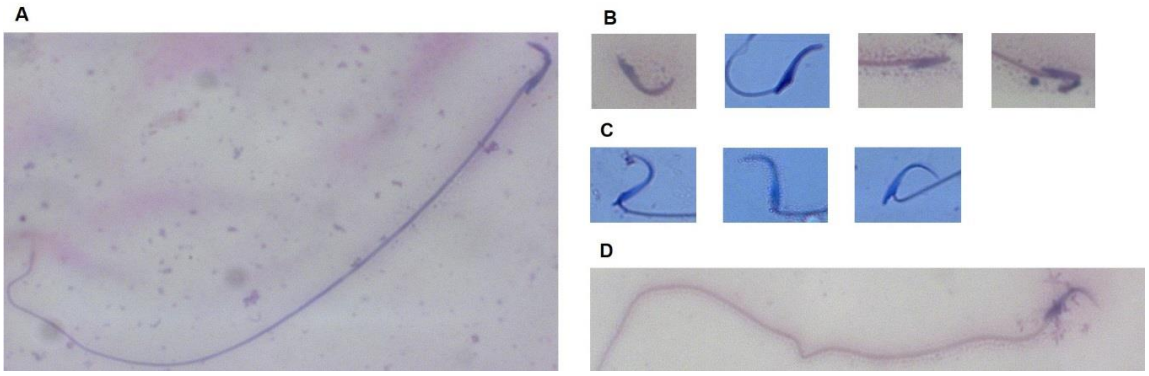

Figure IV-5. Representative photomicrographs of a normal rat sperm (A) and different types of rat sperm abnormalities (B, C and D) stained with Kwik™-Diff stain kit. (B) Head defects; (C) Bent neck defects; (D) Tail defect (1000x magnification; Zeiss).

At ten weeks of recovery after radiation treatment, the percentage of normal epididymal spermatozoa showed a significant decline both in Wt and Tg-RGN comparatively with the respective controls ($p<0.001$) (Figure IV-6). In Wt irradiated rats, very few normal sperm were found and the percentage of normal morphology diminished to 0.50 % ± 0.22. In contrast, the percentage of normal sperm in the Tg-RGN irradiated animals suffered a smaller decrease, 13.07 % ± 12.05. Thus, despite no significantly difference, the percentage of normal sperm was ≈26.1–fold higher in Tg-RGN irradiated relatively to Wt irradiated.

The percentage of epididymal sperm with head defects (Figure IV-6) was significantly lower in Tg-RGN rats when compared with their Wt littermates (4.96 % ± 1.11 vs. 10.00 % ± 2.43, $p<0.05$). Relatively to the damage caused by the radiation, the percentage of epididymal spermatozoa with head defects significantly augmented both in Wt and Tg-RGN groups comparatively with the respective controls ($p<0.001$). In Wt irradiated rats, the percentage of head defects was 98.33 % ± 0.67, whereas in Tg-RGN irradiated rats it was 80.93 % ± 10.32 (Figure IV-6). Thus, the percentage of head defects was ≈0.8–fold lower in Tg-RGN irradiated relatively to Wt irradiated.

For neck/midpiece defects, it was observed a diminution of the percentage of epididymal spermatozoa with bent neck defects in Wt irradiated group (0.83 % ± 0.54) comparatively to the respective control ($p<0.01$) (Figure IV-6). On the other hand, in Tg-RGN irradiated no significant difference was observed in the percentage of sperm with bent neck in comparison with the respective control. Comparing the control groups, the Tg-RGN animals displayed a significantly lower percentage of sperm with bent neck when compared with their Wt counterparts (2.18 % ± 0.47 vs. 8.20 % ± 2.63, $p<0.05$).

Regarding tail defects, the percentage of epididymal spermatozoa exhibited a pronounced decline both in Wt and Tg-RGN rats comparatively with the respective controls ($p<0.001$). No statistical differences were observed between the two groups of control conditions, as well as between Wt and Tg-RGN irradiated animals. In Wt irradiated rats, the percentage of tail defects diminished to 0.33 % ± 0.21, whereas in Tg-RGN irradiated rats, it decreased to 1.46 % ± 1.36 (Figure IV-6).

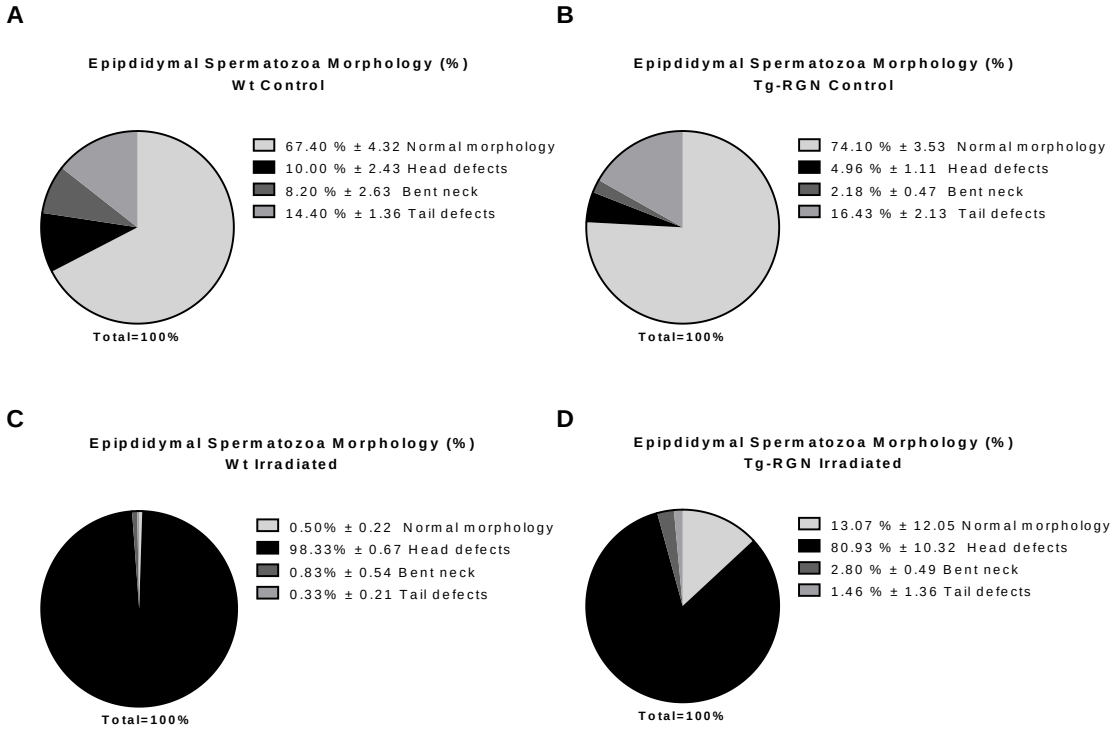

Figure IV-6. Mean distribution of the epididymal sperm morphology in Wt and Tg-RGN animals under control condition and ten weeks of recovery after radiation treatment. Epididymal sperm morphology of Wt and Tg-RGN under control condition are shown in panels A and B, respectively. Epididymal sperm morphology of Wt and Tg-RGN at ten weeks of recovery after radiation treatment are shown in panels C and D, respectively. Results are expressed as mean ± S.E.M. (n≥5 in each group).

3. Testicular expression of cell cycle and apoptosis regulators at ten weeks of recovery after radiation treatment

3.1 The expression of p53 and p21 was decreased in the testis in response to radiation treatment

The tumor suppressor protein p53 has a critical role in the regulation of the Bcl-2 family members and also upregulates the expression of p21, a cyclin-dependent kinase inhibitor, which may induce cell cycle arrest at G1 phase in response to a variety of stress stimuli (reviewed by (Schuler and Green 2001; Speidel 2010)), such as radiation (Shaha et al. 2010).

WB analysis showed that radiation treatment significantly decreased protein levels of p53 both in Wt (0.52 ± 0.10 fold variation, $p<0.01$) and Tg-RGN rats (0.52 ± 0.10 fold variation, $p<0.01$) in comparison with the respective controls (Figure IV-7A and C).

A similar pattern of expression was observed for p21 protein, which significantly decrease both in Wt (0.20 ± 0.05 fold variation, $p<0.01$) and Tg-RGN (0.40 ± 0.17 fold variation, $p<0.05$) rats in response to radiation treatment in comparison with the respective controls (Figure IV-

7B and D). The expression of p53 showed no significant differences between Wt and Tg-RGN rats.

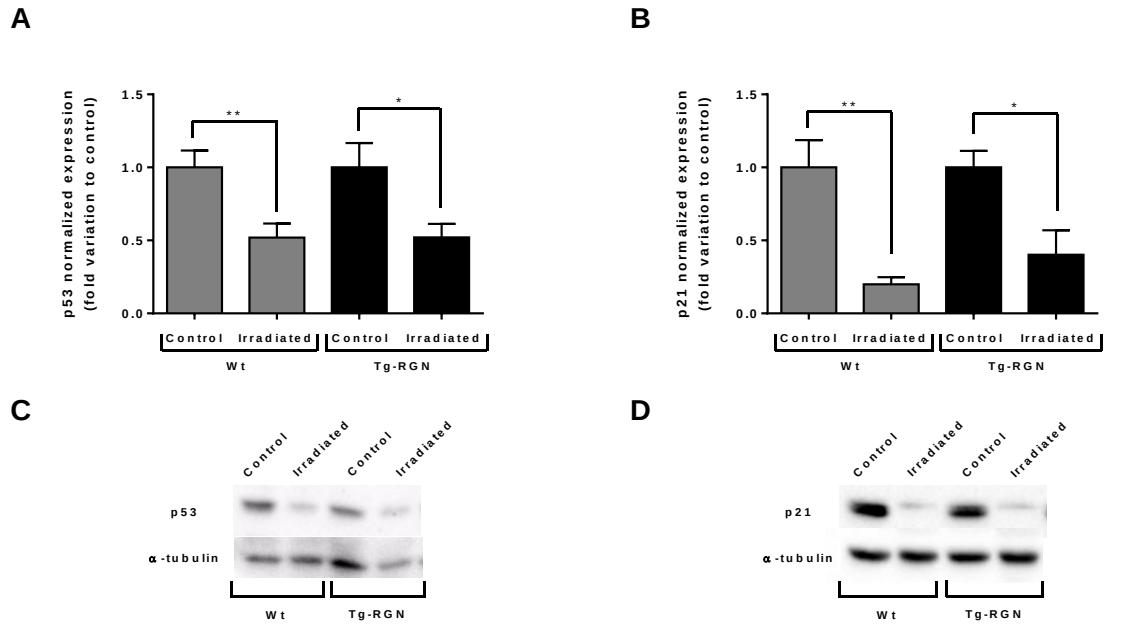

Figure IV-7. Effect of radiation treatment on p53 (A) and p21 (B) protein expression, determined by WB analysis, in the testis of Wt and Tg-RGN animals. Results are expressed as fold variation relatively to respective control group after normalization with α-tubulin. Representative immunoblots for p53 and p21 are shown in panels C and D, respectively. Error bars indicate mean ± S.E.M. (n≥5 in each group). * p<0.05; ** p<0.01; *** p<0.001 when compared with the respective control group.

3.2 The Bcl-2/Bax protein ratio is higher in rats exposed to radiation treatment

As in many tissues throughout the body, the number of cells in the seminiferous tubules of the testis is controlled by the dynamic balance between cell proliferation and apoptotic cell death (Russell et al. 2002). Several stimuli may increase cell death, including the chemotherapeutic drugs and radiation (Shaha et al. 2010).

The Bcl-2 and Bax proteins are, respectively, anti-apoptotic and pro-apoptotic members of the Bcl-2 family of apoptosis regulators (Lawen 2003), that regulate the apoptotic pathway triggered at the mitochondria (Youle and Strasser 2008). The Bax protein is involved in the permeabilization of mitochondrial pores, allowing the release of cytochrome c, while Bcl-2 is a recognized repressor of this process (Jurgensmeier et al. 1998; Rosse et al. 1998). Therefore, the ratio of Bcl-2/Bax protein has been accepted to dictate the cell susceptibility to apoptosis (Mackey et al. 1998).

At ten weeks of recovery after radiation treatment, the expression of Bcl-2 protein was significantly increased both in Wt (3.0 ± 0.6 fold variation, p<0.01) and Tg-RGN animals (2.9 ± 0.5 fold variation, p<0.01) relatively to the respective controls (Figure IV-8A). Regarding the

expression of Bax protein, no significant differences were observed in irradiated animals when compared with the respective control groups (Figure IV-8B). However, the Bcl-2/Bax protein ratio was significantly increased in both irradiated groups when compared with the respective controls (Figure IV-8E). In Wt animals, it was observed a 2.7 ± 0.7 fold variation ($p<0.05$) relatively to the respective control, whereas Tg-RGN animals displayed a 5.9 ± 1.4 fold variation ($p<0.01$) relatively to the respective control. Thus, this anti-apoptotic/pro-apoptotic ratio was found to be ≈2.2-fold higher in Tg-RGN irradiated comparatively to Wt irradiated ($p<0.05$).

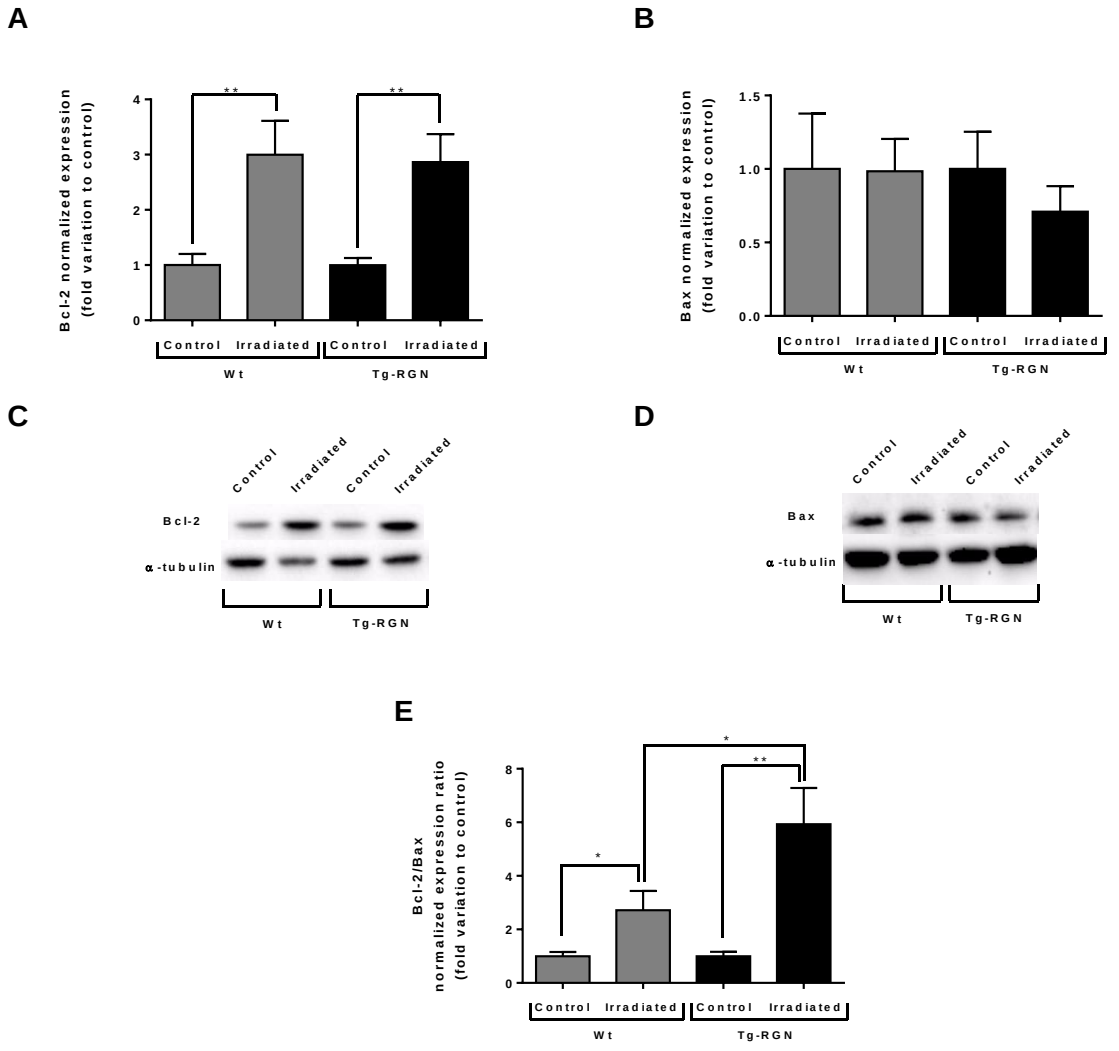

Figure IV-8. Effect of radiation treatment on Bcl-2 (A) and Bax (B) expression and Bcl-2/Bax protein ratio (E) in the testis of Wt and Tg-RGN animals determined by WB analysis. Results are expressed as fold variation relatively to respective control group after normalization with α-tubulin. Representative immunoblots for Bcl-2 and Bax are shown in panels C and D, respectively. Error bars indicate mean ± S.E.M. ($n\geq5$ in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

3.3 The expression of FasL and FasR was decreased in the testis of Wt and Tg-RGN rats after radiation treatment

Germ cell death in human, rat, and mouse testis has been associated with the activation of the extrinsic pathway of apoptosis involving the death factors FasL (ligand) and FasR (receptor) (Lee et al. 1997; Kim et al. 2007).

WB analysis showed that the protein levels of FasL and FasR have a similar expression pattern (Figure IV-9). In the Wt irradiated group, neither the protein expression of ligand nor receptor was detected (respectively, $p<0.001$ and $p<0.01$ relatively to the respective control). In Tg-RGN irradiated group, both ligand and receptor were detected though with slight expression, respectively, 0.19 ± 0.12 ($p<0.01$) and 0.24 ± 0.16 ($p<0.05$) fold variation relatively to the respective control (Figure IV-9A and B). Thus, the FasL and FasR are not expressed in Wt after radiation treatment, but a slight expression was detected in Tg-RGN.

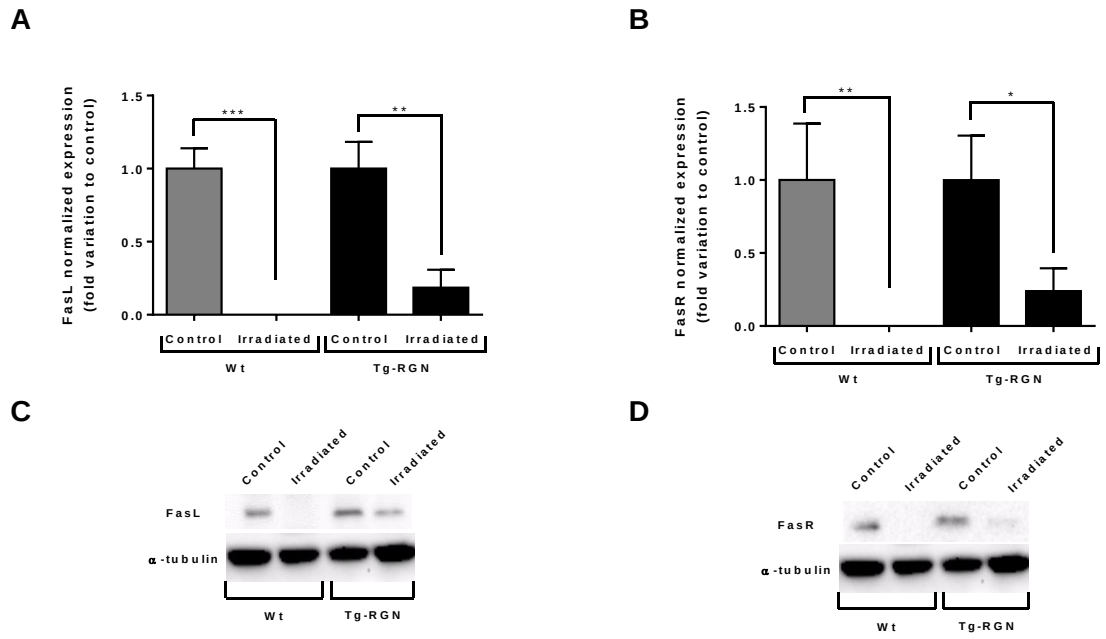

Figure IV-9. Effect of radiation treatment on FasL (A) and FasR (B) expression in the testis of Wt and Tg-RGN animals determined by WB analysis. Results are expressed as fold variation relatively to respective control group after normalization with α-tubulin. Representative immunoblots for FasL and FasR are shown in panels C and D, respectively. Error bars indicate mean ± S.E.M. ($n\geq5$ in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

3.4 The expression of caspase-8 was lower in the testis of Tg-RGN rats comparatively with Wt rats after radiation treatment

Apoptosis can be induced by intrinsic and extrinsic pathways, with both pathways converging to the activation of caspase proteolytic cascade (Lawen 2003). Caspase-8 is the essential mediator of the extrinsic pathway of apoptosis, and interacts with death receptors and

activates the downstream effectors of apoptosis, such as caspase-3 (van Raam and Salvesen 2012).

At ten weeks of recovery after radiation treatment, the protein expression of caspase-8 was significantly increased in both irradiated groups when compared with the respective control groups (Figure IV-10A). In Wt rats, it was observed a 1.59 ± 0.03 fold variation relatively to respective control ($p<0.01$), whereas Tg-RGN showed a 1.30 ± 0.05 fold variation relatively to respective control ($p<0.01$). Hence, the expression of caspase-8 was ≈0.82-fold lower in Tg-RGN irradiated comparatively with Wt irradiated group ($p<0.05$).

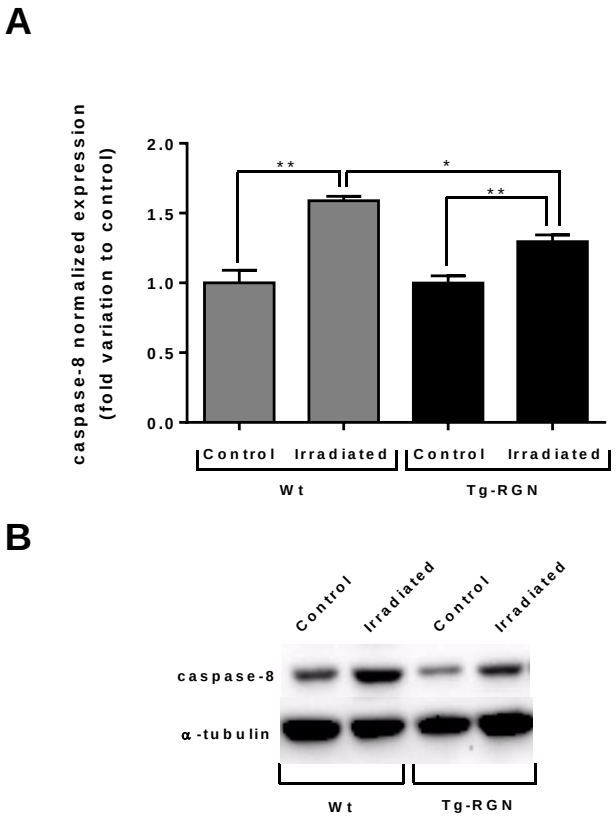

Figure IV-10. Effect of radiation treatment on the protein expression of caspase-8 in the testis of Wt and Tg-RGN animals determined by WB analysis. Results are expressed as fold variation relatively to respective control group after normalization with α-tubulin. Representative immunoblots are shown in panel B. Error bars indicate mean ± S.E.M. ($n\geq5$ in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

3.5 The enzymatic activity of caspase-3 was diminished in Tg-RGN after radiation treatment

Independently of the triggered pathway of apoptosis, caspase-3 is one of the latest activated caspases, designated as effector caspases, which cleave important cellular substrates that irreversibly lead to apoptosis. Because of that, the activation of caspase-3 is a recognized

end-point of apoptosis (Lawen 2003), and the enzymatic activity of caspase-3 is an important tool to measure apoptosis.

In control non-irradiated animals, the enzymatic activity of caspase-3 (Figure IV-11) was significantly lower in Tg-RGN animals relatively to their Wt littermates (46.99 ± 9.75 vs. 74.60 ± 10.49, $p<0.05$). At ten weeks of recovery after testicular irradiation, Wt irradiated rats displayed a significant increase of caspase-3 activity comparatively with the control group (133.3 ± 31.32 vs. 74.60 ± 10.49, $p<0.05$, Figure IV-11). However, no statistical significant difference was observed in Tg-RGN irradiated comparatively with the respective control. Moreover, the activity of caspase-3 was significantly lower in Tg-RGN irradiated animals in comparison with Wt irradiated (63.49 ± 6.86 vs. 133.3 ± 31.32, $p<0.05$).

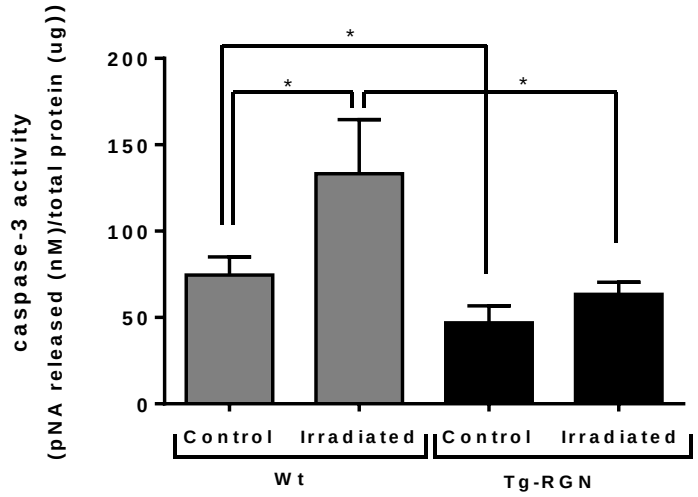

Figure IV-11. Effect of radiation treatment on caspase-3 activity in the testis of Wt and Tg-RGN animals, measured by spectrophotometric analysis. Error bars indicate mean ± S.E.M. (n≥5 in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

3.6 The expression of RGN is enhanced in both experimental groups after radiation treatment

RGN is expressed in Leydig and Sertoli cells, as well as in the entire germ cell line, both in human and rat (Laurentino et al. 2011). Besides its recognized role in the maintenance of intracellular Ca^{2+} homeostasis (Marques et al. 2014), RGN is also associated with the control of cell proliferation and apoptosis (Nakagawa and Yamaguchi 2005; Correia et al. 2014a; Vaz et al. 2014; Marques et al. 2015b).

The protein levels of RGN were enhanced both in Wt and Tg-RGN irradiated rats in comparison with the respective controls, respectively, 1.83 ± 0.17 ($p<0.01$) and 1.51 ± 0.16 ($p<0.05$) fold variation (Figure IV-12A). Moreover, as expected, it was confirmed the higher

expression of RGN in the testis of Tg-RGN animals relatively to their Wt counterparts (Figure IV-12B).

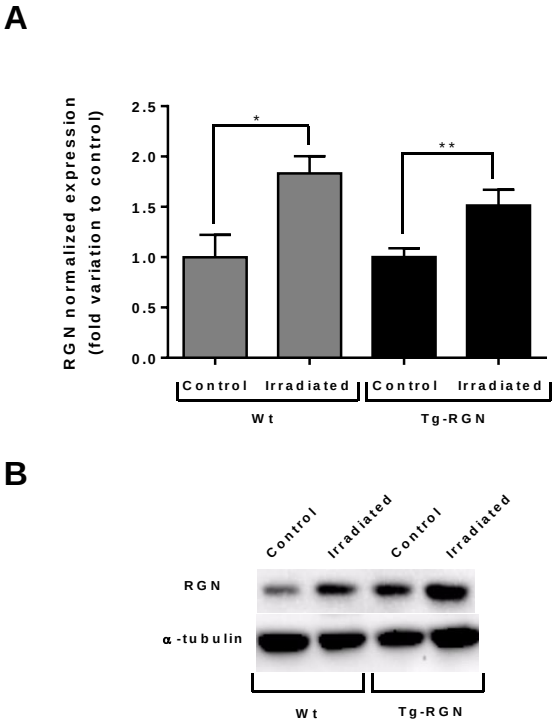

Figure IV-12. Effect of radiation treatment on the expression of RGN in the testis of Wt and Tg-RGN animals determined by WB analysis. Results are expressed as fold variation relatively to respective control group after normalization with α-tubulin. Representative immunoblots are shown in panel B. Error bars indicate mean ± S.E.M. (n≥5 in each group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the respective control group.

V. Discussion

The oncological status and the most common used therapies in cancer treatment are largely known as being deleterious for reproductive function, which is an issue of great concern for patients and clinicians. The increasing number of cancer diagnoses in patients under the age of 35 years, and the, fortunately, increasing survival rates (Knopman et al. 2010), turn the preservation of male fertility upon oncological treatments a matter of uttermost importance.

In late years, some endogenous factors have been identified as protective agents or having beneficial effects on the recovery of reproductive function after testicular injury and cancer. However, there is no established clinical strategy currently used to restrain the undesirable secondary effects of oncological treatments on male reproductive function.

RGN is widely expressed in male sexual tract and, among other functions, it is involved in suppression of cell proliferation and apoptosis (Nakagawa and Yamaguchi 2005; Correia et al. 2014a; Vaz et al. 2014; Marques et al. 2015b). In the testis, the tight control of germ cells survival and apoptosis maintains the proper ratio between developing germ cell and somatic supporting cells (Print and Loveland 2000), which is of paramount importance for a successful spermatogenesis (Orth et al. 1988). Thus, the present dissertation investigated the effect of RGN overexpression in spermatogenesis recovery after radiation treatment, by comparing the testicular status and the sperm parameters of Tg-RGN rats with their Wt counterparts.

Cancer therapies, namely radiation, frequently lead to a temporary disruption or a complete arrest of spermatogenesis (Bujan et al. 2013). Several variables can interfere with the gonadal injury sustained by radiation therapy, including total dose, source of radiation, gonadal protection, scatter radiation, and individual susceptibility (Williams 2013). Indeed, we observed that the exposure to a 6 Gy dose of X-rays provoked testicular and epididymal damage, as discussed below.

First of all, assessing the GI and TDI (Figure IV-1C and D, respectively), we confirmed the significant deleterious effect of radiation treatment in the testis weight and spermatogonial differentiation, as it has been previously reported (Topcu-Tarladacalisir et al. 2009; Li et al. 2014; Demir et al. 2015). In fact, the testis is one of the most radiosensitive organs in the body because of the high mitotic rate of the germinal epithelium. Thus, immature spermatogonia are the most sensitive to the adverse effects of radiation (Osterberg et al. 2014), whereas only high doses (>15 Gy) of radiation are able to affect Leydig cells (Colpi et al. 2004).

Although the prominent diminution of GI and TDI in both irradiated groups, the % of reduction was lower in Tg-RGN rats. Previously, our research group have demonstrated that RGN is expressed in spermatogonia (Laurentino et al. 2011), and considering that the GI and TDI were less affected in Tg-RGN animals, these results suggest that RGN may lead to an accelerated recovery of spermatogenesis. In order to evaluate the output of spermatogenic process, several parameters related with sperm quality were determined. The basic parameters used worldwide in standard testing of semen quality are sperm concentration, motility, viability and morphology (Cooper et al. 2010). Our results showed that Tg-RGN animals display lower sperm counts and motility under normal conditions (Figure IV-2 and

Figure IV-4A, respectively). On the other hand, a higher level of viable sperm (Figure IV-4B) and morphologically normal sperm (Figure IV-6) were observed in Tg-RGN rats. These results are in accordance with previous findings of our research group (Correia et al. 2013).

Although sperm count and motility are generally reduced in Tg-RGN, these animals had less damage in both parameters after radiation treatment (Figure IV-2 and Figure IV-4A, respectively). It should be noticed that motile sperm were found in Tg-RGN irradiated rats whereas no single motile sperm was found in Wt irradiated animals (Figure IV-4A). Concerning the sperm viability, similar results were observed, i.e., some viable sperm was found in Tg-RGN irradiated but not in Wt irradiated rats (Figure IV-4B).

Regarding sperm morphology (Figure IV-6), both Wt and Tg-RGN showed a higher incidence of abnormal sperm due to radiation treatment, which is a result that follows that described by other research groups (Bruce et al. 1974; Wyrobek 1979; Dobrzynska and Gajewski 2000). After radiation treatment, the percentage of head defects have risen sharply, which logically lead to a diminution in the percentage of less dangerous defects, i.e., bent neck and tail defects (Figure IV-6). Although no statistical significance in some results, it is important to underline that Tg-RGN rats displayed 26.1-fold higher percentage of normal sperm and 0.8-fold lower percentage of head defects than their Wt littermates under the same post-irradiation condition.

It is well described that the cellular content within the seminiferous tubules is controlled by an equilibrium between cell proliferation and death (Russell et al. 2002). Taking into account that RGN plays an important role in cell cycle and apoptosis (Nakagawa and Yamaguchi 2005; Correia et al. 2014a; Vaz et al. 2014; Marques et al. 2015b), the expression of several cell cycle and apoptosis modulators was evaluated.

Considering the tumor suppressor gene p53, a significant diminished expression was found in the testis of irradiated rats, but no differences were found between Wt and Tg-RGN irradiated animals (Figure IV-7A). The p53 plays different roles in cells, such as the regulation of cell-cycle inhibitor p21. Therefore, the levels of p21 were determined, and in agreement with p53 levels, low levels of p21 were observed in Wt and Tg-RGN irradiated rats when compared to respective control (Figure IV-7B). These results suggest that the radiation decrease the levels of p53 independently of RGN levels. As previously reported, after low-dose X-rays exposure, no p53 accumulation was observed in the testis, spleen and kidney of Wt mice (Wang et al. 1996).

Spermatogenic arrest is an interruption of male germ cell development at any stage before achievement of the complete maturation. The causes for spermatogenic arrest are primarily genetic or a result of secondary influences from other diseases, or iatrogenic (radiation, chemotherapy or certain antibiotics) (Jahnukainen and Stukenborg 2012). Attending to the diminished expression of p53 and p21 in the present work, it is liable to propose that a reactivation of cell cycle and probably a reinitiation of spermatogenesis might be occurring at ten weeks of recovery after radiation treatment.

One effect triggered by radiation is the massive spermatogonial apoptosis (Beumer et al. 1997), which can be divided into intrinsic (mitochondrial) or extrinsic (mediated by death receptors) (Shukla et al. 2012). However, a cross-talk occurs among the different pathways of germ cell apoptosis, and pathways that conventionally are thought to be activated by extracellular signals may also be integrated into the pathways that are activated by intracellular signals (Liu et al. 2007). Thus, the expression of factors involved in both apoptotic pathways was evaluated.

Our results showed no changes in the amount of testicular Bax protein after radiation treatment (Figure IV-8B), which is in accordance with what was reported by others (Beumer et al. 2000). Nevertheless, we found an increased expression of the antiapoptotic Bcl-2 protein (Figure IV-8A), and consequently, a significant increased Bcl-2/Bax ratio in response to radiation treatment both in Wt and Tg-RGN rats (Figure IV-8E). Comparing the effect of testicular irradiation between Wt and Tg-RGN, a ≈2.2-fold significant increase of Bcl-2/Bax ratio is observed in Tg-RGN rats. Once Bcl-2/Bax protein ratio is considered to be indicative of cell susceptibility to apoptosis (Mackey et al. 1998), the obtained results suggest a lower rate of apoptosis in the testes overexpressing RGN. In fact, several studies have pointed out that RGN turn the cells more resistant to apoptosis. Recently, our research group demonstrated that the *in vivo* overexpression of RGN protects testicular cells from apoptosis induced by thapsigargin or actinomycin D, which may support its role as a germ cell survival factor alone or as a mediator in androgen signaling pathways (Correia et al. 2014a). Others studies have described Bcl-2 as a protein involved in the inhibition of p53 upregulation in response to various stimuli (Reed 1994; Correia et al. 2014a), which also may explain the reduced levels of both p53 and p21 after irradiation simultaneously with the increased Bcl-2 levels.

Regarding the intrinsic pathway of apoptosis, it is described that FasL and FasR are mainly expressed in Sertoli and germ cells, respectively, and that their levels may respond to environmental conditions initiating germ cell death (Campion et al. 2010). Moreover, patients with postmeiotic germ cell arrest showed changes in the expression of FasR in germ cells, indicating the involvement of the FasR/FasL system in the control of quality of gametes production (Francavilla et al. 2002). At ten weeks of recovery after irradiation, the expression of FasR and FasL was completely abolished in Wt animals whereas in Tg-RGN a slight expression was detected (Figure IV-9). The statistically significant reduction of FasR/FasL system in both irradiated experimental groups is unexpected considering the recognized increase of apoptosis due to radiation exposure. Nevertheless, other studies have shown that caspase-8 can be activated by other death receptor/ligand system apart from the traditional FasR/FasL (Deng et al. 2002; Grataroli et al. 2004).

Comparing the effect of radiation between Wt and Tg-RGN animals, we demonstrated that the increase of capase-8 expression was lower in Tg-RGN (Figure IV-10), suggesting a diminished susceptibility to apoptosis in these animals. In fact, it was previously shown that

apoptotic-inducers, such as actinomycin D or tumor necrosis factor-α, induced the activity of caspase-8 in RGN knockout hepatocytes (Ishigami et al. 2002).

The execution of apoptotic cell-death depends on the activation of caspase-3, which is as remarkable end point of apoptosis (Lawen 2003). We demonstrated that radiation treatment increased the activity of caspase-3 in Wt but not in Tg-RGN rats (Figure IV-11). It is important to highlight that the reduced activity of caspase-3 in Tg-RGN animals is concordant with the low levels of caspase-8 and the increased Bcl-2/Bax ratio. Moreover, these findings suggest that Tg-RGN rats are more resistant to apoptosis in response to radiation. An earlier study in rat kidney epithelial cells also described that the RGN overexpression was associated with the modulation of expression of apoptosis-related genes, increasing the expression of Bcl-2 and declining the expression of caspase-3 (Nakagawa and Yamaguchi 2005). This study is also consistent with our present findings, indicating that RGN overexpression leads to lower caspase-3 activity and, consequently, might be able to repress apoptosis.

Considering that several studies indicated that apoptotic-stimuli increases the expression of RGN (Son et al. 2009; Fukui et al. 2011), we decided to evaluate the effect of radiation treatment in RGN expression in the testis. As expected, the expression of RGN increased in both Wt and Tg-RGN animals (Figure IV-12). In fact, our results are supported by previous studies showing that RGN is overexpressed in radioresistant cell lines established by fractionated irradiation (Ogawa et al. 2006). Also, another study in mice liver showed accelerated healing of radiation-induced injury concomitantly with significantly increased levels of RGN (Jeong et al. 2008).

Regarding the evidence about the role of RGN in repressing apoptosis triggered by different apoptosis inducers in various cells type (Izumi and Yamaguchi 2004; Nakagawa and Yamaguchi 2005), including rat testicular cells (Correia et al. 2014a), a suppressive effect on radiation-induced apoptosis modulated by RGN overexpression seems to be supported. Thus, these facts suggest the involvement of RGN in the anti-apoptotic response and its possible role in the development of a mechanism of resistance to apoptosis against external damage.

However, it remains unclear the mechanism underlying the protective role of RGN against radiation. It is well described that ionizing radiation, such as X-rays, results in the production of chemical and biological changes and the generation of reactive chemical species by stimulation of oxidases and NOSs (Azzam et al. 2012). Oxidative stress is one of major causes of defective sperm morphology and function (Aitken and Curry 2011), and it is suggested that the epididymal epithelium should be able to protect sperm against oxidative damage (Jervis and Robaire 2001). Recently, it was demonstrated that RGN play an important protective role against oxidative stress, by enhancing the activity of antioxidant SOD enzyme (Fukaya and Yamaguchi 2004) and reducing the generation of ROS (Handa et al. 2009). Thus, the protective effect of RGN on sperm parameters may possible be explained by the lower levels of oxidative stress previously found in biological models with RGN overexpression, which includes higher antioxidant potential in the epididymal fluid (Handa et al. 2009; Correia et al. 2013).

Nevertheless, further research, including oxidative assays, have to be performed in order to disclose the mechanism behind the protection of RGN against X-rays in male reproductive function.

VI. Conclusions and Future Perspectives

Reproductive problems with impairment of fertility are among the most common and distressing consequences of cancer treatment. In this thesis we confirmed that radiation treatment with ten weeks of recovery, still presented deleterious signals of X-rays injury at reproductive level. However, both epididymal sperm and spermatogenesis were less affected by radiation treatment in Tg-RGN, which also displayed a lower susceptibility to testicular apoptosis induced by X-rays. Thus, the present findings support the involvement of RGN in the anti-apoptotic response.

The present study strengthened the previous findings of our research group about the role of RGN protein in the male gonads. The protective effect of RGN against chemical inducers of apoptosis was recently showed, and this dissertation addressed that RGN also plays a role in the protection against radiation-induced testicular apoptosis. Altogether, the obtained results suggest that the overexpression of RGN had a protective effect against radiation-induced testicular injury, likely by suppressing both apoptosis and oxidative damage of testicular cells and epididymal sperm.

Despite further research is needed, the present discoveries raise the curiosity about the manipulation of RGN levels in the testes as a potential mechanism to preserve fertility in male patients undergoing oncological treatment. In a near future, disclosing the molecular mechanisms behind the suppressive action of RGN in radiation-induced apoptosis in male reproductive tissues, as well as to understand how to enhance its protective effects, will be of uttermost importance to identify solutions for the current oncofertility concerns. Furthermore, it is expected that such promising properties of RGN would also be employed to develop simpler and less expensive strategies of ART.

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VIII. Publications and Communications

1. Publication in International Peer-Reviewed Journal

Silva A.M.S., Correia S., Socorro S.[1], Maia C.J.[1] Endogenous factors in the recovery of reproductive function after testicular injury and cancer. *Current Molecular Medicine* (submitted, under revision). IF: 3.621

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2. Oral Communication

Silva A.M.S.[#], Correia S.[#], Casalta-Lopes J.E., Mamede A.C., Cavaco J.E.B., Botelho M.F., Maia C.J.[*], Socorro S.[*] The protective effect of regucalcin against radiation-induced testicular damage. *X Annual CICS-UBI Symposium*. 6th and 7th July 2015, Covilhã, Portugal.

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