UNIVERSIDADE BEIRA INTERIOR

Regucalcin as one of the guardians of the male reproductive function

Ana Manuela dos Santos Silva

Tese para obtenção do Grau de Doutor em
Biomedicina
(3º ciclo de estudos)

Orientador: Prof. Doutor Cláudio Jorge Maia Baptista
Coorientadora: Doutora Sara Carina de Lima Correia

outubro de 2020

Data de realização das provas:

- 15 de outubro de 2020.

Constituição do júri:

- Doutor Miguel Castelo Branco Craveiro de Sousa, professor catedrático da Universidade da Beira Interior;

- Doutora Sílvia Cristina da Cruz Marques Socorro, professora catedrática da Universidade da Beira Interior;

- Doutor Cláudio Jorge Maia Baptista, professor auxiliar da Universidade da Beira Interior;

- Doutora Sandra Maria Tavares da Costa Rebelo, professora auxiliar da Universidade de Aveiro;

- Doutora Maria Alexandra Barreto Amaral, investigadora especialista do *Max Planck Institute for Molecular Genetics*, Berlim, Alemanha;

- Doutora Sandra Filipa Tomás Sequeira Laurentino, investigadora principal da *Westfälische Wilhelms-Universität Münster*, Alemanha.

Dedication

To all who face distressing reproductive problems.

To all the curious and brave researchers that keep refusing to quit in spite of the obstacles.

To all those who do science with a sparkle in their eyes, a contagious smile, and a cheerful spirit.

To all the talented people that bring out the best in me at both scientific and personal level.

To my loving and inspiring family.

“The important thing is not to stop questioning. Curiosity has its own reason for existence.”

— Albert Einstein

“One of the beautiful things about science is that it allows us to bumble along, getting it wrong time after time, and feel perfectly fine as long as we learn something each time.”

— Martin A. Schwartz

“Somewhere, something incredible is waiting to be known.”

— Carl Sagan

Acknowledgments

First of all, my greatest acknowledgments must go to Professor Cláudio Maia, my supervisor, and to Doctor Sara Correia, my co-supervisor, who have always shown interest and dedication. I would like to express my sincere gratitude for being so available and for turning all of our brainstorming sessions into a masterpiece. Also, for all the knowledge you have shared with me. I deeply appreciate all the suggestions and constructive criticism without which it would have been impossible to evolve. For having guided me and also for teaching me how to uncomplicate things. I shall highlight the scientific competence and rigor, which have motivated me to overcome myself. No words can express how thankful I am to you to have supported my strong will to learn more about male reproduction, allowing my first steps in this incredible branch of research. For having gifted me with new ideas when I felt stuck and for accepting mine so well, allowing me to grow as researcher.

I would like to extend a great word of gratitude to Professor Sílvia Socorro, who regardless of not being an official element of the supervisory team, had a vital contribution during all these years. I am grateful for all the shared knowledge, wise insights, and interest in our results. I also would like to thank the opportunity and trust to have participated in several other works that enriched me so much on so many levels.

To the laboratory technicians from the Health Sciences Research Centre, namely Sofia Duarte, Margarida Carrilho, Maria José Eusébio, Maria João Silva and Dulce Anastácio for their constant kindness, flexibility and availability. To Catarina Ferreira and Ana Borges for their precious technical assistance in the histological processing of samples, and tireless help in acquisition of fluorescence photomicrographs, as well as for their availability and enthusiastic energy together with useful tips and advice.

To Professor Filomena Botelho, Doctor João Casalta, Doctor Ana Mamede and Professor José Eduardo Cavaco whose collaboration was crucial to make part of this project viable.

To the financial support provided by Santander Totta (Bolsa BID/ICI-FCS/CICS/Santander Universidades-UBI/2016). Especially, to Professor Ana Paula Duarte and her team for all the collaboration, shared know-how and sympathy.

To the researchers from the Health Sciences Research Centre and to everyone at the Faculty of Health Sciences of the University of Beira Interior who contribute daily to a friendly work environment.

I am extremely grateful to my laboratory colleagues Cátia, Ricardo, Luís, Henrique, Marília, Tânia, Mariana, Roberta, Tiago, Inês, Margarida, Carlos, Gonçalo, Alexandra, Anabela, Rodrigo, Ana Hurtado, Rúben, Filipe, Sandra, Ziyanda, among many others, with whom I had the pleasure to work with. Thank you very much for the invaluable moments we had even when nothing seemed to work at

all. Furthermore, I have to refer to all the shared knowledge, particularly in experimental techniques, team spirit, outstanding exchange of scientific opinions, optimism, and mutual encouragement. Each one of you have left a great mark.

To my closest friends (you know who you are!), I will be forever in debt for everything you have ever done. To those who accompanied me since always, and those I made during the last years. This hard journey has shown me who really supports me no matter what happens, and I couldn't feel more blessed. I am so fortunate to have true friends like you. Thank you for all your patience, understanding, motivation, sweet and eye-opening words, empathy, for being there when I needed the most, and for never ever letting me doubt my purpose.

To my parents and grandparents, for your unconditional love, care, and support that I will never be able to describe properly. You are the biggest gift I could ever receive. Definitely, you are the main booster to make me pursue my dreams with persistence, always keeping my feet on the ground. Thank you for having taught me to think by myself and to remain faithful to my own ideals. You are my foremost example.

Finally, to every single person who somehow imparted to me any kind of knowledge throughout life, thereby contributing to my personal and professional education, even if not mentioned herein. No matter if you had cross paths with me during a time in which I was far away from beginning this scientific expedition or during it, you have my sincere appreciation. This work is also yours.

Preface

Once upon a time, in a small town touching the sky, a young girl who was just a master's student had the chance to work in the field of male reproduction …

Well, that girl is me! And I still remember like it was today that in that year there was only one project proposal available in this area. The truth is that I feared that I would have to go for another project, because, honestly, I didn't want to choose anything else. In fact, by that time, I already knew, from the bottom of my heart, that reproductive biology was my predilection. Now, some years later, it is even clearer that I have totally fallen for it, and there is no way out. My passion about the mechanisms behind reproductive processes grows, for example, every time I read a new paper on the topic. It is just amazing how it could be so well coordinated, even mind-blowing sometimes. Also, it is really funny when you can watch the sperm swimming or when you have the terrific opportunity to see so beautiful images from sperm or all the male germ line in testicular sections, either assisted by optic or fluorescence microscopy. And then, I think to myself that I am really lucky to study something I really enjoy. You know when you just contemplate? Well, I use to be like that, forgetting the clock for a moment, just letting myself be amazed by such stunning images … But right after, I hear a voice on my mind saying "Keep focus, Ana!"

However, it was not only about happiness and joy. I have been through a lot of ups and far more downs along this process. I have always believed in the importance of tiny progress and celebrating the small triumphs (sometimes some jumps and hugs in the lab for 2 minutes are enough), and now I am absolutely sure that they are a huge booster of this work. Perhaps the most significant conviction of my own that I confirmed during these years was that, in science, as so in life, we have to readjust accordingly as many times as needed.

Finally, I hope that these years of hard work may serve its ultimate true goal. Indeed, I will be genuinely happy that someday the knowledge herein generated may humbly contribute to minimize the rising trend of infertility which undeniably affects our society. This is my way to show empathy and respect, and also to give somehow hope and strength to those people.

And there is still so much more to do …

Resumo

A função reprodutora masculina é altamente sensível quer a danos externos quer a modificações internas que, consequentemente, afetam a espermatogénese levando ao declínio da qualidade espermática e ao comprometimento da fertilidade. Estas alterações a nível reprodutor podem ser devidas a um efeito colateral resultante, por exemplo, de um tratamento oncológico, ou ser uma consequência natural da vida, como o envelhecimento. Devido ao atual diagnóstico precoce e à alta taxa de sobrevivência, os doentes oncológicos jovens têm vidas mais longas. No entanto, é necessária a identificação de estratégias que diminuam com sucesso os efeitos secundários indesejáveis provocados pelos tratamentos oncológicos. Além disso, até há pouco tempo, o declínio da função reprodutora masculina com o avançar da idade era frequentemente negligenciado. Ultimamente, dada a tendência da sociedade moderna para adiar a conceção de um filho biológico, a diminuição da qualidade espermática em indivíduos mais velhos tem vindo a atrair a atenção da comunidade científica. Portanto, estes são assuntos emergentes na área da fertilidade masculina. Sabe-se que o stresse oxidativo (OS) está, por si só, associado a casos de infertilidade masculina. Além disso, sabe-se ainda que o OS está também implicado na radioterapia e no envelhecimento. Nesta tese de doutoramento, pretendeu-se desvendar o potencial da proteína regucalcina (RGN) para contrariar os efeitos nefastos da radioterapia testicular, bem como para atenuar as alterações relacionadas com a idade que ocorrem no tracto reprodutor masculino. Nos últimos anos, a proteína RGN tem vindo a mostrar ser importante na espermatogénese e na fertilidade masculina, estando envolvida na supressão quer de estímulos pró-oxidantes quer da apoptose induzida quimicamente nos túbulos seminíferos. A RGN está amplamente presente nas células testiculares, e diversos estudos apontam-lhe inúmeras funções biológicas. Para além disso, a RGN é também conhecida como Proteína Marcadora da Senescência-30 (SMP-30), resultado da diminuição da sua expressão com o envelhecimento. Recentemente, elevados níveis de RGN amenizaram alterações associadas ao envelhecimento na próstata. Recorrendo a uma abordagem *in vivo*, na presente tese estudou-se o papel da RGN comparando ratos transgénicos que sobre-expressam a RGN (Tg-RGN) com os seus homólogos selvagens (Wt). Dez semanas após os ratos terem sido sujeitos a radioterapia testicular, verificou-se que a RGN foi capaz de mitigar o dano testicular induzido pela radiação, visto que os animais Tg-RGN apresentaram parâmetros espermáticos menos afetados, bem como uma menor taxa de apoptose testicular comparando com os ratos Wt. Além disso, os animais Tg-RGN apresentaram também parâmetros espermáticos não afetados e níveis mais baixos de OS apesar do envelhecimento. Curiosamente, detetou-se uma expressão aumentada da RGN no testículo irradiado e no espermatozoide senescente quer nos ratos Wt quer nos Tg-RGN, o que reforça o envolvimento da RGN na resposta a estímulos ou modificações que possam afetar negativamente a reprodução masculina. De forma geral, estes resultados representam uma forte evidência relativamente ao papel benéfico da RGN como uma guardiã da função reprodutora masculina, sugerindo que esta proteína tem elevado potencial para ser incluída em estratégias para melhorar, proteger e/ou recuperar a fertilidade masculina em homens submetidos a tratamento oncológico ou com idade avançada que pretendam ainda ter descendência biológica.

Palavras-chave

Fertilidade masculina; Regucalcina; Radioterapia; Apoptose; Envelhecimento; Stresse oxidativo; Qualidade espermática; Preservação da fertilidade.

Resumo Alargado

A função reprodutora masculina é altamente sensível quer a danos testiculares externos quer a modificações intrínsecas que, consequentemente, afetam a espermatogénese levando ao declínio da qualidade espermática e ao comprometimento da fertilidade. Estas alterações a nível reprodutor podem ser devidas a um efeito colateral resultante, por exemplo, de um tratamento oncológico, ou ser uma consequência natural da vida, como o envelhecimento. Atualmente, devido ao diagnóstico precoce e à alta taxa de sobrevivência, os doentes oncológicos jovens têm vidas mais longas. No entanto, é necessária a identificação de estratégias que diminuam com sucesso os efeitos secundários indesejáveis provocados pelos tratamentos oncológicos. A radioterapia, que é uma das opções terapêuticas no cancro testicular, já demonstrou ser nociva para a fertilidade masculina, uma vez que os testículos são dos órgãos mais sensíveis à radiação dada a elevada taxa de divisão das células germinativas. Além disso, até há pouco tempo, o declínio da função reprodutora masculina com o avançar da idade era frequentemente negligenciado. O impacto da idade do homem no sucesso da conceção de uma criança é menor quando comparado com o impacto da idade da mulher. No entanto, existem evidências que a idade do homem também é relevante e deve ser tida em conta. Em casais com fertilização natural, com homens com idade superior a 40 anos, verificou-se que o início da gravidez era mais tardio e também a existência de dificuldades de conceção. Além disso, dada a tendência atual da sociedade moderna para adiar a conceção de um filho biológico, a diminuição da qualidade espermática em indivíduos mais velhos tem vindo a atrair a atenção da comunidade científica. Considerando a taxa de incidência do cancro testicular, que é mais frequente nos jovens, bem como a sua elevada taxa de sobrevivência, a maioria dos doentes ambiciona ainda ter filhos no futuro, levando a que os problemas reprodutivos sejam considerados pelos doentes uma das consequências mais frequentes e angustiantes do tratamento oncológico. Por outro lado, nos últimos anos tem-se verificado uma tendência para adiar a paternidade, apesar da diminuição das valências reprodutivas. Portanto, estes são assuntos emergentes na área da fertilidade masculina, tornando-se necessária a identificação de estratégias para a proteger, melhorar e/ou recuperar. Sabe-se que o stresse oxidativo (OS) está, por si só, associado a casos de infertilidade masculina. Além disso, sabe-se ainda que tanto a radioterapia como o envelhecimento provocam um aumento de OS. Nesta tese de doutoramento, ambicionou-se principalmente averiguar se a proteína regucalcina (RGN) tinha potencial para contrariar os efeitos nefastos da radioterapia testicular, bem como para atenuar as alterações relacionadas com a idade que ocorrem no tracto reprodutor masculino. A regucalcina (RGN) é uma proteína de ligação ao cálcio (Ca^{2+}) que se encontra amplamente expressa no sistema reprodutor masculino quer em rato quer em humano, incluindo nas vesículas seminais, epidídimos e testículos. Na última década testemunhou-se a emergência da RGN como uma proteína multifuncional, estando envolvida na regulação de vários processos biológicos para além da manutenção da concentração intracelular de Ca^{2+} ($[Ca^{2+}]_i$). 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 da RGN teve efeitos benéficos na espermatogénese, nomeadamente na supressão da apoptose induzida por fármacos como a tapsigargina ou a actinomicina D, e também na

inibição de OS induzido por hidroperóxido de *tert*-butilo e cloreto de cádmio. Embora já tivesse sido demonstrado que a RGN é regulada positivamente em linhas celulares pancreáticas radiorresistentes, sugerindo que esta proteína pode proteger de danos causados pela radiação, desconhecia-se o seu comportamento em células testiculares irradiadas. Para além disso, a RGN é também conhecida como Proteína Marcadora da Senescência-30 (SMP-30), resultado da sua regulação negativa com o aumento da idade em vários tecidos, inclusive em tecidos do sistema reprodutor masculino, tais como a próstata e o testículo. Recentemente, o nosso grupo de investigação demonstrou que elevados níveis da RGN amenizaram alterações relacionadas com o envelhecimento, melhorando as defesas antioxidantes e controlando a apoptose e a proliferação na próstata, o que indica que a RGN pode ter um papel na atenuação da normal deterioração da função celular devida ao envelhecimento. Recorrendo a uma abordagem *in vivo*, na presente tese estudou-se o papel da RGN comparando ratos transgénicos que sobre-expressam a RGN (Tg-RGN) com os seus homólogos selvagens (Wt). Neste sentido, ratos com três meses de idade, quer Tg-RGN quer Wt, foram submetidos a uma dose única de 6 Gray (Gy) de raios-X, tendo-se posteriormente aguardado um período de recuperação de 10 semanas. Parâmetros como a motilidade e a viabilidade dos espermatozoides parecem ter sido menos afetados pela radiação nos ratos Tg-RGN comparando com os ratos Wt. Quanto à morfologia dos espermatozoides, também parece ter sido menos afetada pela radiação nos Tg-RGN, uma vez que se observou uma maior percentagem de espermatozoides morfologicamente normais e uma reduzida percentagem de defeitos de cabeça nos espermatozoides dos animais Tg-RGN quando comparados com os Wt. Estes resultados nos parâmetros espermáticos foram sustentados por uma menor taxa de apoptose testicular, evidenciada por uma inferior atividade da caspase-3, que é um reconhecido ponto comum à via intrínseca e extrínseca da apoptose, assim como pelo rácio aumentado da expressão das proteínas Bcl-2 (anti-apoptótica)/Bax (pró-apoptótica). Curiosamente, verificou-se ainda que a exposição à radiação aumentou significativamente a expressão da RGN nos dois grupos irradiados, o que suporta o envolvimento desta proteína na resposta anti-apoptótica. No seguimento desta experiência que demonstrou o papel positivo da RGN contra o efeito nefasto da radiação ao nível testicular, recorrendo a ratos jovens-adultos e senescentes, verificou-se que a RGN desempenha também um papel crucial perante as alterações induzidas pelo envelhecimento. Verificou-se, nos animais Tg-RGN, a manutenção do índice gonado-somático (GSI) e da contagem de espermatozoides, assim como a manutenção dos níveis normais de expressão testicular dos importantes marcadores de proliferação celular *stem cell factor* (SCF), c-Kit, e Akt, apesar do aumento da idade. O facto de não ter ocorrido alteração significativa destes parâmetros sugere que nos Tg-RGN senescentes houve preservação do potencial espermatogénico com o avançar da idade. Já nos ratos Wt, verificou-se que o envelhecimento originou a desregulação dos referidos parâmetros. Os ratos Tg-RGN senescentes apresentaram maior viabilidade espermática, maior percentagem de espermatozoides morfologicamente normais, bem como menor percentagem de espermatozoides com defeitos de cabeça e de colo/peça intermédia comparativamente com ratos Wt, simultaneamente com níveis inferiores de peroxidação lipídica e um menor estado oxidante total que podem, por sua vez, justificar o menor impacto do envelhecimento na qualidade espermática dos Tg–RGN. É de realçar que, surpreendentemente, observou-se também um aumento da expressão da RGN nos espermatozoides dos animais senescentes, o que vem desafiar o nome de SMP-30 pelo qual também é conhecida esta proteína. Resumindo, os resultados obtidos nesta

tese indicam que a sobre-expressão da RGN atuou como protetora das células germinativas contra o dano induzido pela radiação, provavelmente por suprimir a apoptose das células testiculares, o que vem reforçar o papel desta proteína como citoprotetora contra o dano provocado por agentes externos. Além disso, estes resultados representam novas pistas acerca da ligação da RGN ao processo de envelhecimento, desta vez ao nível reprodutor masculino, uma vez que a sobre-expressão da RGN parece minimizar o declínio da qualidade espermática e alterações testiculares típicas do aumento da idade. Assim, tendo em conta os principais resultados desta tese, é plausível apontar a RGN como uma guardiã da função reprodutora masculina, dada a ação fulcral demonstrada contrariando ameaças à reprodução masculina. Apesar de ainda ser necessária mais investigação no sentido de estudar como tirar o máximo partido das promissoras propriedades da RGN, a presente tese constitui uma base fundamental que levanta as expetativas no que respeita à futura inclusão desta proteína em estratégias clínicas para melhorar, proteger e/ou recuperar a fertilidade de doentes do sexo masculino submetidos a tratamentos oncológicos ou de homens que desejem, por diversos motivos, ser pais em idade mais tardia.

Abstract

Male reproductive function is highly sensitive to extrinsic testicular damage or intrinsic modifications, which consequently affects spermatogenesis leading to declined sperm quality and compromised fertility. These reproductive modifications can represent a collateral effect, for example from an oncological therapy, or a natural consequence of living, such as ageing. Currently, due to early diagnostic and high survival rate, young cancer patients are living longer. Nevertheless, approaches to successfully restrain the undesirable secondary effects of oncological therapies are still missing. Furthermore, until recently, the decline in male reproductive function with the advance of age was frequently neglected. Lately, given the trend of modern society to delay the conception of a biological child, the decrease in sperm quality in older subjects has been drawing attention from the scientific community. Indeed, these problems are emerging topics on male fertility. Oxidative stress (OS) is known to be for itself associated with male infertility cases. Also, OS is implicated in radiotherapy and ageing. In this doctoral thesis, we aimed to disclose the potential of regucalcin (RGN) protein to counteract the damaging effects of testicular radiotherapy as well as to attenuate the ageing-associated changes in male reproductive tract. In recent years, RGN protein has been showing to be an important player in spermatogenesis and male fertility, managing the suppression of pro-oxidant and chemical apoptotic stimulus in seminiferous tubules. RGN is a protein widely expressed in testicular cells, and its biological function seems to be vast. Moreover, RGN is also known as Senescence Marker Protein-30 (SMP-30) as a result of its decreased expression with ageing. Recently, high levels of RGN prevented ageing-associated changes in the prostate. Using an *in vivo* approach, we studied the role of RGN by comparing transgenic rats overexpressing RGN (Tg-RGN) with their wild-type (Wt) counterparts. Ten weeks after rats were subjected to radiotherapy, we verified that RGN was able to mitigate radiation-induced testicular damage because Tg-RGN animals presented less affected sperm parameters as well as lower rate of testicular apoptosis in comparison with Wt rats. Moreover, Tg-RGN animals also presented unaffected sperm parameters in spite of the ageing concomitantly with lower OS levels. Interestingly, we detected an enhanced expression of RGN in irradiated testis as well as in senescent sperm both in Wt and Tg-RGN rats, reinforcing the involvement of RGN in response to stimuli or modifications that can negatively impact male reproduction. Overall, our results are strong evidence about the beneficial role of RGN as a guardian of male reproductive function, suggesting that RGN protein has great potential to be included in strategies to improve, protect, and/or recover male fertility in men undergoing oncological treatment or with advanced age who still intend to have biological descent.

Keywords

Male fertility; Regucalcin; Radiotherapy; Apoptosis; Ageing; Oxidative stress; Sperm quality; Fertility preservation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

$[Ca^{2+}]i$	Intracellular calcium concentration
3D	Three-dimensional
AMPK	Adenosine monophosphate-activated protein kinase
ART	Assisted Reproductive Techniques
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CDK	Cyclin-dependent kinase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CREM	cAMP-response-element modulator
CT	Computerized tomography
DHEA	Dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DTT	Dithiothreitol
E_2	17β-estradiol
EDS	Ethane dimethane sulfonate
EGF	Epidermal growth factor
FasL	Fas ligand
FasR	Fas receptor
FSH	Follicle-stimulating hormone
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GHS-R	GH secretagogue receptor
GnRH	Gonadotropin releasing hormone
GPX	Glutathione Peroxidase
GSI	Gonadosomatic index
Gy	Gray
H&E	Hematoxylin and eosin
HBSSf	Filtered Hank's buffered salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HP	Hypospermatognesis
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IL	Interleukin
LGF	Liver growth factor
LH	Luteinizing hormone
LPO	Lipid peroxidation
M	month-old
MDA	Malondialdehyde
Mg^{2+}	Magnesium
Mn^{2+}	Manganese

MT-I	Metallothionein-I
MT-II	Metallothionein-II
MT-III	Metallothionein-III
MTs	Metallothioneins
NC	Negative controls
NO	Nitric oxide
NOS	Nitric oxide synthases
OS	Oxidative stress
PBA	PBS containing 1% (w/v) BSA
PBS	Phosphate buffer saline
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
pNA	p-nitro-aniline
PVDF	Polyvinylidene difluoride
RGN	Regucalcin
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMP-30	Senescence Marker Protein-30
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TDI	Tubular differentiation index
Tg-RGN	Transgenic rats overexpressing RGN
TOS	Total oxidant status
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
Wt	Wild-type
Zn^{2+}	Zinc

Chapter I
Male Reproductive Anatomy and Physiology

1. Overview of the testicular structure and physiology

1.1 Testicular 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 [1].

In mammals, the central functions of the testes are: 1) steroid synthesis and secretion; and 2) spermatozoa production [2]. The structural organization of testes determines the physical division of their dual function. The interstitial area is coated by a tough fibrous membrane called tunica albuginea (Figure I.1) [3]. Also, there is 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 [1, 4]. 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) [4, 5]. Seminiferous tubules are highly convoluted structures (Figure I.1) and represent the functional units of the testis. Spermatogenesis occurs within the seminiferous tubules (avascular compartment) whereas steroidogenesis takes place in the interstitium (vascularized region). These testicular functions are coordinated by the communication between hormone and gamete-producing compartments [6].

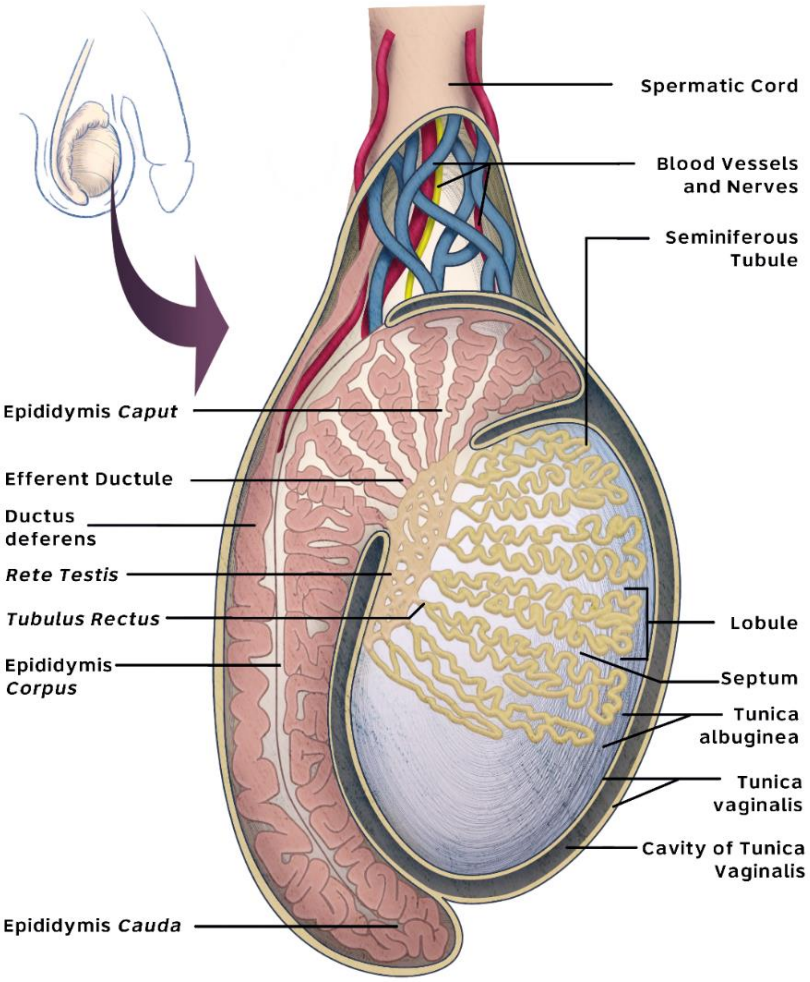

Figure I.1. Schematic representation of the mammalian testis and its anatomical relationship with the epididymis. The testis is encased by two tissue layers, from the inside to the outside, tunica albuginea and tunica vaginalis. Various septa extending from the tunica albuginea divide the testis in lobules, 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 receives testicular secretions by several efferent ductules. The spermatic cord contains the ductus deferens, blood vessels, and nerves (adapted from [7]).

The inside of each seminiferous tubule 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 (Figure I.2) [8]. Externally, the seminiferous tubules are surrounded by mesenchymal cells, including the peritubular myoid cells whose contractile elements produce peristaltic waves along the tubules [9, 10]. 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) [11]. The seminiferous tubules, which contain germ cells in different stages of development and Sertoli cells (Figure I.2), represent about 80% of the testicular mass [12-14]. The somatic Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact (Figure I.2) [15]. The cytoplasm of Sertoli cells extends as thin arms around the germ cells, spanning the thickness of the seminiferous epithelium. These cells are involved in several steps of spermatogenesis by providing structural and nutritional support to the germ cells [15, 16]. 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.2) [16].

The interstitium contains blood and lymphatic vessels and various cell types, including fibroblasts, leukocytes and macrophages. [12, 13]. The somatic Leydig cells also are located in the interstitial space between seminiferous tubules and have an endocrine function [17]. The major source of the testosterone are the Leydig cells [17], 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 [14, 18].

1.2 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 [19]. Sperm cells develop from the primordial germ cells and move towards the lumen of seminiferous tubule as they undergo a series of mitosis followed by the meiotic divisions (Figure I.2) [20, 21].

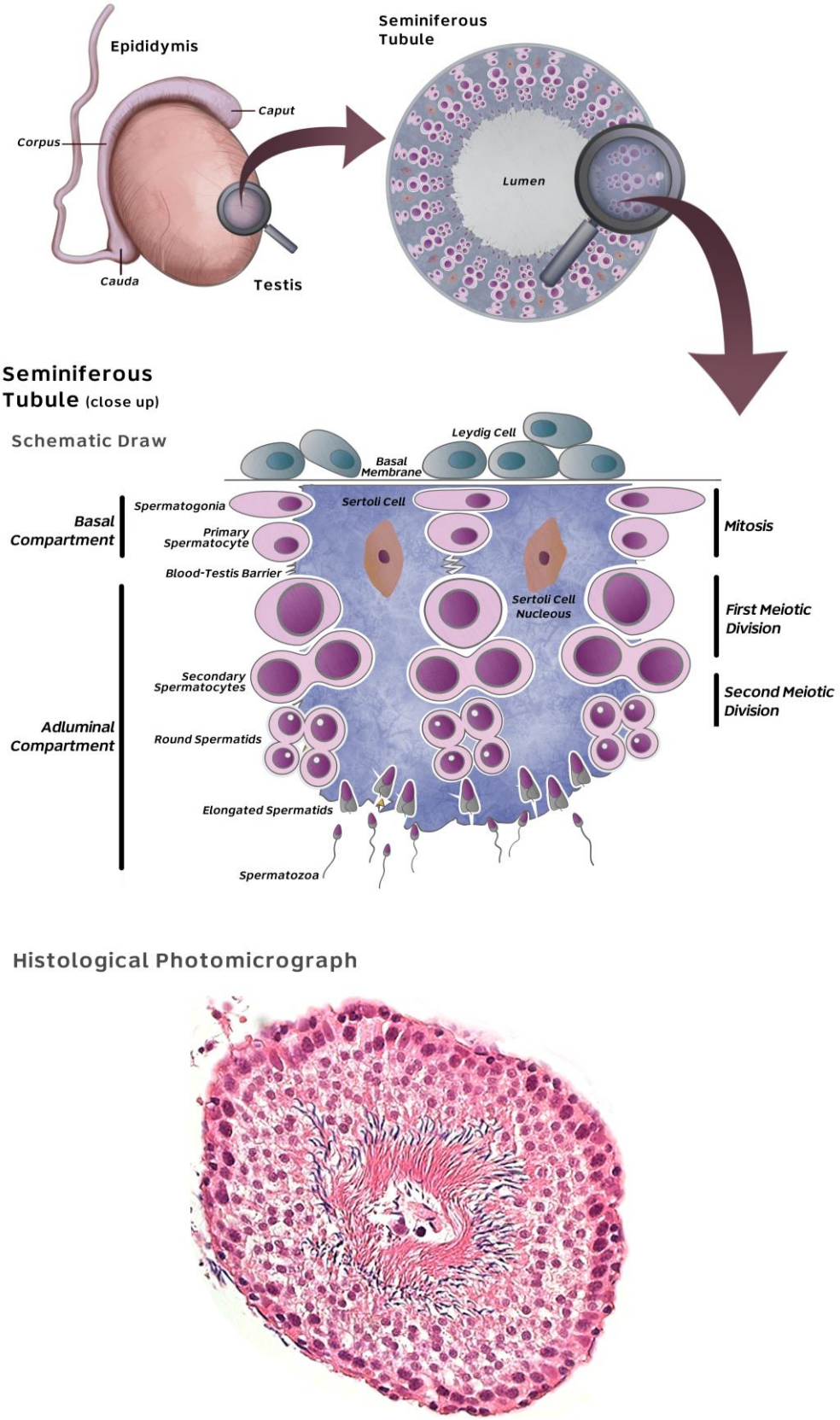

Figure I.2. Schematic drawing of the mammalian spermatogenesis and histological photomicrograph of rat testis. The anatomic relationship between epididymis and testis as well as mammalian spermatogenesis are schematically drawn. The distinct functional regions of the epididymis are also illustrated. A representative photomicrograph of rat testicular section stained with hematoxylin and eosin (H&E) evidencing the histology of a differentiating seminiferous tubule (400x magnification; Zeiss) is shown below.

The expression of a large number of genes is developmentally regulated during spermatogenesis [22], with both transcriptional and translational control mechanisms being responsible for temporal and stage-specific expression pattern [23]. Each spermatogenic cycle in the seminiferous tubules comprises three main phases: mitosis, meiosis, and the final stage of cell differentiation, spermiogenesis [19]. Spermatogonial stem cells are localized at the basal membrane of the seminiferous tubules as single cells and upon division originate daughter cells, the spermatogonia (Figure I.2) [8]. Spermatogenesis (Figure I.2) begins with the proliferation of diploid spermatogonia and, after a species-specific fixed number of mitotic divisions, spermatogonia differentiate into diploid primary spermatocytes [24]. These proceed to the first division of meiosis resulting in haploid 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 (Figure I.2) [19].

The spermatogenesis process has many common features between rodent and human [21]. 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 [25].

1.3 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 signalling mechanisms [26]. The steroid hormones, androgens and estrogens, produced by the gonads, are normally referred to as sex steroid hormones because of their primary role in sex differentiation, gonadal function and germ cell development. The central player in the hormonal control of spermatogenesis is the hypothalamic-pituitary-gonadal axis [26].

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 testosterone, while FSH acts on Sertoli cells inducing the production of several growth factors and other stimulatory factors required for spermatogenesis [27, 28], which includes the androgen-binding protein (ABP). Moreover, the cytochrome P450 aromatase enzyme, which convert testosterone to estrogens, is produced by Sertoli cells due to FSH stimulation [29, 30] (Figure I.3).

Testosterone is the most abundant androgen in mammals, although other androgens such as androstenedione or dehydroepiandrosterone (DHEA) may circulate in significant amounts [31]. DHEA and androstenedione are considered weaker androgens that can be converted to testosterone [32]. Also, testosterone can be converted to 5α-dihydrotestosterone (DHT), which is more potent than testosterone and some of the androgenic control of spermatogenesis may be via DHT, in the cytosol of target cells by the enzyme 5α-reductase [30, 31]. ABP is secreted into the fluid in the tubular lumen, and has a high affinity for androgens hence binding specifically to DHT and testosterone [30, 33]. 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 [33].Testosterone and ABP are released along with sperm and transported from testis to epididymis [31].

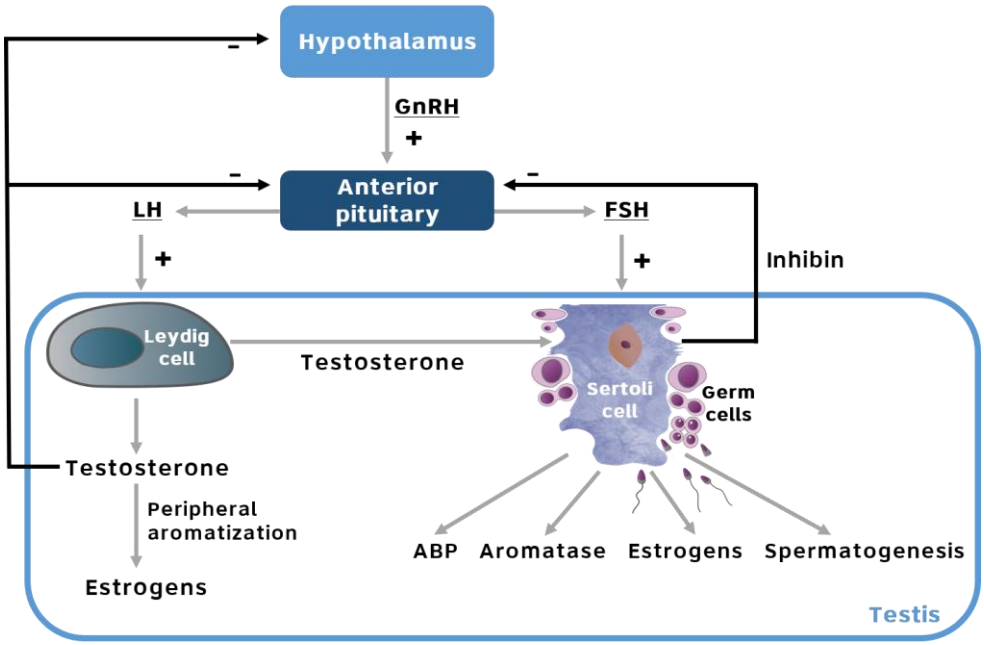

Figure I.3. Schematic representation of the hormonal regulation of spermatogenesis. Leydig cells secrete testosterone in response to the circulating luteinizing hormone (LH). Then, testosterone diffuses into the seminiferous tubules and in Sertoli cells can be converted to estrogens by the aromatase enzyme. Testosterone 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), binding to Sertoli cells, functions together with testosterone to maintain the germ cells and help them to advance through the stages of spermatogenesis. FSH also acts on Sertoli cells to induce the production of inhibin, ABP, and aromatase. Finally, inhibin, estrogens, and testosterone enter the outgoing blood and exert a negative feedback (-) on gonadotropin secretion. Note that estrogens produced by Sertoli cells could stimulate male germ cells directly.

Moreover, testosterone 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 [27, 28, 34]. Besides, testosterone regulates the spermatogenic process by a negative feedback mechanism on the hypothalamus and pituitary inhibiting, respectively, the release of GnRH and LH [26]. Other negative feedback regulatory mechanism is driven by inhibin, which is a member of the transforming growth factor β superfamily, produced by Sertoli cells in response to FSH (Figure I.3). Inhibin represses the production and release of FSH by the pituitary [35, 36] 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 [29, 37]. The rodent and human testis express nuclear and membrane estrogen receptors, and also actively synthesize estrogens, including 17β-estradiol (E_2), which is the most potent of estrogens [29]. The synthesis of estrogens occurs through the aromatization of androgenic precursors by the cytochrome P450 aromatase enzyme [29, 38]. Moreover, a little amount of testosterone (0.1%) is aromatised to estrogens in peripheral tissues (e.g.: fat, bone, brain, mammary gland) [39]. Estrogens can have a direct action on Leydig cells down-regulating the expression of steroidogenic enzymes involved in

testosterone biosynthesis [40]. Since germ cells express estrogens receptors, estrogens produced by Sertoli cells are able to stimulate them directly [41]. Estrogens also can act on the hypothalamus or pituitary exerting a negative feedback, suppressing the production of GnRH and LH, and consequently, decreasing the testosterone levels [42, 43]. Figure I.3 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 [44]. Testicular germ cell apoptosis happens normally and continuously throughout the life [45]. High rates of apoptosis have been associated with the first waves of spermatogenesis [46], and the germ cells that do not achieve the full maturity are more susceptible to die in response to numerous factors [47]. 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 [48]. 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 [49-51]. 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 [52]. 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 [44, 53]. 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.4 [48].

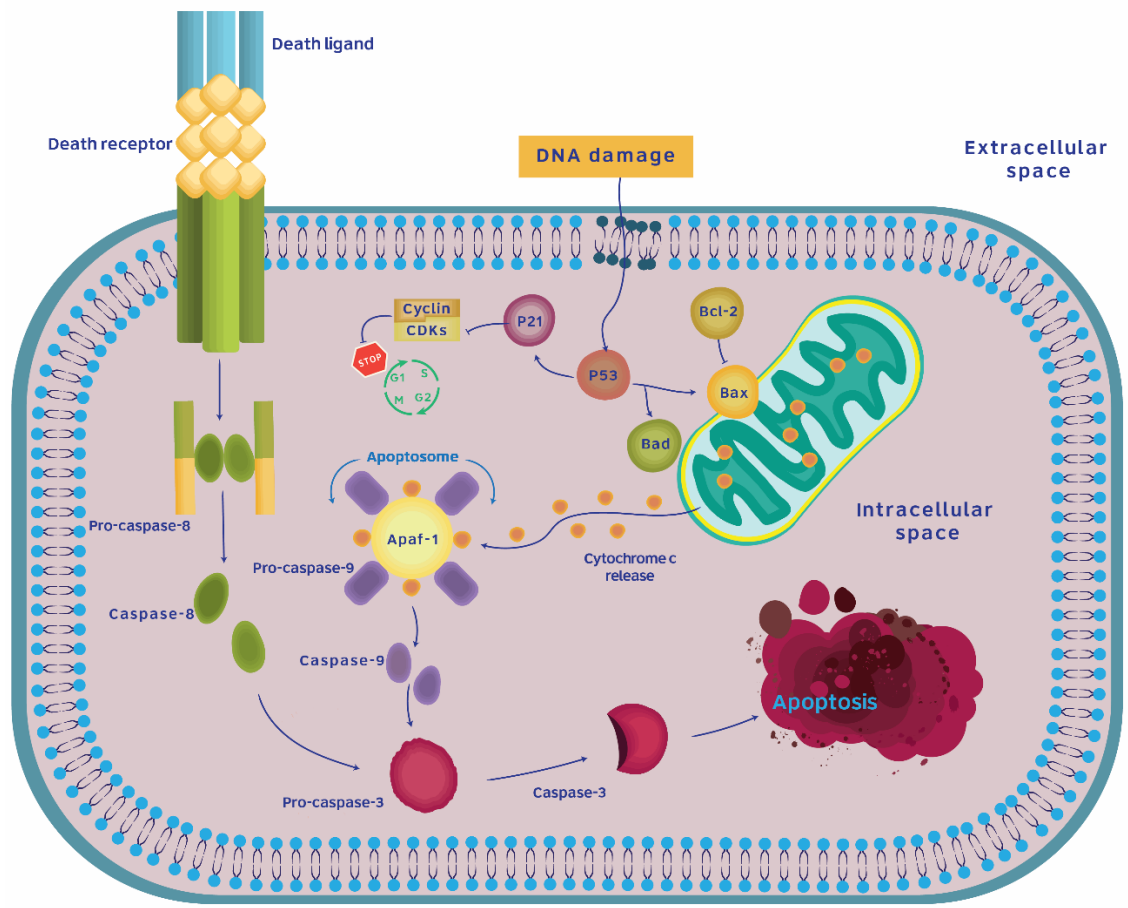

Figure I.4. Illustration of the control of cell cycle and extrinsic and intrinsic pathways of apoptosis. Extracellular ligand binding (FasL or tumor necrosis factor, TNF) to death receptors (FasR and TNF receptor, TNFR) triggers the receptor-mediated (extrinsic) pathway resulting in the direct activation of initiator caspase-8. The mitochondrial (intrinsic) pathway is initiated in response to DNA damage induced by apoptotic stimuli such as radiation, drugs, among others, triggering the activation of the protein p53. In turn, p53 stimulates proapoptotic members of the Bcl-2 protein family, such as Bax and Bad. 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, known as the guardian of the genome, is able to regulate downstream genes important in cell cycle arrest and apoptosis, including p21, Bax, Bad and Bcl-2. The passage through the diverse phases of cell cycle (G1, S, G2, M) is driven by a two-protein complex consisting of cyclin and cyclin-dependent kinase (CDK). Cell cycle arrest is mainly mediated through activation of p21, which stops progression of the cell cycle at the G1 phase by suppressing CDKs. In response to stress signals, p53 induces either cell cycle arrest or apoptosis. Activation and inhibition are indicated by arrows and bar-headed arrows, respectively.

Besides sex steroids, pituitary and hypothalamic hormones, a panoply of other factors play a role in the regulation of spermatogenesis. These other regulatory elements include retinoic acid, the glycoproteins activin and follistatin, and the protein desert hedgehog [21]. In addition, Sertoli cells synthesize transferrin (an iron-transporting protein) and ceruloplasmin (a copper-transporting protein). Furthermore, the Sertoli cells secrete other glycoproteins that function as growth factors or paracrine factors, such as the anti-Müllerian hormone, stem cell factor (SCF), and glial cell line-derived neurotrophic factor [54]. Indeed, the germ cell cycle and movement along the seminiferous tubules is a process under tight control involving distinct mechanisms that include several families of kinases and phosphatases activated, for example, in response to growth factors and cytokines [55]. The SCF is a cytokine that plays a crucial role controlling survival and

proliferation of both spermatogonial stem cells and spermatogonia (Figure I.5) [56, 57]. The SCF, by interaction with its tyrosine kinase receptor, the c-Kit, also seems to control the differentiation of spermatogonia and progression into meiosis [58, 59]. One of the signalling pathways activated by the SCF/c-Kit system involve phosphatidylinositol-3-kinase (PI3K)/Akt (Figure I.5) [60]. Indeed, PI3K and Akt act as downstream molecules of this ligand/receptor system, driving cells to proliferate [61]. The relevance of this pathway was confirmed *in vivo*: the failure of binding of PI3K to c-Kit receptor diminishes activation of Akt, which leads to a reduced proliferation and an increased apoptosis of spermatogonial stem cells and eventually results in an arrest of spermatogenesis [59, 62]. The popular PI3K/Akt pathway is known to mediate self-renewal, survival, and proliferation of spermatogonial stem cells and differentiating spermatogonia (Figure I.5) [55].

Furthermore, germ cells require considerable amounts of iron for proliferation and differentiation [63]. In fact, also other inorganic molecules such as calcium (Ca^{2+}), zinc, selenium, and copper have been implicated in spermatogenesis [64].

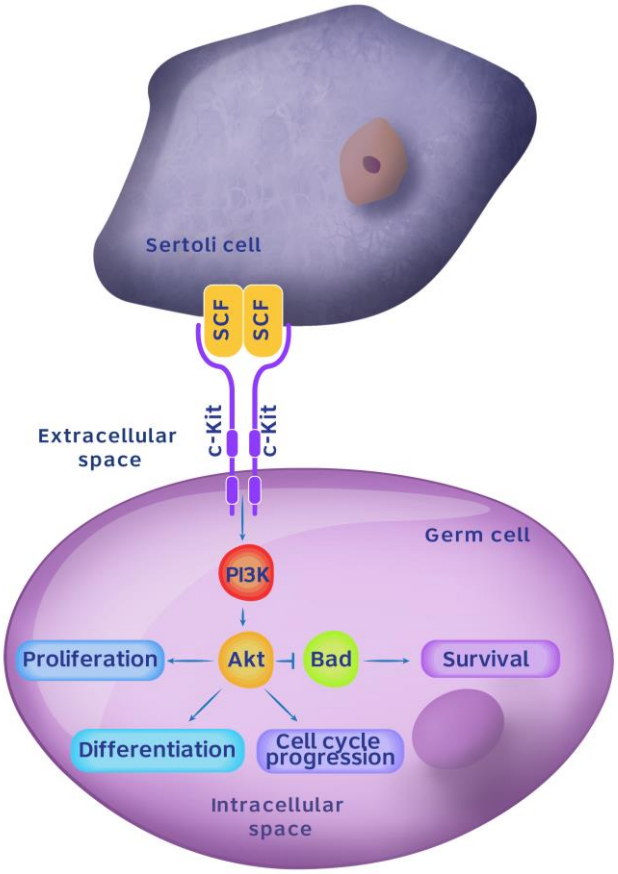

Figure I.5. Illustration of the stem cell factor (SCF)/c-Kit signalling within the seminiferous tubules. The stem cell factor (SCF) is a membrane-bound cytokine present at the surface of Sertoli cells which binds to c-Kit receptor on the surface of adjacent germ cells. One of the downstream-activated signalling cascades include phosphatidylinositol-3-kinase (PI3K)/Akt pathway that regulate germ cell differentiation, proliferation, and cell cycle progression. The protein kinase Akt inactivates the proapoptotic protein Bad through phosphorylation thereby also promoting germ cell survival. Indeed, SCF/c-KIT/PI3K/AKT signalling constitutes is an essential part of Sertoli-germ cell communication network. Activation and inhibition are indicated by arrows and bar-headed arrows, respectively.

2. Epididymal structure and physiology

The efferent ductules carry spermatozoa from the *rete testis* [65]. Moreover, these ductules are mainly involved in fluid homeostasis and reabsorb more than 95% of the luminal fluid released from the seminiferous epithelium, thereby increasing the concentration of sperm as they enter the epididymis [66]. The epididymis is a highly compartmentalized organ that is usually divided in three distinct regions (Figure I.6), the *caput* (head), *corpus* (body) and *cauda* (tail), which cooperate with different functions [67]. 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 (Figure I.6) [68]. 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 that allows the maturation process through the absorptive and secretory activities of the epididymis epithelial cells [67, 69].

The maturation process is androgen-dependent and conducts several biochemical and functional changes in spermatozoa [70]. 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 [41]. The *caput* and *corpus* regions perform early and late sperm maturation events, respectively, while the *cauda* stores the functionally mature spermatozoa [67]. 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 [71].

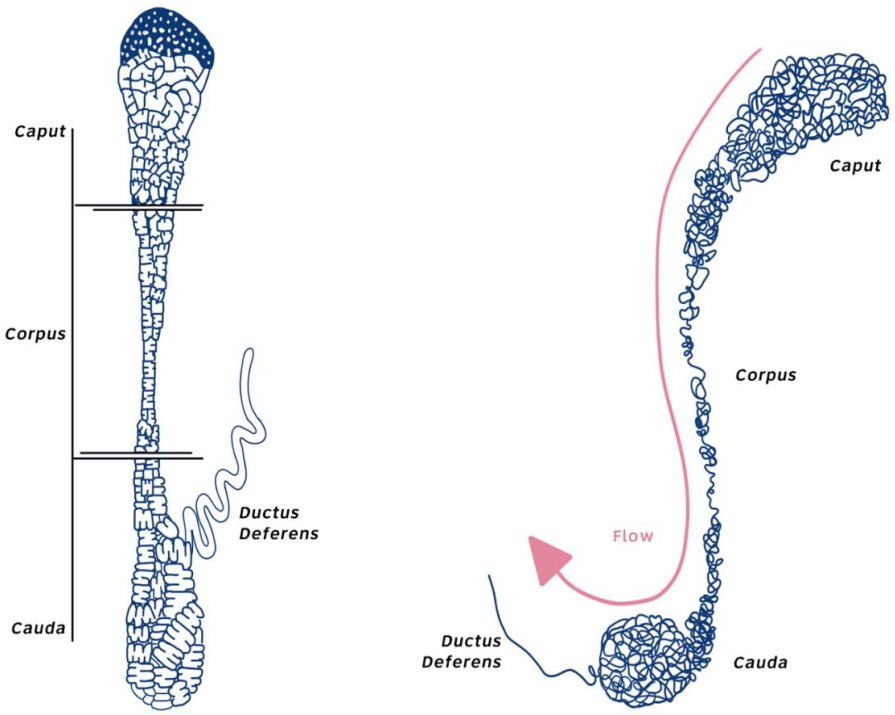

Figure I.6. Schematic organization of the rat epididymis. Frontal view on the left showing the three regions of the epididymis – *caput*, *corpus*, and *cauda*. Oblique lines indicate sites where different regions were segmented. Sagittal view on the right evidencing the transit flow through the epididymis essential for sperm maturation.

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 [68]. The levels of Ca^{2+} in the epididymal fluid are quite low in comparison with other ions, namely sodium, potassium, chloride, ammonium, and magnesium [72]. 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 [73]. 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 [73] 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 [74]. In order to conserve energy and maintain structural integrity, sperm motility needs to be suppressed until it is required [75]. 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 [76-78]. Physiological amounts of reactive oxygen species (ROS) are also involved in the regulation of some sperm functions, such as playing positive effects on maturation [79], capacitation [80], acrosome reaction [81], and sperm-oocyte fusion [82], supporting the importance of preserving seminal ROS at low controlled levels through the delicate balance between ROS production and removal [83].
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 [84].

3. Spermatozoa structure

The sperm cell (Figure I.7) 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.7) [85]. The flagellum of the mammalian spermatozoon consists of four distinct segments: the connecting piece (neck), the midpiece, the principal piece (tail), and the end piece (Figure I.7) [86]. The major motor portion of the flagellum is the axoneme, which is constituted by microtubules and responsible for sperm motility [85, 87]. 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 [87]. The end piece is the thinnest portion of the sperm [88]. The whole sperm cell is delimited by a plasma membrane that presents a high lipid content, especially of polyunsaturated fatty acids [89, 90]. This biochemical composition confers flexibility and fluidity to the sperm plasma membrane, which is crucial for the fusion with the oocyte membrane at fertilization [90].

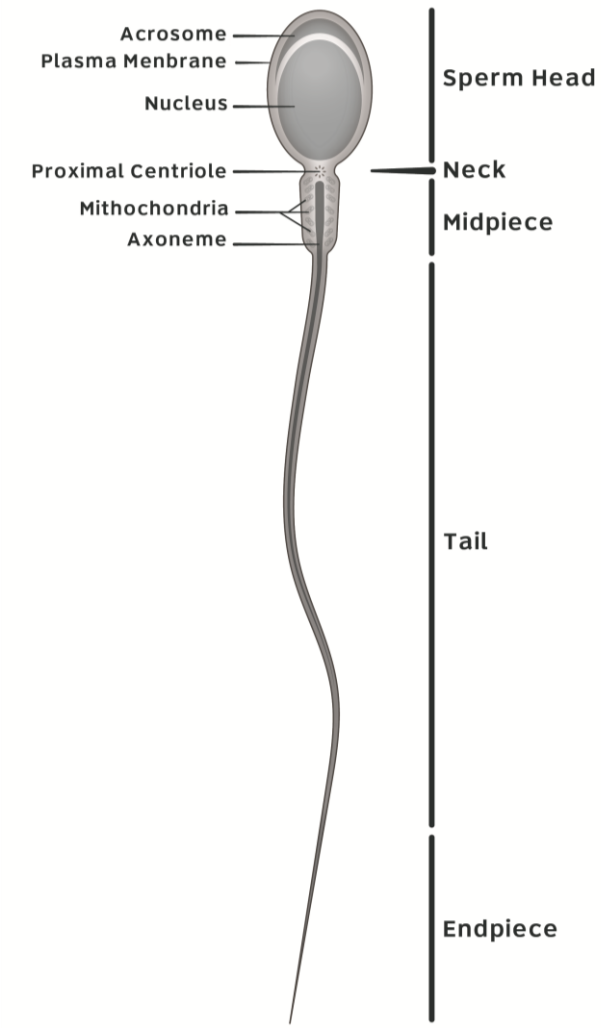

Figure I.7. Schematic representation of the structure of mammalian spermatozoa.

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 [86]. In human, the head of spermatozoa has a spatulate shaped, whereas the rat sperm head is falciform-shaped (Figure I.8) [86].

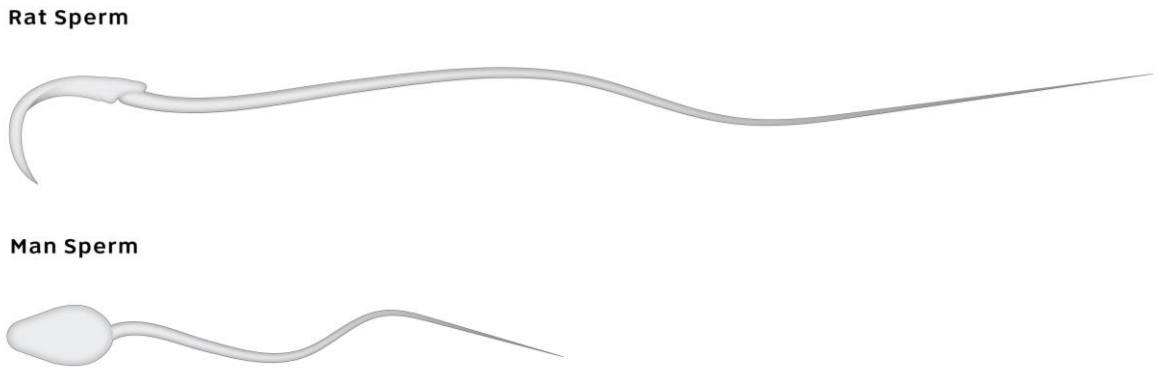

Figure I.8. Schematic representation comparing the rat (up) and human (down) spermatozoa.

References

[1] Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD, editors. The Physiology of Reproduction. Second Edition. New York: Raven Press; 1994. p. 1063–175.

[2] Lara NLM, Costa GMJ, Avelar GF, Lacerda SMSN, Hess RA, de França LR. Testis Physiology—Overview and Histology. In: Skinner MK, editor. Encyclopedia of Reproduction. Second Edition. Oxford: Academic Press; 2018. p. 105-16.

[3] Liu PY, Veldhuis JD. Chapter 12 - Hypothalamo-Pituitary Unit, Testis, and Male Accessory Organs. In: Strauss JF, Barbieri RL, editors. Yen and Jaffe's Reproductive Endocrinology. Eighth Edition. Philadelphia: Elsevier; 2019. p. 285-300.

[4] Van De Graaff KM, Rhees RW, Palmer SL. Human Anatomy and Physiology. Fourth Edition. Eaton K-A, Rothschild R, editors. New York: McGraw-Hill; 2013.

[5] Morton DA, Crawley JL. Chapter 19 - Male Reproductive System. Discovering Anatomy: A Guided Examination of the Cadaver. Englewood: Morton Publishing Company; 2018. p. 359-70.

[6] Schlatt S, Meinhardt A, Nieschlag E. Paracrine regulation of cellular interactions in the testis: factors in search of a function. Eur J Endocrinol. 1997;137:107-17.

[7] Marieb EN, Hoehn K. Chapter 27 - The Reproductive System. Human Anatomy and Physiology. Eleventh Edition. Hoboken: Pearson; 2018. p. 1041-90.

[8] de Rooij DG, Mizrak SC. Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. Development. 2008;135:2207-13.

[9] Gaytan F, Bellido C, Aguilar E, van Rooijen N. Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. J Reprod Fertil. 1994;102:393-9.

[10] Gaytan F, Bellido C, Morales C, Reymundo C, Aguilar E, Van Rooijen N. Effects of macrophage depletion at different times after treatment with ethylene dimethane sulfonate (EDS) on the regeneration of Leydig cells in the adult rat. J Androl. 1994;15:558-64.

[11] Hess RA, Hermo L. Rete Testis: Structure, Cell Biology and Site for Stem Cell Transplantation. In: Skinner MK, editor. Encyclopedia of Reproduction. Second Edition. Oxford: Academic Press; 2018. p. 263-9.

[12] Sharpe RM. Intratesticular factors controlling testicular function. Biol Reprod. 1984;30:29-49.

[13] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect. 1993;101:378-84.

[14] Sikka SC, Wang R. Endocrine disruptors and estrogenic effects on male reproductive axis. Asian J Androl. 2008;10:134-45.

[15] Taylor SL, Weng SL, Fox P, Duran EH, Morshedi MS, Oehninger S, et al. Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. Mol Hum Reprod. 2004;10:825-34.

[16] Griswold MD. The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol. 1998;9:411-6.

[17] Haider SG. Cell biology of Leydig cells in the testis. Int Rev Cytol. 2004;233:181-241.

[18] Akingbemi BT. Estrogen regulation of testicular function. Reprod Biol Endocrinol. 2005;3:51.

[19] Hess R, de Franca L. Spermatogenesis and Cycle of the Seminiferous Epithelium. In: Cheng CY, editor. Molecular Mechanisms in Spermatogenesis. Advances in Experimental Medicine and Biology. New York: Springer; 2009. 636. p. 1-15.

[20] Gilbert SF, Barresi MJF. Chapter 6 - Sex Determination and Gametogenesis. Developmental Biology. Eleventh Edition. Sunderland: Sinauer Associates; 2016. p. 181-215.

[21] Mäkelä J-A, Toppari J. Spermatogenesis. In: Simoni M, Huhtaniemi TI, editors. Endocrinology of the Testis and Male Reproduction. Cham: Springer; 2017. p. 417-56.

[22] Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A, Carreau S. Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. Mol Hum Reprod. 2004;10:535-41.

[23] Angelopoulou R, Plastira K, Msaouel P. Spermatozoal sensitive biomarkers to defective protaminosis and fragmented DNA. Reprod Biol Endocrinol. 2007;5:36.

[24] Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev. 1972;52:198-236.

[25] Adler ID. Comparison of the duration of spermatogenesis between male rodents and humans. Mutat Res. 1996;352:169-72.

[26] Holdcraft RW, Braun RE. Hormonal regulation of spermatogenesis. Int J Androl. 2004;27:335-42.

[27] Walker WH, Cheng J. FSH and testosterone signaling in Sertoli cells. Reproduction. 2005;130:15-28.

[28] Walker WH. Molecular mechanisms of testosterone action in spermatogenesis. Steroids. 2009;74:602-7.

[29] Carreau S, Hess RA. Oestrogens and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 2010;365:1517-35.

[30] Jones RE, Lopez KH. Chapter 4 - The Male Reproductive System. In: Jones RE, Lopez KH, editors. Human Reproductive Biology. Fourth Edition. San Diego: Academic Press; 2014. p. 67-83.

[31] Norris DO, Carr JA. Chapter 10 - The Endocrinology of Mammalian Reproduction. Vertebrate Endocrinology. Fifth Edition. San Diego: Academic Press; 2013. p. 317-74.

[32] Meaden C, Chedrese PJ. Androgens—Molecular Basis and Related Disorders. In: Chedrese PJ, editor. Reproductive Endocrinology: A Molecular Approach. Boston: Springer; 2009. p. 205-14.

[33] Courot M. [ABP: the testicular protein that binds androgens]. Reprod Nutr Dev. 1980;20:587-91.

[34] Erkkila K, Henriksen K, Hirvonen V, Rannikko S, Salo J, Parvinen M, et al. Testosterone regulates apoptosis in adult human seminiferous tubules in vitro. J Clin Endocrinol Metab. 1997;82:2314-21.

[35] Pierik FH, Burdorf A, de Jong FH, Weber RF. Inhibin B: a novel marker of spermatogenesis. Ann Med. 2003;35:12-20.

[36] Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW. Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Mol Cell Endocrinol. 2004;225:29-36.

[37] O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. Endocr Rev. 2001;22:289-318.

[38] Carreau S, Lambard S, Delalande C, Denis-Galeraud I, Bilinska B, Bourguiba S. Aromatase expression and role of estrogens in male gonad: a review. Reprod Biol Endocrinol. 2003;1:35.

[39] Flück CE, Pandey AV. Testicular Steroidogenesis. In: Simoni M, Huhtaniemi IT, editors. Endocrinology of the Testis and Male Reproduction. Cham: Springer International Publishing; 2017. p. 343-71.

[40] Sakaue M, Ishimura R, Kurosawa S, Fukuzawa NH, Kurohmaru M, Hayashi Y, et al. Administration of estradiol-3-benzoate down-regulates the expression of testicular steroidogenic enzyme genes for testosterone production in the adult rat. J Vet Med Sci. 2002;64:107-13.

[41] Correia S, Cardoso HJ, Cavaco JE, Socorro S. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med. 2015;17:e2.

[42] Chimento A, Sirianni R, Casaburi I, Pezzi V. Role of estrogen receptors and g protein-coupled estrogen receptor in regulation of hypothalamus-pituitary-testis axis and spermatogenesis. Front Endocrinol (Lausanne). 2014;5:1.

[43] Hossaini A, Dalgaard M, Vinggaard AM, Pakarinen P, Larsen JJ. Male reproductive effects of octylphenol and estradiol in Fischer and Wistar rats. Reprod Toxicol. 2003;17:607-15.

[44] Pastor LM, Zuasti A, Ferrer C, Bernal-Manas CM, Morales E, Beltran-Frutos E, et al. Proliferation and apoptosis in aged and photoregressed mammalian seminiferous epithelium, with particular attention to rodents and humans. Reprod Domest Anim. 2011;46:155-64.

[45] Wang Q, Zhao XF, Ji YL, Wang H, Liu P, Zhang C, et al. Mitochondrial signaling pathway is also involved in bisphenol A induced germ cell apoptosis in testes. Toxicol Lett. 2010;199:129-35.

[46] Aitken RJ, Findlay JK, Hutt KJ, Kerr JB. Apoptosis in the germ line. Reproduction. 2011;141:139-50.

[47] Shaha C, Tripathi R, Mishra DP. Male germ cell apoptosis: regulation and biology. Philos Trans R Soc Lond B Biol Sci. 2010;365:1501-15.

[48] Shukla KK, Mahdi AA, Rajender S. Apoptosis, spermatogenesis and male infertility. Front Biosci (Elite Ed). 2012;4:746-54.

[49] Allan DJ, Harmon BV, Roberts SA. Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. Cell Prolif. 1992;25:241-50.

[50] Bartke A. Apoptosis of male germ cells, a generalized or a cell type-specific phenomenon? Endocrinology. 1995;136:3-4.

[51] Sinha Hikim AP, Lue Y, Diaz-Romero M, Yen PH, Wang C, Swerdloff RS. Deciphering the pathways of germ cell apoptosis in the testis. J Steroid Biochem Mol Biol. 2003;85:175-82.

[52] Huckins C. The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. Anat Rec. 1978;190:905-26.

[53] Aitken RJ, Baker MA. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. Int J Dev Biol. 2013;57:265-72.

[54] Ross MH, Pawlina W. Male Reproductive System. Histology: A Text and Atlas with Correlated Cell and Molecular Biology. Seventh Edition. Wolters Kluwer; 2016. p. 790-833.

[55] He Z, Kokkinaki M, Dym M. Signaling molecules and pathways regulating the fate of spermatogonial stem cells. Microsc Res Tech. 2009;72:586-95.

[56] Hakovirta H, Yan W, Kaleva M, Zhang F, Vanttinen K, Morris PL, et al. Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium. Endocrinology. 1999;140:1492-8.

[57] Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development. 2000;127:2125-31.

[58] Guerif F, Cadoret V, Rahal-Perola V, Lansac J, Bernex F, Jacques Panthier J, et al. Apoptosis, Onset and Maintenance of Spermatogenesis: Evidence for the Involvement of Kit in Kit-Haplodeficient Mice. Biol Reprod. 2002;67:70-9.

[59] Kissel H, Timokhina I, Hardy MP, Rothschild G, Tajima Y, Soares V, et al. Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. EMBO J. 2000;19:1312-26.

[60] Cardoso HJ, Figueira MI, Correia S, Vaz CV, Socorro S. The SCF/c-KIT system in the male: Survival strategies in fertility and cancer. Mol Reprod Dev. 2014;81:1064-79.

[61] Klippel A, Escobedo MA, Wachowicz MS, Apell G, Brown TW, Giedlin MA, et al. Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. Mol Cell Biol. 1998;18:5699-711.

[62] Blume-Jensen P, Jiang G, Hyman R, Lee KF, O'Gorman S, Hunter T. Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility. Nat Genet. 2000;24:157-62.

[63] Sylvester SR, Griswold MD. The testicular iron shuttle: a "nurse" function of the Sertoli cells. J Androl. 1994;15:381-5.

[64] Camejo MI, Abdala L, Vivas-Acevedo G, Lozano-Hernandez R, Angeli-Greaves M, Greaves ED. Selenium, copper and zinc in seminal plasma of men with varicocele, relationship with seminal parameters. Biol Trace Elem Res. 2011;143:1247-54.

[65] Lee KH, Park JH, Bunick D, Lubahn DB, Bahr JM. Morphological comparison of the testis and efferent ductules between wild-type and estrogen receptor alpha knockout mice during postnatal development. J Anat. 2009;214:916-25.

[66] Clulow J, Jones RC, Hansen LA. Micropuncture and cannulation studies of fluid composition and transport in the ductuli efferentes testis of the rat: comparisons with the homologous metanephric proximal tubule. Exp Physiol. 1994;79:915-28.

[67] Robaire B, Hinton BT. Chapter 17 - The Epididymis. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's Physiology of Reproduction. Fourth Edition. San Diego: Academic Press; 2015. p. 691-771.

[68] Cornwall GA. New insights into epididymal biology and function. Hum Reprod Update. 2009;15:213-27.

[69] Guyonnet B, Dacheux F, Dacheux JL, Gatti JL. The epididymal transcriptome and proteome provide some insights into new epididymal regulations. J Androl. 2011;32:651-64.

[70] Vreeburg JT, Holland MK, Orgebin-Crist MC. Binding of epididymal proteins to rat spermatozoa in vivo. Biol Reprod. 1992;47:588-97.

[71] Dacheux JL, Belleannee C, Jones R, Labas V, Belghazi M, Guyonnet B, et al. Mammalian epididymal proteome. Mol Cell Endocrinol. 2009;306:45-50.

[72] Wales RG, Wallace JC, White IG. Composition of bull epididymal and testicular fluid. J Reprod Fertil. 1966;12:139-44.

[73] Pholpramool C, Borwornpinyo S, Dinudom A. Role of Na+ /H+ exchanger 3 in the acidification of the male reproductive tract and male fertility. Clin Exp Pharmacol Physiol. 2011;38:403-9.

[74] Da Silva N, Pietrement C, Brown D, Breton S. Segmental and cellular expression of aquaporins in the male excurrent duct. Biochim Biophys Acta. 2006;1758:1025-33.

[75] Jones RC. To store or mature spermatozoa? The primary role of the epididymis. Int J Androl. 1999;22:57-67.

[76] Lefebvre J, Boileau G, Manjunath P. Recombinant expression and affinity purification of a novel epididymal human sperm-binding protein, BSPH1. Mol Hum Reprod. 2009;15:105-14.

[77] Cohen DJ, Maldera JA, Vasen G, Ernesto JI, Munoz MW, Battistone MA, et al. Epididymal protein CRISP1 plays different roles during the fertilization process. J Androl. 2011;32:672-8.

[78] Joseph A, Shur BD, Hess RA. Estrogen, efferent ductules, and the epididymis. Biol Reprod. 2011;84:207-17.

[79] Aitken RJ, Baker MA. Oxidative stress and male reproductive biology. Reprod Fertil Dev. 2004;16:581-8.

[80] O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med. 2006;41:528-40.

[81] de Lamirande E, O'Flaherty C. Sperm activation: role of reactive oxygen species and kinases. Biochim Biophys Acta. 2008;1784:106-15.

[82] Riffo MS, Parraga M. Study of the acrosome reaction and the fertilizing ability of hamster epididymal cauda spermatozoa treated with antibodies against phospholipase A2 and/or lysophosphatidylcholine. J Exp Zool. 1996;275:459-68.

[83] Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update. 2008;14:243-58.

[84] Rabbani SI, Devi K, Khanam S. Pioglitazone, a PPAR-gamma ligand inhibited the nicotinamide-streptozotocin induced sperm abnormalities in type-2 diabetic Wistar rats. Pak J Pharm Sci. 2010;23:326-31.

[85] Gilbert SF, Barresi MJF. Chapter 7 - Fertilization: Beginning a New Organism. Developmental Biology. Eleventh Edition. Sunderland: Sinauer Associates; 2016. p. 217-50.

[86] Toshimori K, Eddy EM. Chapter 3 - The Spermatozoon. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's Physiology of Reproduction. Fourth Edition. San Diego: Academic Press; 2015. p. 99-148.

[87] Auger J. Spermatozoa and Sperm Structure. In: Skinner MK, editor. Encyclopedia of Reproduction. Second Edition. Oxford: Academic Press; 2018. p. 62-7.

[88] Saladin K. The Male Reproductive System Anatomy & Physiology: The Unity of Form and Function. Eighth Edition. McGraw-Hill; 2018.

[89] Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta. 2000;1469:197-235.

[90] Lenzi A, Gandini L, Picardo M, Tramer F, Sandri G, Panfili E. Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): scavenger mechanisms and possible scavenger therapies. Front Biosci. 2000;5:E1-15.

Chapter II
Endogenous Factors in the Recovery of Reproductive Function After Testicular Injury and Cancer

Chapter adapted from:

Silva A.M.S., *Correia S., Socorro S.#, Maia C.J.# Endogenous factors in the recovery of reproductive function after testicular injury and cancer. Curr Mol Med. 2016;16:631-49. (DOI: 10.2174/1566524016666160802150400)*

#contributed equally as senior authors

Abstract

The testes are one of the most delicate organs in the male body and highly susceptible to the exogenous influences capable of inducing cell damage. Cancer therapies are well known to negatively affect the male reproductive tract with a severe impairment of spermatogenesis and infertility. The present work aimed to review the available information about the different endogenous factors, hormonal and non-hormonal, which may have protective or advantageous properties on the recovery of male reproductive function after gonadal injury. Furthermore, the perspective that these endogenous molecules could act as cryoprotectants to improve the quality of cryopreserved semen samples was also discussed. The knowledge reviewed herein allowed to identify promising factors able to mitigate the male fertility problems arising either from oncological treatments or other gonadal damage, and opening new possibilities to ameliorate the recovery of spermatogenesis or fertility preservation.

Keywords: Testicular damage; Chemotherapy; Radiotherapy; Male infertility; Fertility preservation.

1. Introduction

Testicles are the crucial organs maintaining male reproductive function, a role that is accomplished by their dual function: i) testosterone synthesis and ii) production of male gametes. Spermatogenesis is the complex and highly regulated process that occurs in the seminiferous tubules of the testis, culminating with the release of spermatozoa into the tubular lumen [1]. The production of spermatozoa depends on a set of coordinated mitotic and meiotic divisions and cell differentiation events [1], and due to its high mitotic rate, the germinal epithelium is very sensitive to exogenous damaging factors [2]. Consequently, cancer treatments, namely chemotherapy and radiotherapy, have several adverse effects on male fertility, mainly by inducing apoptosis of germ cells [3]. Moreover, it has been shown that radiation treatments have a dose-dependent detrimental impact on male reproductive function [3]. Treatment of oncological diseases usually results in temporary or permanent arrest of spermatogenesis [4]. For that reason, sperm cryopreservation preceding cancer treatments remains the standard method for fertility preservation in adult males [5], with other protocols remaining at experimental phase yet [6]. Concerning cryopreservation, one of the major problems is the oxidative stress damage induced to sperm cells [7]. During the freeze-thawing practice, sperm antioxidant defences could not be enough [8] to counteract this damage, resulting in poor sperm quality after cryopreservation and thawing in comparison with fresh sperm [9]. Furthermore, the use of cryopreserved sperm or tissue in assisted reproductive techniques (ART) encompass an economic obstacle due to the high-costs associated [10]. Therefore, it is emergent the demand for upgraded approaches to preserve fertility. The improvement of ART should rely on cheaper and simpler alternatives with good acceptance by patients. Also, effective strategies that would restrain the undesirable adverse effects of oncological

treatments should be developed [11]. This review provides an overview of the endogenous molecules that have been identified as protective agents or having beneficial effects on the recovery of reproductive function after testicular injury and/or may be useful in fertility cryopreservation.

2. Testicular damage and male (in)fertility

2.1 Testicular cancer: from therapy to oncofertility concerns

Fortunately, the survival rate from cancer is continually improving due to the advances in diagnostic tools, treatments and therapeutic options. 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 [12]. Nevertheless, testicular cancer is the most important malignancy in the young male, accounting >10 new cases per 100.000 males per year in Europe and a mortality of 0.3 cases per 100.000 males per year [13, 14]. In fact, testicular germ cell tumours are the most common malignancy in males between 15 and 34 years old, and also, the most frequent cause of death from solid tumours [15]. Depending on the type and stage of the testicular cancer and other factors, the treatment options can include surgery, chemo- and/or radiotherapy [16, 17].

The testes are very sensitive organs and, thus, highly affected by exogenous damaging factors, such as chemo- and radiotherapy treatments [3]. For this reason, the treatment of oncological diseases usually results in temporary or permanent arrest of spermatogenesis [4], as well as in disrupted sex hormone production [18]. 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 [19].

Focusing on radiotherapy, the extent of testicular injury is directly related with the dose of radiation delivered as well as the underlying cell type [20]. The germinal epithelium is more sensitive than the Leydig cells as a result of its high mitotic rate [2]. The use of radiation as therapy for testicular cancer affects spermatogenesis either transiently or permanently, by directly inducing DNA damage [21, 22]. Besides the dose, several variables can affect the deleterious effect of radiation on gonadal function, such as the source of radiation, gonadal shielding, scatter radiation, and individual susceptibility [23, 24]. 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 [25]. Leydig cells are only affected when doses reach >15 Gy [23]. Also, the radiation delivered directly to the testes for treatment of testicular leukaemia, or as part of the total body irradiation prior to bone marrow transplant, involves doses that result in permanent sterility in men [26].

Despite the existing controversy, it is accepted that malignancy itself is associated with male infertility since numerous biological processes are affected in parallel [27]. Indeed, azoospermia is present in ≈ 3% to 18% of men at the moment of cancer diagnosis [28]. In addition, it was shown

that spermatogenesis is affected in oncological patients with lymphoma and leukaemia without testicular pathology, even before the onset of gonadotoxic therapies [5, 29-32]. Also, altered sperm production in patients with testicular cancer before orchiectomy has been detected [33].

2.2 Preservation of male fertility and spermatogenesis recovery after testicular damage

2.2.1 Cryopreservation

Oncological therapies are known to commonly cause provisory or irreversible arrest of spermatogenesis [4]. Currently, sperm cryopreservation preceding cancer treatments remains the only established method for fertility preservation in adult males [5], whereas in the pre-pubertal male the cryopreservation of testicular tissue is an option adopted for preserving fertility [6]. Other procedures are at experimental phase but are not devoid of ethical concerns [6]. In addition, one of the major problems is related to the ability to avoid the oxidative damage that sperm cells and seminal plasma normally retain [7]. During the freeze-thawing practice, the antioxidant defences could be insufficient [8] 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 [8, 34, 35]. Furthermore, the use of cryopreserved sperm or tissue in assisted reproductive techniques (ART) has an economic obstacle due to the high costs associated [10]. Indeed, 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 [11].

2.2.2 Endogenous factors

The following topics explore the available data about several endogenous factors (hormonal and non-hormonal) that may favour the recovery of male reproductive function after gonadal injury or may enhance the quality of cryopreserved sperm.

2.2.2.1 Hormonal factors

Testosterone

Androgens, namely testosterone, are the key spermatogenesis regulators, acting as germ cell survival factors, and also has been shown that testosterone withdrawal induces the apoptosis of support Sertoli cells and germ cells [36]. On the other hand, both endogenous and exogenous testosterone supplementation trigger a negative feedback on the hypothalamic-pituitary-gonadal axis suppressing spermatogenesis. Testosterone action suppresses gonadotropin releasing hormone (GnRH) production, which inhibits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Diminished levels of FSH and LH lead to a decline in

testosterone production and also in intratesticular testosterone levels, diminishing sperm production [37] (Figure II.1).

Since exogenous testosterone therapies are responsible for inhibition of intratesticular testosterone production, spermatogenesis may be compromised [38]. It is well known that intratesticular testosterone is absolutely necessary for normal spermatogenesis, and the reduced levels of intratesticular testosterone may result in azoospermia [39, 40]. However, it has been hypothesized that inhibition of cell division before or during chemotherapy may protect the germ cells, preserving spermatogenesis or improving its recovery. Thus, the administration of testosterone, GnRH antagonists and agonists, as well as estrogens, have been tested with diverse chemotherapeutic drugs, leading to opposite results [41]. The suppression of testosterone has been shown as a helpful agent in the re-establishment of rat spermatogenesis after damage induced by environmental reproductive toxicants [42]. Men, who were administered with low systemic dose of testosterone to suppress intratesticular testosterone levels, also exhibited restoration of spermatogenesis in response to the treatment with cyclophosphamide [43]. Later, the influence of exogenous testosterone on the recovery of spermatogenesis was evaluated after cisplatin chemotherapy, which had a proven harmful consequence on fertility [44]. The obtained results demonstrated that administration of exogenous testosterone during chemotherapy had protective effects on spermatogenesis in mice subjected to high dose chemotherapy, and no additional long-term effects in animals receiving low dose cisplatin were observed. Comparing the effects between testosterone and GnRH agonists, testosterone was considered advantageous due to the highest androgen suppression observed at 2 to 3 weeks after treatment. During this latency time, testosterone levels and active spermatogenesis are paradoxically high. Even a single dose of chemotherapy throughout latency time had serious destructive effects on germ cells [44]. Moreover, GnRH analogues, which can decrease serum gonadotropin levels [45], induced andropause when administered during chemotherapy, but it did not happen with testosterone supplementation [44].

Gonadotropin-releasing hormone agonists and antagonists

By suppressing testosterone production with GnRH agonists or antagonists, the damage of spermatogenesis process and fertility after irradiation or chemotherapeutic agents can be repaired (Figure II.1) [42, 46, 47]. However, these hormonal treatments are generally accompanied by several side effects and should be avoided or applied in a dose as low as possible [46]. Nevertheless, if spermatogonial stem cells survive after cancer treatment, there is a chance for hormonal manipulation towards resumption of sperm production. The testosterone suppression given before or after the cytotoxic insult stimulated the recovery of spermatogenesis from both endogenous and transplanted spermatogonial stem cells, with the reestablishment of fertility [48]. Therefore, the effect of hormonal suppression and transplantation has been evaluated on spermatogenesis recovery. In a study reported by Shetty *et al.* [49], the testes of 12 adult cynomolgus monkeys were irradiated, and 6 of them were treated with a GnRH antagonist. In contrast to the minimal effects of hormone suppression or transplantation alone on the spermatogenic recovery, enhanced recovery occurred when transplantation and GnRH antagonist were combined, since transplanted

testes of GnRH antagonist-treated monkeys displayed i) higher weight; ii) improved percentage of seminiferous tubule cross-sections presenting spermatogenesis; iii) detectable sperm in 5 of 6 animals; and iv) higher sperm counts. Furthermore, hormone suppression also had encouraging results on homing of transplanted spermatogonial stem cells in non-human primate models [49], but it is imperative to remember the inherent existing risks related to reintroduction of tumoral cells in such experimental procedures [50].

Nevertheless, there are several similarities between rodents and men in what concerns spermatogenesis, as well as in its hormonal regulation [46]. In a mouse model, it was demonstrated that the manipulation of hormonal levels can decrease the toxic effects triggered by chemotherapy in spermatogenesis. It was found that the administration of GnRH-agonist during treatment with cyclophosphamide protected spermatogenesis [51].

Several studies have shown that the reduction of intratesticular testosterone levels, induced by GnRH analogues, has a protective role against spermatogenesis injury when administered previously to chemo- or radiotherapy. If administration occurs after cytotoxic damage, this hormonal treatment stimulates the recovery of spermatogenesis and, consequently improves fertility [47, 52, 53]. Posteriorly, it was demonstrated that a strong hormonal suppression using a GnRH antagonist (acyline) plus flutamide (an androgen receptor antagonist) had positive effects in re-establishing spermatogenesis and improving colonization of transplanted spermatogonial stem cell in irradiated mouse testes [48]. Hormonal suppression treatment was administered immediately after irradiation (doses of 10.5 to 13.5 Gy) and successfully enhanced spermatogenesis recovery with a significantly reduced recovery period. In addition, this treatment also improved the effectiveness of transplanted cell colonization. It also should be pointed out that the duration, timing, and degree of hormonal suppression seemed to influence the success of gonadal recovery [48].

Estrogens

In the last years, estrogens have been recognized as important regulators of spermatogenesis and male fertility. Among other roles, estrogens are able to regulate both survival and death of germ cells having, depending on the dose, beneficial or detrimental effects on spermatogenesis [54, 55].

Estrogens also can have a direct action on the endocrine Leydig cells by down-regulating the expression of the steroidogenic enzymes involved in testosterone biosynthesis [56]. These hormones also can act on the hypothalamus or pituitary to reduce FSH and LH levels and subsequently decrease testosterone levels [57] (Figure II.1). Considering that 17β-estradiol (E_2) might induce secondary effects at cardiovascular level [58] or gynecomastia [59], its use in the treatment of gonadotoxic–induced azoospermia represent some weaknesses. The development of selective estrogen receptor modulators, with specific functions in the testis but without estrogen side effects, should be explored for application in the recovery of azoospermia in consequence of cancer therapy [60]. Nevertheless, the androgen-estrogen imbalance, rather than estrogen itself, is more likely to lead to male reproductive diseases [61]. E_2 re-established spermatogenesis in hypogonadal mice and accelerated spermatogenesis onset in immature animals [62, 63]. In irradiated rats, exogenous E_2 accelerated GnRH antagonist-induced spermatogenesis recovery [64].

Furthermore, in rats exposed to testicular irradiation, the administration of E_2 alone was able to provoke spermatogenesis recovery and partially revert testosterone inhibitory effects on spermatogonial differentiation [65]. On the other hand, when administrated to normal adult and pre-pubertal rats, E_2 as well as other estrogens can exert a repressive effect on spermatogenesis [66]. Therapy with E_2 prevented spermatogonial differentiation in neonatal rats [66] and sperm production in juvenile mice [67]. Moreover, estrogens also have been described as apoptosis-inducers of male germ cells and, consequently, as damaging agents for male fertility. In fact, clinical findings in infertile men presenting reduced testosterone/E_2 ratio due to high E_2 levels, are concordant with the apoptotic role of estrogens. Nevertheless, the pro-survival or apoptotic role of estrogens seems to be dependent on the dose. This issue was recently reviewed by Correia and co-authors [54] and it was shown that only E_2 doses above the physiological concentration induce germ cells apoptosis. Accordingly, adjuvant testosterone and E_2 administration exhibited protective action on radiation-induced injury of spermatogenesis [44]. Porter *et al.* [64] showed that E_2 promotes spermatogonial differentiation in irradiated LBNF1 rat gonads separately of its effects on testosterone or gonadotropin concentrations, and also that E_2 is more potent than GnRH antagonist in stimulating spermatogonial restoration. Hormonal manipulation as a therapy to improve spermatogenesis recovery from endogenous surviving stem spermatogonia or transplanted spermatogonia together with E_2 could be a promising prospect for men subjected to gonadotoxic cancer treatments [64, 68]. Using irradiated rats having total block of spermatogonial differentiation, it was shown that about 20 genes were up- or down-regulated by E_2 in testicular cells [69]. Several genes, such as Insulin-like 3 gene that regulates germ cell apoptosis and testicular descent [70], are considered potential candidates to block spermatogonial differentiation and improve spermatogenesis recovery after gonadotoxic effects. Therefore, the identification of downstream genes or signalling pathways involved in the recovery of spermatogenesis in response to hormonal manipulations could unravel novel therapeutic targets for application in men [69].

Growth Hormone and Insulin-Like Growth Factor 1

Growth hormone (GH) is a protein synthesized by the anterior pituitary gland [37], which plays a crucial role in the normal adolescent growth spurt and is also involved in the testes maturation [71] (Figure II.1). In patients with GH deficiency, who experience delayed sexual maturation, supplemental GH can trigger the onset of puberty [72]. Exogenous administration of GH to hypophysectomised rats stimulated the sensitivity of Leydig cells to LH [73]. In addition to the direct effects of GH, mediated by its receptor, several testicular effects are indirectly exerted through the GH-induced secretion of insulin-like growth factor 1 (IGF-1). The IGF-1 increase in the male gonads in response to GH leads to the maturation of sperm cells via a paracrine-autocrine control (Figure II.1) [37, 71]. Regarding the use of GH as therapy, it was shown that human recombinant GH may be an acceptable option for men with idiopathic oligozoospermia when other therapies are unsuccessful [74]. Another study also demonstrated that GH treatment of 18 subfertile males with oligozoospermia or asthenozoospermia significantly increased the levels of IGF-1 and sperm motility, but without differences in sperm counts [75]. However, some doubts

have raised in what concerns GH treatment of male hypogonadotropic hypogonadism. Nevertheless, a possible advantage for sperm motility and sperm counts was also suggested [37].

The protective and regenerative role of GH in testicular tissue after chemotherapy have already been demonstrated in GH-deficient rats by the co-administration of GH and cyclophosphamide [76]. The effect of a co-therapy with GH on the testicular dysfunction induced by methotrexate, an anti-cancer drug acting as a DNA synthesis inhibitor, also was investigated [77]. The impaired spermatogenesis and fertility in consequence of methotrexate treatment are thought to be due to the direct suppression of dihydrofolate reductase, GH deficiency, or other mechanisms. In fact, the administration of GH in combination with methotrexate diminished its detrimental effects on sperm parameters, demonstrating the protective properties of GH [77].

In general, growth factors play an essential role in spermatogenesis, sperm motility, and fertility. Some studies pointed out the possible role of IGF-1 on spermatogenesis, sperm viability and motility in different species, including humans [78-80]. Indeed, considering that IGF-1 receptor is expressed in spermatogonia, spermatocytes, spermatids, and spermatozoa, IGF-1 has been suggested as one of the essential agents for germ cell development and maturation, as well as for sperm motility (Figure II.1) [81]. A beneficial role of seminal plasma IGF-1 as an antioxidant preserving spermatogenesis and the motility of human sperm has been described [75, 78]. Also, it has been proposed that the protective effect of IGF-1 in sperm parameters is due to the diminished levels of lipid peroxidation (LPO) [80]. Considering that sperm cells are highly vulnerable to cryoinjury [8, 34, 35], the effect of IGF-1 in maintaining the quality of cryopreserved buffalos semen was evaluated [81]. It was demonstrated that the *in vitro* incubation of post-thawed sperm with IGF-1 for 2h avoided the deterioration of spermatozoa functional parameters and improved the *in vitro* fertilization outcomes, suggesting that the addition of IGF-1 to the extender before freezing may improve the fertility of subfertile buffalos [81].

Ghrelin

The protein ghrelin was first described as the endogenous ligand of GH secretagogue receptor (GHS-R) [82]. Thereafter, a growing number of studies have been suggesting its potential involvement in male reproduction since rat and human testes express ghrelin and its receptor [83, 84]. Ghrelin might work as a pleiotropic modulator in the testis [85] (Figure II.1). This protein has been mostly detected in the interstitial Leydig cells, and it is capable to inhibit the expression of the stem cell factor (SCF) [86, 87]. The SCF is a membrane-bound cytokine at the surface of Sertoli cells whereas its receptor, the tyrosine kinase receptor c-kit, is present on the surface of adjacent germ cells [88, 89]. The SCF is a powerful paracrine survival factor and the SCF/c-kit communication between Sertoli cells and the germ line is determinant to avoid germ cells death [88]. Furthermore, it has been suggested the involvement of ghrelin in controlling apoptosis of germ cells but it is unknown whether it depends on the down-regulation of SCF levels. For example, ghrelin antioxidant therapy significantly inhibited the proliferation of spermatogonia and spermatocytes (Figure II.1) [90], while the suppression of ghrelin signalling diminished germ cell apoptosis, resulting in better sperm production in leptin-deficient mice [91]. In another study, ghrelin was also able to increase sperm plasma membrane integrity [92].

The role of ghrelin in mice testicular injury caused by ionizing radiation was explored [83]. Exposure to 2 Gy of X-rays induced a prominent increase of nuclear ghrelin expression in differentiating spermatogonia, with an impairment of Leydig cells-expressing ghrelin. Surprisingly, suppression of the ghrelin signalling pathway by administration of a specific GHS-R1a antagonist improved spermatogonia removal via apoptosis during early recovery, resulting in diminished male fertility. These findings suggested that the anti-apoptotic effects of ghrelin have a deep involvement in post-injury repair. In addition, it was also observed that inhibition of ghrelin signalling caused an increase in intratesticular testosterone levels 21 days after irradiation, which may stimulate the recovery of spermatogenesis supported by the surviving spermatogonia. It was also verified that the upregulation and nuclear trafficking of ghrelin may act by a p53-dependent mechanism. The increased levels of ghrelin in response to radiation, as well as the modulation of ghrelin expression by radiation-induced oxidative stress (OS) and the cross-talk between p53 and ghrelin during radiation damage were confirmed in differentiating spermatogonia [83].

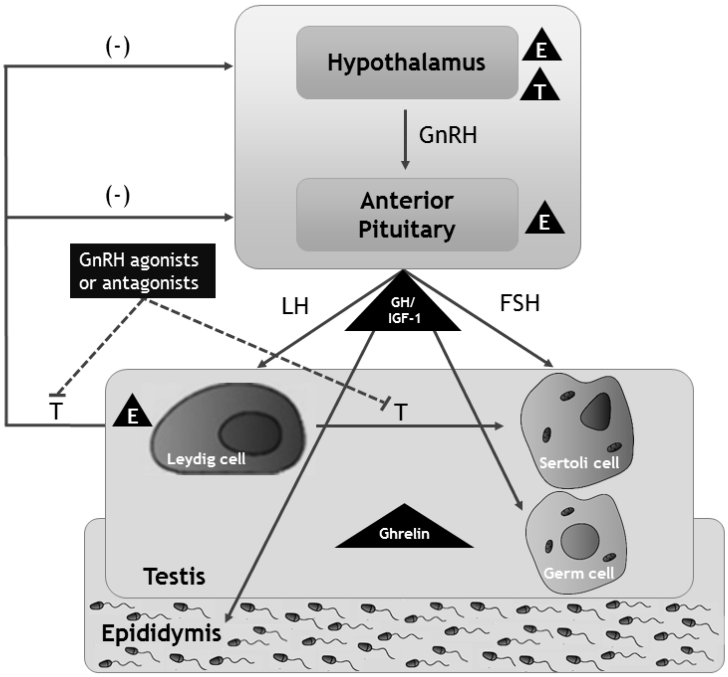

Figure II.1. Hormonal protective factors and their target tissues (cells) in the male reproductive axis. Testosterone produced by Leydig cells has an anti-apoptotic effect on male germ cells. On the other hand, both endogenous testosterone and exogenous testosterone supplementation trigger a negative feedback (-) on the hypothalamic-pituitary-gonadal axis suppressing spermatogenesis. Testosterone action suppresses gonadotropin releasing hormone (GnRH) production, which inhibits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Diminished levels of FSH and LH lead to a decline in testosterone production, diminishing sperm production. By suppressing testosterone biosynthesis with GnRH agonists or antagonists, the damage in spermatogenesis and fertility after irradiation or chemotherapy can be repaired. Estrogens (E) can act on Leydig cells down-regulating the expression of steroidogenic enzymes involved in testosterone biosynthesis, and also on the hypothalamus or pituitary to reduce FSH and LH levels, subsequently decreasing testosterone levels. Growth hormone (GH), synthesized by the anterior pituitary gland, is important for testes maturation. GH outcomes could be mediated by the insulin-like growth factor 1 (IGF-1), which stimulates the maturation of sperm cells, sperm counts and sperm motility via a paracrine-autocrine stimulation. Ghrelin has anti-apoptotic and anti-proliferative effects in germ cells, being involved in gonadal recovery after injury. Ghrelin also has shown to increase sperm plasma membrane integrity. Legend: Induction/stimulation and suppression are indicated by arrows and bar-headed arrows, respectively. Target tissues (cells) of hormonal actions are highlighted by triangles.

Table II.1. Hormonal protective factors and their target tissues (cells) in the male reproductive.

Hormonal Factors	Physiological Effects	References
Testosterone	↓ chemical-induced testicular damage	[43, 44]
	→ spermatogenesis	[43, 44]
GnRH antagonists	→ spermatogenesis	[46-49]
	↓ chemical- and radiation-induced testicular damage	[42, 46, 47, 49]
	↑ testis weight	[47, 49]
	↑ sperm counts	[47, 49]
	↑ colonization of transplanted spermatogonial stem cells	[48, 49]
GnRH agonists/analogues	→ spermatogenesis	[46, 47, 52, 53]
	↑ testis weight	[47]
	↑ sperm counts	[47, 52]
	↓ chemical- and radiation-induced testicular damage	[47, 51-53]
Estrogens	↓ radiation-induced testicular damage	[64, 65]
	→ spermatogonial differentiation	[64]
	modulates SCF gene expression in testis	[55]
	→ spermatogenesis	[62-65]
GH/IGF-1	↓ chemical-induced testicular damage	[76, 77]
	↑ seminiferous epithelial depth and tubular diameter	[77]
	→ sperm maturation	[37, 78, 81]
	↑ sperm motility	[37, 74, 75, 77, 80, 81]
	↑ sperm viability	[77]
	→ testis maturation	[71]
	↓ LPO in sperm	[80]
	→ spermatogenesis	[74, 78]
	↓ post-thawed sperm parameters and *in vitro* fertility deterioration	[81]
	↑ sperm plasma membrane integrity	[80]
Ghrelin	involvement in radiation-induced testicular damage	[83]
	↑ antioxidant defence in testis	[90]
	modulates SCF gene expression in testis	[87]
	⊣ proliferation of spermatogonial stem cells and spermatocytes	[90]
	↓ apoptosis in testis	[83]
	↑ sperm plasma membrane integrity	[92]

↑, increased; ↓, reduced

→, induced / stimulated; ⊣, suppressed

Gonadotropin Releasing Hormone (GnRH), Stem Cell Factor (SCF), Growth Hormone (GH), Insulin-like Growth Factor 1 (IGF-1), lipid peroxidation (LPO).

2.2.2.2 Non-hormonal factors

Arginine and Nitric Oxide

Arginine is a dibasic amino acid required for synthesis of nitric oxide (NO), a highly reactive free radical involved in several biological functions, which acts also as a second messenger. NO is a remarkable signalling molecule involved in neurotransmission, maintenance of normal blood pressure, myocardial function, immune regulation and inflammatory response, apoptosis, protection against oxidative damage, and it also acts as a modulator in both male and female reproductive functions [93-95]. Dietary arginine intake has been indicated as a nutritional fertility modulator [96]. Since arginine is the precursor for NO synthesis, depending on the activity of a family of isoenzymes known as nitric oxide synthases (NOS) that are expressed in male reproductive system [97], it is thought that the arginine mechanism of action in reproduction is due to the production of NO [98]. At physiologic levels, NO plays an important role in promoting normal sperm production, sperm capacitation and acrosome reaction [99], as well as in the maintenance of sperm viability and motility [100]. These findings demonstrated the relevant role of NO in sperm functionality and are also concordant with its possible anti-apoptotic effects over germ cells [99].

Adult men subjected to an arginine-deficient diet have a huge reduction in sperm cell counts and an increase in the fraction of non-motile sperm [93]. In fact, some authors have described a correlation between arginine deficiency and hampered spermatogenesis, as well as with reduced sperm motility [101]. Furthermore, arginine-HCl oral supplementation significantly improved motility and sperm counts in infertile men, allowing to achieve successful pregnancies [102]. Some amino acids present in seminal plasma, including arginine, seem to play a crucial role in spermatogenesis, sperm metabolism and motility. The administration of L-arginine to asthenospermic and oligospermic men enhanced both sperm counts and motility without adverse effects [93, 103-106]. The arginine power to increase sperm motility has an important clinical application in ART, being used to treat patients with subnormal sperm motility [103]. Even low levels of arginine not only improve sperm metabolism and spermatogenesis, but also augment the synthesis of adenosine triphosphate (ATP) that provides energy for sperm motility [101]. It has been described that arginine enhances the glycolysis rate, with production of greater amounts of ATP and lactate that became available for male gametes [101]. In addition, arginine is also involved in reversing the impairment of spermatogenesis caused by the use of glycolytic inhibitors, which are considered potential contraceptives [107]. Arginine supplementation also had a positive effect on erectile dysfunction in men with abnormal NO metabolism [108].

On the other hand, it was demonstrated that arginine administration had a beneficial role against abdominal irradiation side effects in rats [109]. In methotrexate-induced mucositis in rat, arginine supplementation avoided intestinal injury, diminishing cell death and accelerating intestinal repair, which suggested the protective role against chemotherapy-induced damage [94]. Recently, the effect of supplementation with Prelox®, which is a combination of French maritime pine bark extract (Pycnogenol®), L-arginine, L-citrulline and roburins, was determined on subfertile men.

This mixture was able to improve sperm concentration and volume, but also sperm vitality, morphology and motility [110]. Other authors also evaluated the outcome of arginine therapy on testicular induced torsion before, during and after puberty in rodents [111]. Although arginine supplementation did not enhance sperm quantity or quality in torsed testes, some improvements were observed in the contralateral testes. It was also stated that when rats suffered testicular torsion during puberty, their seminiferous epithelium were less damaged. In contrast, the smallest progresses were observed when testicular torsion occurred before puberty [111].

Arginine can also avoid membrane LPO in sperm cells under different peroxidation conditions, and its protective effects have been connected with NO biosynthesis [100, 112, 113]. NOS are expressed in many biological systems, including acrosome and tail region of non-capacitated spermatozoa [114, 115], which indicates a possible participation of NOS in sperm capacitation and acrosome reaction [98]. Indeed, it was further suggested that presence and activity of NOS is dependent on the maturity of male germ cells [99].

Although NO is required for normal male reproduction, it also can be cytotoxic. High levels of NO have been shown to reduce motility and to induce toxicity in human sperm cells [99]. Moreover, high arginine levels can reversibly inhibit rat fertility [116] and also have adverse effects in human sperm motility and fertility [117]. Also, in contrast with the favourable properties of arginine in spermatogenesis, arginine can be converted into polyamines, which in turn act as growth factors in cancer cells. Therefore, chronic administration of arginine in oncological patients should perhaps be avoided until the clarification of its safety [93].

In what concerns sperm storage, it was shown that arginine has a protective effect on post-thaw spermatozoa motility, viability, membrane integrity and LPO in cryopreserved Murrah buffalo spermatozoa, suggesting that NO may play an essential protective role against LPO during freezing and thawing processes [118]. Therefore, arginine could be added at low levels to the semen extender, since it has advantageous properties improving the conditions of spermatozoa storage, which are crucial for the success of artificial insemination [98, 112].

Carnitine/L-Carnitine

L-carnitine is a vitamin-type molecule structurally similar to amino acids, and approximately 25% of L-carnitine is synthesized endogenously from the essential amino acids lysine and methionine, whereas the other 75% are obtained through diet [119]. L-carnitine is, thus, an endogenous substance that is present in the male gonads [120] and recognized as a potent antioxidant [121]. It is a crucial co-factor for fatty acid metabolism and it has an important influence in energy production by facilitating the fatty acids transport into the mitochondria [120]. This is particularly relevant because sperm cells use fatty acid oxidation as part of their source of energy for motility. Thus, the carnitine present in the epididymal fluid will determine the fatty acids transport into the spermatozoa mitochondria [122]. Accordingly, carnitine is positively linked with sperm counts and motility [123] and its levels in the epididymides and sperm are higher than those in the blood plasma [122]. Therefore, it has been proposed that carnitine has a protective role in energy metabolism, spermatogenic process, and semen quality, and consequently in the preservation of

male fertility [124-128]. Furthermore, there is evidence that the beginning of sperm motility occurs simultaneously with an increase of carnitine levels in the epididymal lumen [122].

Carnitine also contributes to a successful sperm maturation, normal testis function [127], and possesses anti-inflammatory, immunomodulatory, and anti-apoptotic properties [129]. It was also suggested that the addition of high (20 mM), but not low (2 mM), concentrations of carnitine caused an increase of cellular Ca^{2+} transport and inhibition of sperm motility [124].

In addition, nutritional supplementation with carnitine also protects male reproductive function against several physical and chemical insults like heat [130], X-rays [131], γ-rays [129, 132], magnetic field [133], and anti-cancer drugs such as etoposide [127] and methotrexate [134]. Recently, it was demonstrated that mice treatment with carnitine before γ-rays exposure significantly reduced testicular dysfunction and improved testicular activity via regulation of spermatogenesis related genes, such as tumour necrosis factor-α, interferon-γ and interleukin 1 (IL-1)-β [129]. It was also verified a decrease in both sperm head and other morphological abnormalities, as well as a decline in the severity of radiation-induced histopathological modifications. Carnitine was also able to improve serum testosterone levels, which alleviate serum testosterone decrease in response to γ-rays. On the other hand, carnitine did not prevent the presence of giant multinucleated spermatids in seminiferous tubules [129]. It was also demonstrated in rat testis that carnitine therapy increased the recovery of seminiferous tubules and significantly reduced the severity of histopathological and morphometric changes induced by a radiation dose of 10 Gy [132]. This radiation dose induced a great decline in pregnancy, but unfortunately it was not observed any positive outcome of carnitine in respect to pregnancy rate after radiation exposure. Nevertheless, it is believed that carnitine administration would be a useful protective agent against radiation-induced injuries in male gonads, thereby improving patient's quality of life after radiotherapy [132].

Etoposide, a commonly used anti-cancer drug, induces cell death of proliferating cells in general and also of male germ cells [135]. It was shown that administration of carnitine partially protects rat testicular tissue against etoposide at pre-pubertal age, and it was proposed that the protective carnitine role happens through its action on spermatogonial stem cell [127]. Moreover, it was also considered that both deleterious and protective effects of etoposide and carnitine, respectively, might be possibly mediated by its actions via Sertoli cells, because carnitine improves Sertoli cell metabolism [127, 136, 137]. In addition, carnitine protects cell membrane against free oxygen radicals [123], while etoposide induces free radical generation [138]. So, this might represent another mechanism by which carnitine achieve beneficial effects in testicular parameters and fertility recovery, although clarification is needed [127].

Carnitine was also found to ameliorate the secondary effects of methotrexate treatment over spermatogenic cells [139]. Probably through its free radical scavenging and antioxidant features [140], L-carnitine showed to counteract both the increase in malondialdehyde levels and the decrease in superoxide dismutase levels provoked by methotrexate in male genital organs. Also, L-carnitine prevented the testicular histologic injuries induced by methotrexate, considerably diminishing the severity of damage. In the same work, the authors also evaluated the effect of vitamin E against methotrexate exposure. Considering the mitigation of both the increase in

malondialdehyde levels and the decrease in superoxide dismutase levels, vitamin E showed a better protective effect than L-carnitine. Furthermore, vitamin E had a superior outcome in decreasing the severity of interstitial edema and germ cell loss. [134]. In fact, vitamin E protects cell membranes against LPO [141], and ethanol-induced OS and decreased steroidogenesis in rat testis can be reversed by treatment with vitamin E [142]. Thus, carnitine, and also vitamin E, might be used to minimize chemotherapy side effects and, consequently, helping in the fertility preservation of cancer patients.

Carnitine administration has been indicated as a reasonable, safe and effective option to treat male infertility due to its beneficial effects on semen volume, sperm counts, motility, and morphology, as well as in fertilization capacity [143, 144]. Imhof *et al.* [100] explored the influence of a non-prescription active micronutrient compound in sperm quality in men with idiopathic sub-fertility [100]. The tested nutraceutical included eight micronutrients: L-carnitine, L-arginine, zinc, vitamin E, glutathione, selenium, coenzyme Q10, and folic acid. After 3 months of treatment, a significantly improvement in sperm quality was observed. Moreover, it also led to pregnancies without any side effects [100].

Also, there are several reports among different species in respect to carnitine protection against cryopreservation-induced damage [145]. When cryopreservation extender was supplemented with carnitine it provided protection in post-thawed sperm acrosome and total morphology [146], and an improvement in sperm motility [8]. It was also detected an important enhancement in sperm motility during liquid-storage, and a better integrity of acrosomal and plasma membrane using moderately low doses of carnitine, which may be due to its antioxidant activity in combination with its essential role in sperm cells energy and fatty acid metabolism [147].

Metallothioneins

Metallothioneins (MTs) are low molecular weight metal-binding proteins, and its synthesis can be induced either by endogenous or exogenous factors, such as cytokines and heavy metals [148]. In addition, MTs synthesis could be also induced by exposure to ionizing [149, 150] or ultraviolet (UV) radiation [151]. MTs are involved in metal detoxification and homeostasis, as well as in scavenging free radicals during oxidative injury [152]. Thus, MTs are able to defend against DNA damage, OS, apoptosis, and carcinogenesis induced by chemical stimulus and radiation [148, 153, 154]. Moreover, MTs also have the capability to increase cell survival, proliferation and angiogenesis [155].

It has been verified an overexpression of MTs in several human tumours, including testis, prostate and acute lymphoblastic leukaemia [156]. In some cases, the expression of MTs is associated with tumour grade or stage, resistance to chemotherapy or radiation, and poor prognosis [156]. Nevertheless, MTs are probably only one of various factors implicated in the complex regulation of resistance to antineoplastic therapy. In contrast, MTs are downregulated in another types of tumours [155]. Some studies proposed that metallothionein-I (MT-I) and metallothionein-II (MT-II) suppress mutation at initial stages of tumorigenesis. In fact, MT-I and MT-II knockout mice displayed elevated incidence and higher sensitivity to tumour development [157]. MT-I/II null mice are very vulnerable to several carcinogens, namely *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine

[158], 7,12-dimethylbenz[*a*]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate [159], benzo[*a*]pyrene [160], and X-ray [150], which suggests that MT-I and MT-II have antioncogenic properties. On the other hand, another MT, specifically the metallothionein-III (MT-III), was found to be unresponsive to inducing agents of MT-I and MT-II [161, 162], suggesting there are some functional differences between MTs. In fact, it was demonstrated that MT-III null mice presented reduced cadmium-induced testicular toxicity, indicating that the lack of MT-III contributes to the protection of male gonads against noxious agents. Unfortunately, the molecular mechanisms by which cadmium-induced testicular injury is attenuated in MT-III null mice are unknown yet [163]. MT-I and MT-II may also act as negative regulators of nuclear factor-kappaB activity, suppressing the development of carcinogen-induced tumours and cancer cells growth, as well as inducing the apoptosis of cancer cells [153, 155]. Recently, it was proposed that MTs are also involved in the protective effect of zinc against toxic stimuli for male reproductive tract, such as cyclophosphamide and cadmium, but the exact mechanism of action remains to be clarified [164, 165].

An experimental approach to overcome the adverse effects of numerous drugs, particularly anticancer agents, involved the pharmacological modulation of MTs. The pharmacological increase of MTs levels during anticancer drug regime protected the normal cells from chemotherapeutics-induced injury [165]. Considering yet the complex concerns about the antiapoptotic outcomes of MT-I and MT-II during radiation and chemotherapy, another solution has been proposed: to suppress the expression of MT-I and MT-II in malignant cells in order to improve the therapeutic response, while normal expression of MT-I and MT-II in healthy cells or tissue protects from treatment side effects [155]. Although looking promising, the utilization of MTs as protective factors against testicular injury in oncological patients deserves further investigation.

Liver Growth Factor

The liver growth factor (LGF) is an albumin-bilirubin complex with hepatic mitogen activity, purified from serum of rat and patients with hepatobiliary illnesses [166]. LGF promotes cell proliferation without apparent toxicity or tissue degeneration [167], and in the presence of liver injury, the concentration of LGF rises notably, displaying also neuroregenerative, anti-hypertensive, and anti-fibrotic actions [168-174].

Having in consideration the beneficial role of hepatocyte growth factor (HGF) in male fertility (e.g.: sperm motility, involvement in modulation of survival, proliferation and apoptosis of germ cells during postnatal testis development), it has been speculated a possible role for LGF in spermatogenesis [175].

Some experiments have been performed to study the role of LGF in testicular recovery, and it was shown that LGF promotes rat testicular regeneration after ethane dimethane sulfonate (EDS)-induced Leydig cell depletion [168, 170]. EDS momentarily and selectively destroys Leydig cells, diminishing serum testosterone levels, increasing pituitary secretion of FSH and LH, and consequently prejudicing spermatogenesis and causing severe morphologic modifications in testicular interstitial and germinal compartments [176, 177]. Administration of LGF avoids germ cell sloughing and Sertoli cell injury, as well as promotes germ cell growth after EDS injection in rat [178]. In addition, LGF stimulates the synthesis of vascular endothelial growth factor (VEGF) and

its receptors in the testis, what might be related to the progression of spermatogenesis and Leydig cell physiology, since in adult male no active angiogenesis occurs [179].

The dimethanesulfonate busulfan, in combination with other alkylating agents or nucleoside analogues, is considered the cornerstone of high-dose chemotherapy [180]. Busulfan selectively destroys the spermatogonial stem cells in various species, having a harmful impact in mammals' spermatogenesis by preventing cell division [168, 169]. The potential protective role of LGF in response to busulfan treatment was evaluated in mice [168]. The results revealed that LGF seems to induce testis regeneration, reflected by an increased weight of testis and epididymis, accompanied by the partial reestablishment of spermatogenesis with augmented sperm production and motility. After exposure to busulfan, LGF seemed to stimulate the remaining spermatogonial stem cells accelerating the reactivation of spermatogenesis, as well as the appearance of normal histological structure in the majority of seminiferous tubules. LGF also restored the required microenvironment for successful spermatogenesis [168]. This reactivation was also confirmed by the increase in spermatozoa concentration in comparison with mice which only received busulfan. It was concluded that LGF administration could represent a single therapy to induce the recovery of fertility in male patients after busulfan-induced germinal epithelium impairment [168].

Recently, the effect of LGF in testicular regeneration in EDS-treated rats was studied [169]. The protein levels of class B scavenger receptors (SR-BI, SR-BII, and LIMPII) and hormone sensitive lipase in male gonads were assessed, to better understand the influence of these proteins in steroidogenesis and spermatogenesis recovery. As a result, LGF enhanced the recovery of seminiferous epithelium, the appearance of the mature pattern of Leydig cell distribution, and the mature SR-BI expression. Moreover, LGF treatment was responsible for the partial recovery of hormone sensitive lipase expression in spermatogonia and Leydig cells. A progressive increase in serum testosterone levels and 3-β-hydroxysteroid dehydrogenase expression in EDS-treated group were also induced by LGF [169]. In addition, it was shown that LGF provides a faster recovery of spermatogenesis and powerfully increases the protein levels of SR-BI, which participates in cholesterol uptake from high-density lipoprotein required for spermatogenesis and cell division. For those reasons, it was proposed that regained SR-BI helped to expand the remaining testicular cells, what is favourable to the testicular regeneration as well as to the reestablishment of steroidogenesis and spermatogenesis. Furthermore, SR-BI expression in male gonad could be considered an early marker of spermatogenesis recovery after LGF therapy in EDS-castrated rats [169]. Addressing the beneficial effects of LGF in human testis upon oncological treatment and in normal physiological conditions is clearly warranted in a near future to deeply ascertain its potential use in the recovery of spermatogenesis.

Epidermal Growth Factor

Epidermal growth factor (EGF) is a polypeptide of 53 amino acids that was first isolated and purified from the submandibular glands of male mice [181]. EGF activates the tyrosine kinase EGF receptors, which in turn trigger an intracellular signalling cascade that the most known effect is the stimulation of cell growth [182, 183]. Interestingly, EGF signalling has been identified as one of the

earliest signal transduction events occurring near the cell membrane in response to a variety of stressful conditions [184-186].

Emphasizing the importance of EGF in male reproductive function, it was shown that sialoadenectomy (removal of the submandibular glands) despite reducing the amount of circulating EGF to an undetectable level, resulted in a reduction of about 55% in epididymal sperm storage [187]. In addition, overexpression of EGF induced hypospermatogenesis in mice [188]. Thus, it has been suggested that an appropriate expression level of EGF is a requisite for the normal accomplishment of spermatogenesis. In the testis, EGF is located in Sertoli cells, pachytene spermatocytes, and round spermatids [189], and it has been indicated as a regulator of Leydig cell proliferation, steroidogenesis, and Sertoli cell activity [190, 191]. *In vitro*, it was shown that EGF induces DNA synthesis in microdissected segments of rat seminiferous tubules at stage I [192]. The administration of EGF after testicular torsion improved bilateral testicular injury [193], and significantly decreased the quantity of apoptotic germ cells in cryptorchid testis [194]. EGF has an anti-apoptotic/proapoptotic action depending on the dose: low concentrations of EGF promote proliferation, whereas high concentrations of EGF induce cell cycle arrest and activate apoptosis [195].

Emerging evidence also pointed out that EGF signalling is able to decrease reactive oxygen species (ROS) production [196-198], and that testicular ischemia–reperfusion stress cause a significant increase in EGF activity [199]. Accordingly, the lack of endogenous EGF by sialoadenectomy resulted in more dramatic testicular damage after torsion/detorsion [199]. Since acute ischemia–reperfusion stress induces a massive germ cell-specific apoptosis, mainly occurring in the meiotic spermatocytes, it would lead to the perturbation of spermatogenic differentiation, and consequently, to the impairment of male fertility [199]. A recent study proposed that the prominent emergence of endogenous EGF during the early phase of testicular reperfusion may exert its anti-apoptotic effect through the modulation of the functionality of anchoring junctions [199]. Indeed, it has been suggested the involvement of anchoring junctions in the regulation of germ cell apoptosis [200], and also that the functional integrity of anchoring junctions is an indispensable factor for early recovery from testicular ischemia–reperfusion injury [199]. These findings are not totally surprising, since the central function of Sertoli cells-germ cells anchoring junctions is the creation of a network that maintains tissue integrity, and that also plays an important role in signal transduction and germ cell behaviour [201]. Moreover, data provided from rat Sertoli–germ cell co–cultures demonstrated a pro-survival influence of endogenous EGF on germ cells in response to ischemia–reperfusion induced testicular damage, which was partially mediated by phosphatidylinositol 3–kinase (PI3K)/Akt pathway [199].

Despite the late improvements in medical practice, altered testicular function and infertility remain significant sequels of testicular torsion and subsequent detorsion of spermatic cord [202], a surgical intervention conducted among boys and young men [203]. The testicular torsion and detorsion of spermatic cord requires a careful management to prevent future testicular loss [203]. It has been suggested that testicular torsion and classical ischemia–reperfusion injury share important characteristics [199] with EGF displaying beneficial effects, which raises the curiosity

about its therapeutic potential for men suffering from testicular damage induced by other exogenous factors.

Interleukins

Many cytokines are local signalling molecules, which play an important role in spermatogenesis, supplying integration and communication between diverse cell types in testis in the course of the hormonal regulation of germ cell maturation [204]. Also, seminal plasma possesses cytokines that are usually present in the male reproductive tract [205]. Focusing at the seminiferous tubules, interleukin 6 (IL-6), which is only produced by Sertoli cells, suppresses DNA synthesis. IL-1, produced by both Sertoli and germ cells, is a spermatogonial growth factor that stimulates DNA repair [206].

It has been demonstrated that cytokines are involved in the cell response to radiation [207-209]. Indeed, the radioprotective properties of IL-1 and IL-6, especially those related to the whole body irradiation, have been recognized in the mouse [209-211]. It was also shown that subcutaneous injection of IL-6, 3 to 6 days after sublethal γ-radiation exposure, has radioprotective effects [212]. Moreover, it is known that Sertoli cells could respond to chemical stress by increasing the IL-1 and IL-6 activity [213, 214]. The IL-6, but not IL-1, was increased after γ-irradiation *in vitro* of radioresistant rat Sertoli cells [215]. A mechanism pointed out to explain the radioresistance of Sertoli cells to γ-irradiation is that the increase of IL-6 after irradiation contributes to the adaptive response to OS generated by γ-irradiation, because the elevated production of ROS by ionizing irradiation causes cellular damage which can be diminished by specific molecules such as cytokines [216]. Legué and co-authors concluded that only IL-6 is increased after irradiation via a transcriptional mechanism [206]. So, both at high and low doses, IL-6 seems to be a good marker of γ-radiation treatment. Furthermore, it was verified that IL-1 and IL-6 have a radioprotective effect in mice testes, since the administration of IL-1 or/and IL-6 prior to whole body irradiation resulted in lower DNA damage on germ cells. IL-6 exerts a protective effect on the pachytene spermatocytes because 10 days after irradiation the number of round spermatids was significantly increased. Nevertheless, IL-6 should not act directly on the pachytene spermatocytes [206] because this cytokine receptor is absent in this testicular cell type [217]. Indeed, the mechanism responsible for this beneficial action remains to be clarified. To our knowledge, the radioprotective influence of IL-6 in human male gonads was not examined yet.

Granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF) is a member of the hematopoietic growth factor family, which controls proliferation, differentiation and survival of hematopoietic progenitor cells [218]. The first studies about its role in both chemotherapy and radiation-induced myelosuppressed animals revealed that G-CSF can reduce the duration of myelosuppression and increase the number of active neutrophils [219, 220]. In 1993, Schmidberger *et al.* [221] studied eleven patients, going through an isolated leucopenia, who were treated with G-CSF during fractionated radiotherapy. Seven of them received concomitantly radiation and chemotherapeutic drug, and four patients received radiotherapy only. Some secondary effects were observed such as

mild bone pain and transient increase in serum alkaline phosphatase levels. However, ten of eleven treated patients responded with improved leucocyte count. Hence, G-CSF treatment is well accepted during continuous fractionated irradiation, and it can be clinically used to mitigate neutropenia provoked by radiotherapy or by combined radio-chemotherapy [221]. Nowadays, G-CSF is a drug used for mobilization of hematopoietic stem cells from bone marrow in radiation-induced hematopoietic damage and for leucocytopenia therapy [222]. There is also evidence sustaining the diverse non-hematopoietic actions of G-CSF in another tissues. Also, it has been demonstrated that G-CSF can have an anti-apoptotic role in distinct organs, as well as an anti-inflammatory role supported by the down-regulated production of pro-inflammatory cytokines such as IL-6 and tumour necrosis factor-α [223-227]. Therefore, the effect of G-CSF in the recovery of spermatogenesis and on the defence upon pelvic γ-radiation (5 Gy) was explored in mice [226]. It was found that G-CSF notably interrupted apoptosis of germ cells after irradiation, which allowed to conclude that it improves the survival of the germ line by activating anti-apoptotic pathways. Moreover, it is believed that the protective role of G-CSF against apoptosis of germ cells is due to the activation of numerous defensive pathways, which protect spermatogonial stem cells as well as differentiating cells from irradiation-induced damage [226]. G-CSF was capable of diminish inflammation and, consequently, it is likely that it mitigates the detrimental impact of inflammatory response. Also, the protected stem cells could be induced to proliferate by G-CSF, which decreases the radiation-induced decline in the testis weight, seminiferous tubules diameter, seminiferous epithelial depth and sperm head count [226]. In the G-CSF-treated group compared with the irradiated group only, the repopulation and stem cell survival indexes in the seminiferous tubules were improved, and the frequency of abnormal sperm was lower. G-CSF also counteracted the observed reduction in sperm counts caused by γ-radiation. Thereby, G-CSF was pointed out as a radioprotective agent in testicular physiology [226]. Nevertheless, the effect of G-CSF in the protection of human irradiated testis deserve attention.

Calcium

Calcium (Ca^{2+}) is an ubiquitous second messenger involved in the activation of diverse signalling cascades, regulating a wide range of biological functions including cell cycle control, cell proliferation, migration and death [228-230]. The maintenance of low cytosolic concentrations of Ca^{2+} is fundamental for proper cell signalling, since prolonged cytoplasmic increase of free Ca^{2+} is toxic and triggers cell death [230]. Thus, Ca^{2+} homeostasis is essential in normal physiology and its deregulation is associated with pathophysiologic conditions [231]. Some studies have shown that Ca^{2+} can mediate indirectly the activation of cAMP-response-element modulator (CREM), a transcription factor that acts as a master control of spermatogenesis. In turn, CREM drives the expression of key genes required for postmeiotic differentiation including protamines 1 and 2, transition proteins 1 and 2, proacrosin, and calspermin, among others [232]. Moreover, Ca^{2+} was shown to be involved in response to hormones and local regulators [233, 234]. The differentiation and survival of spermatogenic cells implicates the adjustment of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [235-239]. Germ cells produce Ca^{2+} currents that increase their density during the development from spermatogonia to early spermatids [240]. Furthermore, deregulation

of Ca^{2+} homeostasis causes reversible infertility, which was observed when administration of Ca^{2+} channel blockers resulted in defective steroidogenesis and spermatogenesis [241-243], altered gene expression in the testis [242] and diminished sperm fertilizing potential [244, 245]. There are several proteins regulating Ca^{2+} homeostasis, including plasma membrane Ca^{2+} transport ATPase, Na^{+}/Ca^{2+} exchanger, mitochondrial Ca^{2+} uniporter channel, sarcoendoplasmic reticulum Ca^{2+}–ATPase, and regucalcin (RGN) [246, 247]. In fact, a tight control of Ca^{2+} homeostasis is a critical factor for mammalian spermatogenesis [248-251], being necessary in the maintenance of Leydig cell steroidogenesis [252, 253], in Sertoli cell function [254, 255], in the maintenance of Sertoli cells tight junctions and integrity of the blood-testis barrier [256]. The delicate control of Ca^{2+} homeostasis is also essential for adequate sperm function, namely for sperm maturation [257], sperm motility [258, 259], hyperactivation [260-263], chemotaxis [264, 265], capacitation [266] and acrosome reaction [267-271], leading to fertilization.

Regucalcin

RGN protein is another endogenous promising factor with interesting features in view of the recovery of male reproductive function after testicular injury, as well as in sperm cryopreservation [36, 272, 273]. These features concerning the regucalcin protein will be detailed in Chapter III.

Table II.2. Effects of non-hormonal factors in the recovery of male reproductive function.

Non-Hormonal Factors	Physiological Effects	References
Arginine	↓ lipid peroxidation in sperm	[112, 118]
	→ spermatogenesis	[93, 100, 101, 103, 105, 112]
	↓ cryopreservation-induced damage in sperm	[118]
	↑ sperm glycolysis rate	[101, 112]
	↑ sperm plasma membrane integrity	[118]
	↑ sperm motility	[93, 100, 101, 103-106, 112, 117, 118]
	↑ sperm viability	[117, 118]
NO	→ acrosome reaction	[99]
	↓ apoptosis signalling in sperm	[99]
	→ sperm capacitation	[99]
	↑ sperm motility	[100]
	↑ sperm viability	[100]
Carnitine/ L-Carnitine	↑ sperm counts	[123, 143]
	↑ sperm motility	[123, 143, 147]
	→ sperm maturation	[127]
	↓ apoptosis in testis	[129]
	↓ chemical- and/or physical-induced testicular damage	[127, 129-134, 139]
	→ Sertoli cell metabolism	[127]
	↓ malondialdehyde level in testis	[134]
	↑ superoxide dismutase level in testis	[134]
	↓ sperm abnormalities	[129, 144, 146, 147]
	↑ pregnancy rates	[144]
	↓ cryopreservation-induced damage in sperm	[8, 145, 146]
	↑ sperm acrosomal integrity	[146, 147]
	↓ liquid-storage-induced disturbances in sperm	[147]
	↑ sperm plasma membrane integrity	[147]
MTs	MT-I and MT-II ↓ chemical-induced damage	[164, 165]
HGF	↓ germ cell apoptosis	[175]
	↑ sperm motility	[175]
	→ testis maturation	[175]
LGF	↓ chemical-induced testicular damage	[168, 169]
	↑ epididymis weight	[168]
	→ spermatogenesis	[168, 169]
	↑ sperm motility	[168]
	→ synthesis of VEGF and its receptors in testis	[170]
	↑ testis weight	[168]
EGF	→ DNA synthesis in seminiferous tubules	[192]
	↓ testicular torsion-induced injury	[193]
	↓ apoptosis in testis	[194, 199]
	↓ testicular ischemia/reperfusion injury	[199]
	↑ integrity of Sertoli-germ cell anchoring junction	[199]
IL-1	→ DNA repair in seminiferous tubules	[206]
	↓ DNA damage on germ cells	[206]
	↓ chemical- and radiation-induced testicular damage	[206, 213]
IL-6	⊣ DNA synthesis in seminiferous tubules	[206]
	↓ DNA damage on germ cells	[206]
	↓ chemical- and radiation-induced testicular damage	[206, 214, 215]
G-CSF	↓ apoptosis in testis	[226]
	↓ radiation-induced testicular damage	[226]
	↑ seminiferous epithelial depth and tubular diameter	[226]
	→ spermatogenesis	[226]
	↓ sperm abnormalities	[226]
	↑testis weight	[226]
Ca^{2+}	→ differentiation and survival of spermatogenic cells	[235-239]
	→ steroidogenesis	[241, 252, 253]
	→ spermatogenesis	[241-243, 249]
	→ sperm fertilizing potential	[244, 245]
	→ maintenance of Sertoli cells tight junctions	[256]
	→ integrity of the blood-testis barrier	[256]
	→ sperm maturation	[257]
	→ sperm motility	[258, 259]
	→ sperm hyperactivation	[262, 263]
	→ chemotaxis	[264, 265]
	→ sperm capacitation	[266]
	→ acrosome reaction	[267-271, 274]
RGN	↓ OS in seminiferous tubules	[272]
	↓ apoptosis in testis	[36, 247, 272]
	↓ chemical-induced testicular damage	[36, 272]
	↓ sperm abnormalities	[275]
	↑ sperm maturation	[275]
	↑ sperm viability	[275]
	↑ post-thaw sperm progressive motility	[273]
	↑ post-thaw sperm acrosomal integrity	[273]
	↑ post-thaw zona pellucida-bound sperm	[273]

↑, increased; ↓, reduced

→, induced / stimulated / crucial for; ⊣, suppressed

Nitric Oxide (NO), Metallothioneins (MTs), Metallothionein-I (MT-I), Metallothionein-II (MT-II) , Hepatocyte Growth Factor (HGF), Liver Growth Factor (LGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Granulocyte Colony Stimulating Factor (G-CSF), Calcium (Ca^{2+}), Regucalcin (RGN), Oxidative Stress (OS).

3. Conclusion

Various agents have been studied in an attempt to recover or enhance the fertility potential of men who suffered from a set of conditions associated with a deleterious impact in gonadal physiology and sperm function. It is widely accepted that there are endogenous molecules providing some degree of protection against several harmful stimuli. In fact, endogenous antioxidants may offer a large defence range against low-dose cancer treatments, and may also have therapeutic properties. Some of these potential endogenous protective agents have intrinsic antioxidant properties whereas others act on cells through specific receptors, such is the case of hormones, growth factors and cytokines. The evidence here exposed identified potential hormonal (Table II.1) and non-hormonal (Table II.2) molecules able to mitigate male fertility problems arising from the administration of oncological treatments. Also, new perspectives of research for fertility preservation or to improve the recovery of spermatogenesis upon testicular injury were opened. Furthermore, it is expected that molecules with such promising properties would also be employed to develop simpler and less expensive strategies of ART, and to improve the cryopreservation processes. Apart from it, it is important to be aware of the fact that male sperm parameters are severely declining in late years. Hence, any research efforts directed to enhance the testicular outputs are emergent not only to treat medical conditions but also to ameliorate men reproductive function and maybe the continuity of future generations.

References

[1] Hess R, de Franca L. Spermatogenesis and Cycle of the Seminiferous Epithelium. In: Cheng CY, editor. Molecular Mechanisms in Spermatogenesis. Advances in Experimental Medicine and Biology. New York: Springer; 2009. 636. p. 1-15.

[2] Osterberg EC, Ramasamy R, Masson P, Brannigan RE. Current practices in fertility preservation in male cancer patients. Urol Ann. 2014;6:13-7.

[3] Trost LW, Brannigan RE. Oncofertility and the male cancer patient. Curr Treat Options Oncol. 2012;13:146-60.

[4] Stahl O, Eberhard J, Jepson K, Spano M, Cwikiel M, Cavallin-Stahl E, et al. Sperm DNA integrity in testicular cancer patients. Hum Reprod. 2006;21:3199-205.

[5] Hotaling JM, Lopushnyan NA, Davenport M, Christensen H, Pagel ER, Muller CH, et al. Raw and test-thaw semen parameters after cryopreservation among men with newly diagnosed cancer. Fertil Steril. 2013;99:464-9.

[6] Pennings G, Mertes H. Ethical issues in infertility treatment. Best Pract Res Clin Obstet Gynaecol. 2012;26:853-63.

[7] Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update. 2008;14:243-58.

[8] Bucak MN, Tuncer PB, Sariozkan S, Baspinar N, Taspinar M, Coyan K, et al. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. Cryobiology. 2010;61:248-53.

[9] Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod Biomed Online. 2018;37:327-39.

[10] Linkeviciute A, Boniolo G, Chiavari L, Peccatori FA. Fertility preservation in cancer patients: the global framework. Cancer Treat Rev. 2014;40:1019-27.

[11] Ko EY, Sabanegh ES. The role of nutraceuticals in male fertility. Urol Clin North Am. 2014;41:181-93.

[12] Vassilakopoulou M, Boostandoost E, Papaxoinis G, Rouge TdLM, Khayat D, Psyrri A. Anticancer treatment and fertility: effect of therapeutic modalities on reproductive system and functions. Crit Rev Oncol Hematol. 2015;97:328-34.

[13] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.

[14] McGlynn KA, Devesa SS, Sigurdson AJ, Brown LM, Tsao L, Tarone RE. Trends in the incidence of testicular germ cell tumors in the United States. Cancer. 2003;97:63-70.

[15] Chieffi P, Chieffi S. Molecular biomarkers as potential targets for therapeutic strategies in human testicular germ cell tumors: an overview. J Cell Physiol. 2013;228:1641-6.

[16] Albers P, Albrecht W, Algaba F, Bokemeyer C, Cohn-Cedermark G, Horwich A, et al. Guidelines on Testicular Cancer. European Urology. 2005;48:885-94.

[17] Brydoy M, Fossa SD, Dahl O, Bjoro T. Gonadal dysfunction and fertility problems in cancer survivors. Acta Oncol. 2007;46:480-9.

[18] Brauner R, Czernichow P, Cramer P, Schaison G, Rappaport R. Leydig-Cell Function in Children after Direct Testicular Irradiation for Acute Lymphoblastic Leukemia. N Engl J Med. 1983;309:25-8.

[19] Schover LR, van der Kaaij M, van Dorst E, Creutzberg C, Huyghe E, Kiserud CE. Sexual dysfunction and infertility as late effects of cancer treatment. EJC Suppl. 2014;12:41-53.

[20] Zhang Z, Shao S, Meistrich ML. The radiation-induced block in spermatogonial differentiation is due to damage to the somatic environment, not the germ cells. J Cell Physiol. 2007;211:149-58.

[21] Apperley JF, Reddy N. Mechanism and management of treatment-related gonadal failure in recipients of high dose chemoradiotherapy. Blood Rev. 1995;9:93-116.

[22] Lushbaugh CC, Casarett GW. The effects of gonadal irradiation in clinical radiation therapy: a review. Cancer. 1976;37:1111-25.

[23] Colpi GM, Contalbi GF, Nerva F, Sagone P, Piediferro G. Testicular function following chemo-radiotherapy. Eur J Obstet Gynecol Reprod Biol. 2004;113 Suppl 1:S2-6.

[24] Trottmann M, Becker AJ, Stadler T, Straub J, Soljanik I, Schlenker B, et al. Semen quality in men with malignant diseases before and after therapy and the role of cryopreservation. Eur Urol. 2007;52:355-67.

[25] Trost L, Brannigan R. Fertility Preservation in Males. In: Gracia C, Woodruff TK, editors. Oncofertility Medical Practice. New York: Springer; 2012. p. 27-50.

[26] Leonard M, Hammelef K, Smith GD. Fertility considerations, counseling, and semen cryopreservation for males prior to the initiation of cancer therapy. Clin J Oncol Nurs. 2004;8:127-31, 45.

[27] Petersen PM, Skakkebaek NE, Vistisen K, Rorth M, Giwercman A. Semen quality and reproductive hormones before orchiectomy in men with testicular cancer. J Clin Oncol. 1999;17:941-7.

[28] Tournaye H, Goossens E, Verheyen G, Frederickx V, De Block G, Devroey P, et al. Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. Hum Reprod Update. 2004;10:525-32.

[29] Katz DJ, Kolon TF, Feldman DR, Mulhall JP. Fertility preservation strategies for male patients with cancer. Nat Rev Urol. 2013;10:463-72.

[30] Howell SJ, Shalet SM. Spermatogenesis after cancer treatment: damage and recovery. J Natl Cancer Inst Monogr. 2005:12-7.

[31] Rueffer U, Breuer K, Josting A, Lathan B, Sieber M, Manzke O, et al. Male gonadal dysfunction in patients with Hodgkin's disease prior to treatment. Ann Oncol. 2001;12:1307-11.

[32] Bujan L, Walschaerts M, Brugnon F, Daudin M, Berthaut I, Auger J, et al. Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: a multicenter prospective study from the CECOS network. Fertil Steril. 2014;102:667-74.e3.

[33] Rives N, Perdrix A, Hennebicq S, Saias-Magnan J, Melin MC, Berthaut I, et al. The semen quality of 1158 men with testicular cancer at the time of cryopreservation: results of the French National CECOS Network. J Androl. 2012;33:1394-401.

[34] Degl'Innocenti S, Filimberti E, Magini A, Krausz C, Lombardi G, Fino MG, et al. Semen cryopreservation for men banking for oligospermia, cancers, and other pathologies: prediction of post-thaw outcome using basal semen quality. Fertil Steril. 2013;100:1555-63.e1-3.

[35] Sharma R, Kattoor AJ, Ghulmiyyah J, Agarwal A. Effect of sperm storage and selection techniques on sperm parameters. Syst Biol Reprod Med. 2015;61:1-12.

[36] Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, et al. Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology. 2014;2:290-8.

[37] Moss JL, Crosnoe LE, Kim ED. Effect of rejuvenation hormones on spermatogenesis. Fertil Steril. 2013;99:1814-20.

[38] Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology. 1989;124:3043-9.

[39] McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de K, Pratis K, et al. Hormonal regulation of spermatogenesis in primates and man: insights for development of the male hormonal contraceptive. J Androl. 2002;23:149-62.

[40] Weinbauer GF, Nieschlag E. Gonadotrophin-releasing hormone analogue-induced manipulation of testicular function in the monkey. Hum Reprod. 1993;8 Suppl 2:45-50.

[41] Meistrich ML. Hormonal stimulation of the recovery of spermatogenesis following chemo- or radiotherapy. APMIS. 1998;106:37-46.

[42] Meistrich ML, Shetty G. Inhibition of spermatogonial differentiation by testosterone. J Androl. 2003;24:135-48.

[43] Masala A, Faedda R, Alagna S, Satta A, Chiarelli G, Rovasio PP, et al. Use of testosterone to prevent cyclophosphamide-induced azoospermia. Ann Intern Med. 1997;126:292-5.

[44] Aminsharifi A, Shakeri S, Ariafar A, Moeinjahromi B, Kumar PV, Karbalaeedoost S. Preventive role of exogenous testosterone on cisplatin-induced gonadal toxicity: an experimental placebo-controlled prospective trial. Fertil Steril. 2010;93:1388-93.

[45] Wang L, Chadwick W, Park SS, Zhou Y, Silver N, Martin B, et al. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol Disord Drug Targets. 2010;9:651-60.

[46] Meistrich ML, Shetty G. Suppression of testosterone stimulates recovery of spermatogenesis after cancer treatment. Int J Androl. 2003;26:141-6.

[47] Meistrich ML, Wilson G, Shuttlesworth G, Huhtaniemi I, Reissmann T. GnRH agonists and antagonists stimulate recovery of fertility in irradiated LBNF1 rats. J Androl. 2001;22:809-17.

[48] Wang G, Shao SH, Weng CC, Wei C, Meistrich ML. Hormonal suppression restores fertility in irradiated mice from both endogenous and donor-derived stem spermatogonia. Toxicol Sci. 2010;117:225–37.

[49] Shetty G, Uthamanthil RK, Zhou W, Shao SH, Weng CC, Tailor RC, et al. Hormone suppression with GnRH antagonist promotes spermatogenic recovery from transplanted spermatogonial stem cells in irradiated cynomolgus monkeys. Andrology. 2013;1:886-98.

[50] Geens M, Tournaye H, Van de Velde H. Strategies for fertility preservation and restoration in the male. Facts Views Vis Obgyn. 2011;3:302-10.

[51] Glode LM, Robinson J, Gould S. Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet. 1981;317:1132-4.

[52] Meistrich ML, Kangasniemi M. Hormone treatment after irradiation stimulates recovery of rat spermatogenesis from surviving spermatogonia. J Androl. 1997;18:80-7.

[53] Udagawa K, Ogawa T, Watanabe T, Yumura Y, Takeda M, Hosaka M. GnRH analog, leuprorelin acetate, promotes regeneration of rat spermatogenesis after severe chemical damage. Int J Urol. 2001;8:615-22.

[54] Correia S, Cardoso HJ, Cavaco JE, Socorro S. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med. 2015;17:e2.

[55] Correia S, Alves MR, Cavaco JE, Oliveira PF, Socorro S. Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertil Steril. 2014;102:299-306.

[56] Sakaue M, Ishimura R, Kurosawa S, Fukuzawa NH, Kurohmaru M, Hayashi Y, et al. Administration of estradiol-3-benzoate down-regulates the expression of testicular steroidogenic enzyme genes for testosterone production in the adult rat. J Vet Med Sci. 2002;64:107-13.

[57] Hossaini A, Dalgaard M, Vinggaard AM, Pakarinen P, Larsen JJ. Male reproductive effects of octylphenol and estradiol in Fischer and Wistar rats. Reprod Toxicol. 2003;17:607-15.

[58] Henriksson P, Carlström K, Pousette Å, Gunnarsson P, Johansson C, Eriksson B, et al. Time for revival of estrogens in the treatment of advanced prostatic carcinoma? Pharmacokinetics, and endocrine and clinical effects, of a parenteral estrogen regimen. Prostate. 1999;40:76-82.

[59] Johnson RE, Murad MH. Gynecomastia: pathophysiology, evaluation, and management. Mayo Clin Proc. 2009;84:1010-5.

[60] Morris ID, Shalet SM. Protection of gonadal function from cytotoxic chemotherapy and irradiation. Baillieres Clin Endocrinol Metab. 1990;4:97-118.

[61] Rivas A, McKinnell C, Fisher JS, Atanassova N, Williams K, Sharpe RM. Neonatal coadministration of testosterone with diethylstilbestrol prevents diethylstilbestrol induction of most reproductive tract abnormalities in male rats. J Androl. 2003;24:557-67.

[62] Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB. Estrogenic induction of spermatogenesis in the hypogonadal mouse. Endocrinology. 2000;141:2861-9.

[63] Bilinska B, Kotula-Balak M, Gancarczyk M, Sadowska J, Tabarowski Z, Wojtusiak A. Androgen aromatization in cryptorchid mouse testis. Acta Histochem. 2003;105:57-65.

[64] Porter KL, Shetty G, Shuttlesworth GA, Weng CC, Huhtaniemi I, Pakarinen P, et al. Estrogen enhances recovery from radiation-induced spermatogonial arrest in rat testes. J Androl. 2009;30:440-51.

[65] Shetty G, Weng CC, Bolden-Tiller OU, Huhtaniemi I, Handelsman DJ, Meistrich ML. Effects of medroxyprogesterone and estradiol on the recovery of spermatogenesis in irradiated rats. Endocrinology. 2004;145:4461-9.

[66] Kula K, Walczak-Jedrzejowska R, Slowikowska-Hilczer J, Oszukowska E. Estradiol enhances the stimulatory effect of FSH on testicular maturation and contributes to precocious initiation of spermatogenesis. Mol Cell Endocrinol. 2001;178:89-97.

[67] Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science. 1999;285:1259-61.

[68] Shetty G, Meistrich ML. Hormonal approaches to preservation and restoration of male fertility after cancer treatment. J Natl Cancer Inst Monogr. 2005;36-9.

[69] Zhou W, Bolden-Tiller OU, Shao SH, Weng CC, Shetty G, AbuElhija M, et al. Estrogen-regulated genes in rat testes and their relationship to recovery of spermatogenesis after irradiation. Biol Reprod. 2011;85:823-33.

[70] Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, et al. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc Natl Acad Sci U S A. 2004;101:7323-8.

[71] Rogol AD, Roemmich JN, Clark PA. Growth at puberty. J Adolesc Health. 2002;31:192-200.

[72] Hibi I, Tanaka T, Tanae A, Kagawa J, Hashimoto N, Yoshizawa A, et al. The influence of gonadal function and the effect of gonadal suppression treatment on final height in growth hormone (GH)-treated GH-deficient children. J Clin Endocrinol Metab. 1989;69:221-6.

[73] Odell WD, Swerdloff RS. Etiologies of sexual maturation: a model system based on the sexually maturing rat. Recent Prog Horm Res. 1976;32:245-88.

[74] Radicioni A, Paris E, Dondero F, Bonifacio V, Isidori A. Recombinant-growth hormone (rec-hGH) therapy in infertile men with idiopathic oligozoospermia. Acta Eur Fertil. 1994;25:311-7.

[75] Ovesen P, Jorgensen JO, Ingerslev J, Ho KK, Orskov H, Christiansen JS. Growth hormone treatment of subfertile males. Fertil Steril. 1996;66:292-8.

[76] Satoh K, Ohyama K, Nakagomi Y, Ohta M, Shimura Y, Sano T, et al. Effects of growth hormone on testicular dysfunction induced by cyclophosphamide (CP) in GH-deficient rats. Endocr J. 2002;49:611-9.

[77] Nouri HS, Azarmi Y, Movahedin M. Effect of growth hormone on testicular dysfunction induced by methotrexate in rats. Andrologia. 2009;41:105-10.

[78] Glander HJ, Kratzsch J, Weisbrich C, Birkenmeier G. Insulin-like growth factor-I and alpha 2-macroglobulin in seminal plasma correlate with semen quality. Hum Reprod. 1996;11:2454-60.

[79] Henricks DM, Kouba AJ, Lackey BR, Boone WR, Gray SL. Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. Biol Reprod. 1998;59:330-7.

[80] Selvaraju S, Reddy IJ, Nandi S, Rao SB, Ravindra JP. Influence of IGF-I on buffalo (Bubalus bubalis) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro. Anim Reprod Sci. 2009;113:60-70.

[81] Selvaraju S, Nandi S, Subramani TS, Raghavendra BS, Rao SB, Ravindra JP. Improvement in buffalo (Bubalus bubalis) spermatozoa functional parameters and fertility in vitro: Effect of insulin-like growth factor-I. Theriogenology. 2010;73:1-10.

[82] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402:656-60.

[83] Li W, Zeng Y, Zhao J, Zhu CJ, Hou WG, Zhang S. Upregulation and nuclear translocation of testicular ghrelin protects differentiating spermatogonia from ionizing radiation injury. Cell Death Dis. 2014;5:e1248.

[84] Roa J, Tena-Sempere M. Connecting metabolism and reproduction: roles of central energy sensors and key molecular mediators. Mol Cell Endocrinol. 2014;397:4-14.

[85] Tena-Sempere M. Ghrelin and reproduction: ghrelin as novel regulator of the gonadotropic axis. Vitam Horm. 2008;77:285-300.

[86] Barreiro ML, Gaytan F, Caminos JE, Pinilla L, Casanueva FF, Aguilar E, et al. Cellular location and hormonal regulation of ghrelin expression in rat testis. Biol Reprod. 2002;67:1768-76.

[87] Barreiro ML, Gaytan F, Castellano JM, Suominen JS, Roa J, Gaytan M, et al. Ghrelin inhibits the proliferative activity of immature Leydig cells in vivo and regulates stem cell factor messenger ribonucleic acid expression in rat testis. Endocrinology. 2004;145:4825-34.

[88] Cardoso HJ, Figueira MI, Correia S, Vaz CV, Socorro S. The SCF/c-KIT system in the male: Survival strategies in fertility and cancer. Mol Reprod Dev. 2014;81:1064-79.

[89] Figueira MI, Cardoso HJ, Correia S, Maia CJ, Socorro S. Hormonal regulation of c-KIT receptor and its ligand: implications for human infertility? Prog Histochem Cytochem. 2014;49:1-19.

[90] Kheradmand A, Dezfoulian O, Alirezaei M. Ghrelin regulates Bax and PCNA but not Bcl-2 expressions following scrotal hyperthermia in the rat. Tissue Cell. 2012;44:308-15.

[91] Zhu CC, Zhang H, Zhang JS, Li Z, Zhao J, Li W, et al. Inhibition of ghrelin signaling improves the reproductive phenotype of male ob/ob mouse. Fertil Steril. 2013;99:918-26.

[92] Kheradmand A, Taati M, Babaei H. The effects of chronic administration of ghrelin on rat sperm quality and membrane integrity. Anim Biol. 2009;59:159-68.

[93] Appleton J. Arginine: Clinical potential of a semi-essential amino acid. Altern Med Rev. 2002;7:512-22.

[94] Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Dietary L-arginine supplementation reduces Methotrexate-induced intestinal mucosal injury in rat. BMC Gastroenterol. 2012;12:41.

[95] Wang J, He Q, Yan X, Cai Y, Chen J. Effect of exogenous nitric oxide on sperm motility in vitro. Biol Res. 2014;47:44.

[96] Lefevre PL, Palin MF, Murphy BD. Polyamines on the reproductive landscape. Endocr Rev. 2011;32:694-712.

[97] Eskiocak S, Gozen AS, Taskiran A, Kilic AS, Eskiocak M, Gulen S. Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. Braz J Med Biol Res. 2006;39:581-8.

[98] O'Flaherty C, Rodriguez P, Srivastava S. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochim Biophys Acta. 2004;1674:215-21.

[99] Roessner C, Paasch U, Glander H-J, Grunewald S. Activity of nitric oxide synthase in mature and immature human spermatozoa. Andrologia. 2010;42:132-7.

[100] Imhof M, Lackner J, Lipovac M, Chedraui P, Riedl C. Improvement of sperm quality after micronutrient supplementation. e-SPEN Journal. 2012;7:e50-e3.

[101] Patel AB, Srivastava S, Phadke RS, Govil G. Arginine activates glycolysis of goat epididymal spermatozoa: an NMR study. Biophys J. 1998;75:1522-8.

[102] Tanimura J. Studies on arginine in human semen. II. The effects of medication with L-arginine-HCL on male infertility. Bull Osaka Med Sch. 1967;13:84-9.

[103] Keller DW, Polakoski KL. L-arginine stimulation of human sperm motility in vitro. Biol Reprod. 1975;13:154-7.

[104] Scibona M, Meschini P, Capparelli S, Pecori C, Rossi P, Menchini Fabris GF. [L-arginine and male infertility]. Minerva Urol Nefrol. 1994;46:251-3.

[105] Aydin S, Inci O, Alagol B. The role of arginine, indomethacin and kallikrein in the treatment of oligoasthenospermia. Int Urol Nephrol. 1995;27:199-202.

[106] Morales ME, Rico G, Bravo C, Tapia R, Alvarez C, Mendez JD. [Progressive motility increase caused by L-arginine and polyamines in sperm from patients with idiopathic and diabetic asthenozoospermia]. Ginecol Obstet Mex. 2003;71:297-303.

[107] Patel AB, Srivastava S, Phadke RS, Govil G. Arginine acts as a protective and reversal agent against glycolytic inhibitors in spermatozoa. Physiol Chem Phys Med NMR. 1999;31:29-40.

[108] Chen J, Wollman Y, Chernichovsky T, Iaina A, Sofer M, Matzkin H. Effect of oral administration of high-dose nitric oxide donor L-arginine in men with organic erectile dysfunction: results of a double-blind, randomized, placebo-controlled study. BJU International. 1999;83:269-73.

[109] Ersin S, Tuncyurek P, Esassolak M, Alkanat M, Buke C, Yilmaz M, et al. The prophylactic and therapeutic effects of glutamine- and arginine-enriched diets on radiation-induced enteritis in rats. J Surg Res. 2000;89:121-5.

[110] Stanislavov R, Rohdewald P. Sperm quality in men is improved by supplementation with a combination of L-arginine, L-citrullin, roburins and Pycnogenol(R). Minerva Urol Nefrol. 2014;66:217-23.

[111] Ribeiro CT, Milhomem R, De Souza DB, Costa WS, Sampaio FJ, Pereira-Sampaio MA. Effect of antioxidants on outcome of testicular torsion in rats of different ages. J Urol. 2014;191:1578-84.

[112] Srivastava S, Desai P, Coutinho E, Govil G. Mechanism of action of L-arginine on the vitality of spermatozoa is primarily through increased biosynthesis of nitric oxide. Biol Reprod. 2006;74:954-8.

[113] Senbel AM, Omar AG, Abdel-Moneim LM, Mohamed HF, Daabees TT. Evaluation of l-arginine on kidney function and vascular reactivity following ischemic injury in rats: protective effects and potential interactions. Pharmacol Rep. 2014;66:976-83.

[114] Herrero MB, Perez Martinez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev. 1996;8:931-4.

[115] Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod. 1996;2:873-8.

[116] Ratnasooriya WD, Dharmasiri MG. L-arginine, the substrate of nitric oxide synthase, inhibits fertility of male rats. Asian J Androl. 2001;3:97-103.

[117] Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod. 1995;10:1786-90.

[118] Siddique R, Atreja S. Effect of l-Arginine and spermine-NONOate on motility, viability, membrane integrity and lipid peroxidation of Murrah buffalo (Bubalus bubalis) spermatozoa. Livest Sci. 2013;153:147-53.

[119] Rebouche CJ, Seim H. Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr. 1998;18:39-61.

[120] Hinton BT, Snoswell AM, Setchell BP. The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals. J Reprod Fertil. 1979;56:105-11.

[121] Gulcin I. Antioxidant and antiradical activities of L-carnitine. Life Sci. 2006;78:803-11.

[122] Jeulin C, Lewin LM. Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Hum Reprod Update. 1996;2:87-102.

[123] Lenzi A, Lombardo F, Sgro P, Salacone P, Caponecchia L, Dondero F, et al. Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. Fertil Steril. 2003;79:292-300.

[124] Deana R, Rigoni F, Francesconi M, Cavallini L, Arslan P, Siliprandi N. Effect of L-carnitine and L-aminocarnitine on calcium transport, motility, and enzyme release from ejaculated bovine spermatozoa. Biol Reprod. 1989;41:949-55.

[125] Costa M, Canale D, Filicori M, D'Lddio S, Lenzi A. L-carnitine in idiopathic asthenozoospermia: a multicenter study. Italian Study Group on Carnitine and Male Infertility. Andrologia. 1994;26:155-9.

[126] Kanter M, Topcu-Tarladacalisir Y, Parlar S. Antiapoptotic effect of L-carnitine on testicular irradiation in rats. J Mol Histol. 2010;41:121-8.

[127] Okada FK, Stumpp T, Miraglia SM. Carnitine reduces testicular damage in rats treated with etoposide in the prepubertal phase. Cell Tissue Res. 2009;337:269-80.

[128] Dokmeci D, Inan M, Basaran UN, Yalcin O, Aydogdu N, Turan FN, et al. Protective effect of L-carnitine on testicular ischaemia-reperfusion injury in rats. Cell Biochem Funct. 2007;25:611-8.

[129] Ahmed MM, Ibrahim ZS, Alkafafy M, El-Shazly SA. L-carnitine protects against testicular dysfunction caused by gamma irradiation in mice. Acta Histochem. 2014;116:1046-55.

[130] Amendola R, Cordelli E, Mauro F, Spano M. Effects of L-acetylcarnitine (LAC) on the post-injury recovery of mouse spermatogenesis monitored by flow cytometry. 2. Recovery after hyperthermic treatment. Andrologia. 1991;23:135-40.

[131] Amendola R, Bartoleschi C, Cordelli E, Mauro F, Uccelli R, Spano M. Effects of L-acetylcarnitine (LAC) on the post-injury recovery of mouse spermatogenesis monitored by flow cytometry. 1. Recovery after X-irradiation. Andrologia. 1989;21:568-75.

[132] Topcu-Tarladacalisir Y, Kanter M, Uzal MC. Role of L-carnitine in the prevention of seminiferous tubules damage induced by gamma radiation: a light and electron microscopic study. Arch Toxicol. 2009;83:735-46.

[133] Ramadan LA, Abd-Allah AR, Aly HA, Saad-El-Din AA. Testicular toxicity effects of magnetic field exposure and prophylactic role of coenzyme Q10 and L-carnitine in mice. Pharmacol Res. 2002;46:363-70.

[134] Yuncu M, Bukucu N, Bayat N, Sencar L, Tarakcioglu M. The effect of vitamin E and L-carnitine against methotrexate-induced injury in rat testis. Turk J Med Sci. 2015;45:517-25.

[135] Hakovirta H, Parvinen M, Lähdetie J. Effects of etoposide on stage-specific DNA synthesis during rat spermatogenesis. Mutat Res. 1993;301:189-93.

[136] Stumpp T, Freymuller E, Miraglia SM. Sertoli cell function in albino rats treated with etoposide during prepubertal phase. Histochem Cell Biol. 2006;126:353-61.

[137] Stumpp T, Freymuller E, Miraglia SM. Sertoli cell morphological alterations in albino rats treated with etoposide during prepubertal phase. Microsc Microanal. 2008;14:225-35.

[138] Oh SY, Sohn YW, Park JW, Park HJ, Jeon HM, Kim TK, et al. Selective cell death of oncogenic Akt-transduced brain cancer cells by etoposide through reactive oxygen species mediated damage. Mol Cancer Ther. 2007;6:2178-87.

[139] Saxena AK, Dhungel S, Bhattacharya S, Jha CB, Srivastava AK. Effect of chronic low dose of methotrexate on cellular proliferation during spermatogenesis in rats. Arch Androl. 2004;50:33-5.

[140] Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Sirvanci S, Gedik N, et al. L-Carnitine ameliorates methotrexate-induced oxidative organ injury and inhibits leukocyte death. Cell Biol Toxicol. 2006;22:47-60.

[141] Palamanda JR, Kehrer JP. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. Lipids. 1993;28:427-31.

[142] Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. Experimental therapeutic intervention with alpha tocopherol in ethanol induced testicular injuries in rats. Indian J Clin Biochem. 2007;22:138-42.

[143] Moradi M, Moradi A, Alemi M, Ahmadnia H, Abdi H, Ahmadi A, et al. Safety and efficacy of clomiphene citrate and L-carnitine in idiopathic male infertility: a comparative study. Urol J. 2010;7:188-93.

[144] Cavallini G, Ferraretti AP, Gianaroli L, Biagiotti G, Vitali G. Cinnoxicam and L-carnitine/acetyl-L-carnitine treatment for idiopathic and varicocele-associated oligoasthenospermia. J Androl. 2004;25:761-70; discussion 71-2.

[145] Kutluyer F, Kayim M, Ogretmen F, Buyukleblebici S, Tuncer PB. Cryopreservation of rainbow trout Oncorhynchus mykiss spermatozoa: effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility. Cryobiology. 2014;69:462-6.

[146] Bucak MN, Sarıözkan S, Tuncer PB, Sakin F, Ateşşahin A, Kulaksız R, et al. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Res. 2010;89:24-30.

[147] Sariozkan S, Ozdamar S, Turk G, Canturk F, Yay A. In vitro effects of l-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage. Cryobiology. 2014;68:349-53.

[148] Fujiwara Y, Satoh M. Protective role of metallothionein in chemical and radiation carcinogenesis. Curr Pharm Biotechnol. 2013;14:394-9.

[149] Miura N, Satoh M, Imura N, Naganuma A. Protective effect of bismuth nitrate against injury to the bone marrow by γ-irradiation in mice: possible involvement of induction of metallothionein synthesis. J Pharmacol Exp Ther. 1998;286:1427-30.

[150] Shibuya K, Suzuki JS, Kito H, Naganuma A, Tohyama C, Satoh M. Protective role of metallothionein in bone marrow injury caused by X-irradiation. J Toxicol Sci. 2008;33:479-84.

[151] Saito T, Tezuka T, Konno R, Fujii N. Protective effects of metallothionein I and II against metal- and ultraviolet radiation-induced damage in cultured lens epithelial cells. Jpn J Ophthalmol. 2010;54:486-93.

[152] Goossens L. Effects of gamma-ray-induced free radicals on the metal content and amino acid composition of human metallothionein-1. J Biosci. 2011;36:235-41.

[153] McGee HM, Woods GM, Bennett B, Chung RS. The two faces of metallothionein in carcinogenesis: photoprotection against UVR-induced cancer and promotion of tumour survival. Photochem Photobiol Sci. 2010;9:586-96.

[154] Sato M, Kondoh M. Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals. Tohoku J Exp Med. 2002;196:9-22.

[155] Pedersen MO, Larsen A, Stoltenberg M, Penkowa M. The role of metallothionein in oncogenesis and cancer prognosis. Prog Histochem Cytochem. 2009;44:29-64.

[156] Theocharis SE, Margeli AP, Klijanienko JT, Kouraklis GP. Metallothionein expression in human neoplasia. Histopathology. 2004;45:103-18.

[157] Waalkes MP, Liu J, Kasprzak KS, Diwan BA. Hypersusceptibility to cisplatin carcinogenicity in metallothionein-I/II double knockout mice: production of hepatocellular carcinoma at clinically relevant doses. Int J Cancer. 2006;119:28-32.

[158] Kondo Y, Himeno S, Endo W, Mita M, Suzuki Y, Nemoto K, et al. Metallothionein modulates the carcinogenicity of N-butyl-N-(4-hydroxybutyl)nitrosamine in mice. Carcinogenesis. 1999;20:1625-7.

[159] Suzuki JS, Nishimura N, Zhang B, Nakatsuru Y, Kobayashi S, Satoh M, et al. Metallothionein deficiency enhances skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate in metallothionein-null mice. Carcinogenesis. 2003;24:1123-32.

[160] Takaishi M, Sawada M, Shimada A, Suzuki JS, Satoh M, Nagase H. Protective role of metallothionein in benzo[a]pyrene-induced DNA damage. J Toxicol Sci. 2009;34:449-58.

[161] Palmiter RD, Findley SD, Whitmore TE, Durnam DM. MT-III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci U S A. 1992;89:6333-7.

[162] Kramer KK, Liu J, Choudhuri S, Klaassen CD. Induction of metallothionein mRNA and protein in murine astrocyte cultures. Toxicol Appl Pharmacol. 1996;136:94-100.

[163] Honda A, Komuro H, Shimada A, Hasegawa T, Seko Y, Nagase H, et al. Attenuation of cadmium-induced testicular injury in metallothionein-III null mice. Life Sci. 2010;87:545-50.

[164] Kheradmand F, Nourmohammadi I, Ahmadi-Faghih MA, Firoozrai M, Modarressi MH. Zinc and low-dose of cadmium protect sertoli cells against toxic-dose of cadmium: The role of metallothionein. Iran J Reprod Med. 2013;11:487-94.

[165] Maremanda KP, Khan S, Jena G. Zinc protects cyclophosphamide-induced testicular damage in rat: involvement of metallothionein, tesmin and Nrf2. Biochem Biophys Res Commun. 2014;445:591-6.

[166] Diaz-Gil J, Gavilanes J, Sanchez G, Garcia-Canero R, Garcia-Segura J, Santamaria L, et al. Identification of a liver growth factor as an albumin-bilirubin complex. Biochem J. 1987;243:443-8.

[167] Diaz-Gil JJ, Munoz J, Albillos A, Rua C, Machin C, Garcia-Canero R, et al. Improvement in liver fibrosis, functionality and hemodynamics in CCI4-cirrhotic rats after injection of the Liver Growth Factor. J Hepatol. 1999;30:1065-72.

[168] Perez-Crespo M, Pericuesta E, Perez-Cerezales S, Arenas MI, Lobo MV, Diaz-Gil JJ, et al. Effect of liver growth factor on both testicular regeneration and recovery of spermatogenesis in busulfan-treated mice. Reprod Biol Endocrinol. 2011;9:21.

[169] Lobo MV, Arenas MI, Huerta L, Sacristan S, Perez-Crespo M, Gutierrez-Adan A, et al. Liver growth factor induces testicular regeneration in EDS-treated rats and increases protein levels of class B scavenger receptors. Am J Physiol Endocrinol Metab. 2015;308:E111-21.

[170] Gonzalo-Gobernado R, Calatrava-Ferreras L, Perucho J, Reimers D, Casarejos MJ, Herranz AS, et al. Liver Growth Factor as a tissue regenerating factor in neurodegenerative diseases. Recent Pat CNS Drug Discov. 2014;9:173-80.

[171] Reimers D, Osuna C, Gonzalo-Gobernado R, Herranz AS, Diaz-Gil JJ, Jimenez-Escrig A, et al. Liver growth factor promotes the survival of grafted neural stem cells in a rat model of Parkinson's disease. Curr Stem Cell Res Ther. 2012;7:15-25.

[172] Calatrava-Ferreras L, Gonzalo-Gobernado R, Reimers D, Herranz AS, Jimenez-Escrig A, Diaz-Gil JJ, et al. Neuroprotective role of liver growth factor "LGF" in an experimental model of cerebellar ataxia. Int J Mol Sci. 2014;15:19056-73.

[173] Condezo-Hoyos L, Arribas SM, Abderrahim F, Somoza B, Gil-Ortega M, Diaz-Gil JJ, et al. Liver growth factor treatment reverses vascular and plasmatic oxidative stress in spontaneously hypertensive rats. J Hypertens. 2012;30:1185-94.

[174] Diaz-Gil JJ, Garcia-Monzon C, Rua C, Martin-Sanz P, Cereceda RM, Miquilena-Colina ME, et al. Liver growth factor antifibrotic activity in vivo is associated with a decrease in activation of hepatic stellate cells. Histol Histopathol. 2009;24:473-9.

[175] Catizone A, Ricci G, Del Bravo J, Galdieri M. Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. J Endocrinol. 2006;189:137-46.

[176] Bakalska M, Atanassova N, Koeva Y, Nikolov B, Davidoff M. Induction of male germ cell apoptosis by testosterone withdrawal after ethane dimethanesulfonate treatment in adult rats. Endocr Regul. 2004;38:103-10.

[177] Ariyaratne S, Kim I, Mills N, Mason I, Mendis-Handagama C. Effects of ethane dimethane sulfonate on the functional structure of the adult rat testis. Arch Androl. 2003;49:313-26.

[178] Martin-Hidalgo A, Lobo M, Sacristán S, Huerta L, Gómez-Pinillos A, Diaz-Gil J, et al. Rat testicular regeneration after EDS administration is stimulated by the liver growth factor (LGF). FEBS J. 2007;274:296.

[179] Martin-Hidalgo A, Arenas M, Sacristán S, Huerta L, Díaz-Gil J, Carrillo E, et al. Rat testis localization of VEGFs and VEGF receptors in control and testicular regeneration stimulated by the liver growth factor (LGF). FEBS J. 2007;274:296.

[180] Galaup A, Paci A. Pharmacology of dimethanesulfonate alkylating agents: busulfan and treosulfan. Expert Opin Drug Metab Toxicol. 2013;9:333-47.

[181] Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem. 1990;265:7709-12.

[182] Narayanan R, Kim HN, Narayanan NK, Nargi D, Narayanan B. Epidermal growth factor-stimulated human cervical cancer cell growth is associated with EGFR and cyclin D1 activation, independent of COX-2 expression levels. Int J Oncol. 2012;40:13-20.

[183] Tarcic G, Avraham R, Pines G, Amit I, Shay T, Lu Y, et al. EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. FASEB J. 2012;26:1582-92.

[184] Cai B, Tomida A, Mikami K, Nagata K, Tsuruo T. Down-regulation of epidermal growth factor receptor-signaling pathway by binding of GRP78/BiP to the receptor under glucose-starved stress conditions. J Cell Physiol. 1998;177:282-8.

[185] Zhang L, Jope RS. Oxidative stress differentially modulates phosphorylation of ERK, p38 and CREB induced by NGF or EGF in PC12 cells. Neurobiol Aging. 1999;20:271-8.

[186] Murshid A, Theriault J, Gong J, Calderwood SK. Investigating receptors for extracellular heat shock proteins. Methods Mol Biol. 2011;787:289-302.

[187] Liu A, Flores C, Kinkead T, Carboni AA, Menon M, Seethalakshmi L. Effects of sialoadenectomy and epidermal growth factor on testicular function of sexually mature male mice. J Urol. 1994;152:554-61.

[188] Wong RW, Kwan RW, Mak PH, Mak KK, Sham MH, Chan SY. Overexpression of epidermal growth factor induced hypospermatogenesis in transgenic mice. J Biol Chem. 2000;275:18297-301.

[189] Radhakrishnan B, Oke BO, Papadopoulos V, DiAugustine RP, Suarez-Quian CA. Characterization of epidermal growth factor in mouse testis. Endocrinology. 1992;131:3091-9.

[190] Bai S, Wilson EM. Epidermal-growth-factor-dependent phosphorylation and ubiquitinylation of MAGE-11 regulates its interaction with the androgen receptor. Mol Cell Biol. 2008;28:1947-63.

[191] Kuijk EW, Colenbrander B, Roelen BA. The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction. 2009;138:721-31.

[192] Wahab-Wahlgren A, Martinelle N, Holst M, Jahnukainen K, Parvinen M, Soder O. EGF stimulates rat spermatogonial DNA synthesis in seminiferous tubule segments in vitro. Mol Cell Endocrinol. 2003;201:39-46.

[193] Uguralp S, Bay Karabulut A, Mizrak B, Kaymaz F, Kiziltay A, Hasirci N. The effect of sustained and local administration of epidermal growth factor on improving bilateral testicular tissue after torsion. Urol Res. 2004;32:323-31.

[194] Kurokawa S, Kojima Y, Mizuno K, Nakane A, Hayashi Y, Kohri K. Effect of epidermal growth factor on spermatogenesis in the cryptorchid rat. J Urol. 2005;174:2415-9.

[195] Zhao X, Dai W, Zhu H, Zhang Y, Cao L, Ye Q, et al. Epidermal growth factor (EGF) induces apoptosis in a transfected cell line expressing EGF receptor on its membrane. Cell Biol Int. 2006;30:653-8.

[196] Maeda H, Rajesh KG, Maeda H, Suzuki R, Sasaguri S. Epidermal growth factor and insulin inhibit cell death in pancreatic beta cells by activation of PI3-kinase/AKT signaling pathway under oxidative stress. Transplant Proc. 2004;36:1163-5.

[197] Hussein Ael A, Shokeir AA, Sarhan ME, El-Menabawy FR, Abd-Elmoneim HA, El-Nashar EM, et al. Effects of combined erythropoietin and epidermal growth factor on renal ischaemia/reperfusion injury: a randomized experimental controlled study. BJU international. 2011;107:323-8.

[198] Zhang HY, James I, Chen CL, Besner GE. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) preserves gut barrier function by blocking neutrophil-endothelial cell adhesion after hemorrhagic shock and resuscitation in mice. Surgery. 2012;151:594-605.

[199] Zhang S, Zeng Y, Qu J, Luo Y, Wang X, Li W. Endogenous EGF maintains Sertoli germ cell anchoring junction integrity and is required for early recovery from acute testicular ischemia/reperfusion injury. Reproduction. 2013;145:177-89.

[200] Richburg JH, Boekelheide K. Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol. 1996;137:42-50.

[201] Siu MK, Wong CH, Lee WM, Cheng CY. Sertoli-germ cell anchoring junction dynamics in the testis are regulated by an interplay of lipid and protein kinases. J Biol Chem. 2005;280:25029-47.

[202] Taskara E, Gor A, Kutlu O, Karaguzel E, Cobanoglu U, Topbas M, et al. Does propofol prevent testicular ischemia-reperfusion injury due to torsion in the long term? Pediatr Surg Int. 2011;27:1003-7.

[203] Kanter M. Protective effects of Ginkgo biloba (EGb 761) on testicular torsion/detorsion-induced ischemia-reperfusion injury in rats. Exp Mol Pathol. 2011;91:708-13.

[204] Tronchon V, Vialard F, El Sirkasi M, Dechaud H, Rollet J, Albert M, et al. Tumor necrosis factor-alpha-308 polymorphism in infertile men with altered sperm production or motility. Hum Reprod. 2008;23:2858-66.

[205] Shukla KK, Agnihotri S, Gupta A, Mahdi AA, Mohamed EA, Sankhwar SN, et al. Significant association of TNFalpha and IL-6 gene with male infertility--an explorative study in Indian populations of Uttar Pradesh. Immunol Lett. 2013;156:30-7.

[206] Legué F, Guitton N, Brouazin-Jousseaume V, Colleu-Durel S, Nourgalieva K, Chenal C. IL-6 a key cytokine in in vitro and in vivo response of Sertoli cells to external gamma irradiation. Cytokine. 2001;16:232-8.

[207] Van Der Meeren A, Squiban C, Gourmelon P, Lafont H, Gaugler MH. Differential regulation by IL-4 and IL-10 of radiation-induced IL-6 and IL-8 production and ICAM-1 expression by human endothelial cells. Cytokine. 1999;11:831-8.

[208] Kennedy M, Kim KH, Harten B, Brown J, Planck S, Meshul C, et al. Ultraviolet irradiation induces the production of multiple cytokines by human corneal cells. Invest Ophthalmol Vis Sci. 1997;38:2483-91.

[209] Neta R, Perlstein R, Vogel SN, Ledney GD, Abrams J. Role of interleukin 6 (IL-6) in protection from lethal irradiation and in endocrine responses to IL-1 and tumor necrosis factor. J Exp Med. 1992;175:689-94.

[210] Neta R, Douches S, Oppenheim JJ. Interleukin 1 is a radioprotector. J Immunol. 1986;136:2483-5.

[211] Neta R, Vogel SN, Sipe JD, Wong GG, Nordan RP. Comparison of in vivo effects of human recombinant IL 1 and human recombinant IL 6 in mice. Lymphokine Res. 1988;7:403-12.

[212] Patchen ML, MacVittie TJ, Williams JL, Schwartz GN, Souza LM. Administration of interleukin-6 stimulates multilineage hematopoiesis and accelerates recovery from radiation-induced hematopoietic depression. Blood. 1991;77:472-80.

[213] Gerard N, Syed V, Jegou B. Lipopolysaccharide, latex beads and residual bodies are potent activators of Sertoli cell interleukin-1 alpha production. Biochem Biophys Res Commun. 1992;185:154-61.

[214] Syed V, Gerard N, Kaipia A, Bardin CW, Parvinen M, Jegou B. Identification, ontogeny, and regulation of an interleukin-6-like factor in the rat seminiferous tubule. Endocrinology. 1993;132:293-9.

[215] Guitton N, Brouazin-Jousseaume V, Dupaix A, Jegou B, Chenal C. Radiation effect on rat Sertoli cell function in vitro and in vivo. Int J Radiat Biol. 1999;75:327-33.

[216] Brouazin-Jousseaume V, Guitton N, Legue F, Chenal C. GSH level and IL-6 production increased in Sertoli cells and astrocytes after gamma irradiation. Anticancer Res. 2002;22:257-62.

[217] Okuda Y, Morris P. Identification of interleukin-6 receptor (IL-6R) mRNA in isolated Sertoli and Leydig cells: regulation by gonadotropin and interleukins in vitro. Endocrine. 1994:1163–8.

[218] Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood. 1991;78:2791-808.

[219] Shimamura M, Kobayashi Y, Yuo A, Urabe A, Okabe T, Komatsu Y, et al. Effect of human recombinant granulocyte colony-stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood. 1987;69:353-5.

[220] Uckun FM, Souza L, Waddick KG, Wick M, Song CW. In vivo radioprotective effects of recombinant human granulocyte colony-stimulating factor in lethally irradiated mice. Blood. 1990;75:638-45.

[221] Schmidberger H, Hess CF, Hoffmann W, Reuss-Borst MA, Bamberg M. Granulocyte colony-stimulating factor treatment of leucopenia during fractionated radiotherapy. Eur J Cancer. 1993;29:1927-31.

[222] Herodin F, Drouet M. Cytokine-based treatment of accidentally irradiated victims and new approaches. Exp Hematol. 2005;33:1071-80.

[223] Lan X, Qu H, Yao W, Zhang C. Granulocyte-colony stimulating factor inhibits neuronal apoptosis in a rat model of diabetic cerebral ischemia. Tohoku J Exp Med. 2008;216:117-26.

[224] Kudo T, Matsumoto T, Nakamichi I, Yada S, Esaki M, Jo Y, et al. Recombinant human granulocyte colony-stimulating factor reduces colonic epithelial cell apoptosis and ameliorates murine dextran sulfate sodium-induced colitis. Scand J Gastroenterol. 2008;43:689-97.

[225] Hiraumi Y, Iwai-Kanai E, Baba S, Yui Y, Kamitsuji Y, Mizushima Y, et al. Granulocyte colony-stimulating factor protects cardiac mitochondria in the early phase of cardiac injury. Am J Physiol Heart Circ Physiol. 2009;296:H823-32.

[226] Kim J, Lee S, Jeon B, Jang W, Moon C, Kim S. Protection of spermatogenesis against gamma ray-induced damage by granulocyte colony-stimulating factor in mice. Andrologia. 2011;43:87-93.

[227] Kim JS, Yang M, Lee CG, Kim SD, Kim JK, Yang K. In vitro and in vivo protective effects of granulocyte colony-stimulating factor against radiation-induced intestinal injury. Arch Pharm Res. 2013;36:1252-61.

[228] Roderick HL, Cook SJ. Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. Nature reviews Cancer. 2008;8:361-75.

[229] Becchetti A, Arcangeli A. Integrins and Ion Channels in Cell Migration: Implications for Neuronal Development, Wound Healing and Metastatic Spread. In: Becchetti A, Arcangeli A, editors. Integrins and Ion Channels. Advances in Experimental Medicine and Biology. New York: Springer; 2010. p.107-23.

[230] Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol. 2003;4:552-65.

[231] Cheng HP, Wei S, Wei LP, Verkhratsky A. Calcium signaling in physiology and pathophysiology. Acta Pharmacol Sin. 2006;27:767-72.

[232] Kimmins S, Kotaja N, Davidson I, Sassone-Corsi P. Testis-specific transcription mechanisms promoting male germ-cell differentiation. Reproduction. 2004;128:5-12.

[233] Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003;4:517-29.

[234] Steele GL, Leung PC. Intragonadal signalling mechanisms in the control of steroid hormone production. J Steroid Biochem Mol Biol. 1992;41:515-22.

[235] Reyes JG, Herrera E, Lobos L, Salas K, Lagos N, Jorquera RA, et al. Dynamics of intracellular calcium induced by lactate and glucose in rat pachytene spermatocytes and round spermatids. Reproduction. 2002;123:701-10.

[236] Mishra DP, Pal R, Shaha C. Changes in cytosolic Ca2+ levels regulate Bcl-xS and Bcl-xL expression in spermatogenic cells during apoptotic death. J Biol Chem. 2006;281:2133-43.

[237] Reyes JG, Osses N, Knox M, Darszon A, Trevino CL. Glucose and lactate regulate maitotoxin-activated Ca2+ entry in spermatogenic cells: the role of intracellular [Ca2+]. FEBS Lett. 2010;584:3111-5.

[238] Berrios J, Osses N, Opazo C, Arenas G, Mercado L, Benos DJ, et al. Intracellular Ca2+ homeostasis in rat round spermatids. Biol Cell. 1998;90:391-8.

[239] Sanchez-Cardenas C, Guerrero A, Trevino CL, Hernandez-Cruz A, Darszon A. Acute slices of mice testis seminiferous tubules unveil spontaneous and synchronous Ca2+ oscillations in germ cell clusters. Biol Reprod. 2012;87:92.

[240] Hagiwara S, Kawa K. Calcium and potassium currents in spermatogenic cells dissociated from rat seminiferous tubules. J Physiol. 1984;356:135-49.

[241] Lee JH, Ahn HJ, Lee SJ, Gye MC, Min CK. Effects of L- and T-type Ca²(+) channel blockers on spermatogenesis and steroidogenesis in the prepubertal mouse testis. J Assist Reprod Genet. 2011;28:23-30.

[242] Lee JH, Kim H, Kim DH, Gye MC. Effects of calcium channel blockers on the spermatogenesis and gene expression in peripubertal mouse testis. Arch Androl. 2006;52:311-8.

[243] Juneja R, Gupta I, Wali A, Sanyal SN, Chakravarti RN, Majumdar S. Effect of verapamil on different spermatozoal functions in guinea pigs--a preliminary study. Contraception. 1990;41:179-87.

[244] Hershlag A, Cooper GW, Benoff S. Pregnancy following discontinuation of a calcium channel blocker in the male partner. Hum Reprod. 1995;10:599-606.

[245] Benoff S, Cooper GW, Hurley I, Mandel FS, Rosenfeld DL, Scholl GM, et al. The effect of calcium ion channel blockers on sperm fertilization potential. Fertil Steril. 1994;62:606-17.

[246] Bagur R, Hajnóczky G. Intracellular Ca(2+) Sensing: Its Role in Calcium Homeostasis and Signaling. Mol Cell. 2017;66:780-8.

[247] Marques R, Maia CJ, Vaz C, Correia S, Socorro S. The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci. 2014;71:93-111.

[248] Benoff S. Voltage dependent calcium channels in mammalian spermatozoa. Front Biosci. 1998;3:D1220-40.

[249] Feng HL, Hershlag A, Han YB, Zheng LJ. Localizations of intracellular calcium and Ca2+-ATPase in hamster spermatogenic cells and spermatozoa. Microsc Res Tech. 2006;69:618-23.

[250] Publicover S, Harper CV, Barratt C. [Ca2+]i signalling in sperm — making the most of what you've got. Nat Cell Biol. 2007;9:235-42.

[251] Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, et al. Ca2+-stores in sperm: their identities and functions. Reproduction. 2009;138:425-37.

[252] Manna PR, Pakarinen P, El-Hefnawy T, Huhtaniemi IT. Functional assessment of the calcium messenger system in cultured mouse Leydig tumor cells: regulation of human chorionic gonadotropin-induced expression of the steroidogenic acute regulatory protein. Endocrinology. 1999;140:1739-51.

[253] Pandey AK, Li W, Yin X, Stocco DM, Grammas P, Wang X. Blocking L-type calcium channels reduced the threshold of cAMP-induced steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells. J Endocrinol. 2010;204:67-74.

[254] Gorczynska-Fjalling E. The role of calcium in signal transduction processes in Sertoli cells. Reprod Biol. 2004;4:219-41.

[255] Gorczynska E, Handelsman DJ. Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells. Endocrinology. 1995;136:2052-9.

[256] Grima J, Wong CC, Zhu LJ, Zong SD, Cheng CY. Testin secreted by Sertoli cells is associated with the cell surface, and its expression correlates with the disruption of Sertoli-germ cell junctions but not the inter-Sertoli tight junction. J Biol Chem. 1998;273:21040-53.

[257] Vijayaraghavan S, Hoskins DD. Changes in the mitochondrial calcium influx and efflux properties are responsible for the decline in sperm calcium during epididymal maturation. Mol Reprod Dev. 1990;25:186-94.

[258] Prien SD, Lox CD, Messer RH, DeLeon FD. Seminal concentrations of total and ionized calcium from men with normal and decreased motility. Fertil Steril. 1990;54:171-2.

[259] Kiliç S, Sarica K, Yaman O, Soygür T, Göğüş O, Yaman LS. Effect of total and ionized calcium levels of seminal fluid on sperm motility. Urol Int. 1996;56:215-8.

[260] Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update. 2008;14:647-57.

[261] Lishko PV, Kirichok Y. The role of Hv1 and CatSper channels in sperm activation. J Physiol. 2010;588:4667-72.

[262] Alasmari W, Barratt CL, Publicover SJ, Whalley KM, Foster E, Kay V, et al. The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. Human Reprod. 2013;28:866-76.

[263] Achikanu C, Pendekanti V, Teague R, Publicover S. Effects of pH manipulation, CatSper stimulation and Ca2+-store mobilization on [Ca2+]i and behaviour of human sperm. Human Reprod. 2018;33:1802-11.

[264] Teves ME, Guidobaldi HA, Uñates DR, Sanchez R, Miska W, Publicover SJ, et al. Molecular mechanism for human sperm chemotaxis mediated by progesterone. PLoS One. 2009;4:e8211.

[265] Mizuno K, Shiba K, Okai M, Takahashi Y, Shitaka Y, Oiwa K, et al. Calaxin drives sperm chemotaxis by Ca^{2+}-mediated direct modulation of a dynein motor. Proc Natl Acad Sci U S A. 2012;109:20497-502.

[266] Yeste M, Fernández-Novell JM, Ramió-Lluch L, Estrada E, Rocha LG, Cebrián-Pérez JA, et al. Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. Andrology. 2015;3:729-47.

[267] Yanagimachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res. 1974;89:161-74.

[268] Florman HM, Corron ME, Kim TD, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol. 1992;152:304-14.

[269] Fraser LR. Calcium channels play a pivotal role in the sequence of ionic changes involved in initiation of mouse sperm acrosomal exocytosis. Mol Reprod Dev. 1993;36:368-76.

[270] O'Toole CM, Roldan ER, Fraser LR. Role for Ca2+ channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. Mol Reprod Dev. 1996;45:204-11.

[271] Nguyen TMD, Combarnous Y, Praud C, Duittoz A, Blesbois E. Ca2+/Calmodulin-Dependent Protein Kinase Kinases (CaMKKs) Effects on AMP-Activated Protein Kinase (AMPK) Regulation of Chicken Sperm Functions. PLoS One. 2016;11:e0147559.

[272] Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

[273] Pillai H, Parmar MS, Shende AM, Thomas J, Sharma HS, Sharma GT, et al. Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (Bubalus bubalis). Mol Reprod Dev. 2017;84:1133-9.

[274] Nguyen TM, Seigneurin F, Froment P, Combarnous Y, Blesbois E. The 5'-AMP-Activated Protein Kinase (AMPK) Is Involved in the Augmentation of Antioxidant Defenses in Cryopreserved Chicken Sperm. PLoS One. 2015;10:e0134420.

[275] Correia S, Oliveira PF, Guerreiro PM, Lopes G, Alves MG, Canario AV, et al. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca2+-binding protein regucalcin: a hidden role for Ca2+ in sperm maturation? Mol Hum Reprod. 2013;19:581-9.

Chapter III
Regucalcin' Protein Actions in Male Reproduction

Chapter adapted from:

Santos V.M., **Silva A.M.S.***, Correia S., Socorro S. Regucalcin' actions in male reproduction: an update (in preparation for submission)*

Correia S., Pillai H.*, **Silva A.M.S.**, Santos V.M. and Socorro S. (2019) The Horizons of Regucalcin in Male Reproduction. In: "Regucalcin: Metabolic Regulation and Disease". Nova Science Publishers, Inc, New York, USA, in press. (ISBN: 978-1-53616-172-4)*

**contributed equally*

Abstract

Regucalcin (RGN) is a highly conserved calcium (Ca^{2+})-binding protein, present throughout the evolution line, from prokaryotes to eukaryotes. It is highly expressed in the liver and kidney of mammalian vertebrates, but its broad expression in a panoply of other tissues has been reported. Over the years, the RGN role in the regulation of several biological processes apart from Ca^{2+} homeostasis also was being disclosed. RGN was shown to be a multifunctional protein involved in the regulation of intracellular signalling pathways, oxidative stress, cell proliferation, apoptosis, and also energetic metabolism. All these are crucial processes for the successful production of male gametes, which propelled researchers investigating the RGN role in male reproduction. The present chapter describes the existent literature on the actions of RGN supporting spermatogenesis and sperm function, and also discusses the recent experimental advances that sustain the usefulness of this protein in reproductive technology.

Keywords: Regucalcin, Reproduction, Male fertility, Spermatogenesis, Sperm capacitation, Cryopreservation, Sex-steroid target gene, Calcium, Oxidative stress, Apoptosis.

1. Introduction

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein highly conserved throughout evolution, being present from prokaryotes to the different levels of complexity of eukaryotes [1-10]. Its role in the regulation of intracellular Ca^{2+} homeostasis has been shown to occur through the modulation of Ca^{2+}-pumps activity at plasma membrane, endoplasmic reticulum and mitochondria [11, 12]. However, RGN ability to bind other divalent cations, such as zinc (Zn^{2+}), manganese (Mn^{2+}) and magnesium (Mg^{2+}), also has been reported [13]. The full-length RGN protein has 299 amino acid residues and a molecular weight of approximately 33-34 kDa, which together with its downregulation associated with ageing, have led some authors to designate it as Senescence Marker Protein-30 (SMP-30) [5, 14, 15]. Although being highly expressed in the liver and kidney cortex, RGN expression has been detected in a broad range of tissues and body fluids of several vertebrate and invertebrate species [16]. Since 2008, the RGN expression in the male reproductive tract, including, the gonads, accessory glands, excurrent ducts fluids and spermatozoa, has been characterized [17-21]. A panoply of factors has been shown to regulate RGN tissue expression levels, namely, Ca^{2+}, glucose levels, oxidative stress (OS), insulin, thyroid and parathyroid hormones, and steroid hormones [16, 22]. Moreover, RGN expression is modulated in response to different physiological conditions and diseases states [23-28].

The last decade has witnessed the emergence of RGN as a multifunctional protein regulating distinct biological processes besides the maintenance of Ca^{2+} homeostasis. For example, RGN has been shown to regulate intracellular signalling pathways by influencing the activity of kinases,

phosphatases, phosphodiesterase, and proteases [12, 29]. Several reports have pointed out that RGN may present antioxidant properties, reducing the levels of OS, and on the other hand, increasing the antioxidant defence and protecting cells from age deterioration [16, 30]. Interestingly, accumulating evidence has put RGN as an important regulator of tissue homeostasis by its dual action controlling proliferation and apoptosis [30-33]. Therefore, its role as a cytoprotector and anti-tumour protein has been proposed [22, 34, 35]. Recently, the properties of RGN modulating cell metabolism started being disclosed and have been related to the control of glycolytic metabolism and lipid handling [36, 37].

The strict control of OS, the delicate balance between cell death and proliferation, the protection from damaging exogenous factors, as well as the proper metabolic support to ensure successful germ cell development, are determinant issues for successful spermatogenesis and, thus, male fertility. Therefore, in the last years, the role of RGN in the context of male reproduction has deserved the attention of some research groups.

2. Role of RGN in calcium homeostasis

The classical and most well-established function of RGN is as a regulator of Ca^{2+} homeostasis through the enhancement of Ca^{2+} pumping activity in several cell types [12]. RGN was reported to reduce $[Ca^{2+}]_i$ in somatic cells by activation of Ca^{2+}-ATPases in mitochondrial and endoplasmic reticulum membranes [38, 39] and Ca^{2+}/Mg^{2+}-ATPase in the plasma membrane [40]. In spermatozoa, and similar to somatic cells, the increase of $[Ca^{2+}]_i$ results from Ca^{2+} entry into cells through the plasma membrane, as well as its release from intracellular stores [41-45]. The internal storage of Ca^{2+} in sperm is low because mature cells do not contain endoplasmic reticulum, the major Ca^{2+} storage organelle. The local potential for Ca^{2+} storage include the acrosome, a redundant nuclear envelope and the mitochondria packed in the midpiece [46]. Thus, the primary source of Ca^{2+} for spermatozoa is the external environment [47]. Low basal $[Ca^{2+}]_i$ is maintained by Ca^{2+} absorption by mitochondria and active Ca^{2+} extrusion by the Ca^{2+}-pump at plasma membrane [48]. Considering RGN expression in spermatozoa, epididymis and epididymal fluid [18, 19, 21], it is likely that this protein is involved not only in the mechanisms of spermatogenesis, but also in spermatozoa maturation along the passage through the epididymis [21] and in other pre-fertilization Ca^{2+}-dependent events [19, 20]. So far, only the potential role of RGN in spermatozoa epididymis maturation [21] and capacitation have been analysed [20]. This issue will be detailed in topic 5 and 8.2.

3. RGN, an X-linked gene

The fact that RGN is encoded by X chromosome (q11.1-12 and p11.3-q11.2 segments in rat and human, respectively [49, 50]) is very interesting when envisaging its liaison with male reproduction. Initially, it was thought that the regulation of male fertility was exclusively dependent on Y-linked genes. However, the enthusiastic efforts of scientific community allowed to discover that several genes located on X chromosome can modulate fertility [51]. This is, for example, the

emblematic case of androgen receptor [52-54]. In fact, over the years it has been unrevealed that the maternal X chromosome is enriched in genes with high testicular expression, which play important roles in the regulation of spermatogenesis [55, 56]. Genetic studies have shown that several X-linked genes are implicated in the control of meiosis, playing critical functions in male germ cell maturation [56]. In fact, the X chromosome has been considered of particular interest in human infertility attributed to the male factor [56]. Since males possess only one X chromosome, if mutations in any gene linked to the X chromosome occur, males will manifest those mutations [57]. This happens because in the karyotype of a normal male (46,XY) there is no second homologous X chromosome in sexual chromosome pair, contrarily to women that typically have two X chromosomes (46,XX karyotype) [58]. Ergo, any mutation in the single-copy of an X-linked gene would not be compensated by the normal allele present in the other chromosome of the same pair (i.e., in chromosome Y), unlike in the 22 pairs of homologous nonsex chromosomes and, therefore, that mutation will manifest [59]. Such is precisely the case with RGN. Given its role in male reproductive physiology (matter to be detailed in the following topics), mutations in the RGN gene could negatively impact male fertility. However, it is also the information given by the single-copy nature of this gene that can provide the clinical practice with strategies towards the preservation of male fertility.

4. Expression pattern of RGN in male reproductive tract and spermatozoa

In 2004, it was shown that RGN was expressed in the bovine ovarian follicle, and an association with follicular growth and dominance and, consequently, with reproduction was reported. An 8-fold higher expression level of RGN was described in dominant follicles when compared to small follicles, indicating that RGN may play an important role in germ cells [60]. Later, RGN mRNA was identified in mice testis [61], and the first evidence of RGN protein expression in a broad range of male reproductive organs was reported for rat and human by Sílvia Socorro group in 2011 [18]. These findings were followed by the characterization of RGN expression in the testis, epididymis, prostate and seminal vesicles of other mammalian species, namely, buffalo and bovines. Table III.1 summarizes the outcomes from the main studies reporting RGN expression in male reproductive organs and spermatozoa. In the testis, RGN was shown to be expressed in the Leydig cells, in the intratubular somatic cells, the Sertoli cells, as well as in the germline [18].

Pillai *et al.* [19] were pioneer describing the presence of RGN in spermatozoa, namely in acrosome region. As described above, acrosome is the major Ca^{2+}-storage organelle of spermatozoa, and the localization of RGN in the acrosomal region propelled researchers to focus on finding out this protein role in Ca^{2+}-related functions like capacitation, acrosomal reaction and membrane fusion (to be detailed in topic 8). Apart from the three reported isoforms of RGN (33-34 kDa, 28 kDa and 24 kDa) [62], two new isoforms of 48 kDa and 44 kDa were reported in sperm [19]. The same study also showed the association of RGN to spermatozoa membrane and its relocation from the cytosol to the acrosomal region during maturation, i.e. from testicular spermatozoa to ejaculated spermatozoa.

Moreover, RGN has been identified as a secreted protein being detected in several fluids of male reproductive tract, namely, in seminiferous tubules, epididymis, and seminal vesicles fluids [18, 20, 21, 63].

Table III.1. RGN expression in tissues, cells, and fluids of male reproductive tract.

Tissue	Species	Localization	Biological form	Reference
Testis	Human	Cytoplasm and some nuclei of Leydig cells, Sertoli cells and germ cells	mRNA and Protein	[18]
	Bovine	Cytoplasm of Leydig cells and weak staining of nuclei of some spermatogonia	mRNA and Protein	[64]
	Buffalo	Cytoplasm and nucleus of Sertoli cells and germ cells	mRNA and Protein	[20]
	Rat	Cytoplasm and some nuclei of Leydig cells, Sertoli cells and germ cells	mRNA and Protein	[18]
	Mice	-	mRNA	[61]
Seminal vesicles	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	[20]
	Rat	Epithelium	mRNA and Protein	[18]
Epididymis	Buffalo	Cytoplasm and nuclei of glandular epithelial cells, luminal secretion and interstitial tissue	mRNA and Protein	[20]
	Rat	Epithelium, smooth muscles and connective tissue	mRNA and Protein	[18]
Prostate	Bovine	Cytoplasm of glandular epithelial cells		[64]
	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	[20]
	Rat	Epithelium	mRNA and Protein	[18]
Bulbo-urethral glands	Bovine	Nuclei of glandular cells	mRNA and Protein	[64]
	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	[20]
Ampulla of vas deferens	Buffalo	Nuclei of glandular epithelium and interstitial tissue	mRNA and Protein	[20]
Spermatozoa	Buffalo	Acrosome	mRNA and Protein	[19]
Seminiferous Tubule Fluid	Rat	-	Protein	[18]
Epididymal Fluid	Rat	-	Protein	[21]
Seminal Vesicular Fluid	Buffalo	-	Protein	[20, 63]

4.1 RGN expression in distinct phenotypes of human spermatogenesis

As a clue to understand the liaison of RGN to male reproduction, its expression pattern in human testis with normal and abnormal phenotypes of spermatogenesis was investigated. RGN expression levels were analysed in cases of hypospermatognesis (HP) and Sertoli cell-only syndrome in comparison with those of testis with conserved spermatogenesis. Increased expression of RGN was

found in the testis of men with HP comparatively with the cases of obstructive azoospermia with conserved spermatogenesis and Sertoli cell-only syndrome [65].

The aetiology of HP is complex and poorly understood, but it has been established that the development and maintenance of successful spermatogenesis depend on a delicate balance between germ cell proliferation and apoptosis [66]. Indeed, augmented rates of apoptosis and altered expression patterns of a panoply of cell death regulators have been detected in the testes of subfertile and infertile men [67-69]. Moreover, accelerated apoptosis has been implicated as a cause for the decreased number of spermatogonia in HP cases, rather than proliferative dysfunction [68].

Presently, no definitive conclusion can be drawn for the increased expression levels of RGN in HP cases. However, it is liable to speculate that RGN may be acting as a protective molecule counteracting the augmented rates of apoptosis associated with disrupted spermatogenesis. A dual role for RGN controlling cell cycle and apoptosis has been proposed [16, 22], with RGN expression being increased in response to the induction of apoptosis by chemical or physical agents [28, 70-74]. Thus, the equilibrium of RGN expression levels would be determinant for the maintenance of the appropriate germ cell lineage number and, consequently, for successful spermatogenesis. The RGN' roles modulating apoptosis, and as a protective molecule for male germ cells, have been investigated in recent years and will be discussed further in the following sections.

5. Role of RGN in development and ageing

Age has been recognized as a factor affecting semen quality in several species [75-78]. In humans, with advancing age, a decline in ejaculate volume [79], sperm number [80, 81], motility [80-83] and normal morphology [80, 81, 84] has been described. Furthermore, the testicular volume increases during puberty, reach its peak at the age of 30 and decreases after 60 years old [85]. These represent some of the age-related modifications contributing to a reduced fertility in older males.

As previously indicated, RGN expression diminishes with ageing, reason why some authors call this protein as SMP-30 [15, 50, 86, 87]. Initially, studies in rat liver and kidney revealed that, in these tissues, the maximum expression of RGN is reached within the first month after birth and is maintained up to 3 or 6.5 months, respectively, after which occur a marked decrease of its expression with ageing progression [15].

The age-related down-regulation of RGN expression was later corroborated in other tissues, including the prostate and the testis [18, 30]. In rat testis, RGN mRNA expression reaches a maximum at 120 days, which corresponds to rat adulthood, and decreases afterwards with rat ageing [18]. In the prostate, RGN expression reached a peak in young rats with 3 months, maintaining a plateau until the age of 6 months, after which RGN expression decreased [30].

Hence, RGN is an important ageing marker and its down-regulation with ageing suggests a probable role in age-related pathologies. In fact, very recently, a study by Cardoso *et al.*, 2018, concluded that RGN is a molecular frailty biomarker, and therefore, its use in biomarker panels is a valuable method for identifying elderly people at risk for increased vulnerability, disease, injury

and mortality [88]. Conversely, it has also been established that RGN overexpression has a protective role against the development of age-related pathologies in several tissues, including in rat prostate, by preventing changes in OS [30]. Overexpression of RGN counteracted the effects of increased prostate cell proliferation and resistance to apoptosis induced by ageing [30]. In addition, in the prostate of Tg-RGN rats, there was an increase in both the activity of glutathione S-transferase and the antioxidant capacity, which occurred in response to the age-associated increase in OS, and was not observed in Wt [30]. OS is one of the proposed mechanisms responsible for the functional deterioration of cells and tissues related to ageing [89], and represents one of the main factors involved in the sperm dysfunction and sperm DNA damage [90-92]. Regarding the decrease in fertility with ageing and considering OS contribution to poor fertility [93], RGN age-related decrease might explain OS increase with age and, consequently, the worsened fertility rates in older individuals. Since age is a major risk factor for the development of the majority of neoplastic diseases, another main finding is that RGN may act as a tumour suppressor protein. A diminished expression of RGN was found in several animal and human cancer cell lines and tissues, namely the liver, kidney, brain, lung, breast and prostate [22]. However, RGN overexpression protected from carcinogen-induced mammary gland tumour development and was linked with reduced proliferation and increased apoptosis in this tissue [33]. Altogether, RGN seems to be a potent protective molecule against OS, inflammation and tumorigenesis, which are factors that increase with age and contribute to the onset of several pathologies. Indeed, the decrease of RGN with ageing is likely a mechanism that may be associated with human diseases. Accordingly, treatment options exploring RGN may promote better health and fertility in older ages, possibly with emphasis for those options mainly associated with its antioxidant effect. In line with this, beneficial roles of RGN for the fertilization capacity of mammalian spermatozoa will be also covered deeper in topic 8.

6. Evidence of RGN as a sex steroid target gene

Sex steroid hormones, namely androgens and estrogens, exert their biological actions by interaction with androgen and estrogen receptors, respectively. These receptors belong to the superfamily of nuclear transcription factors regulating gene expression network in cells and tissues dependently on environmental conditions and developmental stage [94, 95]. Since the emergence of modern molecular biology techniques in the 80s-90s years, the structure and function of steroid receptors, as well as their molecular targets and tissue expression patterns, have been characterized [96-99]). In the reproduction field, the identification of new steroid target genes helped to clarify the molecular basis of spermatogenesis and the relevance of specific players for male fertility [18, 70, 98, 100, 101]). Thus, several studies aimed to investigate the role of sex steroid hormones regulating RGN expression levels in reproductive tissues. Subcutaneous administration of 17β-estradiol (E_2) augmented RGN mRNA expression in the rat liver [102], whereas producing the opposite effect in the kidney [103], mammary gland and prostate [17]. Also, in bovine bulbourethral glands and prostate, E_2 administration lead to decreased RGN expression [64]. Interestingly, E_2-treated MCF-7 breast cancer cells displayed up-regulated expression of RGN for 6 and 12 h of

stimulation, but longer exposure times lead to a diminished expression of RGN [104], in accordance with the results in the rat mammary gland *in vivo* after 7 days of treatment [17].

The action of estrogens regulating RGN expression in the testis was assessed using the *ex vivo* culture of rat seminiferous tubules. E_2-treatment (100 nM) for 24 hours augmented RGN expression in intratubular testicular cells, which was suggested to be a response mechanism to counteract the enhanced apoptosis driven by estrogens [105]. Contrastingly, a study in bovines demonstrated that *in vivo* weekly treatment, totalizing 190 mg E_2/animal, caused down-regulation of RGN expression in the testis [64].

Concerning the regulation by androgens, orchidectomized rats displayed reduced RGN expression in the kidney, but it was induced after testosterone administration (2 mg/Kg) [106]. However, regarding reproductive tissues, orchidectomy increased RGN expression in rat prostate, and a down-regulation was observed after 5α-dihydrotestosterone (DHT) administration [32]. Accordingly, treatment of LNCaP prostate cancer cells with DHT diminished RGN expression, an effect that seemed to be mediated by the androgen receptor but involving *de novo* protein synthesis [104]. The androgen receptor was also implicated on the effect of DHT (10^{-7} M) up-regulating RGN expression in rat seminiferous tubules cultured *ex vivo* [18]. This was the first evidence confirming RGN as an androgen-target gene. However, this result was not followed by *in vivo* findings. In bovines, weekly testosterone administration, for a total of 1.050 g/animal, decreased RGN expression in the testis [64].

Overall, the existent literature has established RGN both as an estrogen- and androgen-target gene. However, sex steroid hormones actions modulating RGN expression levels seem to be tissue- and species-specific, and time-dependent, which might be due to specificities in the interaction with the DNA or differential recruitment of estrogens and androgen receptors co-regulators. Moreover, changes in RGN expression in response to steroids were suggested to be related to the suppression of germ cells apoptosis with RGN mediating these hormones' functions as survival factors in spermatogenesis [107].

Androgens and estrogens have been proposed to increase intracellular Ca^{2+} levels in several cell types [108-112]. Thus, considering the influence of RGN over Ca^{2+} handling [11, 21] and the importance of this ion in spermatogenesis and sperm function it would be relevant to explore the relationship between sex steroid hormones, RGN and intracellular Ca^{2+} levels in reproductive tissues.

7. RGN's influence in male reproduction: what we have learned from the transgenic rat model

After the description of Ca^{2+}-binding protein RGN in several tissues of male reproductive tract, including testis [18], and its identification as an estrogen- and androgen-target gene [17, 18, 104], the potential role of this protein in testicular function has been exploited. A substantial body of evidence has been produced using a transgenic rat model overexpressing RGN (Tg-RGN), which was originally generated by Yamaguchi M [113] by means of oocyte transgene pronuclear injection. These animals are fertile and their spermatogenic output was first investigated by Correia *et al.*

[21]. Quantitative and qualitative sperm parameters, as well as the morphology and function of epididymis, were compared between Tg-RGN and their wild-type (Wt) littermates. Although able to breed, Tg-RGN animals displayed lower sperm counts and reduced sperm motility (Table III.2). However, these features seem to be counterbalanced by the higher viability and diminished incidence of morphological defects (Table III.2) found in the epididymal sperm of Tg-RGN rats [21].

Table III.2. Sperm parameters in the Tg-RGN rats in comparison with their Wt littermates.

*- Published in [21]; Values represent mean ± SEM.

Sperm parameters*	Tg-RGN	Wt	p-value
Sperm counts	$1.28x10^8 \pm 9.24x10^6$	$1.72x10^8 \pm 1.57x10^7$	*P<0.05*
Motility	47.88 % ± 3.67	64.60 % ± 5.66	*P<0.05*
Viability	38.75 % ± 2.36	28.00 % ± 3.84	*P<0.05*
Normal morphology	74.13 % ± 3.74	57.58 % ± 1.76	*P<0.01*
Tail defects	18.60 % ± 2.60	36.18 % ± 2.04	*P<0.001*

As previously described in the Chapter I, it is through the passage in the epididymis, specifically in the order *caput*, *corpus* and *cauda*, and before being released in the *vas deferens* (Figure I.2), that sperm acquire the ability to move progressively and fertilize an oocyte [114]. The unique microenvironment of the epididymal lumen is mainly maintained by the secretory activity of the *caput* region [115]. The *corpus*' secretory activity is lower, playing a role in late sperm maturation events whereas *cauda* essentially stores the functionally mature sperm [116]. RGN expression was two-fold higher in the *corpus* relatively to *caput* and *cauda* regions, which emphasizes its importance for sperm maturation [21].

Interestingly, an altered morphology of *caput* was found in the epididymis of Tg-RGN rats. Although there were no differences in the tubule area, boundwidth, boundheight, and perimeter between Wt and Tg-RGN groups, the epithelial cell height of this region was significantly decreased in Tg-RGN [21]. The altered morphology indicated important alterations in the reabsorptive/secretory activity of this epididymis region that could have an impact in sperm parameters. Indeed, diminished capacity of Ca^{2+} influx was detected in the epididymis of Tg-RGN rats, which suggested that Ca^{2+} concentrations are augmented in the epididymal fluid and that might be responsible for the reduced sperm motility observed in these animals [21].

Moreover, epididymal tissues of Tg-RGN animals showed a higher antioxidant potential compared with Wt [21]. Oxidative damage is one of the main factors leading to male germ cell death and increased incidence of defects [117]. It is also known that RGN has been linked with the decreased

generation of reactive oxygen species (ROS) and increased activity of antioxidant defence systems in several cell types, including liver, lung, heart and brain cells [29, 118-121]. This led the authors to assume that the higher sperm viability and the diminished incidence of morphological defects exhibited by Tg-RGN animals (Table III.2) may be a consequence of the RGN' role protecting sperm from OS.

Therefore, the antioxidant role of RGN in testicular cells was deepened in further research by exposing seminiferous tubules of Tg-RGN rats and controls to pro-oxidant stimuli, namely, *tert*-butyl hydroperoxide and cadmium chloride [70]. As hypothesized, Tg-RGN animals displayed increased protection against oxidative damage, exhibiting lower levels of OS demonstrated by diminished lipid peroxidation (LPO) levels [70]. Also, antioxidant defences, such as glutathione S-transferase, were augmented in the Tg-RGN when exposed to oxidant conditions [70]. As expected, the lower oxidative damage in the seminiferous tubules of Tg-RGN rats was translated in reduced rates of apoptotic cell death [70].

Germ cells are highly sensitive to endogenous and exogenous damaging factors and, as discussed previously in topic 4.1, increased apoptosis has been indicated as a cause of male infertility. The anti-apoptotic effect of RGN has been suggested in different cell lines and *in vivo* models involving multiple pathways and molecular targets, such as Akt, p53, Fas, transforming growth factor-β, tumor necrosis factor-α, caspases and several Bcl-2 family members [16, 22, 31]. The overexpression of RGN was shown to suppress thapsigargin- and actinomycin D-induced apoptosis in rat seminiferous tubules cultured *ex vivo* by modulating the expression and activity of key apoptotic and anti-apoptotic factors [71]. Reduced expression and activity of the effector caspase-3 were observed together with increased expression of p53 and Bcl-2 [71].

Sertoli cells are the somatic cells responsible for providing the germ cells with physical support, as well as with the adequate supply of growth factors and nutrients, namely lactate that has been indicated as the preferred energy source for germ cells [122]. Very recently, the metabolic status of primary Sertoli cells cultures obtained from the testis of Tg-RGN rats was characterized. These cells, though consuming less glucose, produced high levels of lactate, displayed increased expression of alanine transaminase, and augmented glutamine consumption indicating high plasticity of metabolic routes in response to RGN overexpression [123]. Moreover, the lactate produced seems to be consumed by the germ cells with a consequent diminution of apoptotic rate [123].

In sum, the Tg-RGN model has been contributing to establish the basis of the RGN' role in male reproduction. Also, it allowed a better understanding of the molecular regulation of spermatogenesis and sperm function opening new avenues of research, as well as novel perspectives for the development of infertility treatments and contraceptive methods.

8. Protective roles of RGN for the fertilization capacity of mammalian spermatozoa

8.1 Anti-oxidant effect

Mammalian spermatozoa are highly susceptible to oxidative damage due to their higher content of polyunsaturated fatty acids. Therefore, the presence of antioxidants in the seminal fluid is very important to maintain the highly sensitive redox equilibrium of spermatozoa and their functionality. Superoxide dismutase, catalase and glutathione peroxidase (GPX) are the major antioxidant enzymes present in semen playing a relevant role to reduce OS [124].

RGN is broadly present in male reproductive tract tissues and secretions till ejaculation (Table III.1; Figure III.1) [18, 20, 21]. After ejaculation, RGN disappears from seminal plasma whereas spermatozoa maintain RGN expression localized at the acrosomal region [19, 20]. The antioxidant properties of RGN have been demonstrated by many studies in different cell lines and *in vivo* models [125-127]. RGN has been shown to reduce intracellular levels of OS through modulation of enzymes involved in the generation of free radicals, as well as in the antioxidant defence [29, 118, 119, 128]. It has been demonstrated that superoxide dismutase activity was enhanced in normal rat liver and heart in the presence of exogenous RGN [29, 119] and the GPX activity was reduced in animals without RGN [125, 126]. Also, RGN overexpression *in vivo* was capable of counteracting ageing-associated changes in rat prostate, maintaining low levels of OS, reducing LPO and sustaining high activity levels of glutathione-S-transferase [30].

Furthermore, RGN has been identified as a gluconolactonase [129], an enzyme involved in the penultimate step of L-ascorbic acid synthesis in the liver. Ascorbic acid, a cofactor in metal-dependent oxygenases [130], has been indicated as an important antioxidant in semen that protects spermatozoa during cryopreservation [131]. As discussed earlier, a higher antioxidant potential was reported in the epididymis of Tg-RGN rats, which was linked to the higher sperm viability, higher percentage of normal morphology and diminished incidence of tail defects compared to Wt counterparts [21]. Moreover, the testis of Tg-RGN animals was shown to display increased antioxidant capacity and enhanced activity of glutathione-S-transferase [70]. Future research efforts are warranted to disclose the role of RGN as a gluconolactonase and modulating the activity of superoxide dismutase and GPX in the testis and epididymis, as well as in sperm storage and cryopreservation.

The anti-oxidative properties of RGN also may account for the maintenance of the fertility potential of spermatozoa from spermatogenesis until fertilization of ovum, as well as in sperm cryopreservation (to be detailed in topic 8.3) (Figure III.1).

8.2 Anti-capacitatory effect

The capacitation process represents the set of biochemical, structural and physiological changes that sperm undergo in the female reproductive tract rendering them able to fertilize an oocyte [132]. Seminal plasma and its components help to prevent the premature onset of sperm capacitation. Several decapacitation factors like phosphatidylcholine-binding proteins [133], semenogelin [134], cholesterol [135] and Zn^{2+} [136] are present in seminal plasma. Moreover, the capacitation-associated tyrosine phosphorylation of sperm proteins is also shown to be inhibited by seminal plasma [137].

The decapacitation factors should be removed after ejaculation for the occurrence of capacitation and the subsequent acrosomal reaction, which ultimately allows spermatozoa to fertilize the ovum. The proteolytic enzymes released upon acrosome reaction enable the fusion of sperm and oocyte membranes [138]. RGN is present in the testis, sperm and throughout male excurrent ducts, including epididymal and vesicular fluids, and disappearing in the ejaculated seminal plasma (Figure III.1). These findings indicated the potential anti-capacitatory role of RGN, which was investigated by Pillai *et al.* [20]. Co-incubation of recombinant RGN with buffalo seminal plasma confirmed the degradation of protein in the presence of seminal components [20]. Another evidence for the anti-capacitatory property of RGN is that the epididymal spermatozoa of Tg-RGN showed a reduction in motility compared to their Wt counterparts [21].

The suppressive effect of RGN on *in vivo* capacitation was further studied in buffalo using the fluorescent cholortetracyclin assay to detect capacitated spermatozoa [20]. The addition of recombinant RGN to capacitating media significantly reduced the percentage of capacitated spermatozoa compared to the untreated group.

The presence of RGN in spermatozoa cytoplasm, nucleus and acrosome suggest it might be involved in the efflux of Ca^{2+} thereby reducing $[Ca^{2+}]_i$. Therefore, the anti-capacitatory function indicated for RGN may be due to Ca^{2+}-efflux, as many studies have shown that elevation of sperm intracellular Ca^{2+} (Ca^{2+} influx) is required for hyperactivation, capacitation, and acrosome reaction [47, 139-142]. In fact, control mechanisms for $[Ca^{2+}]_i$ are fundamental in capacitation process and it was already demonstrated that Ca^{2+}-ATPases play an important role in sperm motility and capacitation [143, 144]. RGN control of Ca^{2+}-ATPases might, therefore, also be part of the mechanism by which it acts in capacitation, as Ca^{2+}-ATPase function is inhibited during capacitation, leading to an increase of acrosomal Ca^{2+} that results in the triggering of acrosomal exocytosis [145].

Overall, gathered information indicating the anti-capacitatory role of RGN raises the curiosity about its action in human sperm and the benefits of this protein in assisted reproductive techniques (ART), for example to achieve the on-time sperm capacitation improving success outcomes. Further research should be done to determine RGN role in other pre-fertilization events, since RGN localization in the acrosomal region of ejaculated spermatozoa [19] in conjunction with Ca^{2+} requirement for hyperactivation, capacitation, and acrosome reaction leads to speculate that this protein may also have a role in other Ca^{2+}-dependent pre-fertilization processes.

8.3 Cryoprotective role of RGN in spermatozoa

Cryopreservation of semen and artificial insemination are important procedures that allowed significant benefits to the livestock industry [146], as well as introduced important advances in human reproduction technology [147]. Cryopreservation of human sperm started in the 1960s, and over the years it has been noted that the fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm [148]. Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease their fertility potential [149], as described above in topic 2.2.1 of Chapter II. Factors as sudden temperature changes, ice formation and osmotic stress have been proposed as the main reasons for poor sperm quality after cryopreservation and thawing [147]. In addition, the procedures of cryopreservation induce premature capacitation of spermatozoa, which is known as cryo-capacitation [146]. These alterations negatively affect the ability to interact with the female reproductive tract and reduce sperm fertility. For example, 8 times more cryopreserved bovine spermatozoa were required to achieve equivalent fertilization rates *in vivo* compared to fresh sperm [146].

A cytoprotective role has been proposed for RGN by its actions counteracting deregulated proliferation and apoptosis, as well as minimizing Ca^{2+}-related stress and oxidative damage (Figure III.1) [16, 128]. Noteworthy, deregulation of Ca^{2+} levels and abnormal levels of ROS are the main cyto-damaging factors during sperm cryopreservation. Most importantly, RGN was reported as a putative cold tolerance gene in *Drosophila montana* [150], which supported the advantageous role of this protein in sperm cryopreservation. Indeed, the cryoprotective role of recombinant RGN in buffalo spermatozoa was recently reported. Supplementation of buffalo semen extenders with 1 μM of recombinant RGN during freezing resulted in significant increases in the post-thaw progressive motility, acrosome integrity, and zona pellucida binding of spermatozoa compared to control conditions without RGN [151].

Disrupted Ca^{2+}-channel or –pump activity also can be occurring in sperm cryopreservation, given that thawed bull and human sperm have shown to contain increased $[Ca^{2+}]_i$ compared to those before cryopreservation [152, 153]. These findings reflect impaired membrane-selective permeability and/or an inability to maintain physiological concentrations of Ca^{2+} [152, 153]. Moreover, this disruption has consequences, as elevated Ca^{2+} can lead to premature acrosome reaction as well as poor fertility outcomes for the post-thaw sperm. Conversely, the proper regulation of sperm Ca^{2+} channel function can reduce the rate of premature acrosome reaction [154]. Therefore, it is likely to assume that RGN by regulating Ca^{2+} handling proteins can reduce the $[Ca^{2+}]_i$ and minimize Ca^{2+}-related stress thereby protecting the spermatozoa from premature capacitation in reproductive ducts as well as from cryo-capacitation during cryopreservation.

Another possibility in preventing premature capacitation/cryo-capacitation may be due to the Ca^{2+}-binding ability of RGN. Extracellular RGN can bind Ca^{2+} ions and thus reduce $[Ca^{2+}]_i$ by limiting the availability of extracellular Ca^{2+} that would otherwise enter the sperm through the compromised membrane pumps or cation channels. In turn, the degradation of RGN in ejaculated seminal plasma may release the bound Ca^{2+} that can be further used for capacitation [20]. Indeed,

the ability of RGN to counteract the excessive production of ROS and Ca^{2+}-related stress may be accountable to its cryoprotective effect in spermatozoa (Figure III.1).

Altogether, research conducted to date suggest that RGN has very important roles in male reproduction from spermatogenesis to fertilization (Figure III.1). Further research would help to clarify the mechanisms underlying RGN actions in sperm physiology.

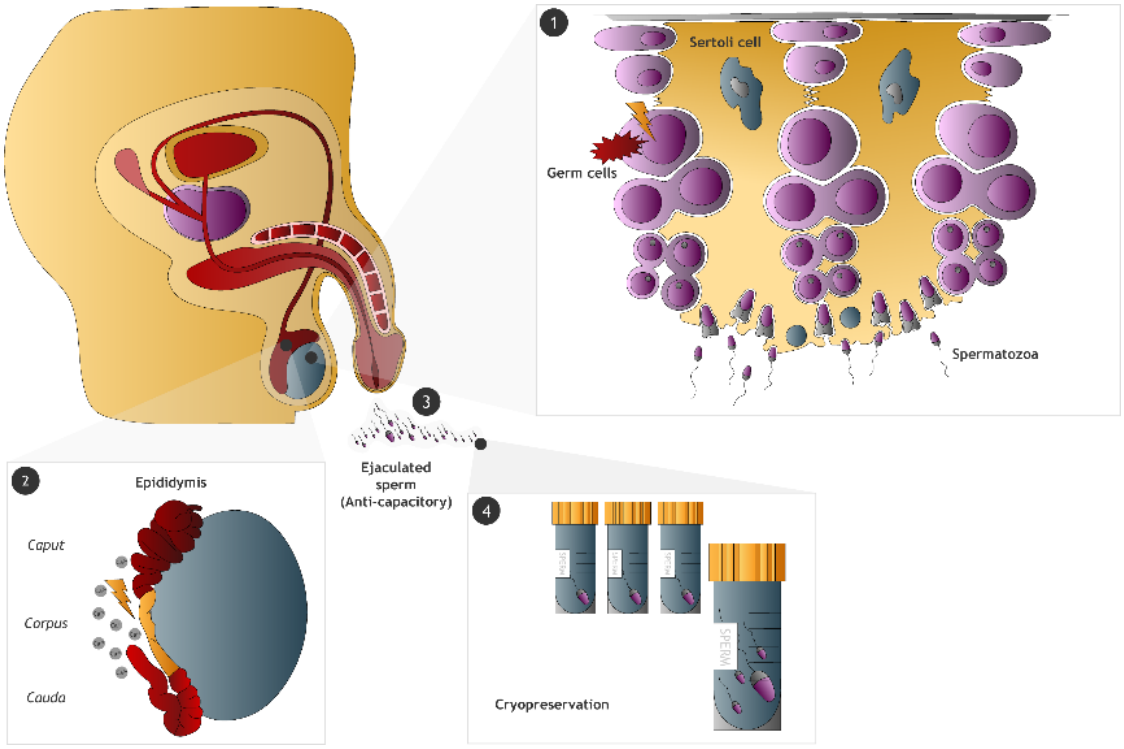

Figure III.1. The broad range of RGN actions in the male reproductive tract from spermatogenesis to the fertility potential of spermatozoa. (1) RGN is expressed in testicular cells (both in germ and somatic Sertoli cells) exerting a protective function over apoptosis and oxidative damage of the germline; it also contributes to the high plasticity of Sertoli cells metabolism ensuring the supply of lactate for germ cells. As an epididymal protein (2), RGN is differentially expressed in the *caput*, *corpus* and *cauda* regions, which is related to the protection against oxidative stress and maintenance of Ca^{2+}-levels in the epididymal lumen contributing for sperm maturation. RGN also is a protein detected in the seminiferous tubules, epididymis, and seminal vesicles fluids, though it is removed from the seminal plasma after ejaculation being maintained in the acrosomal region of ejaculated sperm. (3) Moreover, RGN seems to be an anti-capacitatory agent having a beneficial role in sperm cryopreservation, preventing premature capacitation/cryo-capacitation, Ca^{2+}-related stress, and oxidative damage.

9. Conclusion

Evidence of the anti-apoptotic and anti-proliferative functions, together with the androgenic regulation of RGN expression, first pointed out the role of this protein in spermatogenesis. The anti-oxidant properties of RGN and its capacity to supress oxidative damage also have been described and may account for the maintenance of viability and fertility potential of spermatozoa from spermatogenesis until fertilization of ovum, as well as in sperm cryopreservation. Another relevant aspect of RGN's biological actions is related to the control of Ca^{2+} homeostasis, which is crucial for an accurate sperm function and, consequently, for a healthy male fertility. Overall, it can

be concluded that RGN play a very important role in male reproduction from spermatogenesis to fertilization. Further research would help to clarify the mechanisms underlying RGN's actions in sperm physiology.

References

[1] Misawa H, Yamaguchi M. The gene of Ca2+-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med. 2000;6:191-6.

[2] Shimokawa N, Isogai M, Yamaguchi M. Specific species and tissue differences for the gene expression of calcium-binding protein regucalcin. Mol Cell Biochem. 1995;143:67-71.

[3] Goto SG. Expression of Drosophila homologue of senescence marker protein-30 during cold acclimation. J Insect Physiol. 2000;46:1111-20.

[4] Fujita T, Shirasawa T, Maruyama N. Isolation and characterization of genomic and cDNA clones encoding mouse senescence marker protein-30 (SMP30). Biochim Biophys Acta. 1996;1308:49-57.

[5] Fujita T, Shirasawa T, Uchida K, Maruyama N. Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution. Biochim Biophys Acta. 1992;1132:297-305.

[6] Maruyama N, Ishigami A, Kondo Y. Pathophysiological significance of senescence marker protein-30. Geriatr Gerontol Int. 2010;10 Suppl 1:S88-98.

[7] Nikapitiya C, De Zoysa M, Kang HS, Oh C, Whang I, Lee J. Molecular characterization and expression analysis of regucalcin in disk abalone (Haliotis discus discus): intramuscular calcium administration stimulates the regucalcin mRNA expression. Comp Biochem Physiol B Biochem Mol Biol. 2008;150:117-24.

[8] Wu YD, Jiang L, Zhou Z, Zheng MH, Zhang J, Liang Y. CYP1A/regucalcin gene expression and edema formation in zebrafish embryos exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin. Bull Environ Contam Toxicol. 2008;80:482-6.

[9] Nakajima Y, Natori S. Identification and characterization of an anterior fat body protein in an insect. J Biochem. 2000;127:901-8.

[10] Gomi K, Hirokawa K, Kajiyama N. Molecular cloning and expression of the cDNAs encoding luciferin-regenerating enzyme from Luciola cruciata and Luciola lateralis. Gene. 2002;294:157-66.

[11] Yamaguchi M. Role of regucalcin in calcium signaling. Life Sci. 2000;66:1769-80.

[12] Yamaguchi M. Role of regucalcin in maintaining cell homeostasis and function (review). Int J Mol Med. 2005;15:371-89.

[13] Chakraborti S, Bahnson BJ. Crystal structure of human senescence marker protein 30: insights linking structural, enzymatic, and physiological functions. Biochemistry. 2010;49:3436-44.

[14] Shimokawa N, Yamaguchi M. Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett. 1993;327:251-5.

[15] Fujita T, Shirasawa T, Uchida K, Maruyama N. Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation with tissue maturation and gradual down-regulation with aging. Mech Ageing Dev. 1996;87:219-29.

[16] Marques R, Maia CJ, Vaz C, Correia S, Socorro S. The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci. 2014;71:93-111.

[17] Maia CJ, Santos CR, Schmitt F, Socorro S. Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17β-estradiol. Mol Cell Biochem. 2008;311:81-6.

[18] Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, et al. Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction. 2011;142:447-56.

[19] Pillai H, Shende AM, Parmar MS, Thomas J, Kartha HS, Taru Sharma G, et al. Detection and localization of regucalcin in spermatozoa of water buffalo (Bubalus bubalis): A calcium-regulating multifunctional protein. Reprod Domest Anim. 2017;52:865-72.

[20] Pillai H, Shende AM, Parmar MS, Anjaneya A, Sreela L, Kumaresan A, et al. Regucalcin is widely distributed in the male reproductive tract and exerts a suppressive effect on in vitro sperm capacitation in the water buffalo (Bubalus bubalis). Mol Reprod Dev. 2017;84:212-21.

[21] Correia S, Oliveira P, Guerreiro P, Lopes G, Alves M, Canário A, et al. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca^{2+}-binding protein regucalcin: a hidden role for Ca^{2+} in sperm maturation? Mol Hum Reprod. 2013;19:581-9.

[22] Vaz CV, Correia S, Cardoso HJ, Figueira MI, Marques R, Maia CJ, et al. The Emerging Role of Regucalcin as a Tumor Suppressor: Facts and Views. Curr Mol Med. 2016;16:607-19.

[23] Son TG, Park HR, Kim SJ, Kim K, Kim MS, Ishigami A, et al. Senescence marker protein 30 is up-regulated in kainate-induced hippocampal damage through ERK-mediated astrocytosis. J Neurosci Res. 2009;87:2890-7.

[24] Doran P, Dowling P, Donoghue P, Buffini M, Ohlendieck K. Reduced expression of regucalcin in young and aged MDX diaphragm indicates abnormal cytosolic calcium handling in dystrophin-deficient muscle. Biochim Biophys Acta. 2006;1764:773-85.

[25] Lv Sa, Wang J-h, Liu F, Gao Y, Fei R, Du S-c, et al. Senescence marker protein 30 in acute liver failure: validation of a mass spectrometry proteomics assay. BMC Gastroenterol. 2008;8:17.

[26] Jung KJ, Maruyama N, Ishigami A, Yu BP, Chung HY. The redox-sensitive DNA binding sites responsible for age-related downregulation of SMP30 by ERK pathway and reversal by calorie restriction. Antioxid Redox Signal. 2006;8:671-80.

[27] van Dijk KD, Berendse HW, Drukarch B, Fratantoni SA, Pham TV, Piersma SR, et al. The proteome of the locus ceruleus in Parkinson's disease: relevance to pathogenesis. Brain Pathol. 2012;22:485-98.

[28] Jeong D-H, Goo M-J, Hong I-H, Yang H-J, Ki M-R, DO S-H, et al. Inhibition of radiation-induced apoptosis via overexpression of SMP30 in Smad3-knockout mice liver. J Radiat Res. 2008;49:653-60.

[29] Fukaya Y, Yamaguchi M. Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull. 2004;27:1444-6.

[30] Vaz CV, Marques R, Maia CJ, Socorro S. Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin. Transl Res. 2015;166:693-705.

[31] Yamaguchi M. The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis. 2013;18:1145-53.

[32] Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves MG, et al. Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate. 2014;74:1189-98.

[33] Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, et al. Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res. 2015;330:325-35.

[34] Silva AM, Correia S, Socorro S, Maia CJ. Endogenous Factors in the Recovery of Reproductive Function After Testicular Injury and Cancer. Curr Mol Med. 2016;16:631-49.

[35] Yamaguchi M. Involvement of regucalcin as a suppressor protein in human carcinogenesis: insight into the gene therapy. J Cancer Res Clin Oncol. 2015;141:1333-41.

[36] Yamaguchi M. Regucalcin and metabolic disorders: osteoporosis and hyperlipidemia are induced in regucalcin transgenic rats. Mol Cell Biochem. 2010;341:119-33.

[37] Vaz CV, Marques R, Cardoso HJ, Maia CJ, Socorro S. Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation. Transgenic Res. 2016;25:139-48.

[38] Takahashi H, Yamaguchi M. Role of regucalcin as an activator of Ca^{2+}-ATPase activity in rat liver microsomes. J Cell Biochem. 1999;74:663-9.

[39] Yamaguchi M, Mori S. Activation of hepatic microsomal Ca^{2+}-adenosine triphosphatase by calcium-binding protein regucalcin. Chem Pharm Bull. 1989;37:1031-4.

[40] Takahashi H, Yamaguchi M. Regulatory effect of regucalcin on (Ca^{2+}– Mg^{2+})-ATPase in rat liver plasma membranes: comparison with the activation by Mn^{2+} and Co^{2+}. Mol Cell Biochem. 1993;124:169-74.

[41] Publicover SJ, Barratt CL. Voltage-operated Ca2+ channels and the acrosome reaction: which channels are present and what do they do? Hum Reprod. 1999;14:873-9.

[42] Benoff S, Chu CC, Marmar JL, Sokol RZ, Goodwin LO, Hurley IR. Voltage-dependent calcium channels in mammalian spermatozoa revisited. Front Biosci. 2007;12:1420-49.

[43] Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Mol Cell Endocrinol. 2002;187:139-44.

[44] Darszon A, Lopez-Martinez P, Acevedo JJ, Hernandez-Cruz A, Trevino CL. T-type Ca2+ channels in sperm function. Cell Calcium. 2006;40:241-52.

[45] Florman HM, Jungnickel MK, Sutton KA. Regulating the acrosome reaction. Int J Dev Biol. 2008;52:503-10.

[46] Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, et al. Ca2+-stores in sperm: their identities and functions. Reproduction. 2009;138:425-37.

[47] Foresta C, Rossato M. Calcium influx pathways in human spermatozoa. Mol Hum Reprod. 1997;3:1-4.

[48] Wennemuth G, Babcock DF, Hille B. Calcium clearance mechanisms of mouse sperm. J Gen Physiol. 2003;122:115-28.

[49] Shimokawa N, Matsuda Y, Yamaguchi M. Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem. 1995;151:157-63.

[50] Fujita T, Mandel JL, Shirasawa T, Hino O, Shirai T, Maruyama N. Isolation of cDNA clone encoding human homologue of senescence marker protein-30 (SMP30) and its location on the X chromosome. Biochim Biophys Acta. 1995;1263:249-52.

[51] Shamsi MB, Kumar K, Dada R. Genetic and epigenetic factors: Role in male infertility. Indian J Urol. 2011;27:110-20.

[52] Brown CJ, Goss SJ, Lubahn DB, Joseph DR, Wilson EM, French FS, et al. Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. Am J Hum Genet. 1989;44:264-9.

[53] Ferlin A, Vinanzi C, Garolla A, Selice R, Zuccarello D, Cazzadore C, et al. Male infertility and androgen receptor gene mutations: clinical features and identification of seven novel mutations. Clin Endocrinol. 2006;65:606-10.

[54] O'Hara L, Smith LB. The Genetics of Androgen Receptor Signalling in Male Fertility. In: Vogt PH, editor. Genetics of Human Infertility. Basel: Karger; 2017. 21. p. 86-100.

[55] Stouffs K, Tournaye H, Liebaers I, Lissens W. Male infertility and the involvement of the X chromosome. Hum Reprod Update. 2009;15:623-37.

[56] Zheng K, Yang F, Wang PJ. Regulation of Male Fertility by X-Linked Genes. J Androl. 2010;31:79-85.

[57] Vockel M, Riera-Escamilla A, Tüttelmann F, Krausz C. The X chromosome and male infertility. Hum Genet. 2019.

[58] Nussbaum RL, McInnes RR, Willard HF. Chapter 5 - Principles of Clinical Cytogenetics and Genome Analysis. Thompson & Thompson Genetics in Medicine. Eighth Edition. Philadelphia: Elsevier; 2016. p. 57-74.

[59] Zheng K, Yang F, Wang PJ. Regulation of male fertility by X-linked genes. J Androl. 2010;31:79-85.

[60] Fayad T, Lévesque V, Sirois J, Silversides DW, Lussier JG. Gene expression profiling of differentially expressed genes in granulosa cells of bovine dominant follicles using suppression subtractive hybridization. Biol Reprod. 2004;70:523-33.

[61] Mori T, Ishigami A, Seyama K, Onai R, Kubo S, Shimizu K, et al. Senescence marker protein-30 knockout mouse as a novel murine model of senile lung. Pathol Int 2004;54:167-73.

[62] Arun P, Aleti V, Parikh K, Manne V, Chilukuri N. Senescence marker protein 30 (SMP30) expression in eukaryotic cells: existence of multiple species and membrane localization. PLoS One. 2011;6:e16545.

[63] Harikrishna P, Shende AM, Reena KK, Thomas J, Bhure SK. Purification of Regucalcin from the Seminal Vesicular Fluid: A Calcium Binding Multi-Functional Protein. Protein J. 2016;35:310-7.

[64] Starvaggi Cucuzza L, Divari S, Mulasso C, Biolatti B, Cannizzo FT. Regucalcin expression in bovine tissues and its regulation by sex steroid hormones in accessory sex glands. PLoS One. 2014;9:e113950.

[65] Laurentino SS, Correia S, Cavaco JE, Oliveira PF, de Sousa M, Barros A, et al. Regucalcin, a calcium-binding protein with a role in male reproduction? Mol Hum Reprod. 2012;18:161-70.

[66] Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays. 2000;22:423-30.

[67] Lin WW, Lamb DJ, Wheeler TM, Abrams J, Lipshultz LI, Kim ED. Apoptotic frequency is increased in spermatogenic maturation arrest and hypospermatogenic states. J Urol. 1997;158:1791-3.

[68] Takagi S, Itoh N, Kimura M, Sasao T, Tsukamoto T. Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia. Fertil Steril. 2001;76:901-7.

[69] Bozec A, Amara S, Guarmit B, Selva J, Albert M, Rollet J, et al. Status of the executioner step of apoptosis in human with normal spermatogenesis and azoospermia. Fertil Steril. 2008;90:1723-31.

[70] Correia S, Vaz CV, Silva A, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

[71] Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, et al. Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology. 2014;2:290-8.

[72] Silva AM, Correia S, Casalta-Lopes JE, Mamede AC, Cavaco JE, Botelho MF, et al. The protective effect of regucalcin against radiation-induced damage in testicular cells. Life Sci. 2016;164:31-41.

[73] Ogawa K, Utsunomiya T, Mimori K, Tanaka F, Haraguchi N, Inoue H, et al. Differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation. Int J Oncol. 2006;28:705-13.

[74] Fukui M, Senmaru T, Hasegawa G, Yamazaki M, Asano M, Kagami Y, et al. 17beta-Estradiol attenuates saturated fatty acid diet-induced liver injury in ovariectomized mice by up-regulating hepatic senescence marker protein-30. Biochem Biophys Res Commun. 2011;415:252-7.

[75] Wang C, Sinha Hikim AP, Lue YH, Leung A, Baravarian S, Swerdloff RS. Reproductive aging in the Brown Norway rat is characterized by accelerated germ cell apoptosis and is not altered by luteinizing hormone replacement. J Androl. 1999;20:509-18.

[76] Paul C, Nagano M, Robaire B. Aging results in differential regulation of DNA repair pathways in pachytene spermatocytes in the Brown Norway rat. Biol Reprod. 2011;85:1269-78.

[77] Tesi M, Sabatini C, Vannozzi I, Di Petta G, Panzani D, Camillo F, et al. Variables affecting semen quality and its relation to fertility in the dog: A retrospective study. Theriogenology. 2018;118:34-9.

[78] Wolf KN, Wildt DE, Vargas A, Marinari PE, Kreeger JS, Ottinger MA, et al. Age-dependent changes in sperm production, semen quality, and testicular volume in the black-footed ferret (Mustela nigripes). Biol Reprod. 2000;63:179-87.

[79] Hellstrom WJ, Overstreet JW, Sikka SC, Denne J, Ahuja S, Hoover AM, et al. Semen and sperm reference ranges for men 45 years of age and older. J Androl. 2006;27:421-8.

[80] Stone BA, Alex A, Werlin LB, Marrs RP. Age thresholds for changes in semen parameters in men. Fertil Steril. 2013;100:952-8.

[81] Kumar N, Singh AK, Choudhari AR. Impact of age on semen parameters in male partners of infertile couples in a rural tertiary care center of central India: A cross-sectional study. Int J Reprod Biomed. 2017;15:497-502.

[82] Sloter E, Schmid TE, Marchetti F, Eskenazi B, Nath J, Wyrobek AJ. Quantitative effects of male age on sperm motion. Hum Reprod. 2006;21:2868-75.

[83] Mukhopadhyay D, Varghese AC, Pal M, Banerjee SK, Bhattacharyya AK, Sharma RK, et al. Semen quality and age-specific changes: a study between two decades on 3,729 male partners of couples with normal sperm count and attending an andrology laboratory for infertility-related problems in an Indian city. Fertil Steril. 2010;93:2247-54.

[84] Molina RI, Martini AC, Tissera A, Olmedo J, Senestrari D, de Cuneo MF, et al. Semen quality and aging: analysis of 9.168 samples in Cordoba. Argentina. Arch Esp Urol. 2010;63:214-22.

[85] Johnson L, Petty CS, Neaves WB. Age-related variation in seminiferous tubules in men. A stereologic evaluation. J Androl. 1986;7:316-22.

[86] Fujita T, Shirasawa T, Uchida K, Maruyama N. Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution. Biochim Biophys Acta. 1992;1132:297-305.

[87] Fujita T, Uchida K, Maruyama N. Purification of senescence marker protein-30 (SMP30) and its androgen-independent decrease with age in the rat liver. Biochim Biophys Acta. 1992;1116:122-8.

[88] Cardoso AL, Fernandes A, Aguilar-Pimentel JA, de Angelis MH, Guedes JR, Brito MA, et al. Towards frailty biomarkers: candidates from genes and pathways regulated in aging and age-related diseases. Ageing Res Rev. 2018;47:214-77.

[89] Harman D. Free radical theory of aging. Mutat Res. 1992;275:257-66.

[90] Aitken RJ. The role of free oxygen radicals and sperm function. Int J Androl. 1989;12:95-7.

[91] Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertility. 1987;81:459-69.

[92] Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. Reprod Biomed Online. 2014;28:684-703.

[93] Aitken RJ, Findlay JK, Hutt KJ, Kerr JB. Apoptosis in the germ line. Reproduction. 2011;141:139-50.

[94] Hewitt SC, Korach KS. Estrogen Receptors: New Directions in the New Millennium. Endocr Rev. 2018;39:664-75.

[95] Pinto P, Estêvão M, Socorro S. Androgen receptors in non-mammalian vertebrates: Structure and function. In: Socorro S, editor. Androgen Receptor: Structural Biology, Genetics and Molecular Defects. New York: Nova Science Publishers; 2014. p. 1-26.

[96] Figueira M, Cardoso H, Socorro S. The Role of GPER Signaling in Carcinogenesis: a Focus on Prostate Cancer. In: Fayyaz S, Farooqi A, editors. Recent Trends in Cancer Biology: Spotlight on Signaling Cascades and microRNAs. New York: Springer; 2018. p. 59-117.

[97] Socorro S, Power DM, Olsson PE, Canario AV. Two estrogen receptors expressed in the teleost fish, Sparus aurata: cDNA cloning, characterization and tissue distribution. J Endocrinol. 2000;166:293-306.

[98] Correia S, Cardoso HJ, Cavaco JE, Socorro S. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med. 2015;17:e2.

[99] Oliveira PF, Alves MG, Martins AD, Correia S, Bernardino RL, Silva J, et al. Expression pattern of G protein-coupled receptor 30 in human seminiferous tubular cells. Gen Comp Endocrinol. 2014;201:16-20.

[100] Laurentino S, Gonçalves J, Cavaco JE, Oliveira PF, Alves MG, de Sousa M, et al. Apoptosis-inhibitor Aven is downregulated in defective spermatogenesis and a novel estrogen target gene in mammalian testis. Fertil Steril. 2011;96:745-50.

[101] Correia S, Alves MR, Cavaco JE, Oliveira PF, Socorro S. Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertil Steril. 2014;102:299-306.

[102] Yamaguchi M, Oishi K. 17 beta-Estradiol stimulates the expression of hepatic calcium-binding protein regucalcin mRNA in rats. Mol Cell Biochem. 1995;143:137-41.

[103] Kurota H, Yamaguchi M. Steroid hormonal regulation of calcium-binding protein regucalcin mRNA expression in the kidney cortex of rats. Mol Cell Biochem. 1996;155:105-11.

[104] Maia C, Santos C, Schmitt F, Socorro S. Regucalcin is under-expressed in human breast and prostate cancers: effect of sex steroid hormones. J Cell Biochem. 2009;107:667-76.

[105] Correia S. Estrogens and regucalcin in testicular apoptosis and sperm function: “a matter of life and death". Doctoral Thesis, University of Beira Interior; 2014.

[106] Lin PH, Jian CY, Chou JC, Chen CW, Chen CC, Soong C, et al. Induction of renal senescence marker protein-30 (SMP30) expression by testosterone and its contribution to urinary calcium absorption in male rats. Sci Rep. 2016;6:32085.

[107] Laurentino SS, Correia S, Cavaco JE, Oliveira PF, de Sousa M, Barros A, et al. Regucalcin, a calcium-binding protein with a role in male reproduction? Mol Hum Reprod. 2012;18:161-70.

[108] Gorczynska E, Handelsman DJ. Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells. Endocrinology. 1995;136:2052-9.

[109] Audy MC, Vacher P, Duly B. 17 beta-estradiol stimulates a rapid Ca^{2+} influx in LNCaP human prostate cancer cells. Eur J Endocrinol. 1996;135:367-73.

[110] Picotto G, Vazquez G, Boland R. 17beta-oestradiol increases intracellular Ca^{2+} concentration in rat enterocytes. Potential role of phospholipase C-dependent store-operated Ca2+ influx. Biochem J. 1999;339:71-7.

[111] Azenabor AA, Hoffman-Goetz L. 17 beta-estradiol increases $Ca(^{2+})$ influx and down regulates interleukin-2 receptor in mouse thymocytes. Biochem Biophys Res Commun. 2001;281:277-81.

[112] Sun YH, Gao X, Tang YJ, Xu CL, Wang LH. Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells. J Androl. 2006;27:671-8.

[113] Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R. Role of endogenous regucalcin in transgenic rats: suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal Ca^{2+}-ATPase activity. J Cell Biochem. 2002;86:520-9.

[114] Gervasi MG, Visconti PE. Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. Andrology. 2017;5:204-18.

[115] Dacheux J-L, Belleannée C, Jones R, Labas V, Belghazi M, Guyonnet B, et al. Mammalian epididymal proteome. Mol Cell Endocrinol 2009;306:45-50.

[116] Robaire B, Hinton BT. Chapter 17 - The Epididymis. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's Physiology of Reproduction. Fourth Edition. San Diego: Academic Press; 2015. p. 691-771.

[117] Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redo Signal. 2011;14:367-81.

[118] Handa S, Maruyama N, Ishigami A. Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. Biol Pharm Bull. 2009;32:1645-8.

[119] Ichikawa E, Yamaguchi M. Regucalcin increases superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats. Int J Mol Med. 2004;14:691-5.

[120] Son TG, Zou Y, Jung KJ, Yu BP, Ishigami A, Maruyama N, et al. SMP30 deficiency causes increased oxidative stress in brain. Mech Ageing Dev. 2006;127:451-7.

[121] Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, et al. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. Am J Respir Crit Care Med. 2006;174:530-7.

[122] Alves MG, Rato L, Carvalho RA, Moreira PI, Socorro S, Oliveira PF. Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cell Mol Life Sci. 2013;70:777-93.

[123] Mateus I, Feijo M, Espinola LM, Vaz CV, Correia S, Socorro S. Glucose and glutamine handling in the Sertoli cells of transgenic rats overexpressing regucalcin: plasticity towards lactate production. Sci Rep. 2018;8:10321.

[124] Maneesh M, Jayalekshmi H. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian J Clin Biochem. 2006;21:80-9.

[125] Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, et al. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008;375:346-50.

[126] Kondo Y, Sasaki T, Sato Y, Amano A, Aizawa S, Iwama M, et al. Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008;377:291-6.

[127] Kim HS, Son TG, Park HR, Lee Y, Jung Y, Ishigami A, et al. Senescence marker protein 30 deficiency increases Parkinson's pathology by impairing astrocyte activation. Neurobiol Aging. 2013;34:1177-83.

[128] Son TG, Kim SJ, Kim K, Kim MS, Chung HY, Lee J. Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress. Arch Pharm Res. 2008;31:872-7.

[129] Kondo Y, Inai Y, Sato Y, Handa S, Kubo S, Shimokado K, et al. Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy. Proc Natl Acad Sci U S A. 2006;103:5723-8.

[130] Linster CL, Van Schaftingen E. Vitamin C. Biosynthesis, recycling and degradation in mammals. FEBS J. 2007;274:1-22.

[131] Paudel KP, Kumar S, Meur SK, Kumaresan A. Ascorbic acid, catalase and chlorpromazine reduce cryopreservation-induced damages to crossbred bull spermatozoa. Reprod Domest Anim. 2010;45:256-62.

[132] Puga Molina LC, Luque GM, Balestrini PA, Marin-Briggiler CI, Romarowski A, Buffone MG. Molecular Basis of Human Sperm Capacitation. Frontiers in cell and developmental biology. 2018;6:72.

[133] Desnoyers L, Manjunath P. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. J Biol Chem. 1992;267:10149-55.

[134] de Lamirande E, Yoshida K, Yoshiike TM, Iwamoto T, Gagnon C. Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. J Androl. 2001;22:672-9.

[135] Cross NL. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. Biol Reprod. 1996;54:138-45.

[136] Andrews JC, Nolan JP, Hammerstedt RH, Bavister BD. Role of zinc during hamster sperm capacitation. Biol Reprod. 1994;51:1238-47.

[137] Tomes CN, Carballada R, Moses DF, Katz DF, Saling PM. Treatment of human spermatozoa with seminal plasma inhibits protein tyrosine phosphorylation. Mol Hum Reprod. 1998;4:17-25.

[138] Hirohashi N, Yanagimachi R. Sperm acrosome reaction: its site and role in fertilization. Biol Reprod. 2018;99:127-33.

[139] Mannowetz N, Naidoo NM, Choo S-A, Smith JF, Lishko PV. Slo1 is the principal potassium channel of human spermatozoa. eLife. 2013;2:e01009.

[140] Kwon W-S, Park Y-J, Mohamed E-S, Pang M-G. Voltage-dependent anion channels are a key factor of male fertility. Fertil Steril. 2013;99:354-61.

[141] Lishko PV, Kirichok Y. The role of Hv1 and CatSper channels in sperm activation. J Physiol. 2010;588:4667-72.

[142] Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update. 2008;14:647-57.

[143] Triphan J, Aumuller G, Brandenburger T, Wilhelm B. Localization and regulation of plasma membrane Ca(2+)-ATPase in bovine spermatozoa. Eur J Cell Biol. 2007;86:265-73.

[144] Sengupta T, Ghoshal S, Dungdung SR, Majumder GC, Sen PC. Structural and functional characterization and physiological significance of a stimulator protein of Mg2+-independent Ca2-ATPase isolated from goat spermatozoa. Mol Cell Biochem. 2008;311:93-103.

[145] Feng HL, Hershlag A, Han YB, Zheng LJ. Localizations of intracellular calcium and Ca2+-ATPase in hamster spermatogenic cells and spermatozoa. Microsc Res Tech. 2006;69:618-23.

[146] Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl. 2000;21:1-7.

[147] Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod Biomed Online. 2018;37:327-39.

[148] Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander HJ. Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates. Fertil Steril. 2005;83:1442-6.

[149] Chatterjee S, de Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol Reprod Dev. 2001;60:498-506.

[150] Vesala L, Salminen TS, Kankare M, Hoikkala A. Photoperiodic regulation of cold tolerance and expression levels of regucalcin gene in Drosophila montana. J Insect Physiol. 2012;58:704-9.

[151] Pillai H, Parmar MS, Shende AM, Thomas J, Sharma HS, Sharma GT, et al. Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (Bubalus bubalis). Mol Reprod Dev. 2017;84:1133-9.

[152] Bailey JL, Buhr MM. Ca^{2+} regulation by cryopreserved bull spermatozoa in response to A23187. Cryobiology. 1993;30:470-81.

[153] McLaughlin EA, Ford WC. Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone. Mol Reprod Dev. 1994;37:241-6.

[154] Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. Biol Reprod. 1998;59:12-6.

Chapter IV
Aim and Outline of the Thesis

The calcium (Ca^{2+})-binding protein regucalcin (RGN) is known to be linked to ageing for years. As suggested by its alternative name, Senescence Marker Protein-30, the expression of this protein decreases markedly with ageing in several tissues, including testis. RGN has been indicated as cytoprotective and antioxidant protein, protecting male germ cells against noxious stimuli. Moreover, RGN overexpression was associated with increased sperm viability and lower incidence of sperm tail defects. Male fertility is negatively affected by either oncological treatments like radiotherapy or by the natural and unavoidable ageing occurring throughout life. Depending on the dose, radiation therapy induces temporary or permanent azoospermia. Conversely, the impact of ageing on male fertility is gradual, but a growing body of evidence supports the age-related decrease in sperm quality. Therefore, since the conception of a biological child can be at risk in both situations, more investigation is needed in order to overcome these problems. Although RGN has been showing interesting features, it is entirely unknown to date whether RGN may counteract the noxious effects of testicular irradiation. Furthermore, there are no available data clarifying if RGN may be involved in mitigating the decline of male reproductive capacity that spontaneously occur with the advance of age. In fact, the literature is beyond scarce when it comes to RGN behaviour and/or action under radiation exposure, especially in such specific tissues like those involved in male reproduction. On the other hand, in spite of the well-known link of RGN to the ageing process, the effect of ageing on male fertility only recently has been considered a matter of concern. This is essentially the reason why the role of RGN in male reproductive ageing is still poorly studied. Indeed, RGN potential action as one of the guardians of male reproductive function under such conditions remains to disclose. Accordingly, the first aim of this doctoral thesis is to investigate whether RGN overexpression ameliorates the spermatogenic status and reproductive parameters after radiation treatment. Secondly, we also intended to analyse the influence of maintaining high expression levels of RGN in attenuating the ageing-associated decline of male reproductive function.

This thesis is organised in seven chapters, being the **Chapter I** dedicated to male reproductive anatomy and physiology, an overview of the testicular, epididymal and sperm structure, and also to explore the concepts and processes inherent to a successful spermatogenesis and normal male fertility.

Chapter II describes the testicular damage and male (in)fertility as a consequence of cancer treatments, and also includes strategies of sperm cryopreservation. Besides, it addresses knowledge about several promising endogenous factors that seem to have protective or confer advantageous properties to help on the recovery of male reproductive function after gonadal injury.

Chapter III explores the currently known actions of RGN in male reproduction, from the very first clue of RGN connection to reproductive tissues to more specific findings about its relevance for male fertility, and also briefly includes some general aspects regarding RGN role in molecular and cellular biology.

The main objectives described above were addressed in the following **Chapters V** and **VI**, representing the results obtained during the course of this doctoral thesis.

In **Chapter V**, we explored the role of RGN in the response to *in vivo* testicular irradiation after a 10-week recovery period. The gonadosomatic index (GSI), the germ cell differentiation status, and sperm parameters were compared between transgenic rats overexpressing RGN (Tg-RGN) and their

wild-type (Wt) counterparts. Moreover, the expression of several key modulators of cell cycle and apoptosis in the testis were evaluated.

Then, considering the results obtained in Chapter V, in **Chapter VI** we continued to explore the range of actions of RGN protein using again an *in vivo* overexpression approach. This chapter addresses the role of RGN in the modulation of oxidative stress (OS) and sperm quality of older rats. The sperm parameters and OS analysis were assessed in young-adult and senescent Tg-RGN rats comparatively with their Wt littermates. Furthermore, the GSI and the expression of proliferation regulators with ageing were evaluated. Noteworthy, we were pioneers in analysing the expression of RGN with ageing in sperm.

Finally, **Chapter VII** states the main conclusions of this research work, attempting to provide an integrative view of all the findings, and also covers future perspectives taking into account the generated knowledge.

Chapter V
The Protective Effect of Regucalcin Against Radiation-Induced Damage in Testicular Cells

Chapter published in:

Silva A.M.S., Correia S.*, Casalta-Lopes J.E., Mamede A.C., Cavaco J.E., Botelho M.F., Socorro S. #, Maia C.J.# The protective effect of regucalcin against radiation-induced damage in testicular cells. Life Sci. 2016;164:31-41. (DOI: 10.1016/j.lfs.2016.09.003)*

**contributed equally*

#contributed equally as senior authors

Abstract

Aims: Regucalcin (RGN), a protein broadly expressed in the male reproductive tract, has shown to have beneficial effects on spermatogenesis suppressing chemical-induced apoptosis. This study aimed to evaluate whether RGN overexpression ameliorates the spermatogenic phenotype after radiation treatment.

Main methods: Transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts were exposed to a single dose of X-rays (6 Gy), and at ten weeks after irradiation, the testicular status and the epididymal sperm parameters were evaluated. The expression of RGN and several cell cycle and apoptosis regulators, the enzymatic activity of caspase-3, and RGN immunostaining were also assessed.

Key findings: Tg-RGN animals displayed higher gonadosomatic index, and augmented sperm viability and motility relatively to their Wt counterparts after irradiation, as well as higher frequency of normal sperm morphology and a diminished incidence of head-defects. The differences in reproductive parameters were underpinned by a lower rate of apoptosis, as evidenced by the reduced activity of caspase-3, lower levels of caspase-8, and increased Bcl-2/Bax ratio in the testis of Tg-RGN animals. Supporting the involvement of RGN in the anti-apoptotic response, an enhanced expression of RGN was observed in irradiated rats.

Significance: Transgenic-overexpression of RGN protected against radiation-induced testicular damage, which strengthens the role of this protein protecting cells from the damage of external agents. These findings also indicated that the modulation of RGN testicular levels would be a mechanism for fertility preservation in men undergoing oncological treatment.

Keywords: Radiation therapy; Male fertility preservation; Regucalcin; Testicular damage.

1. Introduction

Testicles are the crucial organs for maintenance of male fertility, a role that is accomplished by their dual function of testosterone synthesis and male gametes production. Spermatogenesis is a complex and highly regulated process occurring in the seminiferous tubules of the testis that, after coordinated mitotic and meiotic divisions and cell differentiation events, culminates with the release of sperm into the lumen [1]. The germinal epithelium is very sensitive to exogenous damaging factors [2] due to its high mitotic rate, and for this reason, cancer treatments frequently have several adverse effects on male fertility, which implicate permanent or transitory impairment of spermatogenesis [3]. Concerning radiotherapy, it is also known both in rodents and humans that the extent of testicular injury is directly related to the dose of radiation delivered [4, 5].

As a result of early detection and successful treatments, young cancer patients are living longer and the development of effective strategies that would restrain the undesirable secondary effects of oncological treatments on fertility is clearly warranted.

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein involved in the control of intracellular Ca^{2+} homeostasis, and several other biological functions [6-9]. RGN has been indicated as a protein that suppresses apoptosis [10-12], and has cytoprotective and antioxidant effects [13-17], protecting cells from noxious stimuli. At testicular level, the RGN protein is expressed in Leydig and Sertoli cells, as well as in all the germ line both in human and rat [18]. Moreover, RGN overexpression was associated with an increased number of viable sperm cells and lower incidence of morphological defects, concomitantly with lower sperm counts and motility [19]. The higher sperm viability and the diminished incidence of morphological defects exhibited by the sperm of transgenic animals overexpressing (Tg-RGN) may be a consequence of the RGN ability to counteract oxidative stress (OS), as recently showed by our research group [20, 21]. On the other hand, the lower sperm motility in Tg-RGN was explained by the altered Ca^{2+}-influx rates observed in the epididymal tissues of these animals [19]. These findings led us to hypothesize that RGN may have a role counteracting the damaging effects of radiation on testicular cells. The present work aims to evaluate the spermatogenic status and reproductive parameters of Tg-RGN comparatively with their wild-type (Wt) counterparts ten weeks after radiation treatment with a single dose of 6 Gy, which is known to cause a significant depletion of germ cells in rat testis [5, 22]. The gonadosomatic index (GSI), tubular differentiation index (TDI), epididymal sperm parameters, and the expression of several modulators of cell cycle and apoptosis were evaluated.

2. Materials and methods

2.1 Animals

Three-month-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 [23]. 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. Both Wt and Tg-RGN rats were randomly divided into two experimental groups: the control group receiving no treatment and the irradiated group ($n \geq 5$ in each group).

2.2 Radiation treatment

Irradiation of Wt and Tg-RGN rats was performed at the Radiotherapy Service, Coimbra Hospital and University Centre, in a linear accelerator Varian Clinac 600 C (Varian Medical Systems, Palo Alto, CA, USA), using a 4 MV photon beam.

For radiotherapy planning, each rat was anesthetized by an intramuscular injection of ketamine (Clorketam 1000; Vetoquinol, Lure, France), and a computerized tomography (CT) was acquired keeping the animal immobilized in lateral decubitus position. After CT acquisition, target volume (testicles) delineation and three-dimensional (3D) computerized planning were performed using Eclipse™ Planning System (Varian Medical Systems) as illustrated in Figure V.1. The goal was to have a homogeneous dose coverage of the target volume, with the dose within volume ranging from 95% to 107% of the prescribed dose (6 Gy). In order to obtain the desired dose in our planning volume, a pair of parallel opposed fields was used. Table, gantry and collimator positions were determined after 3D-planning, as well as field size and monitor units for each field (Figure V.1).

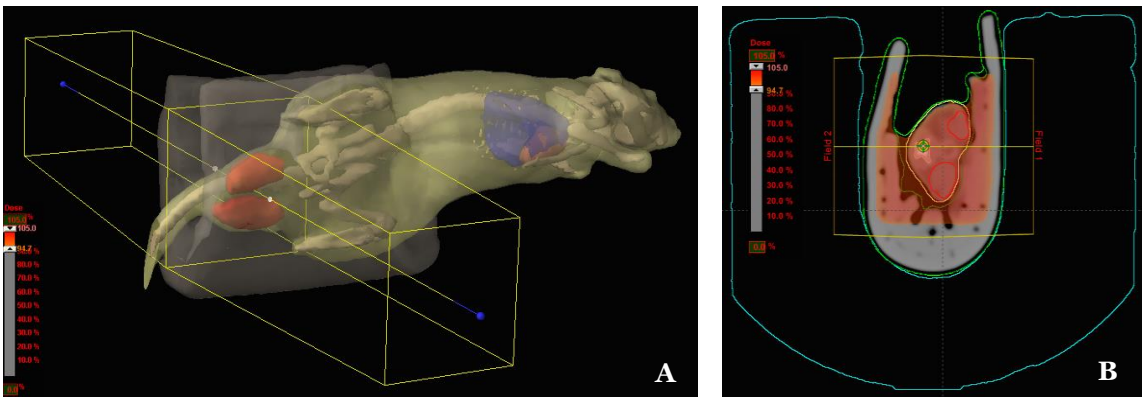

Figure V.1. Planning representation for testicular irradiation. (A) 3D representation of animal positioning, volume delineation (testicles represented in red), and the irradiation fields. (B) CT axial slice with field and dose representation.

2.3 Tissue collection

Ten weeks after radiation treatment, rats were anesthetized by means of an intraperitoneal injection with 100 µL of ketamine (Clorketam 1000) and Xylazine (20 mg/mL; Rompun, Bayer, Mississauga, Ontario, Canada) mixture (2:1) per 100 g of animal weight, and euthanized by cervical dislocation.

Testicles from Wt and Tg-RGN animals were collected, and both gonads and body mass were measured for the calculation of GSI. Then, testicles were immediately frozen on liquid nitrogen and stored at -80ºC until total protein extraction or alternatively fixed in 4% paraformaldehyde for histological processing. Also, epididymides were immediately removed and dissected free from fat, and the *cauda* region of the epididymis from each animal was used for the determination of sperm parameters.

2.4 Histological analysis

Paraffin sections (5 μm) of Wt and Tg-RGN rat testis were deparaffinized in xylene, rehydrated in graded alcohols and stained with hematoxylin (Leica Biosystems, Peterborough, Cambridgeshire, UK) and eosin (Leica Biosystems). The TDI, which is the percentage of seminiferous tubules showing germ cell differentiation, was determined to evaluate the recovery of spermatogenesis ten weeks after radiation treatment. Tubules were scored as differentiating or non-differentiating if containing or not germ cells in at least 3 different stages of differentiation: spermatogonia, spermatocyte and spermatid (round or elongated), or later stage. A minimum of 70 seminiferous tubules per section of each animal was scored using an optical microscope (400x magnification; Zeiss, Jena, Germany, Axio Image A1 microscope). The same procedure was applied both in irradiated and control groups of Tg-RGN and Wt animals.

2.5 Epididymal sperm count and motility

Cauda segments of the epididymis were minced with a sterilized scissor in 3 mL of filtered Hank's buffered salt solution (HBSSf; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C to obtain a sperm suspension. The suspension was incubated at 37 ºC for 5 minutes and used to analyse sperm counts, motility, viability and morphology as previously described [19]. In brief, an aliquot of sperm suspension was diluted in HBSSf, and sperm cells count using a Neubauer's counting chamber (Labor Optik, Balgach, Switzerland). Sperm motility was determined in a 37 °C pre-warmed slide covered with a coverslip using 100 μL of the sperm suspension. At least 10 random fields were assessed for each semen sample using an optical microscope (1000x magnification, Primo Star, Zeiss) with closed diaphragm and the percentage of motile sperm was calculated.

2.6 Sperm viability and morphology analysis

Sperm viability was assessed by using the one-step eosin-nigrosin staining technique [24]. A sample of 5 μL of sperm suspension was mixed with 10 μL of a stain mixture composed by 0.6% eosin (Fisher Scientific, Geel, Belgium) and 5% nigrosin (Acros Organics, Morris Plains, NJ, USA) and placed on a pre-warmed slide.

Sperm morphology was evaluated using the Kwik-Diff™ Staining Kit (Thermo Scientific, Pittsburgh, PA, USA) using standard procedures [19]. Briefly, smears were performed using 5 μL of sperm suspension, air dried and, then, slides were immersed in staining solutions for at least 1 minute and dipped rapidly in water. Afterwards, sperm was classified as normal or abnormal, and the abnormalities were divided into head, neck/midpiece or tail defects. There are sperm cells with only one abnormality, but there are also cells presenting various types of defects simultaneously. In such case, if one of the defects is on the head, it accounted exclusively for the number of head-defects.

Sperm viability and morphology were assessed for a total of 100 sperm cells in random fields (1000x magnification) of each semen sample under a light microscope (Primo Star, Zeiss).

2.7 Western blot (WB)

Total proteins were extracted from testicular tissues using radioimmunoprecipitation assay (RIPA) buffer supplemented with a cocktail of protease inhibitors (Sigma-Aldrich). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) and 50 or 75 μg of protein extracts were resolved on 12.5% polyacrylamide gels by sodium dodecyl sulfate electrophoresis (SDS-PAGE). Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and incubated overnight at 4 ºC with rabbit *anti*-p53 (1:500, FL-393: sc-6243; Santa Cruz Biotechnology, CA, USA), 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, Beverly, MA, USA), rabbit *anti*-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology), mouse *anti*-caspase-8 (1:500, D-8: sc-5263; Santa Cruz Biotechnology), rabbit *anti*-caspase-9 (1:500, H-170: sc-8355; Santa Cruz Biotechnology), or mouse *anti*-RGN (1:1000, ab81721, Abcam, Cambridge, UK) antibodies. A mouse *anti*-α-tubulin monoclonal antibody (1:5000, T9026, Sigma-Aldrich) was used for protein loading control in all 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.

2.8 Immunohistochemistry

The immunohistochemistry expression of RGN was performed as described before [18, 19]. Paraffin sections (5 μm) of Wt and Tg-RGN rat testis were deparaffinized in xylene and rehydrated in graded alcohols. After heat-induced antigen retrieval in citric acid (10 mM, pH 6.0) for 30 minutes at 80–85 ºC, sections were permeabilized with 1% Triton X-100 for 15 minutes at room temperature (RT). Then, endogenous peroxidase was blocked by incubating samples in 3% (v/v) H_2O_2 (Panreac, Barcelona, Spain) for 10 minutes at RT. Unspecific staining was blocked by incubation with 1% (w/v) BSA and 0.3 M glycine (Fisher Scientific, NJ, USA) in phosphate buffer saline (PBS) for 30 minutes at RT. Sections were incubated overnight at 4 ºC with a rabbit polyclonal *anti*-RGN primary antibody (SML-ROI001-EX, COSMOBIO CO., LTD., Tokyo, Japan) diluted 1:50 in PBA. Sections were then incubated with secondary goat *anti*-rabbit biotinylated antibody (B6648, Sigma-Aldrich) diluted 1:200 in PBA for 60 minutes at RT, followed by incubation with ExtrAvidin Peroxidase (E2886, Sigma-Aldrich) diluted 1:20 in PBA. Immunological reaction was detected using HRP substrate solution (Dako, Glostrup, Denmark). Sections were slightly counterstained with filtered Mayer's hematoxylin (05-06002/L, Bio-Optica Milano SPA, Milan, Italy), dehydrated, cleared and mounted. The specificity of the staining was assessed by the omission of primary antibody. Images were acquired using an optical microscope (400x magnification; Zeiss).

2.9 Caspase-3 activity assay

Caspase-3 activity assay was performed as previously described [25]. Briefly, 25 µg of total protein extracted from testicles were incubated with reaction buffer (25 mM HEPES, 0.1% CHAPS, 10% sucrose and 10 mM DTT, pH 7.5) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 hours at 37 ºC. The product of caspase-3 cleavage reaction, *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.

2.10 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 for $p < 0.05$. All experimental data are shown as mean ± standard error of the mean (SEM).

3. Results

3.1 Gonadosomatic index (GSI) and tubular differentiation index (TDI)

The GSI is commonly used as an indicator of the testis condition because gonad weight and size are mainly controlled by the secretory activity of the somatic cells within seminiferous tubules, the Sertoli cells, which determines sperm production and thus, testis volume [26]. At ten weeks of recovery after radiation treatment, the GSI was found to be significantly decreased both in Wt and Tg-RGN animals comparatively with the respective controls ($p<0.001$, Figure V.2A). However, the GSI fold variation in the Wt irradiated rats relatively to the control was 0.39 ± 0.02, whereas Tg-RGN irradiated rats displayed a 0.59 ± 0.09 fold variation (Figure V.2A). Thus, the GSI of irradiated Tg-RGN animals was higher than that of the Wt irradiated group ($p<0.05$).

The TDI that indicates the percentage of seminiferous tubules presenting successive germ cell differentiation stages was used to evaluate the spermatogenic status in Wt and Tg-RGN animals ten weeks after irradiation. At this recovery time, TDI was found to be significantly reduced both in Wt (0.23 ± 0.04 fold variation, $p<0.001$) and Tg-RGN animals (0.32 ± 0.11 fold variation, $p<0.001$) in comparison with the respective controls (Figure V.2B). No statistically significant difference was found in the TDI between the Wt and Tg-RGN irradiated groups (Figure V.2B).

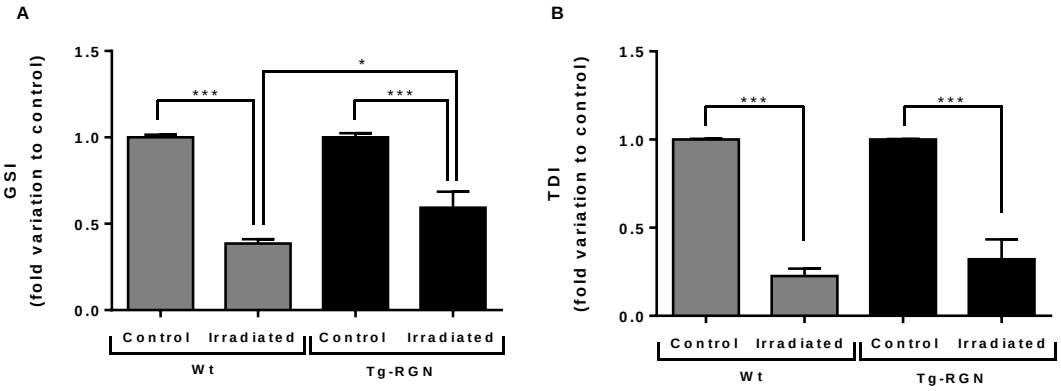

Figure V.2. GSI (A) and TDI (B) in Wt and Tg-RGN animals ten weeks after radiation treatment. Results are expressed as fold variation relatively to the respective control group. Error bars indicate mean ± S.E.M. (n≥5 in each group). *p<0.05; ***p<0.001.

3.2 Epididymal sperm count, motility and viability

Sperm leave the testis as non-functional gametes, acquiring the capacity to move progressively, and eventually to fertilize, during the passage through the different functional regions of the epididymis [27]. Storage of mature sperm occurs in the *cauda* of the epididymis [28], and, for this reason, *cauda* sperm are used to evaluate cell number, motility and viability.

At ten weeks after radiation treatment, sperm counts were significantly decreased both in Wt and Tg-RGN animals comparatively with the respective controls (p<0.001, Figure V.3A). The number of sperm cells/mL decreased to $3.2 \times 10^7 \pm 2.4 \times 10^6$ and $3.1 \times 10^7 \pm 1.2 \times 10^7$ cells/mL in Wt and Tg-RGN irradiated rats, respectively, and, thus, no significant differences were observed between both irradiated groups (Figure V.3A). However, it should be noted that epididymal sperm counts at control conditions were significantly lower in Tg-RGN rats when compared to their Wt littermates ($1.7 \times 10^8 \pm 8.7 \times 10^6$ *vs.* $2.4 \times 10^8 \pm 1.2 \times 10^7$, p<0.001) (Figure V.3A).

Also, the percentage of motile sperm (Figure V.3B) was significantly lower in control non-irradiated Tg-RGN rats comparatively to their Wt littermates (44.0% ± 1.0 *vs.* 72.6% ± 1.3, p<0.001). At ten weeks of recovery after radiation treatment, the percentage of epididymal motile sperm was significantly declined both in Wt and Tg-RGN comparatively to the respective controls (p<0.001, Figure V.3B). Drastically, none motile sperm was found in Wt irradiated animals, but, noteworthy Tg-RGN irradiated rats showed a significant percentage of motile sperm (10.3% ± 5.9, p<0.05) (Figure V.3B).

Concerning sperm viability, control non-irradiated Tg-RGN showed a significant higher percentage of viable sperm comparatively to Wt animals (29.0% ± 1.6 *vs.* 23.8% ± 1.7, p<0.05) (Figure V.3C), which was maintained upon radiation treatment. Ten weeks after irradiation, a significant reduction of sperm viability was found both in Wt (p<0.001) and Tg-RGN (p<0.05) animals in comparison with the respective controls (Figure V.3C). However, Tg-RGN irradiated rats displayed 12.3% ± 6.6 of viable sperm, contrastingly with the total absence of viable sperm in Wt irradiated. Thus, Tg-RGN showed a significantly higher percentage of viable sperm relatively to the Wt rats (Figure V.3C; p<0.05) not only in control conditions but also after irradiation.

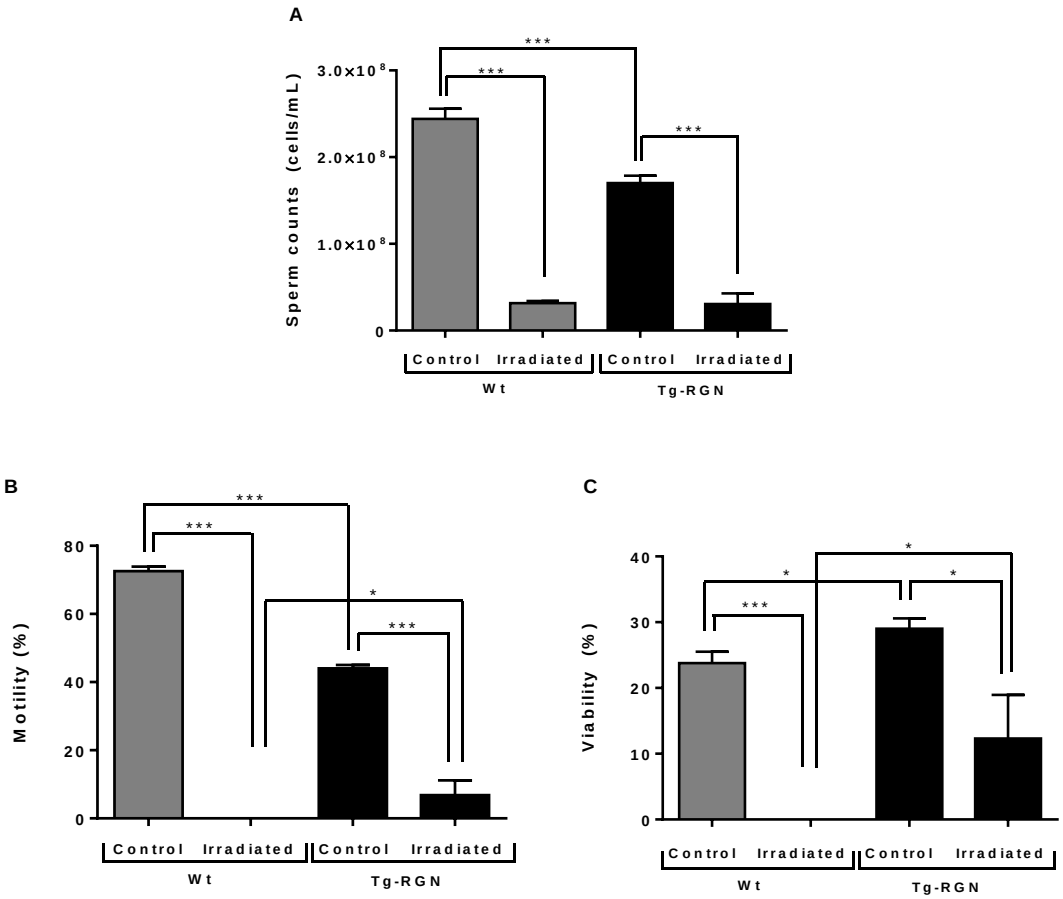

Figure V.3. Epididymal sperm counts (A), and sperm motility (B) and viability (C) in Wt and Tg-RGN animals ten weeks after radiation treatment. Error bars indicate mean ± S.E.M. (n≥5 in each group). *p<0.05; ***p<0.001.

3.3 Epididymal sperm morphology

Sperm morphology, namely the presence of head, midpiece and tail abnormalities, is another important parameter in sperm analysis, which has been shown to reflect the influence of negative stress factors [29].

Within ten weeks of recovery after radiation treatment, the percentage of normal sperm in the epididymis showed a significant decline both in Wt and Tg-RGN rats comparatively with the respective controls (p<0.001, Figure V.4A). However, very few sperm with normal morphology were found in the Wt irradiated rats, only 0.5% ± 0.2, against 22.2% ± 12.3 in the Tg-RGN irradiated animals (Figure V.4A). Therefore, the percentage of normal epididymal sperm was significantly higher in Tg-RGN irradiated animals relatively to Wt irradiated (p<0.05). This followed the status under control conditions since non-irradiated Tg-RGN animals showed a higher percentage of normal sperm relatively to the Wt (74.1% ± 3.5 *vs*. 64.0% ± 3.4, p<0.05) (Figure V.4A).

The percentage of sperm with head-defects (Figure V.4B) 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 radiation-induced damage, a significant augment in the percentage of sperm with head-defects was found both in Wt and Tg-RGN groups comparatively with the respective controls (p<0.001, Figure

V.4B). Moreover, sperm of Tg-RGN irradiated animals showed a significantly lower incidence of head-defects relatively to Wt irradiated rats (77.52% ± 11.93 *vs*. 98.33% ± 0.67, $p<0.05$) (Figure V.4B). For neck/midpiece defects (Figure V.4C), comparison of non-irradiated control groups showed that Tg-RGN animals displayed a significantly lower percentage of sperm with neck/midpiece defects (2.18% ± 0.47 *vs*. 8.20% ± 2.63, $p<0.05$). In the Wt irradiated group, the percentage of sperm with neck/midpiece defects diminished comparatively to the respective control (0.83% ± 0.54 *vs*. 8.20% ± 2.63, $p<0.01$), an effect not seen in Tg-RGN. Nevertheless, a significantly higher percentage of neck/midpiece defects was observed in the Tg-RGN irradiated rats comparatively to Wt irradiated (2.64% ± 0.45 *vs*. 0.83% ± 0.54, $p<0.05$) (Figure V.4C).

Regarding tail defects (Figure V.4D), no statistical differences were perceived between the Wt and Tg-RGN groups in control conditions, as well as after radiation treatment. Nevertheless, the percentage of epididymal sperm with tail defects exhibited a pronounced decline both in Wt and Tg-RGN irradiated rats comparatively with the respective controls ($p<0.001$, Figure V.4D). In Wt irradiated rats, the percentage of tail defects diminished to 0.33% ± 0.21, whereas in Tg-RGN irradiated rats it decreased to 1.65% ± 1.04, though not significantly different relatively to Wt (Figure V.4D).

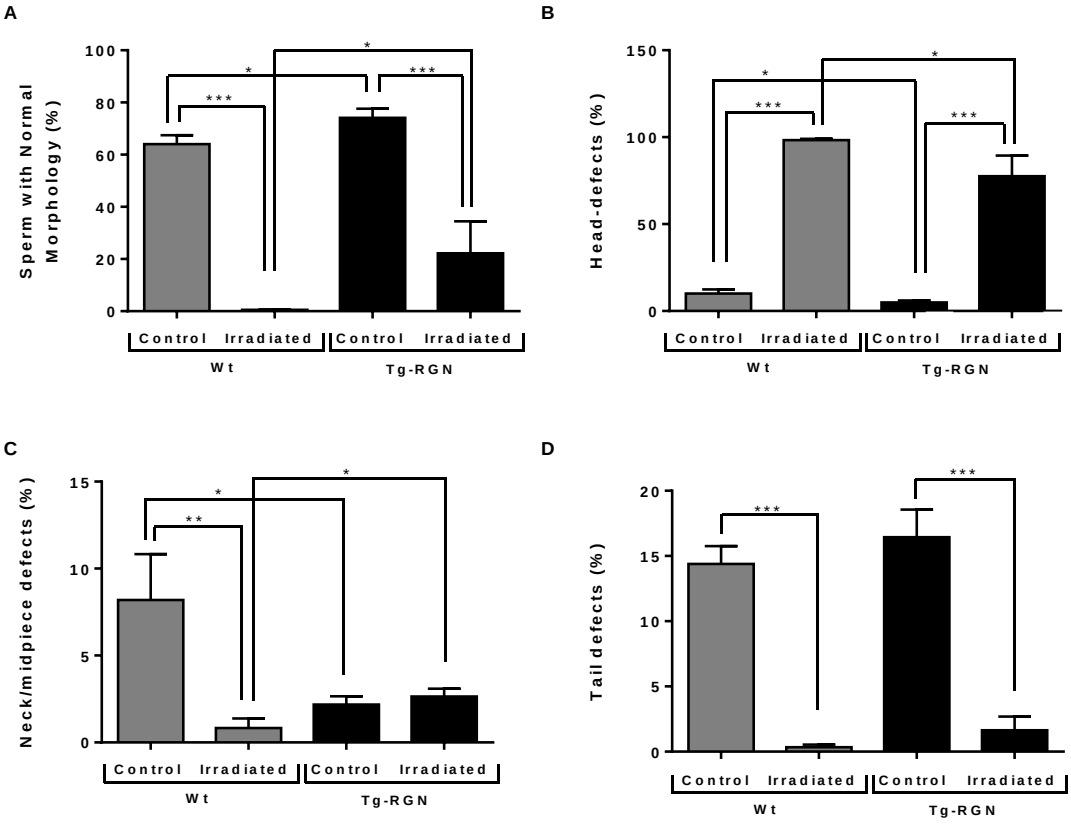

Figure V.4. Epididymal sperm morphology in Wt and Tg-RGN animals ten weeks after radiation treatment. The percentage of normal morphology, head-defects, neck/midpiece defects, and tail defects are shown in panels (A), (B), (C), and (D), respectively. Error bars indicate mean ± S.E.M. (n≥5 in each group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.4 Expression of p53 and p21

The p53 protein has a critical role in the regulation of cell fate, inducing cell cycle arrest at G1 phase and/or cell death by apoptosis in response to a variety of stress stimuli [30, 31], such as radiation [32]. p53 actions are mediated by the regulation of Bcl-2 family members, and it also upregulates the expression of p21, a cyclin-dependent kinase inhibitor [30, 33].

WB analysis showed that at ten weeks after irradiation p53 levels were significantly decreased both in Wt (0.52 ± 0.10 fold variation, $p<0.01$) and Tg-RGN rats (0.52 ± 0.09 fold variation, $p<0.05$) in comparison with the respective non-irradiated controls (Figure V.5A). A similar pattern of expression was observed for the 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, $p<0.05$) irradiated rats comparatively with the respective controls (Figure V.5B). In addition, no significant differences were found on the expression of p53 and p21 between the two irradiated groups (Figure V.5A and B, respectively). Representative immunoblot images are shown in Figure V.5C.

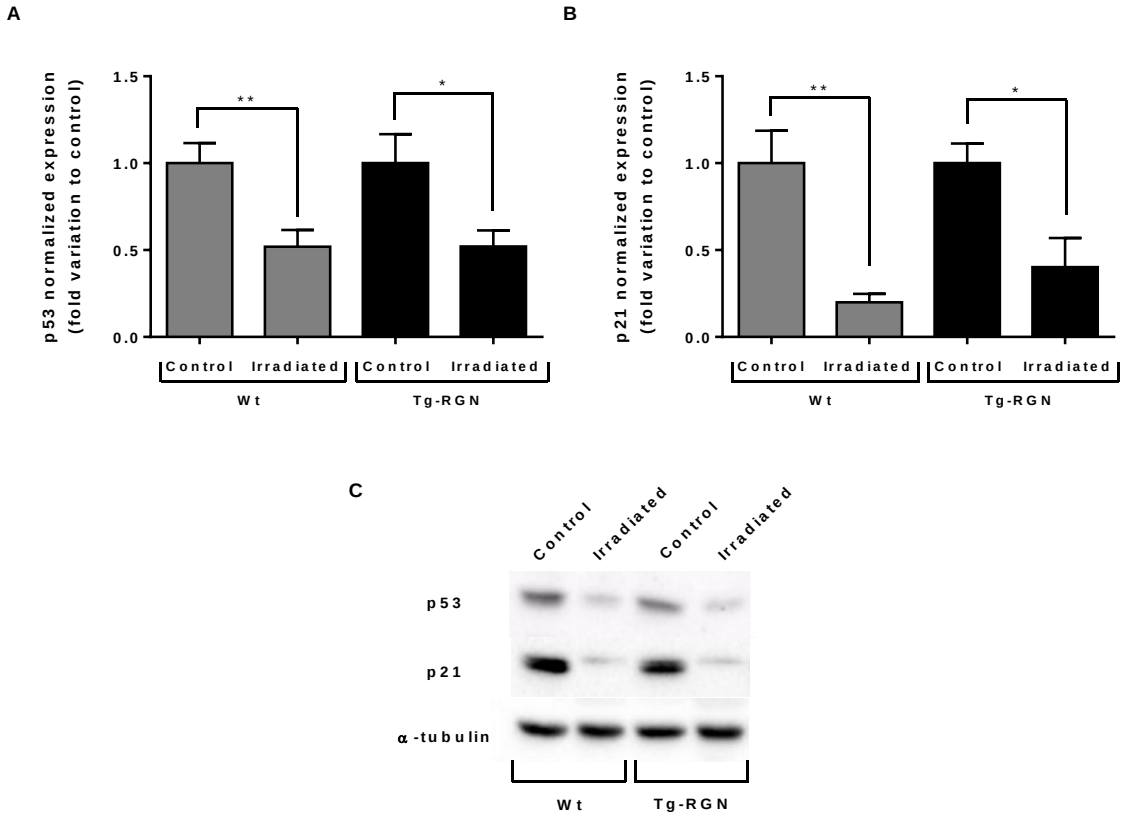

Figure V.5. p53 (A) and p21 (B) protein expression in the testis of Wt and Tg-RGN animals ten weeks after radiation treatment determined by WB analysis. Results are expressed as fold variation relatively to the respective control group after normalization with α-tubulin. Representative immunoblots for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

3.5 Bcl-2/Bax protein ratio

The use of chemotherapeutic drugs and radiation are known to induce germ cell death by apoptosis [32]. The apoptotic process can be triggered by two distinct pathways, the receptor-mediated (or extrinsic) and the mitochondrial (or intrinsic) [34].

The Bcl-2 and Bax proteins are, respectively, anti-apoptotic and pro-apoptotic members of the Bcl-2 family of mitochondrial apoptosis regulators [34]. 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 [35, 36]. Therefore, the ratio of Bcl-2/Bax proteins has been accepted to dictate the cell susceptibility to apoptosis [37].

At ten weeks 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 V.6A). Regarding the expression of Bax protein, no significant differences were observed in irradiated animals when compared with the respective control groups (Figure V.6B). Thus, the Bcl-2/Bax protein ratio (Figure V.6C) was significantly increased in both irradiated groups when compared to the respective controls. In Wt animals, it was observed a 2.7 ± 0.7 fold variation ($p<0.05$), whereas Tg-RGN animals displayed a 5.9 ± 1.4 fold variation ($p<0.01$) relatively to the respective control (Figure V.6C).This means that the anti-apoptotic/pro-apoptotic ratio was ≈2.2-fold higher in Tg-RGN irradiated animals comparatively to Wt irradiated ($p<0.05$). Representative immunoblot images for Bcl-2, Bax, and α-tubulin are shown in Figure V.6F.

3.6 FasL and FasR protein levels

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) in response to a variety of factors [38-40].

WB analysis showed that the protein expression of FasL and FasR upon irradiation treatment and 10 weeks of recovery followed a similar pattern (Figure V.6D and E). Contrastingly with the non-irradiated control, neither the protein expression of ligand ($p<0.001$) nor receptor ($p<0.01$) was detected in the Wt irradiated animals. In the Tg-RGN irradiated group, a slight expression of both FasL and FasR was detected, but still much lower than that of the respective controls (0.19 ± 0.12 ($p<0.01$) and 0.24 ± 0.16 ($p<0.05$) fold variation, respectively). However, the expression of FasL (Figure V.6D) and FasR (Figure V.6E) displayed no significant differences between the two irradiated groups. Representative immunoblot images for FasL, FasR, and α-tubulin are shown in Figure V.6F.

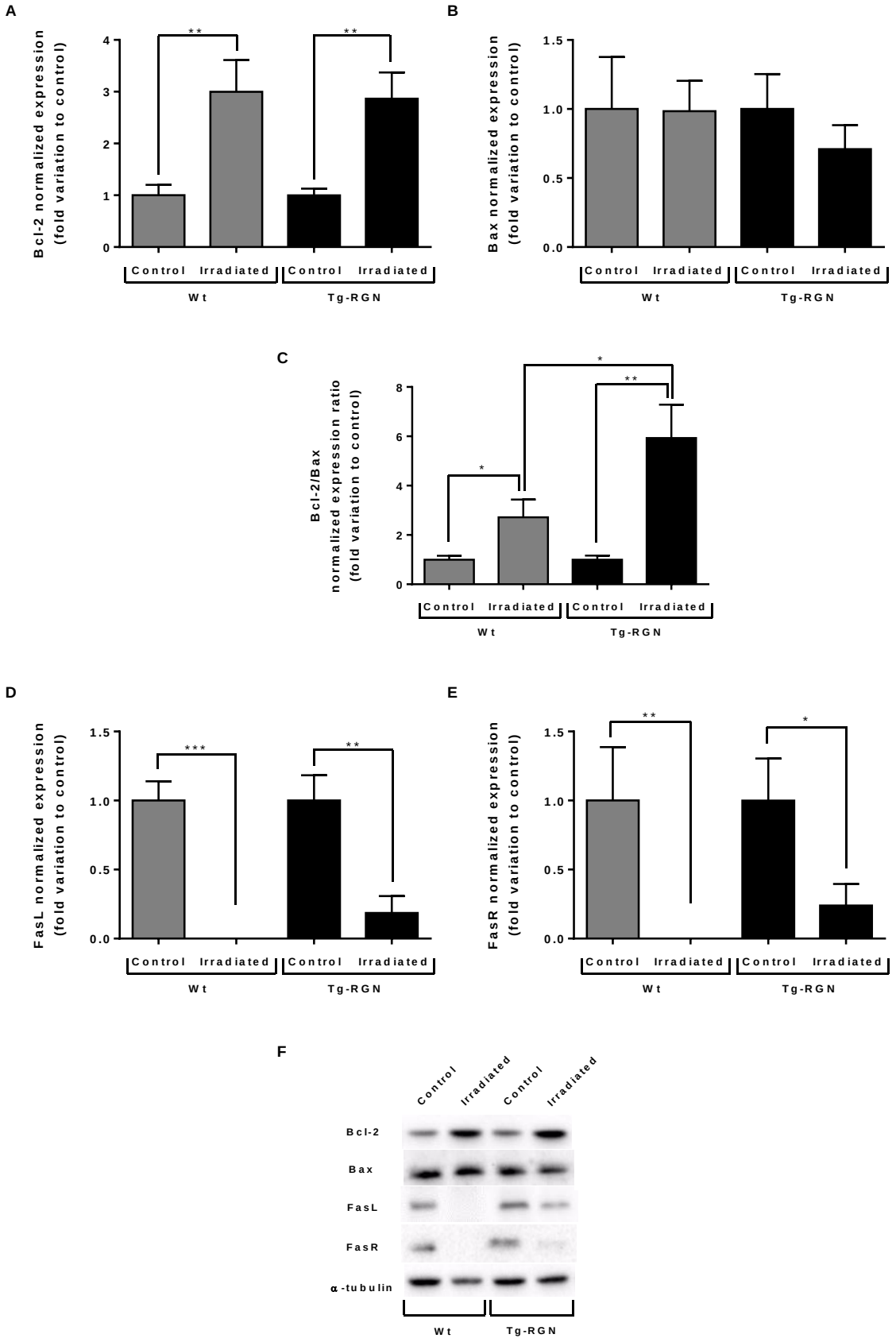

Figure V.6. Bcl-2 (A), Bax (B), FasL (D) and FasR (E) protein expression, and Bcl-2/Bax protein ratio (C) in the testis of Wt and Tg-RGN animals ten weeks after radiation treatment determined by WB analysis. Results are expressed as fold variation relatively to the respective control group after normalization with α-tubulin. Representative immunoblots for Bcl-2, Bax, FasL, FasR, and α-tubulin are shown in panel (F). Error bars indicate mean ± S.E.M. (n≥5 in each group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.7 Expression of caspase-8 and -9, and activity of caspase-3

Caspase-8 is the essential mediator of the extrinsic pathway of apoptosis by interacting with the death receptors and activating the downstream effectors of apoptosis [41]. In turn, the mitochondrial pathway of apoptosis is mediated by the activation of initiator caspase-9 [42, 43]. However, both apoptosis pathways converge at the activation of effector caspase-3, which is recognized as an end-point of apoptosis, independently of the activated pathway [34]. Therefore, the enzymatic activity of caspase-3 has been widely used to measure apoptosis [12, 40, 44].

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 V.7A); 1.59 ± 0.03 fold variation in Wt rats ($p<0.01$) *vs.* 1.30 ± 0.05 fold variation in Tg-RGN ($p<0.01$). Hence, the expression of caspase-8 was significantly lower in Tg-RGN irradiated comparatively with Wt irradiated group ($p<0.05$). Representative immunoblots for caspase-8 and α-tubulin are shown in Figure V.7C.

The expression of caspase-9 protein (Figure V.7B), at 10 weeks of recovery post-irradiation, was significantly reduced both in Wt and Tg-RGN animals relatively to the respective non-irradiated controls, being undetectable in the Wt irradiated rats ($p<0.001$ relatively to the respective control). In Tg-RGN irradiated rats, caspase-9 expression showed a 0.05 ± 0.03 fold variation ($p<0.01$) relatively to the respective control. Moreover, no statistical difference was found between Wt irradiated and Tg-RGN irradiated animals (Figure V.7B). Representative immunoblots for caspase-9 and α-tubulin are shown in Figure V.7C.

At ten weeks of recovery after testicles irradiation, a significant increase of caspase-3 activity was observed in Wt irradiated rats comparatively with the control group (133.3 ± 31.32 *vs.* 74.60 ± 10.49, $p<0.05$, Figure V.7D), an effect not seen in Tg-RGN animals. Thus, 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, $p<0.05$). Moreover, the diminished enzymatic activity of caspase-3 was already evident (Figure V.7D) in non-irradiated Tg-RGN animals (46.99 ± 9.75 *vs.* 74.60 ± 10.49 in Wt littermates, $p<0.05$).

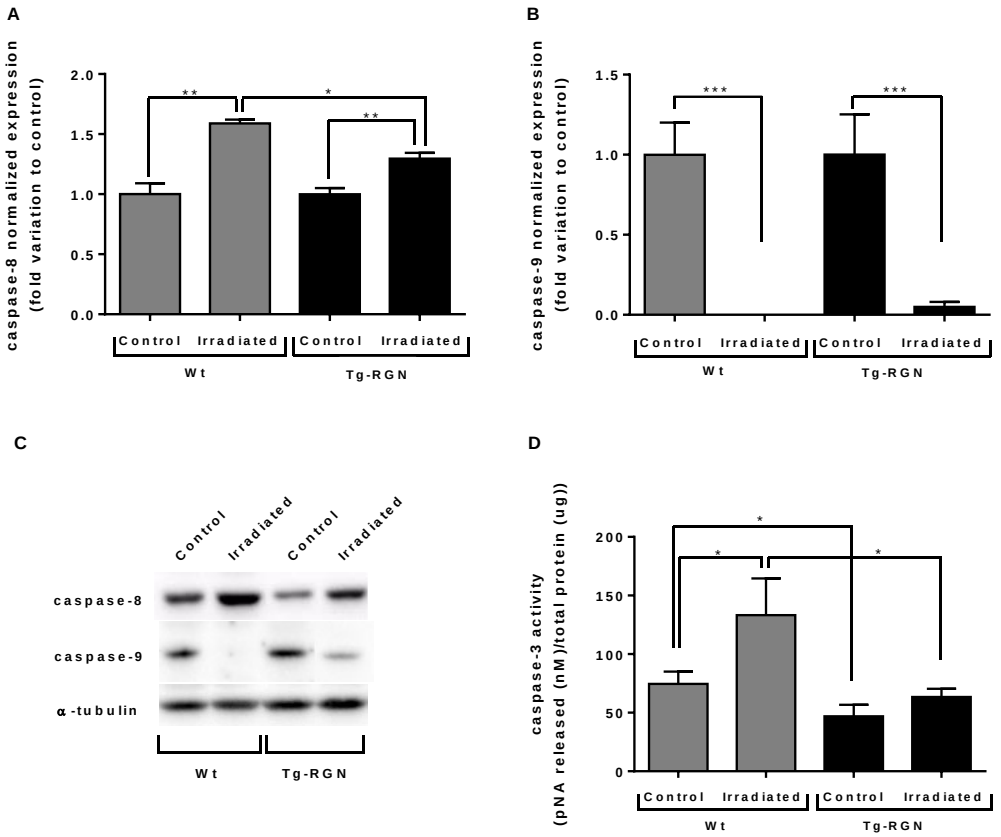

Figure V.7. Caspase-8 (A) and caspase-9 (B) protein expression determined by WB analysis, and caspase-3 activity (D), measured spectrophotometrically, in the testis of Wt and Tg-RGN animals ten weeks after radiation treatment. WB results are expressed as fold variation relatively to the respective control group after normalization with α-tubulin. Representative immunoblots for caspase-8, caspase-9, and α-tubulin are shown in panel (C). Error bars indicate mean ± S.E.M. (n≥5 in each group). *p<0.05; **p<0.01; ***p<0.001.

3.8 RGN expression

RGN has been shown to suppress apoptosis induced by noxious stimuli [10, 12], and an increased expression of RGN in response to radiation was detected in pancreatic cell lines [45]. Therefore, we decided to analyse RGN expression after radiation treatment. The protein levels of RGN were enhanced both in Wt irradiated and Tg-RGN irradiated rats in comparison with the corresponding controls, respectively, 1.83 ± 0.17 (p<0.05) and 1.51 ± 0.16 (p<0.01) fold variation (Figure V.8A). Representative immunoblot images for RGN and α-tubulin are shown in the Figure V.8B.

As expected, the immunohistochemistry analysis confirmed that Tg-RGN rats displayed stronger RGN staining when compared to Wt animals (Figure V.8C). Regarding the effect of irradiation treatment, immunohistochemistry highlighted the expression pattern determined by WB, showing higher RGN expression both in Wt and Tg-RGN irradiated rats comparatively with the respective controls (Figure V.8C).

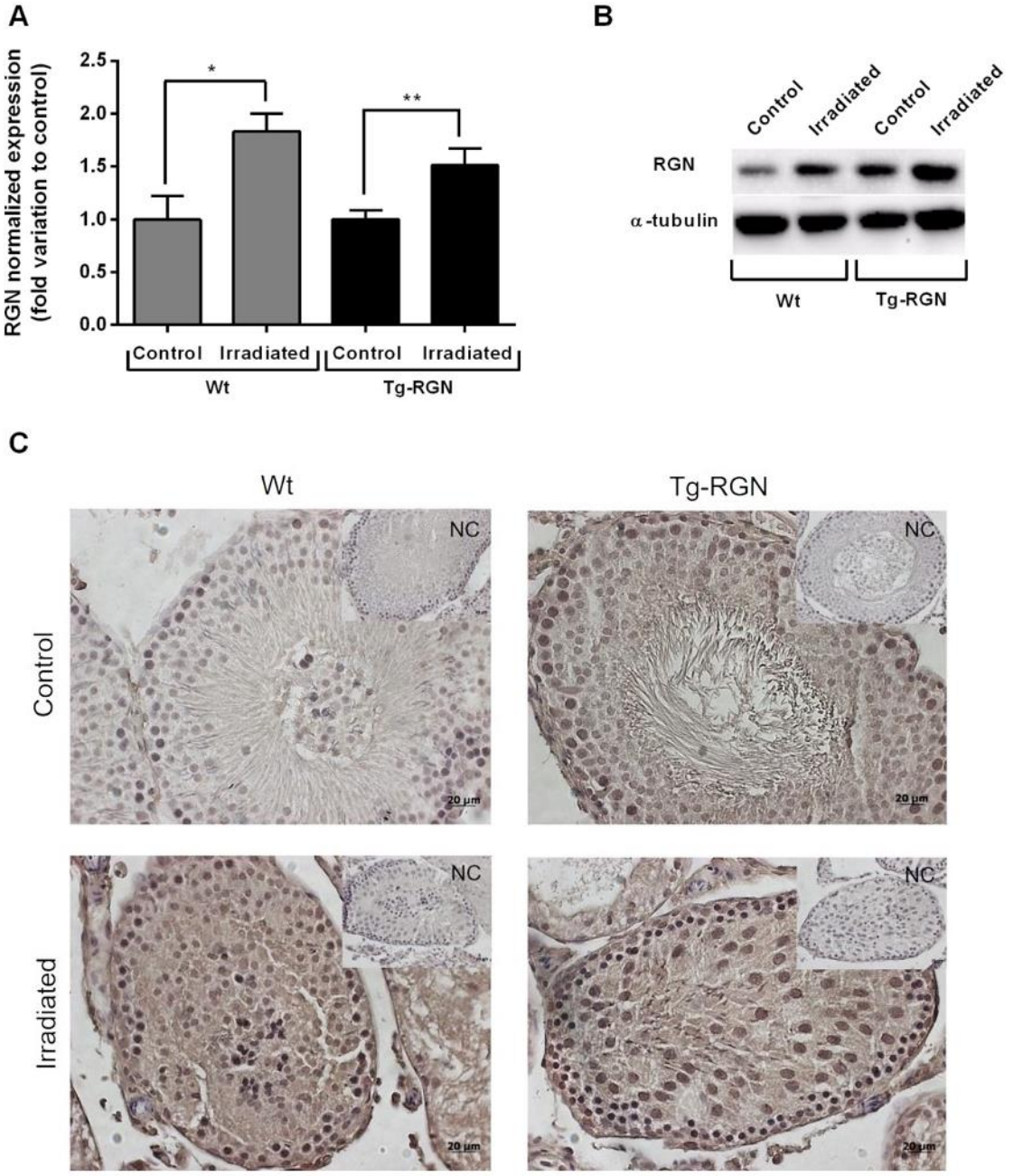

Figure V.8. RGN protein expression in the testis of Wt and Tg-RGN animals ten weeks after radiation treatment determined by WB analysis (A, B) and immunohistochemistry (C). (A) WB results are expressed as fold variation relatively to the respective control group after normalization with α-tubulin. Representative immunoblots are shown in panel (B). Error bars indicate mean ± S.E.M. (n≥5 in each group). *$p<0.05$; **$p<0.01$. (C) Scale bar indicates 20 µm. Representative negative controls (NC) obtained by omission of the primary antibody are provided as insert panels.

4. Discussion

Cancer therapies, namely radiation, frequently lead to a temporary disruption or complete arrest of spermatogenesis due to its damaging effects inducing germ cell death by apoptosis [3]. Indeed, the testis is one of the most radiosensitive organs in the body because of the high mitotic rate of the germinal epithelium, with immature spermatogonia being extremely sensitive to the adverse effects of radiation [2].

Considering the cytoprotective roles suggested for RGN and its ability to suppress apoptosis in response to noxious stimuli, the present work investigated the *in vivo* effect of RGN overexpression in spermatogenesis after radiotherapy.

As expected, the single exposure to a 6 Gy dose of X-rays provoked evident testicular damage. The deleterious effect of radiation treatment on the testis weight and spermatogonial differentiation was confirmed by assessing the GSI and TDI (Figure V.2A and B, respectively), and followed other previously published reports [46-48]. However, and despite no differences were perceived in the TDI between Tg-RGN and Wt irradiated animals, at ten weeks of recovery after irradiation the GSI was significantly higher in Tg-RGN rats (Figure V.2A). These results indicate that RGN overexpression accelerated the recovery of spermatogenesis, which is further supported by our previous results showing that RGN is expressed in spermatogonial cells [18].

In order to evaluate the output of the spermatogenic process in Tg-RGN and Wt animals, several parameters related with sperm quality were determined. The obtained results confirmed our previous findings [19] showing that Tg-RGN display lower sperm counts and motility (Figure V.3A and Figure V.3B, respectively), but contrastingly, higher numbers of viable (Figure V.3C) and morphologically normal (Figure V.4A) sperm, as well as lower incidence of sperm defects comparatively with Wt animals. Despite the initially reduced sperm motility, Tg-RGN animals displayed a higher percentage of motile sperm after irradiation indicating a lower damage of radiation in this parameter upon overexpression of RGN (Figure V.3B). Moreover, it should be noticed that motile sperm were only found in the Tg-RGN irradiated whereas no single motile sperm was found in Wt irradiated (Figure V.3B). Concerning sperm viability, similar results were observed, i.e., higher percentage of viable sperm after radiotherapy in Tg-RGN rats, with the total absence of viable sperm in Wt irradiated (Figure V.3C).Thus, although no differences were observed in sperm counts, Tg-RGN irradiated rats presented higher motility and viability than their Wt irradiated counterparts.

Regarding sperm morphology (Figure V.4), both Wt and Tg-RGN animals showed a higher incidence of abnormal sperm after radiotherapy, which is in agreement with previous reports describing the effects of X-rays on spermatogenesis [49-51]. Nevertheless, it is important to highlight that Tg-RGN rats displayed higher percentage of normal sperm (Figure V.4A) and lower percentage of head-defects (Figure V.4B) than Wt counterparts under the same post-irradiation period. The precise mechanisms underlying this protective role of RGN against radiation damage remain unclear but it is likely that it would be related with the OS levels, a major cause responsible for defective sperm morphology [52]. It is well established that ionizing radiation, such as X-rays, results in chemical and biological changes and in the generation of reactive chemical species by stimulation of oxidases and nitric oxide synthases

(NOS) [53]. On the other hand, RGN seems to play a relevant role against OS, by enhancing the activity of antioxidant superoxide dismutase [54] and glutathione-S-transferase [20, 21] enzymes and reducing the generation of reactive oxygen species (ROS) [55]. RGN overexpression in distinct biological models, including the rat seminiferous tubules, also was associated with a higher antioxidant potential and diminished lipid peroxidation (LPO) [20, 21, 55].

Moreover, the activity of epididymal epithelium contributes to the establishment of a proper luminal milieu that protects sperm against oxidative damage [56]. Interestingly, our recent work showed the differential expression of RGN in the functional regions of the epididymis associated and the higher antioxidant potential in the epididymal fluid under RGN overexpression conditions [19]. Thus, the protective effect of RGN on sperm parameters upon irradiation may possible be explained by lower levels of OS.

It is well described that the cellular content within the seminiferous tubules and the proper spermatogenic output are maintained by the delicate equilibrium between cell proliferation and death [32, 57]. Moreover, it is known that RGN plays an important role in the regulation of cell cycle and apoptosis [10-12, 58]. Therefore, the expression of several cell cycle and apoptosis modulators was evaluated in Tg-RGN and Wt animals.

Considering the tumor suppressor gene p53, a diminished expression was found in the testis of irradiated rats comparatively with controls, but no differences were found between Wt and Tg-RGN (Figure V.5A). Since p53 regulates the expression of cell-cycle inhibitor p21 [30, 33], its expression levels were also determined; p21 expression followed p53, i.e. low expression was observed in the testis of Wt and Tg-RGN rats after irradiation (Figure V.5B). These results suggest that irradiation decreased the levels of p53 and p21 independently of RGN. These findings are further supported by previous studies describing no accumulation of p53 in the mice testis in response to low dose of X-rays [59].

Relatively to the apoptotic process, no changes in the amount of testicular Bax protein after radiation treatment were shown (Figure V.6B), which is in agreement with what was reported by others [60]. Nevertheless, an increased expression of the antiapoptotic Bcl-2 protein was found (Figure V.6A) concomitantly with a decreased expression of activated caspase-9 (Figure V.7B). Consequently, an increased Bcl-2/Bax ratio in response to radiotherapy was observed both in Wt and Tg-RGN rats (Figure V.6C). However, the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio is ≈2.2-fold higher in irradiated Tg-RGN relatively to irradiated Wt, which indicates a lower rate of apoptosis in these animals. Bcl-2 has been implicated in the inhibition of p53 upregulation in response to various stimuli [12, 61], which may explain the reduced levels of p53, p21, and caspase-9 after irradiation. In addition, Bcl-2 is a recognized repressor of the release of cytochrome c to the cytosol [36], inhibiting the formation of the apoptosome and, consequently, the activation of caspase-9. In fact, it has been pointed out that RGN turn the cells more resistant to apoptosis [62]. Recently, we demonstrated that the overexpression of RGN protects testicular cells from apoptosis induced by thapsigargin or actinomycin D, supporting its role as a germ cell survival factor alone or as a mediator in androgen signalling pathways [12].

Regarding the extrinsic 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 [63]. 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 [64]. After irradiation, the expression of FasL and FasR (Figure V.6D and E, respectively) was undetectable in Wt animals whereas in Tg-RGN a slight expression still was evident. The reduction of FasR/FasL system in both irradiated groups is somehow unexpected considering the recognized increase of apoptosis in consequence of radiation exposure. Even considering the diminished expression of FasL and FasR, an increase of caspase-8 expression was observed in both Wt and Tg-RGN irradiated rats (Figure V.7A). This is in line with studies showing that caspase-8 can be activated by other death receptor/ligand system apart from the traditional FasR/FasL, such as the ligands TRAIL and tumor necrosis factor-α and its respective receptors [65-67]. However, the increase of caspase-8 expression was lower in Tg-RGN (Figure V.7A), which also supports a diminished rate of apoptosis in these animals. In accordance, it was previously shown that apoptotic-inducers, namely actinomycin D and tumor necrosis factor-α, were capable to induce caspase-8 activity in the hepatocytes of RGN knockout mice [68].

The execution of apoptotic cell-death, independently of the activated pathway, depends on the activation of caspase-3, which is a remarkable end point of apoptosis [34]. Caspase-3 activity was increased in Wt rats at ten weeks after irradiation, an effect not seen in Tg-RGN (Figure V.7D). It is important to highlight that the reduced activity of caspase-3 in Tg-RGN irradiated animals is concordant with the low levels of caspase-8 and the increased Bcl-2/Bax ratio. Altogether these findings concur indicating that testicular cells of Tg-RGN rats are more resistant to apoptosis induced by radiotherapy. Indeed, prior *in vivo* and *in vitro* studies have shown that RGN overexpression was associated with the modulation of expression of apoptosis-related genes, increasing the expression of Bcl-2 and declining the expression and/or activity of caspase-3 [10-12].

Since several studies indicated that apoptotic-stimuli, including radiation, increase the expression of RGN [45, 69, 70], we decided to evaluate the expression of RGN in the testis of irradiated animals. RGN expression was increased in the testis of both Wt and Tg-RGN animals at ten weeks after irradiation (Figure V.8), which followed a previous study showing that RGN is overexpressed in radioresistant pancreatic cell lines established by fractionated irradiation [45]. Also, another study showed accelerated healing of radiation-induced injury in mice liver concomitant with increased levels of RGN [71].

5. Conclusions

Overall, the obtained results suggest that the overexpression of RGN had a protective effect against radiation-induced testicular damage, likely by suppressing apoptosis and OS. Moreover, the present results strengthened our previous findings describing the protective role of RGN protein against chemical-induced apoptosis in the male gonad and strongly support the involvement of RGN in the anti-apoptotic response and resistance to external damage. Despite further research is needed, the present findings also raised the curiosity about the manipulation of RGN levels in the testicles as a potential mechanism to preserve fertility in male patients undergoing oncological treatment. In a near future, disclosing the mechanisms behind RGN protection against noxious stimuli for the male reproductive function will be of uttermost importance to identify solutions for the current oncofertility concerns.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709). Sara Correia is recipient of a fellowship from FCT (SFRH/BPD/104820/2014).
The authors also acknowledge Catarina Ferreira for technical assistance in tissue processing for histological analysis.

References

[1] Hess R, de Franca L. Spermatogenesis and Cycle of the Seminiferous Epithelium. In: Cheng CY, editor. Molecular Mechanisms in Spermatogenesis. Advances in Experimental Medicine and Biology. New York: Springer; 2009. 636. p. 1-15.

[2] Osterberg EC, Ramasamy R, Masson P, Brannigan RE. Current practices in fertility preservation in male cancer patients. Urol Ann. 2014;6:13-7.

[3] Trost LW, Brannigan RE. Oncofertility and the male cancer patient. Curr Treat Options Oncol. 2012;13:146-60.

[4] Kuban DA, Meistrich ML, Rex Cheung M, Hussey DH. Chapter 27 - The Testicle In: Cox JD, Ang KK, editors. Radiation Oncology. Ninth Edition. Philadelphia: Elsevier; 2010. p. 653-75.

[5] Abuelhija M, Weng CC, Shetty G, Meistrich ML. Rat models of post-irradiation recovery of spermatogenesis: interstrain differences. Andrology. 2013;1:206-15.

[6] Yamaguchi M, Yamamoto T. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull. 1978;26:1915-8.

[7] Yamaguchi M. Role of regucalcin in maintaining cell homeostasis and function (Review). Int J Mol Med. 2005;15:371-89.

[8] Marques R, Peres CG, Vaz CV, Gomes IM, Figueira MI, Cairrao E, et al. 5alpha-Dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth. Med Oncol. 2015;32:676.

[9] Marques R, Maia CJ, Vaz C, Correia S, Socorro S. The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci. 2014;71:93-111.

[10] Nakagawa T, Yamaguchi M. Overexpression of regucalcin suppresses apoptotic cell death in cloned normal rat kidney proximal tubular epithelial NRK52E cells: change in apoptosis-related gene expression. J Cell Biochem. 2005;96:1274-85.

[11] Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves MG, et al. Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate. 2014;74:1189-98.

[12] Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, et al. Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology. 2014;2:290-8.

[13] Kondo Y, Sasaki T, Sato Y, Amano A, Aizawa S, Iwama M, et al. Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008;377:291-6.

[14] Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, et al. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun. 2008;375:346-50.

[15] Son TG, Zou Y, Jung KJ, Yu BP, Ishigami A, Maruyama N, et al. SMP30 deficiency causes increased oxidative stress in brain. Mech Ageing Dev. 2006;127:451-7.

[16] Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, et al. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. Am J Respir Crit Care Med. 2006;174:530-7.

[17] Son TG, Kim SJ, Kim K, Kim MS, Chung HY, Lee J. Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress. Arch Pharm Res. 2008;31:872-7.

[18] Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, et al. Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction. 2011;142:447-56.

[19] Correia S, Oliveira PF, Guerreiro PM, Lopes G, Alves MG, Canario AV, et al. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca2+-binding protein regucalcin: a hidden role for Ca2+ in sperm maturation? Mol Hum Reprod. 2013;19:581-9.

[20] Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

[21] Vaz CV, Marques R, Maia CJ, Socorro S. Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin. Transl Res. 2015;166:693-705.

[22] Trost L, Brannigan R. Fertility Preservation in Males. In: Gracia C, Woodruff TK, editors. Oncofertility Medical Practice. New York: Springer; 2012. p. 27-50.

[23] Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R. Role of endogenous regucalcin in transgenic rats: suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal Ca2+-ATPase activity. J Cell Biochem. 2002;86:520-9.

[24] Dott HM, Foster GC. Technique for Studying Morphology of Mammalian Spermatozoa Which Are Eosinophilic in a Differential Live-Dead Stain. J Reprod Fertil. 1972;29:443-5.

[25] Cardoso HJ, Vaz CV, Correia S, Figueira MI, Marques R, Maia CJ, et al. Paradoxical and contradictory effects of imatinib in two cell line models of hormone-refractory prostate cancer. Prostate. 2015;75:923-35.

[26] Griswold MD. Interactions between germ cells and Sertoli cells in the testis. Biol Reprod. 1995;52:211-6.

[27] Cornwall GA. New insights into epididymal biology and function. Hum Reprod Update. 2009;15:213-27.

[28] Robaire B, Hinton BT. Chapter 17 - The Epididymis. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's Physiology of Reproduction. Fourth Edition. San Diego: Academic Press; 2015. p. 691-771.

[29] Menkveld R, Holleboom CA, Rhemrev JP. Measurement and significance of sperm morphology. Asian J Androl. 2011;13:59-68.

[30] Speidel D. Transcription-independent p53 apoptosis: an alternative route to death. Trends Cell Biol. 2010;20:14-24.

[31] Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. Biochem Soc Trans. 2001;29:684-8.

[32] Shaha C, Tripathi R, Mishra DP. Male germ cell apoptosis: regulation and biology. Philos Trans R Soc Lond B Biol Sci. 2010;365:1501-15.

[33] Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, et al. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. 1995;9:935-44.

[34] Lawen A. Apoptosis-an introduction. Bioessays. 2003;25:888-96.

[35] Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A. 1998;95:4997-5002.

[36] Rosse T, Olivier R, Monney L, Rager M, Conus S, Fellay I, et al. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature. 1998;391:496-9.

[37] Mackey TJ, Borkowski A, Amin P, Jacobs SC, Kyprianou N. Bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with prostate cancer. Urology. 1998;52:1085-90.

[38] Kim SK, Yoon YD, Park YS, Seo JT, Kim JH. Involvement of the Fas-Fas ligand system and active caspase-3 in abnormal apoptosis in human testes with maturation arrest and Sertoli cell-only syndrome. Fertil Steril. 2007;87:547-53.

[39] Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology. 1997;138:2081-8.

[40] Correia S, Alves MR, Cavaco JE, Oliveira PF, Socorro S. Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertil Steril. 2014;102:299-306.

[41] van Raam BJ, Salvesen GS. Proliferative versus apoptotic functions of caspase-8 Hetero or homo: the caspase-8 dimer controls cell fate. Biochim Biophys Acta. 2012;1824:113-22.

[42] Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91:479-89.

[43] Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell. 1998;1:949-57.

[44] Figueira MI, Correia S, Vaz CV, Cardoso HJ, Gomes IM, Marques R, et al. Estrogens down-regulate the stem cell factor (SCF)/c-KIT system in prostate cells: evidence of antiproliferative and proapoptotic effects. Biochem Pharmacol. 2016;99:73-87.

[45] Ogawa K, Utsunomiya T, Mimori K, Tanaka F, Haraguchi N, Inoue H, et al. Differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation. Int J Oncol. 2006;28:705-13.

[46] Topcu-Tarladacalisir Y, Kanter M, Uzal MC. Role of L-carnitine in the prevention of seminiferous tubules damage induced by gamma radiation: a light and electron microscopic study. Arch Toxicol. 2009;83:735-46.

[47] Li W, Zeng Y, Zhao J, Zhu CJ, Hou WG, Zhang S. Upregulation and nuclear translocation of testicular ghrelin protects differentiating spermatogonia from ionizing radiation injury. Cell Death Dis. 2014;5:e1248.

[48] Demir A, Karadag MA, Cecen K, Tanidir Y, Turkeri L, Atasoy BM, et al. Effects of testosterone treatment on recovery of rat spermatogenesis after irradiation. J Pak Med Assoc. 2015;65:300-5.

[49] Bruce WR, Furrer R, Wyrobek AJ. Abnormalities in the shape of murine sperm after acute testicular X-irradiation. Mutat Res. 1974;23:381-6.

[50] Dobrzynska MM, Gajewski AK. Induction of micronuclei in bone marrow and sperm head abnormalities after combined exposure of mice to low doses of X-rays and acrylamide. Teratog Carcinog Mutagen. 2000;20:133-40.

[51] Wyrobek AJ. Changes in mammalian sperm morphology after X-ray and chemical exposures. Genetics. 1979;92:s105-19.

[52] Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal. 2011;14:367-81.

[53] Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012;327:48-60.

[54] Fukaya Y, Yamaguchi M. Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull. 2004;27:1444-6.

[55] Handa S, Maruyama N, Ishigami A. Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. Biol Pharm Bull. 2009;32:1645-8.

[56] Jervis KM, Robaire B. Dynamic changes in gene expression along the rat epididymis. Biol Reprod. 2001;65:696-703.

[57] Correia S, Cardoso HJ, Cavaco JE, Socorro S. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med. 2015;17:e2.

[58] Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, et al. Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res. 2015;330:325-35.

[59] Wang X, Matsumoto H, Takahashi A, Nakano T, Okaichi K, Ihara M, et al. p53 accumulation in the organs of low-dose X-ray-irradiated mice. Cancer Lett. 1996;104:79-84.

[60] Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB, De Rooij DG. Apoptosis regulation in the testis: involvement of Bcl-2 family members. Mol Reprod Dev. 2000;56:353-9.

[61] Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol. 1994;124:1-6.

[62] Yamaguchi M. The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis. 2013;18:1145-53.

[63] Campion SN, Sandrof MA, Yamasaki H, Boekelheide K. Suppression of radiation-induced testicular germ cell apoptosis by 2,5-hexanedione pretreatment. III. Candidate gene analysis identifies a role for fas in the attenuation of X-ray-induced apoptosis. Toxicol Sci. 2010;117:466-74.

[64] Francavilla S, D'Abrizio P, Cordeschi G, Pelliccione F, Necozione S, Ulisse S, et al. Fas expression correlates with human germ cell degeneration in meiotic and post-meiotic arrest of spermatogenesis. Mol Hum Reprod. 2002;8:213-20.

[65] Deng Y, Lin Y, Wu X. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev. 2002;16:33-45.

[66] Grataroli R, Vindrieux D, Selva J, Felsenheld C, Ruffion A, Decaussin M, et al. Characterization of tumour necrosis factor-alpha-related apoptosis-inducing ligand and its receptors in the adult human testis. Mol Hum Reprod. 2004;10:123-8.

[67] Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science. 1998;281:1305-8.

[68] Ishigami A, Fujita T, Handa S, Shirasawa T, Koseki H, Kitamura T, et al. Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis. Am J Pathol. 2002;161:1273-81.

[69] Fukui M, Senmaru T, Hasegawa G, Yamazaki M, Asano M, Kagami Y, et al. 17beta-Estradiol attenuates saturated fatty acid diet-induced liver injury in ovariectomized mice by up-regulating hepatic senescence marker protein-30. Biochem Biophys Res Commun. 2011;415:252-7.

[70] Son TG, Park HR, Kim SJ, Kim K, Kim MS, Ishigami A, et al. Senescence marker protein 30 is up-regulated in kainate-induced hippocampal damage through ERK-mediated astrocytosis. J Neurosci Res. 2009;87:2890-7.

[71] Jeong DH, Goo MJ, Hong IH, Yang HJ, Ki MR, Do SH, et al. Inhibition of radiation-induced apoptosis via overexpression of SMP30 in Smad3-knockout mice liver. J Radiat Res. 2008;49:653-60.

Chapter VI
Overexpression of Regucalcin Mitigates the Ageing-Related Changes in Oxidative Stress and Sperm Quality

Chapter submitted for publication (under revision):

Silva A.M.S., Socorro S., Hurtado de Llera A., Vaz C.V., Correia S.#, Maia C.J.# Overexpression of regucalcin mitigates the ageing-related changes in oxidative stress and sperm quality.
#contributed equally as senior authors

Abstract

Age-related changes, namely the increase in oxidative stress (OS) with the consequent sperm damage, result in decreased male fertility. Regucalcin (RGN) is a Ca^{2+}-binding protein that has been shown to have beneficial effects on spermatogenesis by suppressing OS and chemical/radiation-induced damage. This work aims to evaluate whether RGN overexpression reduces the ageing-associated decline of male reproductive function. Sperm and testicular function analysis were performed in young-adult and senescent transgenic rats overexpressing RGN (Tg-RGN) comparatively with their wild-type (Wt) littermates. The gonadosomatic index (GSI) and the expression levels of RGN and other proliferation regulators were evaluated. Moreover, the sperm parameters and OS analysis were assessed. Both GSI and sperm counts were reduced in the senescent Wt rats, but maintained in the Tg-RGN. Also, the levels of stem cell factor (SCF), c-Kit, and Akt were maintained in the testis of aged Tg-RGN rats, suggesting that the normal spermatogenic output was preserved over time in these animals, an effect not observed in Wt. Senescent Tg-RGN rats also presented lower sperm lipid peroxidation and total oxidant status relatively to the Wt. Furthermore, aged Tg-RGN rats displayed higher sperm viability, higher frequency of sperm with normal morphology, and reduced incidence of head and neck/midpiece defects when compared with Wt, which may be a consequence of the lower OS levels found in the sperm of these animals. Interestingly, RGN expression increased with ageing in sperm. Altogether, these findings indicate that the modulation of RGN levels may alleviate the age-related decline in sperm quality and testicular function.

Keywords: Ageing; Male reproduction; Oxidative stress; Regucalcin; Sperm.

1. Introduction

Ageing is characterized by a time-dependent progressive increase in susceptibility to permanent and gradual changes, triggered by several endogenous and environmental factors, that leads to impaired physiological function [1, 2]. It is well established that male reproductive capacity decline with age, and this fact is a major problem because of the current trend in the modern societies to conceive at later ages [3]. Age-related alterations in men include changes in hormone levels, deregulation of gene expression, and the augment in oxidative stress (OS), which result in decreased sperm quality compromising fertility [2, 4]. In rodents, several studies have documented significant alterations with ageing, namely testicular atrophy, a decreased number of germ cells, a thinner seminiferous tubule epithelium, a lower sperm production, a decreased antioxidant capacity, among others [5-7].

Regucalcin (RGN) is a Ca^{2+}-binding protein, also known as senescence marker protein-30, whose expression decreases markedly with ageing in several tissues, namely, liver, kidney [8], prostate [9], and testis [10]. At testicular level, the RGN protein is expressed in Leydig and Sertoli cells, as well as in all the germ cells in human and rat [10]. RGN actions have been associated with the control of OS [11], cell proliferation [12], and apoptosis [12, 13], suggesting that its decreased expression with ageing may contribute to the age-associated deterioration of cell function. Our previous findings demonstrated that

RGN overexpression prevents ageing-associated changes in the prostate [9], and also that RGN overexpression has beneficial effects on spermatogenesis by suppressing both chemical- [13] and radiation-induced damage [14]. Moreover, it is well described that the activity of epididymal epithelium protect sperm against oxidative damage [15], and we previously showed the differential expression of RGN in the functional regions of the epididymis associated with a higher antioxidant potential in the epididymis under RGN overexpression [16]. Attending to its protective properties, RGN was proposed as an endogenous molecule with potential application on the recovery of male reproductive function upon a deleterious impact in gonadal physiology and sperm function, triggered by a set of different conditions [17]. However, whether RGN has a role in the ageing process of male reproductive system remains unclear.

The present work aims to investigate the influence of maintaining high expression levels of RGN in preventing the ageing-associated decline of male reproductive function. For this purpose, young-adult and senescent transgenic rats overexpressing RGN (Tg-RGN) and their wild-type (Wt) counterparts were used for sperm and testicular function analysis.

2. Materials and methods

2.1 Animals

Young-adult (3-month-old, 3M) and senescent (9M) Wt and Tg-RGN Sprague Dawley (*Rattus norvegicus*) rats (n ≥ 5 in each group) were obtained, respectively, from Charles River (Barcelona, Spain) and Japan SLC (Hamamatsu, Japan). The age of 3M was selected to ensure that the rats have completed at least one spermatogenic cycle and were sexually mature [18, 19], whereas 9M old represents a stage of reproductive senescence [20, 21]. In fact, various studies demonstrated that as early as 6M several aspects associated with the male reproductive potential were affected to some extent in rats [20-22], including the first modifications in the levels of genes and enzymes involved in steroidogenesis [21], suggesting that the onset of compromised spermatogenesis may occur at 6M. Therefore, using rats with 9M ensure an ageing-altered reproductive function.

This project was approved by the Animal-Welfare Body of the Health Research Centre at the University of Beira Interior (CICS-UBI). Animals were handled in compliance with the European Union rules for the care and handling of laboratory animals (Directive number 2010/63/EU) and housed under a 12 h light:12 h darkness cycle, with food and water available *ad libitum* during the course of the experiment.

2.2 Tissue collection

Rats were anesthetized intraperitoneally with ketamine (50 mg/Kg; Clorketam 1000) plus xylazine (20 mg/Kg; Rompun), and euthanized by cervical dislocation.

Testes from Wt and Tg-RGN animals were collected, and the gonadosomatic index (GSI) was determined from the body and testicular mass. Testes were frozen on liquid nitrogen and stored at -80ºC until protein extraction. Also, epididymides were removed and dissected free from fat, and the *cauda* region of the epididymis from each animal was used for the determination of sperm parameters.

2.3 Epididymal sperm counts and motility

Cauda segments of the epididymis were minced in 3 mL of filtered Hank's buffered salt solution (HBSSf; Sigma-Aldrich, St. Louis, MO, USA) at 37ºC to obtain a sperm suspension. The suspension was incubated at 37ºC for 5 minutes and used to analyse sperm counts, motility, viability and morphology, as previously described [14, 16]. The remaining sperm suspension was frozen on liquid nitrogen and stored at -80ºC until protein extraction or determination of total oxidant status (TOS). Briefly, an aliquot of sperm suspension was diluted in HBSSf, and sperm cells counted using a Neubauer´s chamber (Labor Optik, Balgach, Switzerland). Sperm motility was determined in a 37ºC pre-warmed slide covered with a coverslip using 100 µL of the sperm suspension. At least 10 random fields were assessed for each semen sample using an optical microscope (1000x magnification, Primo Star, Zeiss), and the percentage of motile sperm was calculated.

2.4 Sperm viability and morphology analysis

Sperm viability was assessed by using the one-step eosin-nigrosin staining technique, as previously described [14]. A sample of 5 µL of sperm suspension was mixed with 10 µL of a stain mixture composed by 0.6% eosin (Fisher Scientific, Geel, Belgium) and 5% nigrosin (Acros Organics, Morris Plains, NJ, USA) and placed on a pre-warmed slide.

Sperm morphology was evaluated using the Kwik-Diff™ staining kit (Thermo Scientific, Pittsburgh, PA, USA) using standard procedures [14, 16]. Briefly, smears were performed using 5 µL of sperm suspension, air dried, and underwent a three-step differential staining. Afterwards, sperm was classified as normal or abnormal, and the abnormalities were divided into head, neck/midpiece or tail defects. There are sperm with only one abnormality, but there are also cells presenting various types of defects simultaneously. In such case, if one of the defects is on the head, it accounted exclusively for the number of head-defects.

Sperm viability and morphology were assessed for a total of 100 sperm cells in random fields of each semen sample under a light microscope (Primo Star, Zeiss).

2.5 Total protein extraction

For western blot (WB) analysis, total proteins were extracted from testicular tissue and sperm cells using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1% protease inhibitors cocktail (Sigma-Aldrich) and 10% PMSF 10 mM (Sigma-Aldrich). For glutathione peroxidase (GPX) activity assay, total proteins were isolated from sperm cells without adding PMSF. Briefly, 300 µL of sperm suspension was centrifuged at 700 RCF for 40 minutes at 4ºC to separate the HBSS from the sperm. The pellet (sperm cells) was then homogenized with 90 µL of RIPA Buffer supplemented with 1% protease inhibitors cocktail. For thiobarbituric acid reactive substances (TBARS) assay, total proteins from sperm cells were obtained using a different RIPA buffer composition (1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, and 0.1% SDS diluted in phosphate-buffered saline 1x). Protein concentration in each sample extract was determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

2.6 Total oxidant status (TOS) assay

The TOS was determined using a commercial kit based in a method developed by Erel O [23] (RL0024, Rel Assay Diagnostics, Gaziantep, Turkey). In Erel's method, oxidants present in the sample oxidize Fe^{2+} to Fe^{3+}. The ferric ion produces a coloured complex with chromogen in an acidic medium. The colour intensity, measured spectrophotometrically, is associated with the total amount of oxidant molecules present in the sample. HBSS from sperm suspension of each sample was used to determine indirectly the TOS in sperm using H_2O_2 as standard. Briefly, 100 µL of sperm suspension was centrifuged at 700 RCF for 10 minutes at 4°C to separate the HBSS from the sperm. The supernatant (HBSS) was then transferred to a clean tube and 25 µL (in duplicate) of sample or standard (H_2O_2 at 3 different concentrations) were pipetted into a 96-well microplate. Then, sample (or standard) and buffer solution were mixed and the initial absorbance at 530 nm was read after 30 seconds using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The reactional plate was incubated 10 minutes at 37°C. After that, the prochromogen was added to the mixture, the reactional plate was immediately incubated for 5 minutes at 37°C, and the final absorbance was read at 530 nm. Results were expressed in µmoL H_2O_2 Equivalents L^{-1}.

2.7 Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation (LPO) was assessed by the colorimetric measurement of malondialdehyde-thiobarbituric acid (MDA–TBA) adduct formation using the TBARS Assay Kit (10009055; Cayman Chemical, Ann Arbor, MI, USA). Briefly, the MDA–TBA adduct was formed under high temperature and acidic conditions. At the end, vials were placed on ice to stop the reaction and centrifuged. Next, 150 µL (in duplicate) of each reaction was added to a clear plate, and the absorbance at 532 nm was measured using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The concentration of TBARS in each protein sample was calculated from the standard curve and expressed as µM MDA μg^{-1} total protein.

2.8 Glutathione peroxidase (GPX) activity assay

The GPX activity was determined using a commercial kit (703102; Cayman Chemical) according to the manufacturer's protocol. The activity of GPX was measured indirectly by a coupled reaction with glutathione reductase. The oxidation of NADPH to $NADP^+$, catalysed by glutathione reductase, is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A_{340} is directly proportional to the GPX activity under conditions in which the GPX activity is rate limiting. Briefly, 20 µL of each sample was pipetted in triplicate into a 96-well microplate. All reagents were stabilized at 25°C for 30 minutes. The decrease in absorbance was read in intervals of 30 seconds during 5 minutes, which allowed to determine the rate of ΔA_{340} per minute, and the calculation of the enzymatic activity of GPX. Results were expressed as U/µg total protein.

2.9 Western blot (WB)

Total proteins (25 or 50 μg of each extract) were resolved in 12.5% polyacrylamide gels by sodium dodecyl sulfate electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were incubated overnight at 4ºC with primary antibodies, and 1 hour at room temperature with secondary antibodies (Table VI.1). Afterwards, membranes were incubated 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™ software Version 5.1 (Bio-Rad) and normalized by division with the respective β-actin (Table VI.1) band density.

Table VI.1. Antibodies' specifications used for western blot (WB) studies.

Antibody	Host Species	Dilution	Reference	Manufacturer
RGN	Mouse	1:1000	ab81721	Abcam
AMPKα1/2	Rabbit	1:500	no. 2532	Cell Signaling Technology
SCF	Rabbit	1:500	H-189: sc-9132	Santa Cruz Biotechnology
c-Kit	Rabbit	1:500	C-19: sc-168	Santa Cruz Biotechnology
Akt	Rabbit	1:1000	no. 9272	Cell Signaling Technology
β-actin	Mouse	1:10000	A1978	Sigma-Aldrich
Rabbit IgG	Goat	1:40000	sc-2004	Santa Cruz Biotechnology
Mouse IgG	Goat	1:40000	sc-2005	Santa Cruz Biotechnology

Abbreviations: RGN, regucalcin; AMPKα1/2, adenosine monophosphate-activated protein kinase; SCF, stem cell factor; IgG, Immunoglobulin G.

2.10 Statistical analysis

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

3. Results

3.1 The gonadosomatic index, and the levels of stem cell factor, c-Kit, and Akt were maintained with ageing in the testis of Tg-RGN rats

The GSI is used as an indicator of testis function because gonad weight and size that are mainly controlled by the secretory activity of Sertoli cells determine sperm production capacity [24]. Figure VI.1A shows that 3M Tg-RGN rats display lower GSI relatively to their Wt littermates with the same age (0.004321 ± 0.000158 *vs.* 0.005497 ± 0.000098, respectively, $p<0.001$). Nevertheless, in Wt rats, the GSI significantly decreased to 0.004338 ± 0.000134 with ageing comparatively with the respective

3M controls that displayed a GSI of 0.005497 ± 0.000098 (p<0.001, Figure VI.1A). In Tg-RGN animals, the GSI was not affected by ageing (Figure VI.1A). No statistically significant difference was found in the GSI between the Wt and Tg-RGN 9M groups (Figure VI.1A).

The tyrosine kinase receptor c-Kit and its ligand, the stem cell factor (SCF), have been shown to play an important role in the Sertoli cells-germ cells communication [25], determining the survival of germ line cells [26]. Indeed, the SCF/c-Kit system regulates spermatogonia proliferation in adult testis [27].

WB analysis showed an increased expression of SCF with ageing in Wt rats (1.40 ± 0.07 fold variation, p<0.01), whereas no statistical differences were observed between the young-adult and the aged Tg-RGN (Figure VI.1B). Regarding c-Kit, its expression was decreased in Wt rats with the advance of the age (0.75 ± 0.06 fold variation, p<0.05, Figure VI.1C). However, Tg-RGN rats were able to maintain the expression levels of c-Kit from 3M to 9M (Figure VI.1C).

Moreover, we analysed the protein expression levels of Akt, which also participates in the control of germ cell proliferation [28] and is involved in the testicular ageing process [29]. We found that Akt expression was increased in Wt aged rats comparatively to young-adult Wt (1.43 ± 0.10 fold variation, p<0.01, Figure VI.1D). On the other hand, Tg-RGN rats did not alter Akt expression with ageing. Representative immunoblot images for SCF, c-Kit, Akt, and β-actin are shown in Figure VI.1E.

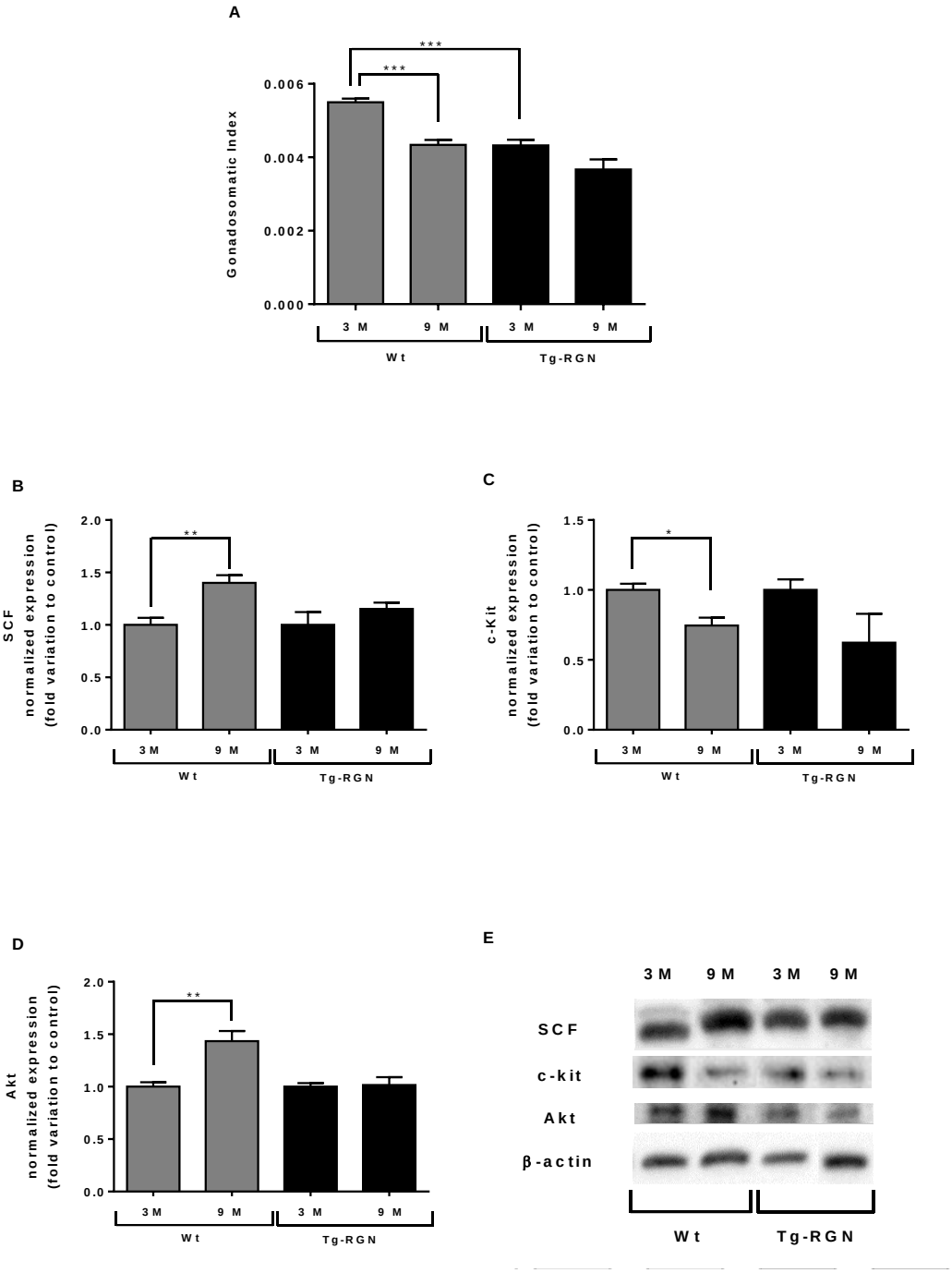

Figure VI.1. Gonadosomatic index (A), and SCF (B), c-Kit (C) and Akt (D) protein expression in the testis of young-adult (3M) and senescent (9M) Wt and Tg-RGN animals. WB results are expressed as fold variation relatively to the respective control group after normalization with β-actin. Representative immunoblots for SCF, c-Kit, Akt and β-actin are shown in panel (E). Error bars indicate mean ± S.E.M. (n≥5 in each group). *p<0.05; **p<0.01; ***p<0.001.

3.2 Ageing did not affect epididymal sperm parameters in the Tg-RGN animals

Sperm leave the testis as non-functional gametes, acquiring the capacity to move progressively, and eventually to fertilize, during the passage through the different functional regions of the epididymis [30]. Storage of mature sperm occurs in the *cauda* of the epididymis [31], and, for this reason, *cauda* sperm was used to evaluate cell number, motility and viability. Analysis of sperm parameters are

conventionally used to predict male fertility potential, being helpful in diagnostics of male subfertility and infertility [32].

Epididymal sperm counts at young condition were significantly lower in Tg-RGN rats when compared to Wt littermates ($1.7 \times 10^8 \pm 9.8 \times 10^6$ *vs.* $2.4 \times 10^8 \pm 1.2 \times 10^7$, $p<0.001$; Figure VI.2A). Sperm counts were significantly decreased (to $2.0 \times 10^8 \pm 6.4 \times 10^6$ cells/mL) in 9M Wt animals comparatively with the 3M control group ($p<0.01$, Figure VI.2A). Contrastingly, 9M Tg-RGN animals maintained the number of sperm cells/mL (Figure VI.2A) found for the respective 3M control. Nevertheless, no significant difference was found in sperm counts between the Wt senescent and Tg-RGN senescent groups (Figure VI.2A).

Also, the percentage of motile sperm (Figure VI.2B) was significantly lower in Tg-RGN rats comparatively to their Wt littermates both at young (47.9% ± 3.7 *vs.* 64.6% ± 5.7, $p<0.05$) and senescent state (45.0% ± 2.3 *vs.* 66.8% ± 1.9, $p<0.001$). No significant difference was found on the percentage of motile sperm with ageing in both the Wt and Tg-RGN groups (Figure VI.2B).

Concerning sperm viability, Figure VI.2C shows that Tg-RGN have a significant higher percentage of viable sperm compared to the 3M (38.8% ± 2.4 *vs.* 28.0% ± 3.8, $p<0.05$) and 9M (33.1% ± 2.4 *vs.* 19.8% ± 2.8, $p<0.05$) Wt animals. The percentage of viable sperm did not change significantly due to ageing in both Wt and Tg-RGN animals (Figure VI.2C).

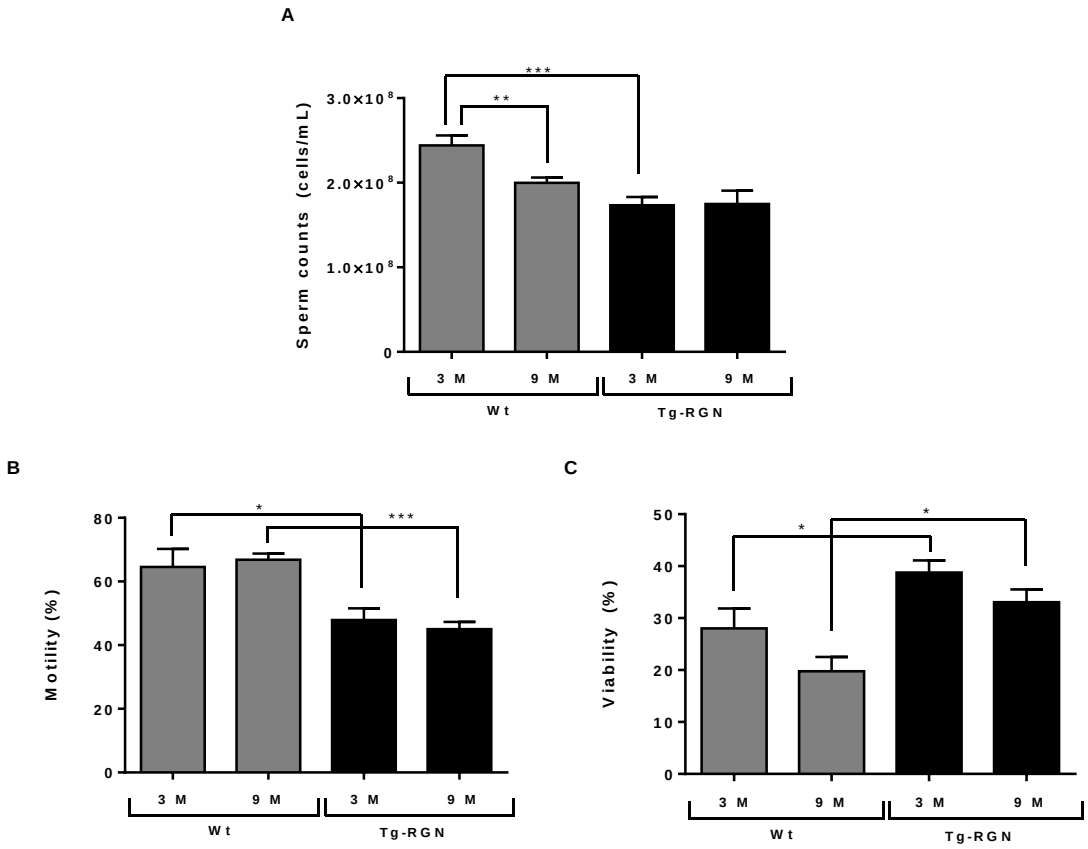

Figure VI.2. Epididymal sperm counts (A), motility (B) and viability (C) in young-adult (3M) and senescent (9M) Wt and Tg-RGN animals. Error bars indicate mean ± S.E.M. (n≥5 in each group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

The percentage of normal sperm in the *cauda* of the epididymis (Figure VI.3A) was higher in Tg-RGN relatively to Wt animals both at 3M (74.1% ± 3.7 *vs*. 57.6% ± 1.8, $p<0.01$) and 9M (74.5% ± 3.3 *vs*. 56.4% ± 4.5, $p<0.05$). No significant differences were found with ageing in both the Wt and Tg-RGN groups in comparison to controls.

Concerning head-defects (Figure VI.3B), the percentage of sperm presenting head-defects significantly increased from 1.7% ± 0.3 (3M) to 11.9% ± 1.4 (9M) ($p<0.01$) in Wt rats. On the other hand, ageing did not alter the percentage of head-defects in Tg-RGN rats. Moreover, a significant lower percentage of head-defects was found in the sperm of senescent Tg-RGN rats, only 5.9% ± 0.9, against 11.9% ± 1.4 in their Wt aged littermates ($p<0.05$).

A similar pattern was observed for neck/midpiece defects (Figure VI.3C). In Wt senescent group, the percentage of sperm with neck/midpiece defects increased comparatively to the respective young control (13.9% ± 1.6 *vs*. 3.7% ± 0.8, $p<0.01$), an effect not observed in Tg-RGN. Furthermore, the comparison of aged groups showed that Tg-RGN animals displayed a significantly lower percentage of sperm with neck/midpiece defects (6.1% ± 1.1 *vs*. 13.9% ± 1.6, $p<0.01$).

Regarding tail defects (Figure VI.3D), the percentage of abnormal sperm at 3M was significantly lower in Tg-RGN rats when compared with the Wt counterparts with the same age (18.6% ± 2.6 *vs*. 36.2% ± 2.0, $p<0.001$). In addition, the percentage of sperm with tail defects suffered a variation of ≈43.8% with ageing in Wt rats ($p<0.001$). No significant differences were perceived between the Wt and Tg-RGN groups in senescent status.

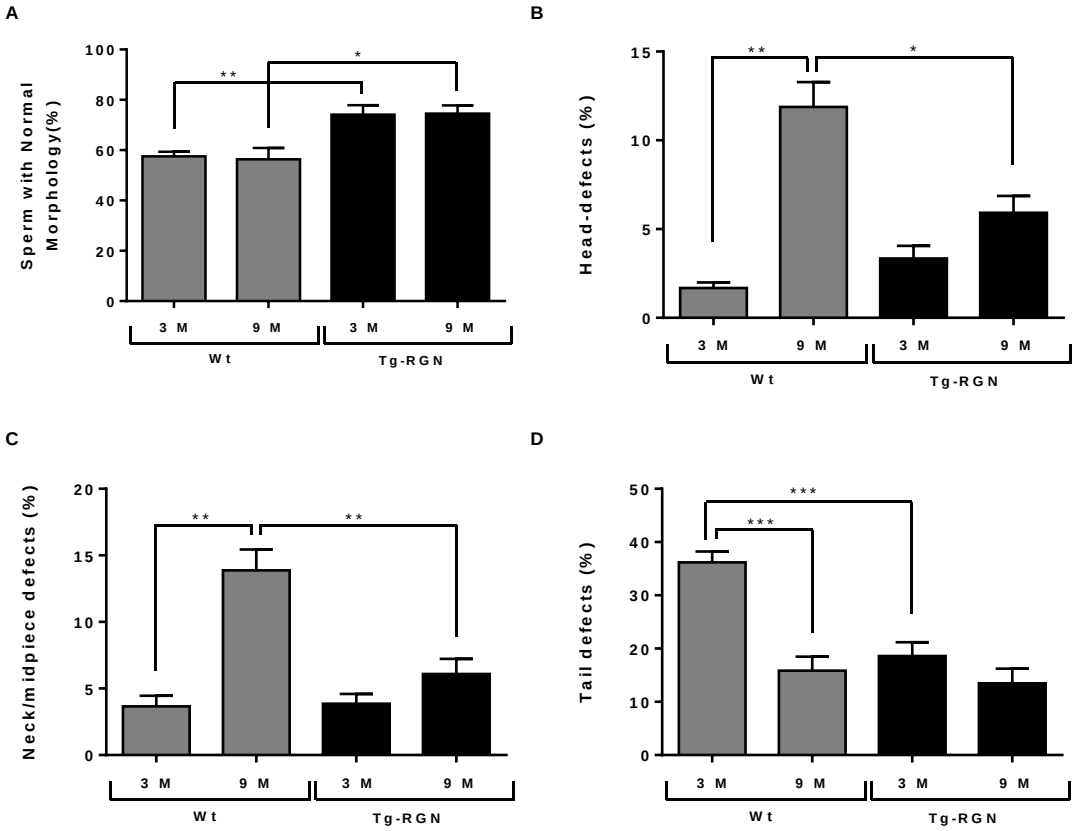

Figure VI.3. Epididymal sperm morphology in young-adult (3M) and senescent (9M) Wt and Tg-RGN animals. The percentage of normal morphology, head-defects, neck/midpiece defects, and tail defects are shown in panels (A), (B), (C), and (D), respectively. Error bars indicate mean ± S.E.M. (n≥5 in each group). *p<0.05; **p<0.01; ***p<0.001.

3.3 Epididymal sperm from Tg-RGN rats presented lower total oxidant status and lower levels of thiobarbituric acid reactive substances

The total oxidant condition may provide more relevant biological information compared to that obtained individually. Considering the cumulative effect of all oxidants present in the sample, the TOS was evaluated (Figure VI.4A). Neither Wt rats nor Tg-RGN rats showed a significant alteration in the TOS with ageing. However, Tg-RGN senescent rats presented a lower total amount of oxidant molecules relatively to Wt senescent rats (24.12 μmoL H_2O_2 Equivalents/L ± 4.11 *vs.* 39.44 μmoL H_2O_2 Equivalents/L ± 2.83, p<0.05).

Sperm is highly susceptible to oxidative deterioration of the polyunsaturated fatty acids present in cell membrane, the so-called LPO [7]. Thus, we quantified the levels of TBARS as an indicator of LPO (Figure VI.4B). TBARS levels increased with ageing in both Wt (0.5 μM MDA/μg total protein ± 0.1 *vs.* 2.1 μM MDA/μg total protein ± 0. 3, p<0.001) and Tg-RGN (0.6 μM MDA/μg total protein ± 0.1 *vs.* 1.3 μM MDA/μg total protein ± 0.2, p<0.01) animals. Also, sperm of Tg-RGN aged rats displayed lower TBARS levels compared to Wt aged (1.3 μM MDA/μg total protein ± 0.2 *vs.* 2.1 μM MDA/μg total protein ± 0.3, p<0.01).

To achieve the essential fine balance between reactive oxygen species (ROS) production and recycling, sperm cells rely on various enzymatic ROS scavengers such as GPX [33]. In fact, the increase in OS and

the weakening of oxidative defence system have been considered a major cause of cell physiological deterioration with ageing [34]. Therefore, the GPX activity was evaluated (Figure VI.4C). The results obtained showed that GPX activity was ≈2.4-fold higher in 3M Tg-RGN rats compared to their Wt littermates with the same age (77.33 U/µg total protein ± 5.25 *vs*. 32.19 U/µg total protein ± 4.68, respectively, $p<0.001$). The level of GPX activity at 3M was maintained similar at 9M in Wt rats. Nevertheless, the GPX activity in Tg-RGN animals presented a decline with ageing (77.33 U/µg total protein ± 5.25 at 3M *vs.* 41.62 U/µg total protein ± 2.40 at 9M, $p<0.01$). No significant differences were found in GPX activity between the Wt aged and Tg-RGN aged groups.

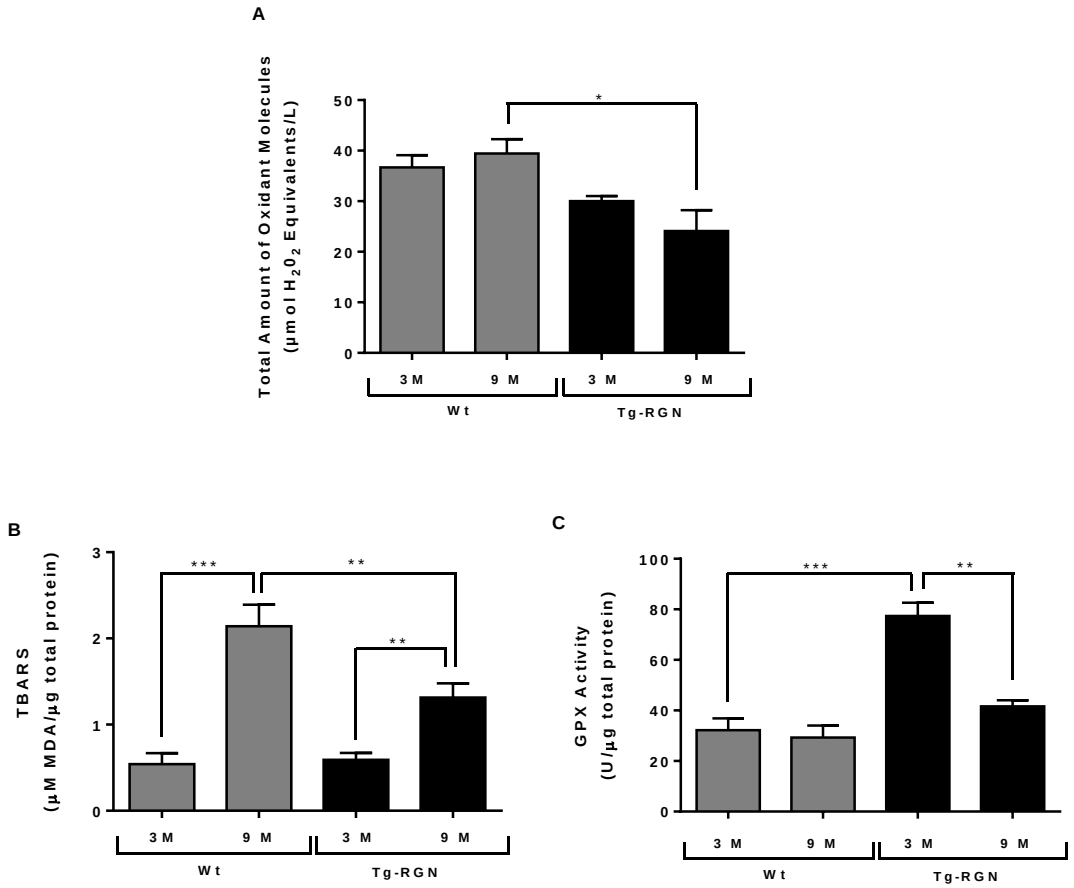

Figure VI.4. Total oxidant status (TOS) (A), thiobarbituric acid reactive substances (TBARS) (B), and glutathione peroxidase (GPX) activity (C), measured spectrophotometrically, in epididymal sperm of young-adult (3M) and senescent (9M) Wt and Tg-RGN animals. Error bars indicate mean ± S.E.M. (n≥5 in each group). *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.4 RGN and AMPKα expression levels were maintained with ageing in the testis of Tg-RGN rats but enhanced in epididymal sperm

RGN expression has been shown to decrease markedly with ageing in several tissues [8-10], and many studies have pointed out its role as a regulator of OS at testicular level [11, 13, 14]. Therefore, we decided to confirm RGN expression in the testis and sperm of young-adult and senescent animals.

RGN levels in testicular tissue were significantly decreased with ageing in the Wt group (0.47 ± 0.10 fold variation, $p<0.05$, Figure VI.5A), but no significant differences were observed between the young and senescent Tg-RGN groups (Figure VI.5A).

Conversely, the protein levels of RGN were enhanced in sperm of both Wt senescent and Tg-RGN senescent rats in comparison with the corresponding young controls, respectively, 1.48 ± 0.05 ($p<0.01$) and 2.05 ± 0.11 ($p<0.001$) fold variation (Figure VI.5D).

Besides its central role as a sensor of cell metabolism, the protein kinase AMPK has been shown to be involved in the control of testis function, namely in sperm production and quality [35]. Thus, AMPKα expression was evaluated in the testis and sperm of Wt and Tg-RGN animals over ageing (Figure VI.5B and 5E). The testis of Wt aged rats displayed increased AMPKα expression compared to the young-adult Wt (2.68 ± 0.15 fold variation, $p<0.01$). Contrarily, no statistically significant difference was observed in AMPKα levels of Tg-RGN with ageing. WB analysis showed that AMPKα levels were significantly enhanced in sperm of 9M Tg-RGN rats in comparison with the respective 3M controls (2.05 ± 0.37 fold variation, $p<0.05$), an effect not observed in Wt animals (Figure VI.5E). Representative immunoblots for RGN, AMPKα and β-actin in testis and sperm are shown in Figure VI.5C and Figure VI.5F, respectively.

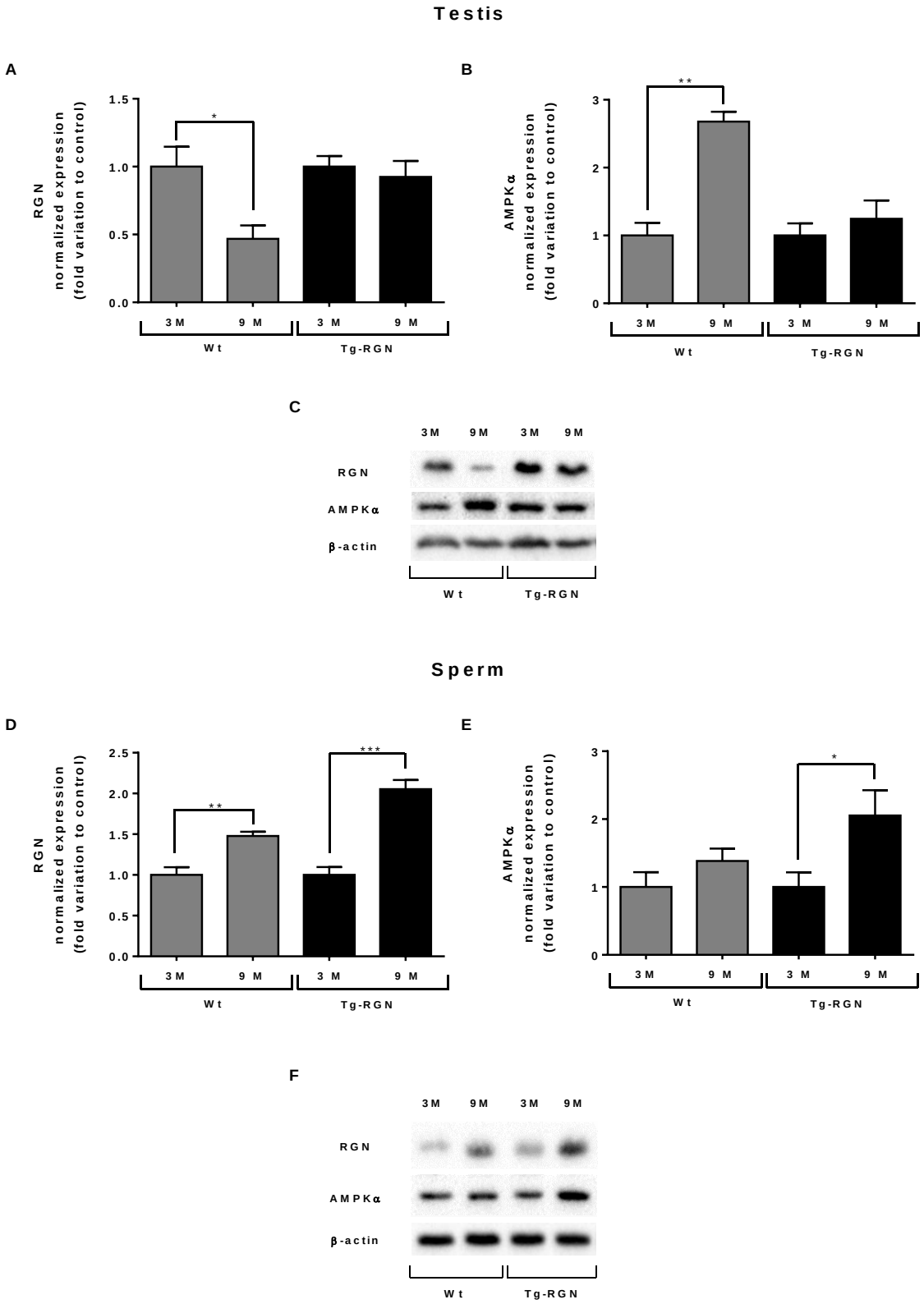

Figure VI.5. RGN and AMPKα protein expression in the testis (A and B) and in the epididymal sperm (D and E) of young-adult (3M) and senescent (9M) Wt and Tg-RGN animals determined by WB analysis. Results are expressed as fold variation relatively to the respective control group after normalization with β-actin. Representative immunoblots for RGN, AMPKα, and β-actin in the testis and in the epididymal sperm are shown in panels (C) and (F), respectively. Error bars indicate mean ± S.E.M. (n≥5 in each group). **p*<0.05; ***p*<0.01; ****p*<0.001.

4. Discussion

RGN downregulated expression with ageing has been indicated as a biomarker of cell senescence [8-10]. On the other hand, the cytoprotective actions of RGN in the male reproductive system have been reported [11, 13, 14]. The present study investigated how RGN overexpression *in vivo* would impact the spermatogenic output over ageing. The obtained findings clearly indicate that ageing triggered evident modifications at testicular and sperm level. Previously, our research group have demonstrated the down-regulation of RGN expression with ageing in the rat testis [10], a result confirmed herein in Wt rats (Figure VI.5A). Also, the same expression pattern was recently identified in the White Yak testis [36]. The reduction of RGN levels with ageing in the testis of Wt rats (Figure VI.5A) follows the pattern of GSI (Figure VI.1A), which is in agreement with other study [10].

Contrarily to what was reported in the testis and other rat tissues [8-10], ageing increased RGN expression in sperm of Wt and Tg-RGN rats (Figure VI.5D). To the best of our knowledge, this is the first report showing an up-regulation of RGN with ageing, indicating that RGN expression seems to be up- or down-regulated during the ageing process in a tissue-specific manner. Nevertheless, this result strongly suggests an involvement of RGN in the preservation of the sperm quality in older males. The up-regulation of RGN in sperm with ageing might be explained by a higher requirement of antioxidant-rich environment in sperm due to its well-known special predisposition to OS comparatively to other tissues [7, 37]. In agreement, the cryoprotective role of RGN in water buffalo sperm was recently shown [38].

The deleterious effect of advanced age on the testis was confirmed by assessing the GSI (Figure VI.1A), which is in accordance with what has been described by others [6, 39]. The present study showed that ageing decreased the GSI in Wt aged rats, but not in Tg-RGN aged animals. Though the GSI was naturally lower in young-adult Tg-RGN animals in comparison with the young-adult Wt, this reflects the lower sperm counts found in these animals in comparison with Wt [16]. Moreover, the testis of Tg-RGN rats showed a stable weight over time, suggesting the preservation of the testicular function. This finding reinforces the action of RGN in the maintenance of testis function indicated by organ weight preservation, which is also supported by our previous study showing a higher GSI in Tg-RGN rats relatively to their Wt counterparts after testicular injury by irradiation [14].

The achievement of successful spermatogenesis requires an accurate regulation of germ cell proliferation and apoptosis, which has been associated with the activity of the SCF/c-Kit system establishing the communication between Sertoli cells (SCF) and germ cells (c-Kit) [26]. Regarding SCF, we confirmed that its expression was increased with ageing in Wt rats (Figure VI.1B), a finding that follows a previous work demonstrating the enhanced SCF levels in the testis of aged rats [40]. Concerning the c-Kit receptor expression, it was decreased with ageing in Wt rats (Figure VI.1C). The expression pattern of SCF/c-Kit system was not deregulated in the testis of Tg-RGN rats, suggesting a proper regulation of germ cell proliferation, contrarily to what happened in Wt, being probably a compensatory mechanism. This result is also supported by evaluation of Akt protein expression, which is a critical regulator of germ cell proliferation [28]. Altogether, with the analysis of GSI, our results

suggest that RGN is an important player in the maintenance of a normal cell proliferation rate, as well as its capacity to preserve the normal spermatogenic output in aged rats.

In order to clarify the role of RGN in spermatogenesis of aged rats, several parameters related with sperm quality were determined. The results acquired in sperm counts (Figure VI.2A) are in accordance with the GSI (Figure VI.1A). Although 3M Tg-RGN rats displayed lower sperm counts and GSI, these animals were able to maintain constant sperm counts with ageing, suggesting that sperm production does not decline under conditions of RGN overexpression. Indeed, Tg-RGN senescent rats seem to preserve the ability to produce as much sperm as in young-adult period as sperm counts were maintained unchanged, which supports the stable GSI found over time.

In accordance with previously described, Tg-RGN animals displayed lower sperm motility compared to Wt, but no differences were observed with ageing. The lower sperm motility in Tg-RGN was previously explained by the altered Ca^{2+}-influx rates observed in the epididymis of these animals [16]. Concerning sperm viability, it is remarkable that, independently of the age, a higher percentage of viable sperm was found in Tg-RGN rats relatively to the Wt (Figure VI.2C). No changes were perceived on sperm motility and viability that can be attributed to the ageing process neither in Wt nor in Tg-RGN rats. Although controversially, some studies have found that age had no influence in sperm motility [41, 42].

One of the main consequences of ageing is the accumulation of ROS generating increased OS in male germ cells throughout the lifespan [2]. The changes in sperm morphology with ageing are associated with the increased OS [43]. Wt animals showed a higher incidence of abnormal sperm with ageing (Figure VI.3), which is in agreement with previous reports [41, 42, 44, 45]. Nevertheless, it should be highlighted that Tg-RGN rats displayed higher percentage of normal sperm (Figure VI.3A) and lower percentage of head and neck/midpiece defects (Figure VI.3B and 3C, respectively) than Wt under the same age status. In fact, we recently demonstrated that Tg-RGN rats presented higher frequency of normal sperm morphology and a diminished incidence of head-defects after testicular irradiation [14]. Thus, although no differences were observed in GSI and sperm counts between the two aged groups, Tg-RGN senescent rats presented higher sperm viability, higher frequency of normal sperm morphology and diminished incidence of head and neck/midpiece defects than their Wt senescent littermates. Changes in sperm morphology can be associated with increased OS, and our results showed augmented LPO levels in sperm of aged Wt and Tg-RGN animals (Figure VI.4B), whose results are in accordance with previous studies [9, 46]. However, the 9M Tg-RGN showed lower LPO relatively to 9M Wt rats. These results are also supported by TOS levels (Figure VI.4A), suggesting that sperm of Tg-RGN senescent rats presented lower levels of OS compared with their age-matched Wt rats.

It is well known that sperm present an increased susceptibility to OS when compared to somatic cells [47]. As an antioxidant defence, GPX primarily protects against LPO of the plasma membrane of sperm [48]. Nevertheless, GPX and superoxide dismutase are known to exhibit lower activity as the sperm ages over time [47]. Predictably, we found a reduced GPX activity with ageing in Tg-RGN rats (Figure VI.4C). However, it should be highlighted that the activity of GPX was higher in 3M Tg-RGN animals relatively to 3M Wt littermates (Figure VI.4C), a result that corroborates our previous works showing lower OS levels and increased antioxidant defences in Tg-RGN rats [11, 16]. Altogether, these results indicate that the diminished OS in the sperm of senescent Tg-RGN may be a consequence of lower

production of ROS and augmented antioxidant defence or scavenge of ROS by enzymatic antioxidant systems. Thus, Tg-RGN animals seem to be more efficient attempting to maintain the ROS closer to the physiological levels in comparison with Wt. RGN overexpression in distinct biological models, including the rat seminiferous tubules and epididymis, was also associated with a higher antioxidant potential and diminished LPO [9, 11, 16] .

AMPK protein is a metabolic sensor that plays an important role in sperm production and function, and its regulatory role in OS and cellular senescence also has been suggested [49]. The AMPKα expression levels were enhanced with ageing in sperm of Tg-RGN rats (Figure VI.5E), but not in Wt. This suggests a metabolic adaptation which may contribute to preserve sperm quality in Tg-RGN aged rats that does not occur in the sperm of their Wt littermates. Supporting our results, it was observed that AMPK stimulated intracellular anti-oxidative defence enzymes in chicken sperm [50]. On the other hand, the AMPKα expression levels were enhanced with ageing in testis of Wt rats (Figure VI.5B), but not in Tg-RGN rats. This is consistent with a previous study showing that AMPKα-1 activation was enhanced in aged skeletal muscle of rat [51]. Also, another research group showed that *in vitro* senescence was accompanied by an elevation in AMPK activity in human fibroblasts [52].

In conclusion, these findings suggest that the overexpression of RGN minimizes the ageing-related decline of sperm quality, likely by suppressing OS and maintaining a proper spermatogenic output.

Declaration of interest

The authors declare that there are no conflicts of interest.

Author contributions

AMSS performed the experiments, assembled and analysed the data, and wrote the manuscript. AHL and CVV collaborated in western blot and OS analysis, respectively, and critically revised the manuscript. SS contributed to experimental conception and design, and critical reading of manuscript. SC and CJM contributed to experimental conception and design, data analysis and interpretation, critical reading and editing of manuscript, and final approval of manuscript.

Acknowledgements

This work was supported by Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (Project PEst-OE/SAU/UI0709/2014). AMSS was founded by Santander Totta (Bolsa BID/ICI-FCS/CICS/Santander Universidades-UBI/2016). AHL is recipient of a post-doctoral fellowship of Junta de Extremadura (Spain) and the Fondo Social Europeo (Ref. PO17022). SC was recipient of a fellowship from FCT (SFRH/BPD/104820/2014) and currently is assistant researcher

under a contract of employment in public functions accordingly to the Portuguese Legislation, namely the Decree-Law n. º 57/2016, of 29 august, changed by the Law n. º 57/2017, of 19 July.

References

[1] López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153:1194-217.

[2] Gunes S, Hekim GNT, Arslan MA, Asci R. Effects of aging on the male reproductive system. J Assist Reprod Genet. 2016;33:441-54.

[3] Yatsenko AN, Turek PJ. Reproductive genetics and the aging male. J Assist Reprod Genet. 2018;35:933-41.

[4] Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S. Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. Ageing Res Rev. 2015;19:22-33.

[5] Tanemura K, Kurohmaru M, Kuramoto K, Hayashi Y. Age-related morphological changes in the testis of the BDF1 mouse. J Vet Med Sci. 1993;55:703-10.

[6] Wang C, Sinha Hikim AP, Lue YH, Leung A, Baravarian S, Swerdloff RS. Reproductive aging in the Brown Norway rat is characterized by accelerated germ cell apoptosis and is not altered by luteinizing hormone replacement. J Androl. 1999;20:509-18.

[7] Weir CP, Robaire B. Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the Brown Norway rat. J Androl. 2007;28:229-40.

[8] Fujita T, Shirasawa T, Uchida K, Maruyama N. Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation with tissue maturation and gradual down-regulation with aging. Mech Ageing Dev. 1996;87:219-29.

[9] Vaz CV, Marques R, Maia CJ, Socorro S. Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin. Transl Res. 2015;166:693-705.

[10] Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, et al. Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction. 2011;142:447-56.

[11] Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

[12] Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves MG, et al. Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate. 2014;74:1189-98.

[13] Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, et al. Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology. 2014;2:290-8.

[14] Silva AM, Correia S, Casalta-Lopes JE, Mamede AC, Cavaco JE, Botelho MF, et al. The protective effect of regucalcin against radiation-induced damage in testicular cells. Life Sci. 2016;164:31-41.

[15] Jervis KM, Robaire B. Dynamic changes in gene expression along the rat epididymis. Biol Reprod. 2001;65:696-703.

[16] Correia S, Oliveira PF, Guerreiro PM, Lopes G, Alves MG, Canario AV, et al. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca2+-binding protein regucalcin: a hidden role for Ca2+ in sperm maturation? Mol Hum Reprod. 2013;19:581-9.

[17] Silva AM, Correia S, Socorro S, Maia CJ. Endogenous Factors in the Recovery of Reproductive Function After Testicular Injury and Cancer. Curr Mol Med. 2016;16:631-49.

[18] Sengupta P. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med. 2013;4:624-30.

[19] Lewis EM, Barnett JFJ, Freshwater L, Hoberman AM, Christian MS. Sexual maturation data for Crl Sprague-Dawley rats: criteria and confounding factors. Drug Chem Toxicol. 2002;25:437-58.

[20] Mendis-Handagama SM, Gelber SJ. Signs of aging are apparent in the testis interstitium of Sprague Dawley rats at 6 months of age. Tissue Cell. 1995;27:689-99.

[21] Sokanovic SJ, Janjic MM, Stojkov NJ, Baburski AZ, Bjelic MM, Andric SA, et al. Age related changes of cAMP and MAPK signaling in Leydig cells of Wistar rats. Exp Gerontol. 2014;58:19-29.

[22] Jarak I, Almeida S, Carvalho RA, Sousa M, Barros A, Alves MG, et al. Senescence and declining reproductive potential: Insight into molecular mechanisms through testicular metabolomics. Biochim Biophys Acta Mol Basis Dis. 2018;1864:3388-96.

[23] Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38:1103-11.

[24] Griswold MD. Interactions between germ cells and Sertoli cells in the testis. Biol Reprod. 1995;52:211-6.

[25] Figueira MI, Cardoso HJ, Correia S, Maia CJ, Socorro S. Hormonal regulation of c-KIT receptor and its ligand: implications for human infertility? Prog Histochem Cytochem. 2014;49:1-19.

[26] Correia S, Alves MR, Cavaco JE, Oliveira PF, Socorro S. Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertil Steril. 2014;102:299-306.

[27] Mauduit C, Hamamah S, Benahmed M. Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update. 1999;5:535-45.

[28] Cardoso HJ, Figueira MI, Correia S, Vaz CV, Socorro S. The SCF/c-KIT system in the male: Survival strategies in fertility and cancer. Mol Reprod Dev. 2014;81:1064-79.

[29] Oldknow KJ, Seebacher J, Goswami T, Villen J, Pitsillides AA, O'Shaughnessy PJ, et al. Follistatin-like 3 (FSTL3) mediated silencing of transforming growth factor β (TGFβ) signaling is essential for testicular aging and regulating testis size. Endocrinology. 2013;154:1310-20.

[30] Cornwall GA. New insights into epididymal biology and function. Hum Reprod Update. 2009;15:213-27.

[31] Robaire B, Hinton BT. Chapter 17 - The Epididymis. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's Physiology of Reproduction. Fourth Edition. San Diego: Academic Press; 2015. p. 691-771.

[32] Chiu YH, Edifor R, Rosner BA, Nassan FL, Gaskins AJ, Minguez-Alarcon L, et al. What does a single semen sample tell you? Implications for male factor infertility research. Am J Epidemiol. 2017;186:918-26.

[33] Drevet JR. The antioxidant glutathione peroxidase family and spermatozoa: a complex story. Mol Cell Endocrinol. 2006;250:70-9.

[34] Salmon AB, Richardson A, Perez VI. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med. 2010;48:642-55.

[35] Martin-Hidalgo D, Hurtado de Llera A, Calle-Guisado V, Gonzalez-Fernandez L, Garcia-Marin L, Bragado MJ. AMPK function in mammalian spermatozoa. Int J Mol Sci. 2018;19:3293.

[36] Ruan CM, Wang J, Yang YX, Hu JJ, Ma YJ, Zhang Y, et al. Proteomic analysis of Tianzhu White Yak (Bos grunniens) testis at different sexual developmental stages. Anim Sci J. 2019;90:333-43.

[37] Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online. 2004;8:616-27.

[38] Pillai H, Parmar MS, Shende AM, Thomas J, Sharma HS, Sharma GT, et al. Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (Bubalus bubalis). Mol Reprod Dev. 2017;84:1133-9.

[39] Mahmoud AM, Goemaere S, El-Garem Y, Van Pottelbergh I, Comhaire FH, Kaufman JM. Testicular volume in relation to hormonal indices of gonadal function in community-dwelling elderly men. J Clin Endocrinol Metab. 2003;88:179-84.

[40] Schoenfeld HA, Hall SJ, Boekelheide K. Continuously proliferative stem germ cells partially repopulate the aged, atrophic rat testis after gonadotropin-releasing hormone agonist therapy. Biol Reprod. 2001;64:1273-82.

[41] Wolf KN, Wildt DE, Vargas A, Marinari PE, Kreeger JS, Ottinger MA, et al. Age-dependent changes in sperm production, semen quality, and testicular volume in the black-footed ferret (Mustela nigripes). Biol Reprod. 2000;63:179-87.

[42] Tesi M, Sabatini C, Vannozzi I, Di Petta G, Panzani D, Camillo F, et al. Variables affecting semen quality and its relation to fertility in the dog: A retrospective study. Theriogenology. 2018;118:34-9.

[43] Menkveld R, Holleboom CA, Rhemrev JP. Measurement and significance of sperm morphology. Asian J Androl. 2011;13:59-68.

[44] Bhanmeechao C, Srisuwatanasagul S, Prapaiwan N, Ponglowhapan S. Reproductive aging in male dogs: The epididymal sperm defects and expression of androgen receptor in reproductive tissues. Theriogenology. 2018;108:74-80.

[45] Stone BA, Alex A, Werlin LB, Marrs RP. Age thresholds for changes in semen parameters in men. Fertil Steril. 2013;100:952-8.

[46] Honma T, Tsuduki T, Sugawara S, Kitano Y, Ito J, Kijima R, et al. Aging decreases antioxidant effects and increases lipid peroxidation in the Apolipoprotein E deficient mouse. J Clin Biochem Nutr. 2013;52:234-40.

[47] Lavranos G, Balla M, Tzortzopoulou A, Syriou V, Angelopoulou R. Investigating ROS sources in male infertility: a common end for numerous pathways. Reprod Toxicol. 2012;34:298-307.

[48] Tavilani H, Goodarzi MT, Doosti M, Vaisi-Raygani A, Hassanzadeh T, Salimi S, et al. Relationship between seminal antioxidant enzymes and the phospholipid and fatty acid composition of spermatozoa. Reprod Biomed Online. 2008;16:649-56.

[49] Han X, Tai H, Wang X, Wang Z, Zhou J, Wei X, et al. AMPK activation protects cells from oxidative stress-induced senescence via autophagic flux restoration and intracellular NAD(+) elevation. Aging Cell. 2016;15:416-27.

[50] Nguyen TM, Seigneurin F, Froment P, Combarnous Y, Blesbois E. The 5'-AMP-activated protein kinase (AMPK) is involved in the augmentation of antioxidant defenses in cryopreserved chicken sperm. PLoS One. 2015;10:e0134420.

[51] Hardman SE, Hall DE, Cabrera AJ, Hancock CR, Thomson DM. The effects of age and muscle contraction on AMPK activity and heterotrimer composition. Exp Gerontol. 2014;55:120-8.

[52] Wang W, Yang X, Lopez de Silanes I, Carling D, Gorospe M. Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. J Biol Chem. 2003;278:27016-23.

Chapter VII
Summarizing Discussion and Future Perspectives

Male reproductive system involves a set of cellular and molecular hormone-dependent events, which are susceptible to external interferences, damage or even to intrinsic modifications, especially taking into consideration the testis and spermatozoa. For instance, pharmacological and recreative drugs [1-3], environmental and occupational toxins [4-6], and even different types of radiation (e.g. X-rays, γ-rays, etc), are known to affect negatively male fertility [7].

Reproductive problems are among the most common and distressing consequences of cancer therapies, especially in men with age within the early 30-40s and who still want, or wish in the future, to conceive children. Although sometimes neglected at the moment of cancer diagnosis, the germ line is very sensitive to exogenous damaging agents due to its high mitotic rate. For this reason, cancer treatments frequently have undesirable secondary effects on male fertility, leading to permanent or temporary impairment of spermatogenesis. Although the recovery of spermatogenesis could occur after delivering lower doses of radiation to the testes, it may take several years. This fact, together with the advisable reproductive age limit for women, can become problematic for couples who wish to father a biological child and consider fatherhood of uttermost importance for a better quality of life. Therefore, there is a recognized need to invest time and resources in research aiming to solve or minimize the negative effects of oncological therapies, which in spite of everything are a “necessary evil”, even regardless of its undesirable effects in male fertility.

The main subject of this thesis is the regucalcin (RGN) protein, which has been studied by some research groups worldwide in different contexts. Many studies suggest that RGN plays a broad range of roles in cells, even leading some authors to refer to RGN as an “handyman” protein. In the last years, our team has been focusing in study its biological function in rat reproductive tissues, including breast, prostate and testis, using the well-established transgenic rat overexpressing RGN (Tg-RGN) model. As explored in Chapter III, RGN has been showing attractive features, namely cytoprotective, antioxidant, and also having a dual action controlling proliferation and apoptosis. Besides being widely present in male reproductive tract, previous studies of our research group showed that RGN also has beneficial effects on spermatogenesis by suppressing chemical-induced apoptosis. The available information about the role of RGN in male reproduction lead us to raise unanswered questions to date. Indeed, the protective role of RGN against damage induced by *in vivo* testicular irradiation was investigated in Chapter V. Tg-RGN animals showed resistance to radiation-induced testicular damage displaying lower rates of apoptosis after irradiation supported mostly by increased antiapoptotic/proapoptotic proteins ratio (Bcl-2/Bax) and reduced activity of caspase-3 (Figure VII.1). Moreover, suppressed apoptosis was concomitant with augmented sperm viability and motility, as well as a higher frequency of normal sperm morphology and diminished incidence of head-defects in comparison with their wild-type (Wt) counterparts (Figure VII.1). This chapter supported our hypothesis, demonstrating the protective role of RGN counteracting apoptosis and decreased sperm quality triggered by X-rays on the male germ line. This work, together with a previous study of our group about the preventive action of RGN relative to ageing-associated changes in the prostate, awakened the curiosity to evaluate whether RGN relieved the ageing-associated decline of male reproductive function. Although males continue to produce sperm throughout their lives [8], ageing does exert in some extent a detrimental effect on male reproductive organs and tissues [9]. In general, reproductive senescence is considered as the decline and/or the end of reproductive activity that occurs with ageing [9]. One of the most typical features of

male reproductive ageing is the gradual fall in testosterone levels along the years, which begins around the 30 years old [10-12]. Furthermore, the testicular volume increases during puberty, reach its peak at the age of 30 and decreases after 60 years old [13]. In Chapter VI, we evaluated a parameter related to testicular weight, the gonadosomatic index (GSI), which was diminished in Wt rats with the advance of the age, but maintained under RGN overexpression (Tg-RGN rats). Sperm production is negatively correlated with age in men, decreasing more than 30% in men over the age of 50 [14-16]. Curiously, we found a preserved sperm count in aged rats when RGN was overexpressed (Chapter VI). This contrasts with the decreased sperm count found in aged animals under normal levels of RGN expression (Wt animals). In humans, after the age of 45, it was shown that semen volume gradually decreases [17]. In Chapter VI, we also verified that the expression levels of stem cell factor (SCF), c-Kit, and Akt were maintained in the aged testis under RGN overexpression (Figure VII.1), contrarily to what was seen in Wt animals that have typical levels of RGN. As referred above, sperm counts and GSI followed the same pattern, i.e., maintained in Tg-RGN but disturbed in Wt rats. Taking into account that SCF, c-Kit and Akt are key players in male germ cell proliferation, these findings suggest that the normal spermatogenic output was preserved under RGN overexpression despite the advance of the age (Figure VII.1). Stone and co-authors demonstrated that sperm concentration and morphology were also affected with ageing, with the percentage of sperm presenting a normal morphology beginning to decline after 40 years old. In the same study, sperm motility fell after 43 years of age [17]. Noteworthy, our results in Chapter VI showed that RGN overexpression minimized the increase of head and neck/midpiece defects in sperm due to ageing, which was concomitantly reflected by a higher percentage of sperm presenting a normal morphology (Figure VII.1). Nevertheless, although the age-associated changes referred above lead to a reduced functionality of the male reproductive system, men are usually fertile until advanced age. In late years, it has been recognized that the impact of father's ageing on conception outcomes is significant and should be considered. Therefore, the age factor is not an exclusive concern for women. The diminished sperm quality in older men has been deserving attention from the scientific community in recent years, because it is warranted to answer to the concern of modern society who father children later and later. Supporting the relevance of men age, a study evaluating paternal age effect on couples with natural fertilization confirmed an increased delay in pregnancy onset as well as conception difficulties in cases where men were older than 40 years old [18]. On the contrary, the latest advances in assisted reproductive techniques (ART) give older men an opportunity to have a child even with poor semen parameters [19], which can be controversial.

In general, it is well known that both radiation and ageing trigger the increase of oxidative stress (OS) in the male reproductive system. OS is one of the main contributors to defective spermatogenesis and male infertility [20], with the sperm membrane itself being highly vulnerable to lipid peroxidation (LPO). Ergo, the increased accumulation of OS in male germ cells, which compromises sperm quality, represents an important consequence of ageing. In Chapter VI, we presented results supporting the antioxidant action that has been attributed to RGN by the scientific community, namely the lower total oxidant status (TOS) and the reduced levels of LPO in aged sperm under RGN overexpression (Figure VII.1). On the other hand, one of the most interesting and unexpected results of Chapter VI was the increase of RGN expression with ageing in sperm, which came to challenge the Senescence Marker Protein-30 (SMP-30) name attributed to RGN due to its decreased expression with the advance of the

age in liver, kidney, prostate and testis. Before our study, RGN was thought to only decrease with the advance of age, not increase. To the best of our knowledge there are no other studies reporting similar results in other tissues or cells, reason why this question should be addressed in the future. It will be interesting to find out in the future if this up-regulation of RGN with ageing is a specific characteristic of sperm cells or not, since in such case it can represent another strong evidence of the important function of RGN as a specific guardian of sperm quality. Furthermore, attending to the antioxidant effect of RGN, explored in Chapter III together with our results of OS analysis presented in Chapter VI, we suggest that the increase of RGN expression in sperm with ageing may be related to minimizing the well-known predisposition to OS that is typical of sperm, also taking into account the increased need to counteract the accumulation of reactive oxygen species (ROS) in male gametes associated to ageing. Chapter VI is the first work reporting the action of RGN in the preservation of the sperm quality in older rats. Moreover, we must be conscious that sperm quality has been decreasing in the last decades, even though this fact is not directly linked to age but to environmental factors, industrial pollution and/or lifestyle [21]. One of the main factors that has been pointed as responsible for the rising incidence of male subfertility/infertility is precisely OS. Also, the fertilizing ability seems to be diminishing over the last decades even in young men [22, 23]. Those findings, together with the age-related decline of sperm quality, have been raising several fertility concerns for men and/or couples who desire to conceive. Given the additional difficulty that age brings relative to younger couples, these concerns are especially pronounced for older men and/or couples in which the male is a cancer survivor.

Overall, in this thesis we detected higher sperm viability, higher frequency of sperm with normal morphology, and reduced incidence of sperm with head defects in both irradiated and aged groups under RGN overexpression (Chapter V and VI, respectively; Figure VII.1). Since ROS production above physiological levels is known to negatively affect sperm parameters [20], and considering the lower TOS and the lower LPO found in aged sperm of Tg-RGN rats comparatively to Wt (Chapter VI; Figure VII.1), we suggest that the lower levels of OS could be one of the reasons for the less affected sperm parameters found when RGN was overexpressed. Reinforcing this possibility, RGN overexpression in several experimental models, including the rat seminiferous tubules and epididymis, was previously associated with a higher antioxidant potential and diminished LPO, contributing to a diminished ROS production [24-26]. Furthermore, increased adenosine monophosphate-activated protein kinase (AMPKα) expression was found in aged sperm under RGN overexpression (Chapter VI; Figure VII.1). Given the relevance of AMPKα for sperm function as well as its stimulating action in sperm antioxidant defences, AMPKα may act as a contributing factor for the less affected sperm quality when RGN was overexpressed. Indeed, it is liable to suggest that lower OS levels may be an explanation for the beneficial effect of RGN in mitigating both irradiation and ageing negative impact on sperm quality. Moreover, given the role of RGN as a tumour suppressor protein, it would be interesting in the future to study its connection with testicular cancer, lymphoma and/or leukaemia because sperm production is known to be affected in such cases even before the onset of cancer therapies. It would be interesting to investigate whether RGN have a dual role protecting from testicular cancer development and concomitantly minimizing the deterioration of sperm quality as a consequence of cancer itself, attending to the findings addressed herein together with data previously published [27-31].

The RGN expression levels increased in irradiated testis and in aged sperm, both in Wt and Tg-RGN rats. This is particularly relevant because Tg-RGN rats, which already have RGN overexpressed, still augmented RGN expression due to radiation or ageing (Chapter V and VI, respectively; Figure VII.1). Such findings are a clear indication about the RGN protective role in male reproductive system, responding to a negative influence or spontaneous physiological modifications through the increase of RGN levels in an attempt to guard sperm production and quality (Figure VII.1).Thus, in light of the new evidence herein revealed, exogenous administration of RGN in infertility cases may be a future perspective to consider, especially in infertility caused by oncological treatments or age-related. In fact, there is a rising body of evidence showing an improvement in semen parameters after oral intake of antioxidants and vitamins, alone or in combination, in infertile men [32].

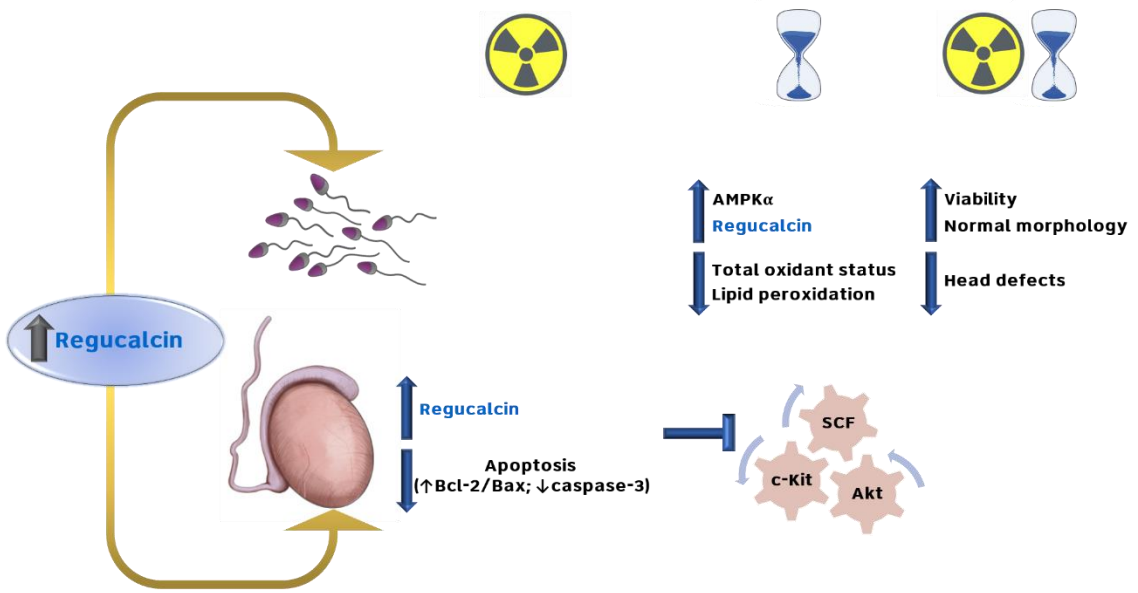

Figure VII.1. Integrative view highlighting the major findings about regucalcin role in rat sperm and testis minimizing the impact of radiation and ageing. Regucalcin overexpression increased sperm viability, augmented the frequency of sperm with normal morphology, and reduced the incidence of sperm with head defects in both irradiated and aged groups. Also, in control groups, regucalcin overexpression increased both sperm viability and the percentage of morphologically normal sperm. Regucalcin protein decreased oxidative stress by diminishing total oxidant status and lowering lipid peroxidation in senescent sperm, and increased adenosine monophosphate-activated protein kinase (AMPKα) expression in aged sperm possibly stimulating antioxidant defences. Moreover, regucalcin reduced testicular apoptosis after radiotherapy mainly through the increase of the protein ratio Bcl-2 (antiapoptotic)/Bax (proapoptotic) and the decrease of caspase-3 activity. In spite of the ageing, regucalcin prevented the modification of the expression levels of stem cell factor (SCF), c-kit, and Akt in testis, which might be associated with the preservation of a normal spermatogenic output. Overall, regucalcin minimizes the detrimental impact of radiotherapy and ageing on male reproduction. Furthermore, both radiation treatment and ageing stimulated the expression of regucalcin in testis and sperm, respectively, even when regucalcin already was transgenically overexpressed. Thus, the enhancement of regucalcin expression seems to take part of a strategy or cascade of mechanisms to protect against circumstances that have a detrimental effect in male reproduction, ultimately pointing regucalcin protein as an important guardian of male fertility. Radiotherapy and ageing are represented by ionizing radiation symbol and hourglass, respectively. Upwards arrows and downwards arrows indicate increase and decrease, respectively. Bar-headed arrow indicates inhibition.

Since the beginning of this project, we have noticed a growing number of studies about the role of RGN protein in male reproduction from other research group, which emphasizes the potential of RGN in this field. Recently, Pillai and co-authors reported that supplementation of recombinant RGN in cryopreservation extender has a protective effect on cryopreserved buffalo spermatozoa [33]. Attending

to their work, another approach to implement the use of RGN in the future of ART may be through the inclusion of RGN as a supplement of the human sperm cryopreservation extender to improve ART outcomes. In fact, there is recombinant human and rat RGN for sale (at the moment for research use only), but no studies with human semen samples were conducted yet. Since such approach seems to have high potential for clinical application of RGN, it should be matter of investigation in future studies.

RGN has an enormous potential in different aspects of male reproduction, namely as a factor to include in new strategies of fertility treatment and/or preservation. Herein, we presented strong evidence that RGN is an important player minimizing interferences that can negatively affect male reproduction, which strengthen previous findings of our research group as well as amplified the set of evidence about the beneficial role of RGN in male fertility. The present results raise expectations about the exploitation of such promising features of RGN in the development of better strategies for male fertility treatment, even if only as adjuvant. Altogether, our results clarified important mechanistic questions, and also opened new and relevant ones. This thesis may be a booster to continue the study of the mechanisms underlying the cytoprotective actions of RGN, which are not totally known, as well as to explore the molecular partners of RGN. We believe that efforts to translate the fundamental knowledge generated during this thesis into a real and fully implemented clinical application should be a priority in near future.

Taking into account that telomere length could be considered a molecular marker of spermatogenesis and sperm quality [34], our results open doors to study if RGN overexpression could influence sperm telomere length with age. Sperm telomeres, despite being longer than in somatic cells, seem to elongate with age in contrast with the shortening of telomere length in somatic cells with age. [35-37]. So, both RGN expression and telomere length decrease with age in other cell types, but in sperm cells they increase. Recent data show that sperm of interfile men have shorter length telomere concomitantly with higher LPO and increased sperm abnormal morphology [38], and we herein showed that RGN overexpression was able to attenuate both LPO and sperm morphological defects (Figure VII.1). Thus, given the protective nature of both telomeres and RGN, our results can represent a basis to further studies about a possible connection between RGN and sperm telomere elongation with ageing. This thesis also came to highlight the importance to characterize RGN's localisation within the sperm cell in the future, preferably in human samples, because until now the localisation of RGN was only characterized in buffalo sperm (see Chapter III; Table III.1). In case RGN is present in the nucleus of sperm (similarly to other cells - see Chapter III; Table III.1), this fact will allocate RGN within the same cellular compartment of chromosomes.

In conclusion, this thesis contributed to give additional evidence-based insights about the role of RGN as a guardian of male reproductive function, and we believe that the discoveries until date are only "the tip of the iceberg".

References

[1] Nudmamud-Thanoi S, Thanoi S. Methamphetamine induces abnormal sperm morphology, low sperm concentration and apoptosis in the testis of male rats. Andrologia. 2011;43:278-82.

[2] Silva AM, Correia S, Socorro S, Maia CJ. Endogenous factors in the recovery of reproductive function after testicular injury and cancer. Curr Mol Med. 2016;16:631-49.

[3] Correia S, Alves MG, Oliveira PF, Alves MR, van Pelt AM, Cavaco JE, et al. Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology. 2014;2:290-8.

[4] Kenkel S, Rolf C, Nieschlag E. Occupational risks for male fertility: an analysis of patients attending a tertiary referral centre. Int J Androl. 2001;24:318-26.

[5] Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). Toxicol Appl Pharmacol. 2005;207:532-7.

[6] Sengupta P, Banerjee R. Environmental toxins: alarming impacts of pesticides on male fertility. Hum Exp Toxicol. 2014;33:1017-39.

[7] Kesari KK, Agarwal A, Henkel R. Radiations and male fertility. Reprod Biol Endocrinol. 2018;16:118.

[8] Amann RP. The cycle of the seminiferous epithelium in humans: A need to revisit? J Androl. 2008;29:469-87.

[9] Noblanc A, Robaire B. Male Reproductive Senescence. In: Skinner MK, editor. Encyclopedia of Reproduction. Second Edition. Oxford: Academic Press; 2018. p. 422-8.

[10] Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. J Clin Endocrinol Metab. 2001;86:724-31.

[11] Morley JE, Kaiser FE, Perry HM 3rd, Patrick P, Morley PM, Stauber PM, et al. Longitudinal changes in testosterone, luteinizing hormone, and follicle-stimulating hormone in healthy older men. Metabolism. 1997;46:410-3.

[12] Wang X, Stocco DM. The decline in testosterone biosynthesis during male aging: a consequence of multiple alterations. Mol Cell Endocrinol. 2005;238:1-7.

[13] Johnson L, Petty CS, Neaves WB. Age-related variation in seminiferous tubules in men. A stereologic evaluation. J Androl. 1986;7:316-22.

[14] Neaves WB, Johnson L, Porter JC, Parker CR Jr., Petty CS. Leydig cell numbers, daily sperm production, and serum gonadotropin levels in aging men. J Clin Endocrinol Metab. 1984;59:756-63.

[15] Homonnai ZT, Fainman N, David MP, Paz GF. Semen quality and sex hormone pattern of 29 middle aged men. Andrologia. 1982;14:164-70.

[16] Johnson L, Petty CS, Neaves WB. Influence of age on sperm production and testicular weights in men. J Reprod Fertil. 1984;70:211-8.

[17] Stone BA, Alex A, Werlin LB, Marrs RP. Age thresholds for changes in semen parameters in men. Fertil Steril. 2013;100:952-8.

[18] de La Rochebrochard E, Thonneau P. Paternal age >or=40 years: an important risk factor for infertility. Am J Obstet Gynecol. 2003;189:901-5.

[19] Gunes S, Hekim GNT, Arslan MA, Asci R. Effects of aging on the male reproductive system. J Assist Reprod Genet. 2016;33:441-54.

[20] Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal. 2011;14:367-81.

[21] Geoffroy-Siraudin C, Loundou AD, Romain F, Achard V, Courbiere B, Perrard MH, et al. Decline of semen quality among 10 932 males consulting for couple infertility over a 20-year period in Marseille, France. Asian J Androl. 2012;14:584-90.

[22] Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, et al. Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update. 2017;23:646-59.

[23] Barratt CL, De Jonge CJ, Sharpe RM. 'Man Up': the importance and strategy for placing male reproductive health centre stage in the political and research agenda. Hum Reprod. 2018;33:541-5.

[24] Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

[25] Handa S, Maruyama N, Ishigami A. Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. Biol Pharm Bull. 2009;32:1645-8.

[26] Correia S, Oliveira PF, Guerreiro PM, Lopes G, Alves MG, Canario AV, et al. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca2+-binding protein regucalcin: a hidden role for Ca2+ in sperm maturation? Mol Hum Reprod. 2013;19:581-9.

[27] Vaz CV, Marques R, Maia CJ, Socorro S. Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin. Transl Res. 2015;166:693-705.

[28] Yamaguchi M. The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis. 2013;18:1145-53.

[29] Vaz CV, Maia CJ, Marques R, Gomes IM, Correia S, Alves MG, et al. Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate. 2014;74:1189-98.

[30] Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, et al. Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res. 2015;330:325-35.

[31] Vaz CV, Correia S, Cardoso HJ, Figueira MI, Marques R, Maia CJ, et al. The emerging role of regucalcin as a tumor suppressor: facts and views. Curr Mol Med. 2016;16:607-19.

[32] Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, et al. A systematic review of the effect of oral antioxidants on male infertility. Reprod Biomed Online. 2010;20:711-23.

[33] Pillai H, Parmar MS, Shende AM, Thomas J, Sharma HS, Sharma GT, et al. Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (Bubalus bubalis). Mol Reprod Dev. 2017;84:1133-9.

[34] Rocca MS, Foresta C, Ferlin A. Telomere length: lights and shadows on their role in human reproduction. Biol Reprod. 2019;100:305-17.

[35] Aston KI, Hunt SC, Susser E, Kimura M, Factor-Litvak P, Carrell D, et al. Divergence of sperm and leukocyte age-dependent telomere dynamics: implications for male-driven evolution of telomere length in humans. Mol Hum Reprod. 2012;18:517-22.

[36] Kimura M, Cherkas LF, Kato BS, Demissie S, Hjelmborg JB, Brimacombe M, et al. Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm. PLoS Genet. 2008;4:e37.

[37] Jorgensen PB, Fedder J, Koelvraa S, Graakjaer J. Age-dependence of relative telomere length profiles during spermatogenesis in man. Maturitas. 2013;75:380-5.

[38] Darmishonnejad Z, Zarei-Kheirabadi F, Tavalaee M, Zarei-Kheirabadi M, Zohrabi D, Nasr-Esfahani MH. Relationship between sperm telomere length and sperm quality in infertile men. Andrologia. 2020;52:e13546.

Appendixes

Appendix I

List of Scientific Publications

Included in this thesis

Articles

1 - **Silva A.M.S.**, Correia S., Socorro S., Maia C.J. Endogenous factors in the recovery of reproductive function after testicular injury and cancer. Curr Mol Med. 2016;16:631-49. (DOI: 10.2174/1566524016666160802150400)

2 - **Silva A.M.S.**, Correia S., Casalta-Lopes J.E., Mamede A.C., Cavaco J.E., Botelho M.F., Socorro S., Maia C.J. The protective effect of regucalcin against radiation-induced damage in testicular cells. Life Sci. 2016;164:31-41. (DOI: 10.1016/j.lfs.2016.09.003)

3 - **Silva A.M.S.**, Socorro S., Hurtado de Llera A., Vaz C.V., Correia S., Maia C.J. Overexpression of regucalcin mitigates the ageing-related changes in oxidative stress and sperm quality. *(submitted, under revision)*

4 - Santos V.M., **Silva A.M.S.**, Correia S., Socorro S. Regucalcin' actions in male reproduction: an update *(in preparation for submission)*

Book Chapter

1 - Correia S., Pillai H., **Silva A.M.S.**, Santos V.M. and Socorro S. (2019) The Horizons of Regucalcin in Male Reproduction. In: "Regucalcin: Metabolic Regulation and Disease". Nova Science Publishers, Inc, New York, USA, *in press*. (ISBN: 978-1-53616-172-4)

Published Abstract

1 - **Silva A.M.S.**, Correia S., Vaz, C.V., Socorro S., Maia C. J. Overexpression of the calcium-binding protein regucalcin mitigates the age-associated changes in oxidative stress and semen quality. J Sex Med. 15(7):S324. 2018 (DOI: 10.1016/j.jsxm.2018.04.447)

Other publications during the doctoral degree

Articles

1 - Correia S., Vaz C.V., **Silva A.M.S.**, Cavaco J.E., Socorro S. Regucalcin counteracts *tert*-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol. 2017;37:159-66.

2 – Hurtado de Llera A., **Silva A.M.S.**, Correia S., Martins R.V.L, Hérnandez-García F.I., Socorro S., Bragado M.J. AMPK expression and localization in male reproductive system of Wistar rats during sexual maturation and estrogens effects *(in preparation for submission)*

3 - Martins R.V.L, **Silva A.M.S.**, Socorro S., Correia S. The protective effect of regucalcin against *tert*-butyl hydroperoxide-induced disruption of testicular glycolytic metabolism. *(in preparation for submission)*

List of Scientific Communications

Oral communications related to this thesis

1 - 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. 6-7 July 2015, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

2 - Ana M. S. Silva, Sara Correia, João E. Casalta-Lopes, Ana C. Mamede, José E.B. Cavaco, Maria F. Botelho, Cláudio J. Maia, Sílvia Socorro. The androgen-target gene regucalcin as an apoptosis modulator in irradiated rat testis. I Congress in Health Sciences Research Towards Innovation and Entrepreneurship - Trends in Endocrinology and Neurosciences. 26-28 November 2015, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

3 - Ana M. S. Silva, Sara Correia, Cátia V. Vaz, Sílvia Socorro, Cláudio J. Maia. Transgenic overexpression of regucalcin protein attenuates ageing-associated changes in the male reproductive function. XIV Annual CICS-UBI Symposium. 4th and 5th July 2019, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

Poster communications related to this thesis

1 - Ana M. S. Silva, Sara Correia, Cátia V. Vaz, Sílvia Socorro, Cláudio J. Maia. Regucalcin overexpression prevents the age-associated decline of sperm quality. II International Congress in Health Sciences Research Towards Innovation and Entrepreneurship - Trends in Biotechnology for Biomedical Applications. 17th to 20th May 2017. Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

2 - Silva A. M. S., Correia S., Vaz, C.V., Socorro S., Maia C.J. Overexpression of the calcium-binding protein regucalcin mitigates the age-associated changes in oxidative stress and semen quality. 20th Congress of the European Society for Sexual Medicine & 21st World Meeting of the International Society for Sexual Medicine. 28th February to 3rd March 2018, Lisbon Congress Center, Lisbon, Portugal.

3 - Silva A.M.S., Socorro S., Hurtado de Llera A., Vaz C.V., Correia S., Maia C.J. Regucalcin protein alleviates ageing-related changes in male reproductive function. III International Congress in Health Sciences Research Towards Innovation and Entrepreneurship - Trends in Aging and Cancer. 14th to 16th November 2019. Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

Other oral communication during the doctoral degree

1 - Martins R.V.L, **Silva A.M.S.**, Socorro S., Correia S. The protective effect of regucalcin against *tert*-butyl hydroperoxide-induced disruption of testicular glycolytic metabolism. III International Congress in Health Sciences Research Towards Innovation and Entrepreneurship - Trends in Aging and Cancer. 14th to 16th November 2019. Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

Other poster communication during the doctoral degree

1 - Roberta V. L. Martins, **Ana M. S. Silva**, Mariana Feijó, Sílvia Socorro, Sara Correia. Metabolic cooperation in the seminiferous tubules of transgenic rats overexpressing regucalcin. XIV Annual CICS-UBI Symposium. 4th and 5th July 2019, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal.

Appendix II

Send Orders for Reprints to reprints@benthamscience.ae

Current Molecular Medicine 2016, *16,* 1-19 1

REVIEW ARTICLE

Endogenous Factors in the Recovery of Reproductive Function After Testicular Injury and Cancer

A.M.S. Silva, S. Correia, S. Socorro* and C.J. Maia*

CICS-UBI- Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

S. Socorro

C.J. Maia

ARTICLE HISTORY

Received: February 2, 2016
Revised: July 19, 2016
Accepted: July 20, 2016

DOI: 10.2174/1566524016666160802150400

Abstract: The testes are one of the most delicate organs in the male body and highly susceptible to the exogenous influences capable of inducing cell damage. Cancer therapies are well known to negatively affect the male reproductive tract with a severe impairment of spermatogenesis and infertility. The present work aimed to systematically review the available information about the different endogenous factors (hormonal and non-hormonal) that may have protective or advantageous properties on the recovery of male reproductive function after gonadal injury. Furthermore, the perspective that these endogenous molecules could act as cryoprotectants to improve the quality of cryopreserved semen samples was also discussed. The knowledge reviewed herein allowed to identify promising factors able to mitigate the male fertility problems arising either from oncological treatments or other gonadal damage, and opened new possibilities to ameliorate the recovery of spermatogenesis or to preserve fertility.

Keywords: Chemotherapy, radiotherapy, male infertility, testicular cancer, fertility preservation.

INTRODUCTION

Testicles are the crucial organs maintaining male reproductive function, a role that is accomplished by their dual function: i) testosterone synthesis, and ii) production of male gametes. Spermatogenesis is the complex and highly regulated process that occurs in the seminiferous tubules of the testis, culminating with the release of spermatozoa into the tubular lumen [1]. The production of spermatozoa depends on a set of coordinated mitotic and meiotic divisions and cell differentiation events [1], and due to its high mitotic rate, the germinal epithelium is very sensitive to exogenous damaging factors [2]. Consequently, cancer treatments, namely chemotherapy and radiotherapy, have several adverse effects on male fertility, mainly by inducing apoptosis of germ cells [3]. Moreover, it has been shown that radiation treatments have a dose-dependent detrimental impact on male reproductive function (reviewed by Trost, LW and Brannigan, RE [3]). Treatment of oncological diseases usually results in temporary or permanent arrest of spermatogenesis [4], as well as in disrupted sex hormone production [5]. Additionally, despite the existing controversy, it is accepted that malignancy itself is associated with male infertility since numerous biological processes are affected in parallel [6]. Indeed, spermatogenesis was shown to be affected in oncological patients with lymphoma and leukemia without testicular pathology, even before the onset of gonadotoxic therapies [7-10]. Sperm cryopreservation preceding cancer treatments remains the only established method for fertility preservation in adult males [8], whereas in the pre-pubertal male the cryopreservation of testicular tissue is the adopted option for preserving fertility [11]. Other procedures are currently at experimental phase but are not devoid of ethical concerns [11].

Concerning cryopreservation, one of the major problems is the oxidative damage induced to sperm cells [12]. During the freeze–thawing practice, sperm antioxidant defenses could not be enough [13] to counteract this damage, which includes a significantly decreased motility, viability, morphology, chromatin integrity, mitochondrial potential, *in vivo* fertilizing capacity, deterioration of acrosomal and plasma membrane integrity, and DNA damage [13-15]. Furthermore, the use of cryopreserved sperm or tissue in assisted reproductive techniques (ART) has an

*Address correspondence to this author at the Faculdade de Ciências da Saúde, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; (C.J. Maia) Tel: +351 275 329 068; Fax: +351 275 329 099; E-mail: cmaia@fcsaude.ubi.pt; (S. Socorro) Tel: +351 275 329 053; Fax: +351 275 329 099; E-mail: ssocorro@fcsaude.ubi.pt

1566-5240/16 $58.00+.00

economic obstacle due to the high-costs associated [16].

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 [17]. This review provides a systematic overview of the endogenous molecules that have been identified as protective agents or having beneficial effects on the recovery of reproductive function after testicular injury and cancer.

HORMONAL FACTORS

Testosterone

Androgens, namely testosterone (T), are the key spermatogenesis regulators, acting as germ cell survival factors, and also has been shown that T withdrawal induces the apoptosis of support Sertoli cells and germ cells [18]. On the other hand, both endogenous and exogenous T supplementation trigger a negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis suppressing spermatogenesis. T action suppresses gonadotropin releasing hormone (GnRH) production, which inhibits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Diminished levels of FSH and LH lead to a decline in T production and also in intratesticular testosterone (ITT) levels, diminishing sperm production (reviewed by [19]) (Fig. **1**).

Since exogenous T therapies are responsible for inhibition of ITT production, spermatogenesis may be compromised [20]. It is well known that ITT is absolutely necessary for normal spermatogenesis, and the reduced levels of ITT may result in azoospermia [21, 22]. However, it has been hypothesized that inhibition of cell division before or during chemotherapy may protect the germ cells, preserving spermatogenesis or improving its recovery. Thus, the administration of T, GnRH antagonists and agonists, as well as estrogens, have been tested with diverse chemotherapeutic drugs, leading to opposite results [23]. The suppression of T has been shown as a helpful agent in the re-establishment of rat spermatogenesis after damage induced by environmental reproductive toxicants [24]. Men, who were administered with low systemic dose of T to suppress ITT levels, also exhibited restoration of spermatogenesis in response to the treatment with cyclophosphamide [25]. Later, the influence of exogenous T on the recovery of spermatogenesis was evaluated after cisplatin chemotherapy, which had a proven harmful consequence on fertility [26]. The obtained results demonstrated that administration of exogenous T during chemotherapy had protective effects on spermatogenesis in mice subjected to high dose chemotherapy, and no additional long-term effects in animals receiving low dose cisplatin were observed. Comparing the effects between T and GnRH agonists, T was considered advantageous due to the highest androgen suppression observed at 2 to 3 weeks after treatment. During this latency time, T levels and active spermatogenesis are paradoxically high. Even a single dose of chemotherapy throughout latency time had serious destructive effects on germ cells [26]. Moreover, GnRH analogues, which can decrease serum gonadotropin levels [27], induced andropause when administered during chemotherapy, but it did not happen with T supplementation [26].

Gonadotropin-Releasing Hormone Agonists and Antagonists

By suppressing T production with GnRH agonists or antagonists, the damage of spermatogenesis process and fertility after irradiation or chemotherapeutic agents can be repaired (Fig. **1**) [24, 28, 29]. However, these hormonal treatments are generally accompanied by several side effects and should be avoided or applied in a dose as low as possible [28]. Nevertheless, if spermatogonial stem cells survive after cancer treatment, there is a chance for hormonal manipulation towards resumption of sperm production. The testosterone suppression given before or after the cytotoxic insult stimulated the recovery of spermatogenesis from both endogenous and transplanted spermatogonial stem cells, with the reestablishment of fertility [30]. Therefore, the effect of hormonal suppression and transplantation has been evaluated on spermatogenesis recovery. In a study reported by Shetty *et al*. [31], the testes of 12 adult cynomolgus monkeys were irradiated, and 6 of them were treated with a GnRH antagonist. In contrast to the minimal effects of hormone suppression or transplantation alone on the spermatogenic recovery, enhanced recovery occurred when transplantation and GnRH antagonist were combined, since transplanted testes of GnRH antagonist-treated monkeys displayed i) higher weight; ii) improved percentage of seminiferous tubule cross-sections presenting spermatogenesis; iii) detectable sperm in 5 of 6 animals; and iv) higher sperm counts. Furthermore, hormone suppression also had encouraging results on homing of transplanted spermatogonial stem cells in non-human primate models [31], but it is imperative to remember the inherent existing risks related to reintroduction of tumoral cells in such experimental procedures [32].

Nevertheless, there are several similarities between rodents and men in what concerns spermatogenesis, as well as in its hormonal regulation [28]. In a mouse model, it was demonstrated that the manipulation of hormonal levels can decrease the toxic effects triggered by chemotherapy in spermatogenesis. It was found that the administration of GnRH-agonist during treatment with cyclophosphamide protected spermatogenesis [33].

Several studies have shown that the reduction of ITT levels, induced by GnRH analogues, has a protective role against spermatogenesis injury when administered previously to chemo- or radiotherapy. If administration occurs after cytotoxic damage, this hormonal treatment stimulates the recovery of

spermatogenesis and, consequently improves fertility [29, 34, 35]. Posteriorly, it was demonstrated that a strong hormonal suppression using a GnRH antagonist (acyline) plus flutamide (an androgen receptor antagonist) had positive effects in reestablishing spermatogenesis and improving colonization of transplanted spermatogonial stem cell in irradiated mouse testes [30]. Hormonal suppression treatment was administered immediately after irradiation (doses of 10.5 to 13.5 Gy) and successfully enhanced spermatogenesis recovery with a significantly reduced recovery period. In addition, this treatment also improved the effectiveness of transplanted cell colonization. It also should be pointed out that the duration, timing, and degree of hormonal suppression seemed to influence the success of gonadal recovery [30].

Estrogens

In the last years, estrogens have been recognized as important regulators of spermatogenesis and male fertility. Among other roles, estrogens are able to regulate both survival and death of germ cells having, depending on the dose, beneficial or detrimental effects on spermatogenesis [36, 37].

Estrogens also can have a direct action on the endocrine Leydig cells by down-regulating the expression of the steroidogenic enzymes involved in T biosynthesis [38]. These hormones also can act on the hypothalamus or pituitary to reduce FSH and LH levels and subsequently decrease T levels [39] (Fig. **1**). Considering that 17β-estradiol (E_2) might induce secondary effects at cardiovascular level [40] or gynecomastia [41], its use in the treatment of gonadotoxic–induced azoospermia represent some weaknesses. The development of selective estrogen receptor modulators, with specific functions in the testis but without estrogen side effects, should be explored for application in the recovery of azoospermia in consequence of cancer therapy [42]. Nevertheless, the androgen-estrogen imbalance, rather than estrogen itself, is more likely to lead to male reproductive diseases [43]. E_2 reestablished spermatogenesis in hypogonadal mice and accelerated spermatogenesis onset in immature animals [44, 45]. In irradiated rats, exogenous E_2 accelerated GnRH antagonist–induced spermatogenesis recovery [46]. Furthermore, in rats exposed to testicular irradiation, the administration of E_2 alone was able to provoke spermatogenesis recovery and partially revert T inhibitory effects on spermatogonial differentiation [47]. On the other hand, when administrated to normal adult and pre-pubertal rats, E_2 as well as other estrogens can exert a repressive effect on spermatogenesis [48]. Therapy with E_2 prevented spermatogonial differentiation in neonatal rats [48] and sperm production in juvenile mice [49]. Moreover, estrogens also have been described as apoptosis-inducers of male germ cells and, consequently, as damaging agents for male fertility. In fact, clinical findings in infertile men presenting reduced T/E_2 ratio due to high E_2 levels, are concordant with the apoptotic role of estrogens. Nevertheless, the pro-survival or apoptotic role of estrogens seems to be dependent on the dose. This issue was recently reviewed by Correia and co-authors [36] and it was shown that only E_2 doses above the physiological concentration induce germ cells apoptosis. Accordingly, adjuvant T and E_2 administration exhibited protective action on radiation-induced injury of spermatogenesis [26]. Porter *et al*. [46] showed that E_2 promotes spermatogonial differentiation in irradiated $LBNF_1$ rat gonads separately of its effects on T or gonadotropin concentrations, and also that E_2 is more potent than GnRH antagonist in stimulating spermatogonial restoration. Hormonal manipulation as a therapy to improve spermatogenesis recovery from endogenous surviving stem spermatogonia or transplanted spermatogonia together with E_2 could be a promising prospect for men subjected to gonadotoxic cancer treatments [46, 50]. Using irradiated rats having total block of spermatogonial differentiation, it was shown that about 20 genes were up- or down-regulated by E_2 in testicular cells [51]. Several genes, such as Insulin-like 3 gene that regulates germ cell apoptosis and testicular descent [52], are considered potential candidates to block spermatogonial differentiation and improve spermatogenesis recovery after gonadotoxic effects. Therefore, the identification of downstream genes or signaling pathways involved in the recovery of spermatogenesis in response to hormonal manipulations could unravel novel therapeutic targets for application in men [51].

Growth Hormone and Insulin-Like Growth Factor 1

Growth hormone (GH) is a protein synthesized by the anterior pituitary gland [19], which plays a crucial role in the normal adolescent growth spurt and is also involved in the testes maturation [53] (Fig. **1**). In patients with GH deficiency, who experience delayed sexual maturation, supplemental GH can trigger the onset of puberty [54]. Exogenous administration of GH to hypophysectomised rats stimulated the sensitivity of Leydig cells to LH [55]. In addition to the direct effects of GH, mediated by its receptor, several testicular effects are indirectly exerted through the GH-induced secretion of insulin-like growth factor 1 (IGF-1). The IGF-1 increase in the male gonads in response to GH leads to the maturation of sperm cells via a paracrine-autocrine control (Fig. **1**) [19, 53]. Regarding the use of GH as therapy, it was shown that human recombinant GH may be an acceptable option for men with idiopathic oligozoospermia when other therapies are unsuccessful [56]. Another study also demonstrated that GH treatment of 18 subfertile males with oligozoospermia or asthenozoospermia significantly increased the levels of IGF-1 and sperm motility, but without differences in sperm counts [57]. However, some doubts have raised in what concerns GH treatment of male hypogonadotropic hypogonadism. Nevertheless, a possible advantage for sperm motility and sperm counts was also suggested [19].

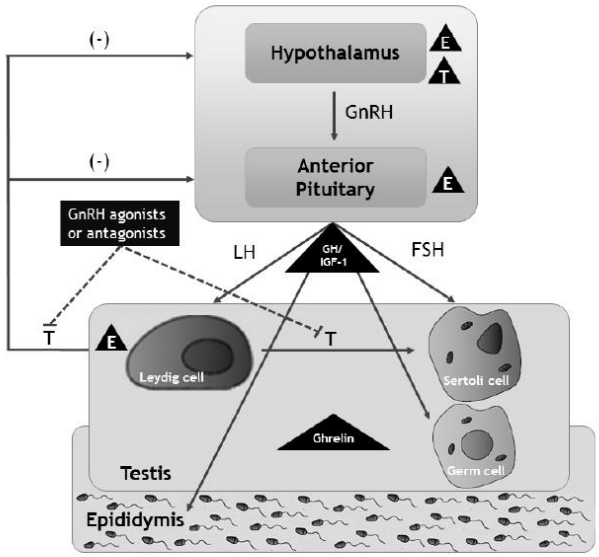

Fig. (1). Hormonal protective factors and their target tissues (cells) in the male reproductive axis. Testosterone (T) produced by Leydig cells has an anti-apoptotic effect on male germ cells. On the other hand, both endogenous T and exogenous T supplementation trigger a negative feedback (-) on the hypothalamic-pituitary-gonadal (HPG) axis suppressing spermatogenesis. T action suppresses gonadotropin releasing hormone (GnRH) production, which inhibits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Diminished levels of FSH and LH lead to a decline in T production, diminishing sperm production. By suppressing T biosynthesis with GnRH agonists or antagonists, the damage in spermatogenesis and fertility after irradiation or chemotherapy can be repaired. Estrogens (E) can act on Leydig cells down-regulating the expression of steroidogenic enzymes involved in T biosynthesis, and also on the hypothalamus or pituitary to reduce FSH and LH levels, subsequently decreasing T levels. Growth hormone (GH), synthesized by the anterior pituitary gland, is important for testes maturation. GH outcomes could be mediated by the insulin-like growth factor 1 (IGF-1), which stimulates the maturation of sperm cells, sperm counts and sperm motility via a paracrine-autocrine stimulation. Ghrelin has anti-apoptotic and anti-proliferative effects in germ cells, being involved in gonadal recovery after injury. Ghrelin also has shown to increase sperm plasma membrane integrity. Legend: Induction/stimulation and suppression are indicated by arrows and bar-headed arrows, respectively. Target tissues (cells) of hormonal actions are highlighted by triangles.

The protective and regenerative role of GH in testicular tissue after chemotherapy have already been demonstrated in GH-deficient rats by the co-administration of GH and cyclophosphamide [58]. The effect of a co-therapy with GH on the testicular dysfunction induced by methotrexate, an anti-cancer drug acting as a DNA synthesis inhibitor, also was investigated [59]. The impaired spermatogenesis and fertility in consequence of methotrexate treatment are thought to be due to the direct suppression of dihydrofolate reductase, GH deficiency, or other mechanisms. In fact, the administration of GH in combination with methotrexate diminished its detrimental effects on sperm parameters, demonstrating the protective properties of GH [59].

In general, growth factors play an essential role in spermatogenesis, sperm motility, and fertility. Some studies pointed out the possible role of IGF-1 on spermatogenesis, sperm viability and motility in different species, including humans [60-62]. Indeed, considering that IGF-1 receptor is expressed in spermatogonia, spermatocytes, spermatids, and spermatozoa, IGF-1 has been suggested as one of the essential agents for germ cell development and maturation, as well as for sperm motility (Fig. **1**) [63]. A beneficial role of seminal plasma IGF-1 as an antioxidant preserving spermatogenesis and the motility of human sperm has been described [57, 60]. Also, it has been proposed that the protective effect of IGF-1 in sperm parameters is due to the diminished levels of lipid peroxidation [62]. Considering that sperm cells are highly vulnerable to cryoinjury [13-15], the effect of IGF-1 in maintaining the quality of cryopreserved buffalos semen was evaluated [63]. It was demonstrated that the *in vitro* incubation of post-thawed sperm with IGF-1 for 2h avoided the deterioration of spermatozoa functional parameters and improved the *in vitro* fertilization outcomes, suggesting that the addition of IGF-1 to the extender before freezing may improve the fertility of subfertile buffalos [63].

Ghrelin

The protein ghrelin was first described as the endogenous ligand of GH secretagogue receptor (GHS-R) [64]. Thereafter, a growing number of studies have been suggesting its potential involvement in male reproduction since rat and human testes express ghrelin and its receptor [65, 66]. Ghrelin might work as a pleiotropic modulator in the testis [67] (Fig. **1**). This protein has been mostly detected in the interstitial Leydig cells, and it is capable to inhibit the expression of the stem cell factor (SCF) [68, 69]. The SCF is a membrane-bound cytokine at the surface of Sertoli cells whereas its receptor, the tyrosine kinase receptor c-kit, is present on the surface of adjacent germ cells [70, 71]. The SCF is a powerful paracrine survival factor and the SCF/c-kit communication between Sertoli cells and the germ line is determinant to avoid germ cells death [70]. Furthermore, it has been suggested the involvement of ghrelin in controlling apoptosis of germ cells but it is unknown whether it depends on the down-regulation of SCF levels. For example, ghrelin antioxidant therapy significantly inhibited the proliferation of spermatogonia and spermatocytes (Fig. **1**) [72], while the suppression of ghrelin signaling diminished germ cell apoptosis, resulting in better sperm production in leptin-deficient mice [73]. In another study, ghrelin was also able to increase sperm plasma membrane integrity [74].

The role of ghrelin in mice testicular injury caused by ionizing radiation was explored [65]. Exposure to 2 Gy of X-rays induced a prominent increase of nuclear ghrelin expression in differentiating spermatogonia, with an impairment of Leydig cells-expressing ghrelin. Surprisingly, suppression of the ghrelin signaling pathway by administration of a specific GHS-R1a antagonist improved spermatogonia removal via apoptosis during early recovery, resulting in diminished male fertility. These findings suggested that the anti-apoptotic effects of ghrelin have a deep involvement in post-injury repair. In addition, it was also observed that inhibition of ghrelin signaling caused an increase in ITT levels 21 days after irradiation, which may stimulate the recovery of spermatogenesis supported by the surviving spermatogonia. It was also verified that the upregulation and nuclear trafficking of ghrelin may act by a p53-dependent mechanism. The increased levels of ghrelin in response to radiation, as well as the modulation of ghrelin expression by radiation-induced oxidative stress and the cross-talk between p53 and ghrelin during radiation damage were confirmed in differentiating spermatogonia [65].

NON-HORMONAL FACTORS

Arginine and Nitric Oxide

Arginine is a dibasic amino acid required for synthesis of nitric oxide (NO), a highly reactive free radical involved in several biological functions, which acts also as a second messenger. NO is a remarkable signaling molecule involved in neurotransmission, maintenance of normal blood pressure, myocardial function, immune regulation and inflammatory response, apoptosis, protection against oxidative damage, and it also acts as a modulator in both male and female reproductive functions [75-77]. Dietary arginine intake has been indicated as a nutritional fertility modulator [78]. Since arginine is the precursor for NO synthesis, depending on the activity of a family of isoenzymes known as nitric oxide synthases (NOS) that are expressed in male reproductive system [79], it is thought that the arginine mechanism of action in reproduction is due to the production of NO [80]. At physiologic levels, NO plays an important role in promoting normal sperm production, sperm capacitation and acrosome reaction [81], as well as in the maintenance of sperm viability and motility [82]. These findings demonstrated the relevant role of NO in sperm functionality and are also concordant with its possible anti-apoptotic effects over germ cells [81].

Adult men subjected to an arginine-deficient diet have a huge reduction in sperm cell counts and an increase in the fraction of non-motile sperm [75]. In fact, some authors have described a correlation between arginine deficiency and hampered spermatogenesis, as well as with reduced sperm motility [83]. Furthermore, arginine-HCl oral supplementation significantly improved motility and sperm counts in infertile men, allowing to achieve successful pregnancies [84]. Some amino acids present in seminal plasma, including arginine, seem to play a crucial role in spermatogenesis, sperm metabolism and motility. The administration of L-arginine to asthenospermic and oligospermic men enhanced both sperm counts and motility without adverse effects [75, 85-88]. The arginine power to increase sperm motility has an important clinical application in ART, being used to treat patients with subnormal sperm motility [85]. Even low levels of arginine not only improve sperm metabolism and spermatogenesis, but also augment the synthesis of adenosine triphosphate (ATP) that provides energy for sperm motility [83]. It has been described that arginine enhances the glycolysis rate, with production of greater amounts of ATP and lactate that became available for male gametes [83]. In addition, arginine is also involved in reversing the impairment of spermatogenesis caused by the use of glycolytic inhibitors, which are considered potential contraceptives [89]. Arginine supplementation also had a positive effect on erectile dysfunction in men with abnormal NO metabolism [90].

On the other hand, it was demonstrated that arginine administration had a beneficial role against abdominal irradiation side effects in rats [91]. In methotrexate-induced mucositis in rat, arginine supplementation avoided intestinal injury, diminishing cell death and accelerating intestinal repair, which suggested the protective role against chemotherapy-induced damage [76]. Recently, the effect of supplementation with Prelox®R, which is a combination of French maritime pine bark extract (Pycnogenol®), L-arginine, L-citrulline and roburins, was determined on subfertile men. This mixture was able to improve sperm concentration and volume, but

also sperm vitality, morphology and motility [92]. Other authors also evaluated the outcome of arginine therapy on testicular induced torsion before, during and after puberty in rodents [93]. Although arginine supplementation did not enhance sperm quantity or quality in torsed testes, some improvements were observed in the contralateral testes. It was also stated that when rats suffered testicular torsion during puberty, their seminiferous epithelium were less damaged. In contrast, the smallest progresses were observed when testicular torsion occurred before puberty [93].

Arginine can also avoid membrane lipid peroxidation in sperm cells under different peroxidation conditions, and its protective effects have been connected with NO biosynthesis [82, 94, 95]. NOS are expressed in many biological systems, including acrosome and tail region of non-capacitated spermatozoa [96, 97], which indicates a possible participation of NOS in sperm capacitation and acrosome reaction [80]. Indeed, it was further suggested that presence and activity of NOS is dependent on the maturity of male germ cells [81].

Although NO is required for normal male reproduction, it also can be cytotoxic. High levels of NO have been shown to reduce motility and to induce toxicity in human sperm cells [81]. Moreover, high arginine levels can reversibly inhibit rat fertility [98] and also have adverse effects in human sperm motility and fertility [99]. Also in contrast with the favorable properties of arginine in spermatogenesis, arginine can be converted into polyamines, which in turn act as growth factors in cancer cells. Therefore, chronic administration of arginine in oncological patients should perhaps be avoided until the clarification of its safety [75].

In what concerns sperm storage, it was shown that arginine has a protective effect on post-thaw spermatozoa motility, viability, membrane integrity and lipid peroxidation in cryopreserved Murrah buffalo spermatozoa, suggesting that NO may play an essential protective role against lipid peroxidation during freezing and thawing processes [100]. Therefore, arginine could be added at low levels to the semen extender, since it has advantageous properties improving the conditions of spermatozoa storage, which are crucial for the success of artificial insemination [80, 94].

Carnitine/L-Carnitine

L-carnitine is a vitamin-type molecule structurally similar to amino acids, and approximately 25% of L-carnitine is synthesized endogenously from the essential amino acids lysine and methionine, whereas the other 75% are obtained through diet [101]. L-carnitine is, thus, an endogenous substance that is present in the male gonads [102] and recognized as a potent antioxidant [103]. It is a crucial co-factor for fatty acid metabolism and it has an important influence in energy production by facilitating the fatty acids transport into the mitochondria [102]. This is particularly relevant because sperm cells use fatty acid oxidation as their main source of energy for motility. Thus, the carnitine present in the epididymal fluid will determine the fatty acids transport into the spermatozoa mitochondria [104]. Accordingly, carnitine is positively linked with sperm counts and motility [105] and its levels in the epididymides and sperm are higher than those in the blood plasma [104]. Therefore, it has been proposed that carnitine has a protective role in energy metabolism, spermatogenic process, and semen quality, and consequently in the preservation of male fertility [106-110]. Furthermore, there is evidence that the beginning of sperm motility occurs simultaneously with an increase of carnitine levels in the epididymal lumen [104].

Carnitine also contributes to a successful sperm maturation, normal testis function [109], and possesses anti-inflammatory, immunomodulatory, and anti-apoptotic properties [111]. It was also suggested that the addition of high (20 mM), but not low (2 mM), concentrations of carnitine caused an increase of cellular Ca^{2+} transport and inhibition of sperm motility [106].

In addition, nutritional supplementation with carnitine also protects male reproductive function against several physical and chemical insults like heat [112], X-rays [113], γ-rays [111, 114], magnetic field [115], and anti-cancer drugs such as etoposide [109] and methotrexate [116]. Recently, it was demonstrated that mice treatment with carnitine before γ-rays exposure significantly reduced testicular dysfunction and improved testicular activity via regulation of spermatogenesis related genes, such as tumor necrosis factor-α, interferon-γ and interleukin 1 (IL-1)-β [111]. It was also verified a decrease in both sperm head and other morphological abnormalities, as well as a decline in the severity of radiation-induced histopathological modifications. Carnitine was also able to improve serum T levels, which alleviate serum T decrease in response to γ-rays. On the other hand, carnitine did not prevent the presence of giant multinucleated spermatids in seminiferous tubules [111]. It was also demonstrated in rat testis that carnitine therapy increased the recovery of seminiferous tubules and significantly reduced the severity of histopathological and morphometric changes induced by a radiation dose of 10 Gy [114]. This radiation dose induced a great decline in pregnancy, but unfortunately it was not observed any positive outcome of carnitine in respect to pregnancy rate after radiation exposure. Nevertheless, it is believed that carnitine administration would be a useful protective agent against radiation-induced injuries in male gonads, thereby improving patient's quality of life after radiotherapy [114].

Etoposide, a commonly used anti-cancer drug, induces cell death of proliferating cells in general and also of male germ cells [117]. It was shown that administration of carnitine partially protects rat testicular tissue against etoposide at pre-pubertal age, and it was proposed that the protective carnitine role

happens through its action on spermatogonial stem cell [109]. Moreover, it was also considered that both deleterious and protective effects of etoposide and carnitine, respectively, might be possibly mediated by its actions via Sertoli cells, because carnitine improves Sertoli cell metabolism [109, 118, 119]. In addition, carnitine protects cell membrane against free oxygen radicals [105], while etoposide induces free radical generation [120]. So, this might represent another mechanism by which carnitine achieve beneficial effects in testicular parameters and fertility recovery, although clarification is needed [109].

Carnitine was also found to ameliorate the secondary effects of methotrexate treatment over spermatogenic cells [121]. Probably through its free radical scavenging and antioxidant features [122], L-carnitine showed to counteract both the increase in malondialdehyde levels and the decrease in superoxide dismutase levels provoked by methotrexate in male genital organs. Also, L-carnitine prevented the testicular histologic injuries induced by methotrexate, considerably diminishing the severity of damage. In the same work, the authors also evaluated the effect of vitamin E against methotrexate exposure. Considering the mitigation of both the increase in malondialdehyde levels and the decrease in superoxide dismutase levels, vitamin E showed a better protective effect than L-carnitine. Furthermore, vitamin E had a superior outcome in decreasing the severity of interstitial edema and germ cell loss. [116]. In fact, vitamin E protects cell membranes against lipid peroxidation [123], and ethanol-induced oxidative stress and decreased steroidogenesis in rat testis can be reversed by treatment with vitamin E [124]. Thus, carnitine, and also vitamin E, might be used to minimize chemotherapy side effects and, consequently, helping in the fertility preservation of cancer patients.

Carnitine administration has been indicated as a reasonable, safe and effective option to treat male infertility due to its beneficial effects on semen volume, sperm counts, motility, and morphology, as well as in fertilization capacity [125, 126]. Imhof *et al*. [82] explored the influence of a non-prescription active micronutrient compound in sperm quality in men with idiopathic sub-fertility [82]. The tested nutraceutical included eight micronutrients: L-carnitine, L-arginine, zinc, vitamin E, glutathione, selenium, coenzyme Q_{10}, and folic acid. After 3 months of treatment, a significantly improvement in sperm quality was observed. Moreover, it also led to pregnancies without any side effects [82].

Also, there are several reports among different species in respect to carnitine protection against cryopreservation-induced damage [127]. When cryopreservation extender was supplemented with carnitine it provided protection in post-thawed sperm acrosome and total morphology [128], and an improvement in sperm motility [13]. It was also detected an important enhancement in sperm motility during liquid-storage, and a better integrity of acrosomal and plasma membrane using moderately low doses of carnitine, which may be due to its antioxidant activity in combination with its essential role in sperm cells energy and fatty acid metabolism [129].

Metallothioneins

Metallothioneins (MTs) are low molecular weight metal-binding proteins, and its synthesis can be induced either by endogenous or exogenous factors, such as cytokines and heavy metals [130]. In addition, MTs synthesis could be also induced by exposure to ionizing [131, 132] or ultraviolet (UV) radiation [133]. MTs are involved in metal detoxification and homeostasis, as well as in scavenging free radicals during oxidative injury [134]. Thus, MTs are able to defend against DNA damage, oxidative stress, apoptosis, and carcinogenesis induced by chemical stimulus and radiation [130, 135, 136]. Moreover, MTs also have the capability to increase cell survival, proliferation and angiogenesis [137].

It has been verified an overexpression of MTs in several human tumors, including testis, prostate and acute lymphoblastic leukemia [138]. In some cases, the expression of MTs is associated with tumor grade or stage, resistance to chemotherapy or radiation, and poor prognosis [138]. Nevertheless, MTs are probably only one of various factors implicated in the complex regulation of resistance to antineoplastic therapy. In contrast, MTs are downregulated in another types of tumors [137]. Some studies proposed that metallothionein-I (MT-I) and metallothionein-II (MT-II) suppress mutation at initial stages of tumorigenesis. In fact, MT-I and MT-II knockout mice displayed elevated incidence and higher sensitivity to tumor development [139]. MT-I/II null mice are very vulnerable to several carcinogens, namely N-butyl-N-(4-hydroxybutyl) nitrosamine [140], 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate [141], benzo[a]pyrene [142], and X-ray [132], which suggests that MT-I and MT-II have antioncogenic properties. On the other hand, another MT, specifically the metallothionein-III (MT-III), was found to be unresponsive to inducing agents of MT-I and MT-II [143, 144], suggesting there are some functional differences between MTs. In fact, it was demonstrated that MT-III null mice presented reduced cadmium-induced testicular toxicity, indicating that the lack of MT-III contributes to the protection of male gonads against noxious agents. Unfortunately, the molecular mechanisms by which cadmium-induced testicular injury is attenuated in MT-III null mice are unknown yet [145]. MT-I and MT-II may also act as negative regulators of nuclear factor-kappaB activity, suppressing the development of carcinogen-induced tumors and cancer cells growth, as well as inducing the apoptosis of cancer cells [135, 137]. Recently, it was proposed that MTs are also involved in the protective effect of zinc against toxic stimulus for male reproductive tract, such as cyclophosphamide and cadmium, but the exact mechanism of action remains to be clarified [146, 147].

An experimental approach to overcome the adverse effects of numerous drugs, particularly anticancer agents, involved the pharmacological modulation of MTs. The pharmacological increase of MTs levels during anticancer drug regime protected the normal cells from chemotherapeutics-induced injury [147]. Considering yet the complex concerns about the antiapoptotic outcomes of MT-I and MT-II during radiation and chemotherapy, another solution has been proposed: to suppress the expression of MT-I and MT-II in malignant cells in order to improve the therapeutic response, while normal expression of MT-I and MT-II in healthy cells or tissue protects from treatment side effects [137]. Although looking promising, the utilization of MTs as protective factors against testicular injury in oncological patients deserves further investigation.

Liver Growth Factor

The liver growth factor (LGF) is an albumin-bilirubin complex with hepatic mitogen activity, purified from serum of rat and patients with hepatobiliary illnesses [148]. LGF promotes cell proliferation without apparent toxicity or tissue degeneration [149], and in the presence of liver injury, the concentration of LGF rises notably, displaying also neuroregenerative, anti-hypertensive, and anti-fibrotic actions [150-156].

Having in consideration the beneficial role of hepatocyte growth factor (HGF) in male fertility (e.g. sperm motility, involvement in modulation of survival, proliferation and apoptosis of germ cells during postnatal testis development), it has been speculated a possible role for LGF in spermatogenesis [157].

Some experiments have been performed to study the role of LGF in testicular recovery, and it was shown that LGF promotes rat testicular regeneration after ethane dimethane sulfonate (EDS)-induced Leydig cell depletion [150, 152]. EDS momentarily and selectively destroys Leydig cells, diminishing serum T levels, increasing pituitary secretion of FSH and LH, and consequently prejudicing spermatogenesis and causing severe morphologic modifications in testicular interstitial and germinal compartments [158, 159]. Administration of LGF avoids germ cell sloughing and Sertoli cell injury, as well as promotes germ cell growth after EDS injection in rat [160]. In addition, LGF stimulates the synthesis of vascular endothelial growth factor (VEGF) and its receptors in the testis, what might be related to the progression of spermatogenesis and Leydig cell physiology, since in adult male no active angiogenesis occurs [161].

The dimethanesulfonate busulfan, in combination with other alkylating agents or nucleoside analogues, is considered the cornerstone of high-dose chemotherapy [162]. Busulfan selectively destroys the spermatogonial stem cells in various species, having a harmful impact in mammals´ spermatogenesis by preventing cell division [150, 151]. The potential protective role of LGF in response to busulfan treatment was evaluated in mice [150]. The results revealed that LGF seems to induce testis regeneration, reflected by an increased weight of testis and epididymis, accompanied by the partial reestablishment of spermatogenesis with augmented sperm production and motility. After exposure to busulfan, LGF seemed to stimulate the remaining spermatogonial stem cells accelerating the reactivation of spermatogenesis, as well as the appearance of normal histological structure in the majority of seminiferous tubules. LGF also restored the required microenvironment for successful spermatogenesis [150]. This reactivation was also confirmed by the increase in spermatozoa concentration in comparison with mice which only received busulfan. It was concluded that LGF administration could represent a single therapy to induce the recovery of fertility in male patients after busulfan-induced germinal epithelium impairment [150].

Recently, the effect of LGF in testicular regeneration in EDS-treated rats was studied [151]. The protein levels of class B scavenger receptors (SR-BI, SR-BII, and LIMPII) and hormone sensitive lipase in male gonads were assessed, to better understand the influence of these proteins in steroidogenesis and spermatogenesis recovery. As a result, LGF enhanced the recovery of seminiferous epithelium, the appearance of the mature pattern of Leydig cell distribution, and the mature SR-BI expression. Moreover, LGF treatment was responsible for the partial recovery of hormone sensitive lipase expression in spermatogonia and Leydig cells. A progressive increase in serum T levels and 3-β-hydroxysteroid dehydrogenase expression in EDS-treated group were also induced by LGF [151]. In addition, it was shown that LGF provides a faster recovery of spermatogenesis and powerfully increases the protein levels of SR-BI, which participates in cholesterol uptake from HDL required for spermatogenesis and cell division. For those reasons, it was proposed that regained SR-BI helped to expand the remaining testicular cells, what is favorable to the testicular regeneration as well as to the reestablishment of steroidogenesis and spermatogenesis. Furthermore, SR-BI expression in male gonad could be considered an early marker of spermatogenesis recovery after LGF therapy in EDS-castrated rats [151]. Addressing the beneficial effects of LGF in human testis upon oncological treatment and in normal physiological conditions is clearly warranted in a near future to deeply ascertain its potential use in the recovery of spermatogenesis.

Epidermal Growth Factor

Epidermal growth factor (EGF) is a polypeptide of 53 amino acids that was first isolated and purified from the submandibular glands of male mice [163]. EGF activates the tyrosine kinase EGF receptors, which in turn trigger an intracellular signaling cascade that the most known effect is the stimulation of cell growth [164, 165]. Interestingly, EGF signaling has been identified as one of the earliest signal transduction events occurring near the cell membrane in response to a variety of stressful conditions [166-168].

Emphasizing the importance of EGF in male reproductive function, it was shown that sialoadenectomy (removal of the submandibular glands) despite reducing the amount of circulating EGF to an undetectable level, resulted in a reduction of about 55% in epididymal sperm storage [169]. In addition, overexpression of EGF induced hypospermatogenesis in mice [170]. Thus, it has been suggested that an appropriate expression level of EGF is a requisite for the normal accomplishment of spermatogenesis. In the testis, EGF is located in Sertoli cells, pachytene spermatocytes, and round spermatids [171], and it has been indicated as a regulator of Leydig cell proliferation, steroidogenesis, and Sertoli cell activity [172, 173]. *In vitro*, it was shown that EGF induces DNA synthesis in microdissected segments of rat seminiferous tubules at stage I [174]. The administration of EGF after testicular torsion improved bilateral testicular injury [175], and significantly decreased the quantity of apoptotic germ cells in cryptorchid testis [176]. EGF has an anti-apoptotic/proapoptotic action depending on the dose: low concentrations of EGF promote proliferation, whereas high concentrations of EGF induce cell cycle arrest and activate apoptosis [177].

Emerging evidence also pointed out that EGF signaling is able to decrease reactive oxygen species (ROS) production [178-180], and that testicular ischemia–reperfusion stress cause a significant increase in EGF activity [181]. Accordingly, the lack of endogenous EGF by sialoadenectomy resulted in more dramatic testicular damage after torsion/detorsion [181]. Since acute ischemia–reperfusion stress induces a massive germ cell-specific apoptosis, mainly occurring in the meiotic spermatocytes, it would lead to the perturbation of spermatogenic differentiation, and consequently, to the impairment of male fertility [181]. A recent study proposed that the prominent emergence of endogenous EGF during the early phase of testicular reperfusion may exert its anti-apoptotic effect through the modulation of the functionality of anchoring junctions [181]. Indeed, it has been suggested the involvement of anchoring junctions in the regulation of germ cell apoptosis [182], and also that the functional integrity of anchoring junctions is an indispensable factor for early recovery from testicular ischemia–reperfusion injury [181]. These findings are not totally surprising, since the central function of Sertoli cells-germ cells anchoring junctions is the creation of a network that maintains tissue integrity, and that also plays an important role in signal transduction and germ cell behavior [183]. Moreover, data provided from rat Sertoli–germ cell co-cultures demonstrated a pro-survival influence of endogenous EGF on germ cells in response to ischemia–reperfusion induced testicular damage, which was partially mediated by phosphatidylinositol 3-kinase/Akt pathway [181].

Despite the late improvements in medical practice, altered testicular function and infertility remain significant sequels of testicular torsion and subsequent detorsion of spermatic cord [184], a surgical intervention conducted among boys and young men [185]. The testicular torsion and detorsion of spermatic cord requires a careful management to prevent future testicular loss [185]. It has been suggested that testicular torsion and classical ischemia–reperfusion injury share important characteristics [181] with EGF displaying beneficial effects, which raises the curiosity about its therapeutic potential for men suffering from testicular damage induced by other exogenous factors.

Interleukins

Many cytokines are local signaling molecules, which play an important role in spermatogenesis, supplying integration and communication between diverse cell types in testis in the course of the hormonal regulation of germ cell maturation [186]. Also, seminal plasma possesses cytokines that are usually present in the male reproductive tract [187]. Focusing at the seminiferous tubules, interleukin 6 (IL-6), which is only produced by Sertoli cells, suppresses DNA synthesis. IL-1, produced by both Sertoli and germ cells, is a spermatogonial growth factor that stimulates DNA repair [188].

It has been demonstrated that cytokines are involved in the cell response to radiation [189-191]. Indeed, the radioprotective properties of IL-1 and IL-6, especially those related to the whole body irradiation, have been recognized in the mouse [191-193]. It was also shown that subcutaneous injection of IL-6, 3 to 6 days after sublethal γ-radiation exposure, has radioprotective effects [194]. Moreover, it is known that Sertoli cells could respond to chemical stress by increasing the IL-1 and IL-6 activity [195, 196]. The IL-6, but not IL-1, was increased after γ-irradiation *in vitro* of radioresistant rat Sertoli cells [197]. A mechanism pointed out to explain the radioresistance of Sertoli cells to γ-irradiation is that the increase of IL-6 after irradiation contributes to the adaptive response to oxidative stress generated by γ-irradiation, because the elevated production of ROS by ionizing irradiation causes cellular damage which can be diminished by specific molecules such as cytokines [198]. Legué and co-authors concluded that only IL-6 is increased after irradiation via a transcriptional mechanism [188]. So, both at high and low doses, IL-6 seems to be a good marker of γ-radiation treatment. Furthermore, it was verified that IL-1 and IL-6 have a radioprotective effect in mice testes, since the administration of IL-1 or/and IL-6 prior to whole body irradiation resulted in lower DNA damage on germ cells. IL-6 exerts a protective effect on the pachytene spermatocytes because 10 days after irradiation the number of round spermatids was significantly increased. Nevertheless, IL-6 should not act directly on the pachytene spermatocytes [188] because this cytokine receptor is absent in this testicular cell type [199]. Indeed, the mechanism responsible for this beneficial action remains to be clarified. To our knowledge, the radioprotective influence of IL-6 in human male gonads was not examined yet.

Granulocyte Colony Stimulating Factor

Granulocyte colony stimulating factor (G-CSF) is a member of the hematopoietic growth factor family, which controls proliferation, differentiation and survival of hematopoietic progenitor cells [200]. The first studies about its role in both chemotherapy and radiation-induced myelosuppressed animals revealed that G-CSF can reduce the duration of myelosuppression and increase the number of active neutrophils [201, 202]. In 1993, Schmidberger *et al*. [203] studied eleven patients, going through an isolated leucopenia, who were treated with G-CSF during fractionated radiotherapy. Seven of them received concomitantly radiation and chemotherapeutic drug, and four patients received radiotherapy only. Some secondary effects were observed such as mild bone pain and transient increase in serum alkaline phosphatase levels. However, ten of eleven treated patients responded with improved leucocyte count. Hence, G-CSF treatment is well accepted during continuous fractionated irradiation, and it can be clinically used to mitigate neutropenia provoked by radiotherapy or by combined radio-chemotherapy [203]. Nowadays, G-CSF is a drug used for mobilization of hematopoietic stem cells from bone marrow in radiation-induced hematopoietic damage and for leucocytopenia therapy [204]. There is also evidence sustaining the diverse non-hematopoietic actions of G-CSF in another tissues. Also, it has been demonstrated that G-CSF can have an anti-apoptotic role in distinct organs, as well as an anti-inflammatory role supported by the down-regulated production of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor-α [205-209]. Therefore, the effect of G-CSF in the recovery of spermatogenesis and on the defense upon pelvic γ-radiation (5 Gy) was explored in mice [208]. It was found that G-CSF notably interrupted apoptosis of germ cells after irradiation, which allowed to conclude that it improves the survival of the germ line by activating anti-apoptotic pathways. Moreover, it is believed that the protective role of G-CSF against apoptosis of germ cells is due to the activation of numerous defensive pathways, which protect spermatogonial stem cells as well as differentiating cells from irradiation-induced damage [208]. G-CSF was capable of diminish inflammation and, consequently, it is likely that it mitigates the detrimental impact of inflammatory response. Also, the protected stem cells could be induced to proliferate by G-CSF, which decreases the radiation-induced decline in the testis weight, seminiferous tubules diameter, seminiferous epithelial depth and sperm head count [208]. In the G-CSF-treated group compared with the irradiated group only, the repopulation and stem cell survival indexes in the seminiferous tubules were improved, and the frequency of abnormal sperm was lower. G-CSF also counteracted the observed reduction in sperm counts caused by γ-radiation. Thereby, G-CSF was pointed out as a radioprotective agent in testicular physiology [208]. Nevertheless, the effect of G-CSF in the protection of human irradiated testis deserve attention.

Regucalcin

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein that plays an important role in intracellular Ca^{2+} homeostasis by regulating the activity of Ca^{2+} pumps localized on cell membrane and endoplasmic reticulum [210]. RGN is expressed in several tissues of human reproductive tract, including the testis, epididymis, seminal vesicles and prostate [211-213]. At testicular level, RGN is expressed in Leydig and Sertoli cells, as well as in all germ cell line both in rat and human [212].

Recently, several studies have demonstrated the role of RGN in modulating the expression of cell proliferation and apoptosis regulators [18, 213-215]. Moreover, the RGN protein has been associated with the suppression of cell proliferation and apoptosis [18, 210, 216-221]. Considering that the success of spermatogenesis depends on the tight balance between germ cell survival and death, a role for RGN in spermatogenesis and male fertility was suggested [220]. In order to investigate the protective function of RGN in testicular apoptosis, the seminiferous tubules of transgenic animals overexpressing RGN (Tg-RGN) and their wild type (Wt) counterparts were cultured *ex vivo* in the presence of apoptosis-inducers thapsigargin (Thap) and actinomycin D (Act D) [18]. Concomitantly with RGN overexpression, it was detected a reduced activity of apoptosis executioner caspase-3, increased expression of anti-apoptotic protein Bcl-2, as well as increased Bcl-2/Bax protein ratio in the seminiferous tubules of Tg-RGN. In addition, the mRNA expression of p53 and p21 was significantly diminished in Tg-RGN treated with Thap or Act D. Altogether, the results of Correia and co-authors allowed consider RGN as a germ cell survival factor with a protective role suppressing apoptosis induced by noxious stimuli [18], which was confirmed by posterior findings [222]. RGN overexpression was shown to improve the total antioxidant capacity, decrease the levels of thiobarbituric acid reactive substances, and diminish the activity of caspase-3 in rat seminiferous tubules exposed to the pro-oxidant agents tert-butyl hydroperoxide and cadmium chloride [222]. The importance of RGN for the maintenance of germ cell survival and spermatogenic output was also established by the fact that infertile men with abnormal spermatogenesis phenotypes exhibited different testicular expression patterns of RGN [220].

Furthermore, significant alterations in the epididymal epithelium and sperm parameters between Tg-RGN and their Wt counterparts were found [221]. Tg-RGN rats have lower sperm counts and reduced sperm motility. However, this result is counterbalanced by a greater sperm viability, higher percentage of normal sperm morphology, and a reduced incidence of tail defects, which also suggests the involvement of RGN in sperm maturation [221]. The beneficial effects of RGN on sperm parameters seemed to be explained by the lower levels of oxidative stress and increased antioxidant defenses found in the biological models of RGN overexpression [221-223]. Augmented levels of RGN have been related to the increased activity of

Table 1. Effects of hormonal factors in the recovery of male reproductive function.

Hormonal Factors	Physiological Effects	References
T	↓ chemical-induced testicular damage	[25, 26]
	→ spermatogenesis	[25, 26]
GnRH antagonists	→ spermatogenesis	[28-31]
	↓ chemical- and radiation-induced testicular damage	[24, 28, 29, 31]
	↑ testis weight	[29, 31]
	↑ sperm counts	[29, 31]
	↑ colonization of transplanted spermatogonial stem cells	[30, 31]
GnRH agonists/analogues	→ spermatogenesis	[28, 29, 34, 35]
	↑ testis weight	[29]
	↑ sperm counts	[29, 34]
	↓ chemical- and radiation-induced testicular damage	[29, 33-35]
Estrogens	↓ radiation-induced testicular damage	[46, 47]
	→ spermatogonial differentiation	[46]
	modulates SCF gene expression in testis	[37]
	→ spermatogenesis	[44-47]
GH/IGF-1	↓ chemical-induced testicular damage	[58, 59]
	↑ seminiferous epithelial depth and tubular diameter	[59]
	→ sperm maturation	[19, 60, 63]
	↑ sperm motility	[19, 56, 57, 59, 62, 63]
	↑ sperm viability	[59]
	→ testis maturation	[53]
	↓ lipid peroxidation in sperm	[62]
	→ spermatogenesis	[56, 60]
	↓ post-thawed sperm parameters and *in vitro* fertility deterioration	[63]
	↑ sperm plasma membrane integrity	[62]
Ghrelin	involvement in radiation-induced testicular damage	[65]
	↑ antioxidant defense in testis	[72]
	modulates SCF gene expression in testis	[69]
	⊣ proliferation of spermatogonial stem cells and spermatocytes	[72]
	↓ apoptosis in testis	[65]
	↑ sperm plasma membrane integrity	[74]

Legend:

↑, increased; ↓, reduced

→, induced / stimulated; ⊣, suppressed

Testosterone (T), Gonadotropin Releasing Hormone (GnRH), Stem Cell Factor (SCF), Growth Hormone (GH), Insulin-like Growth Factor 1 (IGF-1).

superoxide dismutase and glutathione-S-transferase enzymes, and decreased activity of NOS, which contributes to reducing the generation of ROS and attenuating its damaging effects [223-230]. The cytoprotective and antioxidant properties of RGN were also highlighted by studies in the RGN-knockout mice, which showed higher susceptibility to the oxidative stress induced by exposure to cigarette [231], and displayed increased levels of anion superoxide in the brain [232-234]. Also, RGN overexpression in a mouse carcinoma cell line was able to increase cell viability under oxidative damage induced by tert-butyl hydroperoxide [235]. Hence, available studies evidenced the protective effect of RGN against oxidative stress, as well as its importance in order to keep ROS at physiological concentrations.

Overall, the present data strongly support the idea that the manipulation of RGN levels in the testis, by means of its overexpression, would have a positive effect on the preservation of fertility in male patients undergoing treatment of oncological disorders, by reducing both apoptosis and oxidative damage.

CONCLUSION

Various agents have been studied in an attempt to recover or enhance the fertility potential of men who suffered from a set of conditions associated with a deleterious impact in gonadal physiology and sperm function. It is widely accepted that there are endogenous molecules providing some degree of protection against several harmful stimuli. In fact, endogenous antioxidants may offer a large defense

Table 2. Effects of non-hormonal factors in the recovery of male reproductive function.

Non-Hormonal Factors	Physiological Effects	References
Arginine	↓ lipid peroxidation in sperm	[94, 100]
	→ spermatogenesis	[75, 82, 83, 85, 87, 94]
	↓ cryopreservation-induced damage in sperm	[100]
	↑ sperm glycolysis rate	[83, 94]
	↑ sperm plasma membrane integrity	[100]
	↑ sperm motility	[75, 82, 83, 85-88, 94, 99, 100]
	↑ sperm viability	[99, 100]
NO	→ acrosome reaction	[82]
	↓ apoptosis signaling in sperm	[82]
	→ sperm capacitation	[82]
	↑ sperm motility	[81]
	↑ sperm viability	[81]
Carnitine/L-Carnitine	↑ sperm counts	[105, 125]
	↑ sperm motility	[105, 125, 129]
	→ sperm maturation	[109]
	↓ apoptosis in testis	[111]
	↓ chemical- and/or physical-induced testicular damage	[109, 111-116, 121]
	→ sertoli cell metabolism	[109]
	↓ malondialdehyde level in testis	[116]
	↑ superoxide dismutase level in testis	[116]
	↓ sperm abnormalities	[111, 126, 128, 129]
	↑ pregnancy rates	[126]
	↓ cryopreservation-induced damage in sperm	[13, 127, 128]
	↑ sperm acrosomal integrity	[128, 129]
	↓ liquid-storage-induced disturbances in sperm	[129]
	↑ sperm plasma membrane integrity	[129]
MTs	MT-I and MT-II ↓ chemical-induced damage	[146, 147]
HGF	↓ germ cell apoptosis	[157]
	↑ sperm motility	[157]
	→ testis maturation	[157]
LGF	↓ chemical-induced testicular damage	[150, 151]
	↑ epididymis weight	[150]
	→ spermatogenesis	[150, 151]
	↑ sperm motility	[150]
	→ synthesis of VEGF and its receptors in testis	[152]
	↑ testis weight	[150]
EGF	→ DNA synthesis in seminiferous tubules	[174]
	↓ testicular torsion-induced injury	[175]
	↓ apoptosis in testis	[176, 181]
	↓ testicular ischemia/reperfusion injury	[181]
	↑ integrity of Sertoli-germ cell anchoring junction	[181]
IL-1	→ DNA repair in seminiferous tubules	[188]
	↓ DNA damage on germ cells	[188]
	↓ chemical- and radiation-induced testicular damage	[188, 195]
IL-6	⊣ DNA synthesis in seminiferous tubules	[188]
	↓ DNA damage on germ cells	[188]
	↓ chemical- and radiation-induced testicular damage	[188, 196, 197]

(Table 2) Contd....

Non-Hormonal Factors	Physiological Effects	References
G-CSF	↓ apoptosis in testis	[208]
	↓ radiation-induced testicular damage	[208]
	↑ seminiferous epithelial depth and tubular diameter	[208]
	→ spermatogenesis	[208]
	↓ sperm abnormalities	[208]
	↑ testis weight	[208]
RGN	↓ oxidative stress in seminiferous tubules	[222]
	↓ apoptosis in testis	[18, 210, 222]
	↓ chemical-induced testicular damage	[18, 222]
	↓ sperm abnormalities	[221]
	↑ sperm maturation	[221]
	↑ sperm viability	[221]

Legend:

↑, increased; ↓, reduced

→, induced / stimulated; ⊣, suppressed

Nitric Oxide (NO), Metallothioneins (MTs), Metallothionein-I (MT-I), Metallothionein-II (MT-II) , Hepatocyte Growth Factor (HGF), Liver Growth Factor (LGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Granulocyte Colony Stimulating Factor (G-CSF), Regucalcin (RGN).

range against low-dose cancer treatments, and may also have therapeutic properties. Some of these potential endogenous protective agents have intrinsic antioxidant properties whereas others act on cells through specific receptors, such is the case of hormones, growth factors and cytokines. The evidence here exposed identified potential hormonal (Table **1**) and non-hormonal (Table **2**) molecules able to mitigate male fertility problems arising from the administration of oncological treatments. Also, new perspectives of research for fertility preservation or to improve the recovery of spermatogenesis upon testicular injury were opened. Furthermore, it is expected that molecules with such promising properties would also be employed to develop simpler and less expensive strategies of ART, and to improve the cryopreservation processes. Apart from it, it is important to be aware of the fact that male sperm parameters are severely declining in late years. Hence, any research efforts directed to enhance the testicular outputs are emergent not only to treat medical conditions but also to ameliorate men reproductive function and maybe the continuity of future generations.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was partially supported by the Pluriannual Programme of Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-OE/SAU/UI0709/2014). Sara Correia is funded by FCT fellowship (SFRH/BPD/104820/2014).

REFERENCES

[1] Hess R, de Franca L. Spermatogenesis and Cycle of the Seminiferous Epithelium. In: Cheng CY, editor. Molecular Mechanisms in Spermatogenesis. Advances in Experimental Medicine and Biology. 636: Springer New York; 2008. p. 1-15.

[2] Osterberg EC, Ramasamy R, Masson P, Brannigan RE. Current practices in fertility preservation in male cancer patients. Urol Ann 2014; 6(1): 13-7.

[3] Trost LW, Brannigan RE. Oncofertility and the male cancer patient. Curr Treat Options Oncol 2012; 13(2): 146-60.

[4] Stahl O, Eberhard J, Jepson K, *et al.* Sperm DNA integrity in testicular cancer patients. Hum Reprod 2006; 21(12): 3199-205.

[5] Brauner R, Czernichow P, Cramer P, Schaison G, Rappaport R. Leydig-Cell Function in Children after Direct Testicular Irradiation for Acute Lymphoblastic Leukemia. N Engl J Med 1983; 309(1): 25-8.

[6] Petersen PM, Skakkebaek NE, Vistisen K, Rorth M, Giwercman A. Semen quality and reproductive hormones before orchiectomy in men with testicular cancer. J Clin Oncol 1999; 17(3): 941-7.

[7] Katz DJ, Kolon TF, Feldman DR, Mulhall JP. Fertility preservation strategies for male patients with cancer. Nat Rev Urol 2013; 10(8): 463-72.

[8] Hotaling JM, Lopushnyan NA, Davenport M, *et al.* Raw and test-thaw semen parameters after cryopreservation among men with newly diagnosed cancer. Fertil Steril 2013; 99(2): 464-9.

[9] Howell SJ, Shalet SM. Spermatogenesis after cancer treatment: damage and recovery. J Natl Cancer Inst Monogr 2005; (34): 12-7.

[10] Rueffer U, Breuer K, Josting A, *et al.* Male gonadal dysfunction in patients with Hodgkin's disease prior to treatment. Ann Oncol 2001; 12(9): 1307-11.

[11] Pennings G, Mertes H. Ethical issues in infertility treatment. Best Pract Res Clin Obstet Gynaecol 2012; 26(6): 853-63.

[12] Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update 2008; 14(3): 243-58.

[13] Bucak MN, Tuncer PB, Sariozkan S, *et al.* Effects of antioxidants on post-thawed bovine sperm and oxidative

stress parameters: antioxidants protect DNA integrity against cryodamage. Cryobiology 2010; 61(3): 248-53.
[14] Degl'Innocenti S, Filimberti E, Magini A, *et al.* Semen cryopreservation for men banking for oligospermia, cancers, and other pathologies: prediction of post-thaw outcome using basal semen quality. Fertil Steril 2013; 100(6): 1555-63.e1-3.
[15] Sharma R, Kattoor AJ, Ghulmiyyah J, Agarwal A. Effect of sperm storage and selection techniques on sperm parameters. Syst Biol Reprod Med 2015; 61(1): 1-12.
[16] Linkeviciute A, Boniolo G, Chiavari L, Peccatori FA. Fertility preservation in cancer patients: the global framework. Cancer Treat Rev 2014; 40(8): 1019-27.
[17] Ko EY, Sabanegh ES. The role of nutraceuticals in male fertility. Urol Clin North Am 2014; 41(1): 181-93.
[18] Correia S, Alves MG, Oliveira PF, *et al.* Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways. Andrology 2014; 2(2): 290-8.
[19] Moss JL, Crosnoe LE, Kim ED. Effect of rejuvenation hormones on spermatogenesis. Fertil Steril 2013; 99(7): 1814-20.
[20] Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology 1989; 124(6): 3043-9.
[21] McLachlan RI, O'Donnell L, Meachem SJ, *et al.* Hormonal regulation of spermatogenesis in primates and man: insights for development of the male hormonal contraceptive. J Androl 2002; 23(2): 149-62.
[22] Weinbauer GF, Nieschlag E. Gonadotrophin-releasing hormone analogue-induced manipulation of testicular function in the monkey. Hum Reprod 1993; 8 Suppl 2: 45-50.
[23] Meistrich ML. Hormonal stimulation of the recovery of spermatogenesis following chemo- or radiotherapy. APMIS 1998; 106(1): 37-45; discussion 45-6.
[24] Meistrich ML, Shetty G. Inhibition of spermatogonial differentiation by testosterone. J Androl 2003; 24(2): 135-48.
[25] Masala A, Faedda R, Alagna S, *et al.* Use of testosterone to prevent cyclophosphamide-induced azoospermia. Ann Intern Med 1997; 126(4): 292-5.
[26] Aminsharifi A, Shakeri S, Ariafar A, Moeinjahromi B, Kumar PV, Karbalaeedoost S. Preventive role of exogenous testosterone on cisplatin-induced gonadal toxicity: an experimental placebo-controlled prospective trial. Fertil Steril 2010; 93(5): 1388-93.
[27] Wang L, Chadwick W, Park SS, *et al.* Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol Disord Drug Targets 2010; 9(5): 651-60.
[28] Meistrich ML, Shetty G. Suppression of testosterone stimulates recovery of spermatogenesis after cancer treatment. Int J Androl 2003; 26(3): 141-6.
[29] Meistrich ML, Wilson G, Shuttlesworth G, Huhtaniemi I, Reissmann T. GnRH agonists and antagonists stimulate recovery of fertility in irradiated LBNF1 rats. J Androl 2001; 22(5): 809-17.
[30] Wang G, Shao SH, Weng CC, Wei C, Meistrich ML. Hormonal suppression restores fertility in irradiated mice from both endogenous and donor-derived stem spermatogonia. Toxicol Sci 2010; 117(1): 225-37.
[31] Shetty G, Uthamanthil RK, Zhou W, *et al.* Hormone suppression with GnRH antagonist promotes spermatogenic recovery from transplanted spermatogonial stem cells in irradiated cynomolgus monkeys. Andrology 2013; 1(6): 886-98.
[32] Geens M. Strategies for fertility preservation and restoration in the male. Facts Views Vis Obgyn 2011; 3(4): 302-10.
[33] Glode LM, Robinson J, Gould S. Protection from cyclophosphamide-induced testicular damage with an analogue of gonadotropin-releasing hormone. Lancet 1981; 317(8230): 1132-4.
[34] Meistrich ML, Kangasniemi M. Hormone treatment after irradiation stimulates recovery of rat spermatogenesis from surviving spermatogonia. J Androl 1997; 18(1): 80-7.
[35] Udagawa K, Ogawa T, Watanabe T, Yumura Y, Takeda M, Hosaka M. GnRH analog, leuprorelin acetate, promotes regeneration of rat spermatogenesis after severe chemical damage. Int J Urol 2001; 8(11): 615-22.
[36] Correia S, Cardoso HJ, Cavaco JE, Socorro S. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med 2015; 17: e2.
[37] Correia S, Alves MR, Cavaco JE, Oliveira PF, Socorro S. Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertil Steril 2014; 102(1): 299-306.
[38] Sakaue M, Ishimura R, Kurosawa S, *et al.* Administration of estradiol-3-benzoate down-regulates the expression of testicular steroidogenic enzyme genes for testosterone production in the adult rat. J Vet Med Sci 2002; 64(2): 107-13.
[39] Hossaini A, Dalgaard M, Vinggaard AM, Pakarinen P, Larsen JJ. Male reproductive effects of octylphenol and estradiol in Fischer and Wistar rats. Reprod Toxicol 2003; 17(5): 607-15.
[40] Henriksson P, Carlström K, Pousette Å, *et al.* Time for revival of estrogens in the treatment of advanced prostatic carcinoma? Pharmacokinetics, and endocrine and clinical effects, of a parenteral estrogen regimen. Prostate 1999; 40(2): 76-82.
[41] Johnson RE, Murad MH. Gynecomastia: pathophysiology, evaluation, and management. Mayo Clin Proc 2009; 84(11): 1010-5.
[42] Morris ID, Shalet SM. Protection of gonadal function from cytotoxic chemotherapy and irradiation. Baillieres Clin Endocrinol Metab 1990; 4(1): 97-118.
[43] Rivas A, McKinnell C, Fisher JS, Atanassova N, Williams K, Sharpe RM. Neonatal coadministration of testosterone with diethylstilbestrol prevents diethylstilbestrol induction of most reproductive tract abnormalities in male rats. J Androl 2003; 24(4): 557-67.
[44] Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB. Estrogenic induction of spermatogenesis in the hypogonadal mouse. Endocrinology 2000; 141(8): 2861-9.
[45] Bilinska B, Kotula-Balak M, Gancarczyk M, Sadowska J, Tabarowski Z, Wojtusiak A. Androgen aromatization in cryptorchid mouse testis. Acta Histochem 2003; 105(1): 57-65.
[46] Porter KL, Shetty G, Shuttlesworth GA, *et al.* Estrogen enhances recovery from radiation-induced spermatogonial arrest in rat testes. J Androl 2009; 30(4): 440-51.
[47] Shetty G, Weng CC, Bolden-Tiller OU, Huhtaniemi I, Handelsman DJ, Meistrich ML. Effects of medroxyprogesterone and estradiol on the recovery of spermatogenesis in irradiated rats. Endocrinology 2004; 145(10): 4461-9.
[48] Kula K, Walczak-Jedrzejowska R, Slowikowska-Hilczer J, Oszukowska E. Estradiol enhances the stimulatory effect of FSH on testicular maturation and contributes to precocious initiation of spermatogenesis. Mol Cell Endocrinol 2001; 178(1-2): 89-97.
[49] Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 1999; 285(5431): 1259-61.
[50] Shetty G, Meistrich ML. Hormonal approaches to preservation and restoration of male fertility after cancer treatment. J Natl Cancer Inst Monogr 2005; (34): 36-9.
[51] Zhou W, Bolden-Tiller OU, Shao SH, *et al.* Estrogen-regulated genes in rat testes and their relationship to recovery of spermatogenesis after irradiation. Biol Reprod 2011; 85(4): 823-33.
[52] Kawamura K, Kumagai J, Sudo S, *et al.* Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc Natl Acad Sci U S A 2004; 101(19): 7323-8.
[53] Rogol AD, Roemmich JN, Clark PA. Growth at puberty. J Adolesc Health 2002; 31(6 Suppl): 192-200.
[54] Hibi I, Tanaka T, Tanae A, *et al.* The influence of gonadal function and the effect of gonadal suppression treatment on final height in growth hormone (GH)-treated GH-deficient children. J Clin Endocrinol Metab 1989; 69(2): 221-6.

[55] Odell WD, Swerdloff RS. Etiologies of sexual maturation: a model system based on the sexually maturing rat. Recent Prog Horm Res 1976; 32: 245-88.

[56] Radicioni A, Paris E, Dondero F, Bonifacio V, Isidori A. Recombinant-growth hormone (rec-hGH) therapy in infertile men with idiopathic oligozoospermia. Acta Eur Fertil 1994; 25(5): 311-7.

[57] Ovesen P, Jorgensen JO, Ingerslev J, Ho KK, Orskov H, Christiansen JS. Growth hormone treatment of subfertile males. Fertil Steril 1996; 66(2): 292-8.

[58] Satoh K, Ohyama K, Nakagomi Y, *et al.* Effects of growth hormone on testicular dysfunction induced by cyclophosphamide (CP) in GH-deficient rats. Endocr J 2002; 49(6): 611-9.

[59] Nouri HS, Azarmi Y, Movahedin M. Effect of growth hormone on testicular dysfunction induced by methotrexate in rats. Andrologia 2009; 41(2): 105-10.

[60] Glander HJ, Kratzsch J, Weisbrich C, Birkenmeier G. Insulin-like growth factor-I and alpha 2-macroglobulin in seminal plasma correlate with semen quality. Hum Reprod 1996; 11(11): 2454-60.

[61] Henricks DM, Kouba AJ, Lackey BR, Boone WR, Gray SL. Identification of insulin-like growth factor I in bovine seminal plasma and its receptor on spermatozoa: influence on sperm motility. Biol Reprod 1998; 59(2): 330-7.

[62] Selvaraju S, Reddy IJ, Nandi S, Rao SB, Ravindra JP. Influence of IGF-I on buffalo (Bubalus bubalis) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro. Anim Reprod Sci 2009; 113(1-4): 60-70.

[63] Selvaraju S, Nandi S, Subramani TS, Raghavendra BS, Rao SB, Ravindra JP. Improvement in buffalo (Bubalus bubalis) spermatozoa functional parameters and fertility in vitro: Effect of insulin-like growth factor-I. Theriogenology 2010; 73(1): 1-10.

[64] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402(6762): 656-60.

[65] Li W, Zeng Y, Zhao J, Zhu CJ, Hou WG, Zhang S. Upregulation and nuclear translocation of testicular ghrelin protects differentiating spermatogonia from ionizing radiation injury. Cell Death Dis 2014; 5: e1248.

[66] Roa J, Tena-Sempere M. Connecting metabolism and reproduction: roles of central energy sensors and key molecular mediators. Mol Cell Endocrinol 2014; 397(1-2): 4-14.

[67] Tena-Sempere M. Ghrelin and reproduction: ghrelin as novel regulator of the gonadotropic axis. Vitam Horm 2008; 77: 285-300.

[68] Barreiro ML, Gaytan F, Caminos JE, *et al.* Cellular location and hormonal regulation of ghrelin expression in rat testis. Biol Reprod 2002; 67(6): 1768-76.

[69] Barreiro ML, Gaytan F, Castellano JM, *et al.* Ghrelin inhibits the proliferative activity of immature Leydig cells in vivo and regulates stem cell factor messenger ribonucleic acid expression in rat testis. Endocrinology 2004; 145(11): 4825-34.

[70] Cardoso HJ, Figueira MI, Correia S, Vaz CV, Socorro S. The SCF/c-KIT system in the male: Survival strategies in fertility and cancer. Mol Reprod Dev 2014; 81(12): 1064-79.

[71] Figueira MI, Cardoso HJ, Correia S, Maia CJ, Socorro S. Hormonal regulation of c-KIT receptor and its ligand: implications for human infertility? Prog Histochem Cytochem 2014; 49(1-3): 1-19.

[72] Kheradmand A, Dezfoulian O, Alirezaei M. Ghrelin regulates Bax and PCNA but not Bcl-2 expressions following scrotal hyperthermia in the rat. Tissue Cell 2012; 44(5): 308-15.

[73] Zhu CC, Zhang H, Zhang JS, *et al.* Inhibition of ghrelin signaling improves the reproductive phenotype of male ob/ob mouse. Fertil Steril 2013; 99(3): 918-26.

[74] Kheradmand A, Taati M, Babaei H. The effects of chronic administration of ghrelin on rat sperm quality and membrane integrity. Anim Biol 2009; 59(2): 159-68.

[75] Appleton J. Arginine: Clinical potential of a semi-essential amino acid. Altern Med Rev 2002; 7(6): 512-22.

[76] Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Dietary L-arginine supplementation reduces Methotrexate-induced intestinal mucosal injury in rat. BMC Gastroenterol 2012; 12: 41.

[77] Wang J, He Q, Yan X, Cai Y, Chen J. Effect of exogenous nitric oxide on sperm motility in vitro. Biol Res 2014; 47: 44.

[78] Lefevre PL, Palin MF, Murphy BD. Polyamines on the reproductive landscape. Endocr Rev 2011; 32(5): 694-712.

[79] Eskiocak S, Gozen AS, Taskiran A, Kilic AS, Eskiocak M, Gulen S. Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. Braz J Med Biol Res 2006; 39(5): 581-8.

[80] O'Flaherty C, Rodriguez P, Srivastava S. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochim Biophys Acta 2004; 1674(2): 215-21.

[81] Roessner C, Paasch U, Glander HJ, Grunewald S. Activity of nitric oxide synthase in mature and immature human spermatozoa. Andrologia 2010; 42(2): 132-7.

[82] Imhof M, Lackner J, Lipovac M, Chedraui P, Riedl C. Improvement of sperm quality after micronutrient supplementation. Clin Nutr ESPEN 2012; 7(1): e50-e3.

[83] Patel AB, Srivastava S, Phadke RS, Govil G. Arginine activates glycolysis of goat epididymal spermatozoa: an NMR study. Biophys J 1998; 75(3): 1522-8.

[84] Tanimura J. Studies on arginine in human semen. II. The effects of medication with L-arginine-HCL on male infertility. Bull Osaka Med Sch 1967; 13(2): 84-9.

[85] Keller DW, Polakoski KL. L-arginine stimulation of human sperm motility in vitro. Biol Reprod 1975; 13(2): 154-7.

[86] Scibona M, Meschini P, Capparelli S, Pecori C, Rossi P, Menchini Fabris GF. [L-arginine and male infertility]. Minerva Urol Nefrol 1994; 46(4): 251-3.

[87] Aydin S, Inci O, Alagol B. The role of arginine, indomethacin and kallikrein in the treatment of oligoasthenospermia. Int Urol Nephrol 1995; 27(2): 199-202.

[88] Morales ME, Rico G, Bravo C, Tapia R, Alvarez C, Mendez JD. [Progressive motility increase caused by L-arginine and polyamines in sperm from patients with idiopathic and diabetic asthenozoospermia]. Ginecol Obstet Mex 2003; 71: 297-303.

[89] Patel AB, Srivastava S, Phadke RS, Govil G. Arginine acts as a protective and reversal agent against glycolytic inhibitors in spermatozoa. Physiol Chem Phys Med NMR 1999; 31(1): 29-40.

[90] Chen J, Wollman Y, Chernichovsky T, Iaina A, Sofer M, Matzkin H. Effect of oral administration of high-dose nitric oxide donor L-arginine in men with organic erectile dysfunction: results of a double-blind, randomized, placebo-controlled study. BJU Int 1999; 83(3): 269-73.

[91] Ersin S, Tuncyurek P, Esassolak M, *et al.* The prophylactic and therapeutic effects of glutamine- and arginine-enriched diets on radiation-induced enteritis in rats. J Surg Res 2000; 89(2): 121-5.

[92] Stanislavov R, Rohdewald P. Sperm quality in men is improved by supplementation with a combination of L-arginine, L-citrullin, roburins and Pycnogenol(R). Minerva Urol Nefrol 2014; 66(4): 217-23.

[93] Ribeiro CT, Milhomem R, De Souza DB, Costa WS, Sampaio FJ, Pereira-Sampaio MA. Effect of antioxidants on outcome of testicular torsion in rats of different ages. J Urol 2014; 191(5 Suppl): 1578-84.

[94] Srivastava S, Desai P, Coutinho E, Govil G. Mechanism of action of L-arginine on the vitality of spermatozoa is primarily through increased biosynthesis of nitric oxide. Biol Reprod 2006; 74(5): 954-8.

[95] Senbel AM, Omar AG, Abdel-Moneim LM, Mohamed HF, Daabees TT. Evaluation of l-arginine on kidney function and vascular reactivity following ischemic injury in rats: protective effects and potential interactions. Pharmacol Rep 2014; 66(6): 976-83.

[96] Herrero MB, Perez Martinez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev 1996; 8(5): 931-4.

[97] Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod 1996; 2(11): 873-8.

[98] Ratnasooriya WD, Dharmasiri MG. L-arginine, the substrate of nitric oxide synthase, inhibits fertility of male rats. Asian J Androl 2001; 3(2): 97-103.

[99] Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod 1995; 10(7): 1786-90.

[100] Siddique RA, Atreja SK. Effect of L-Arginine and spermine-NONOate on motility, viability, membrane integrity and lipid peroxidation of Murrah buffalo (Bubalus bubalis) spermatozoa. Livestock Sci 2013; 153(1-3): 147-53.

[101] Rebouche CJ, Seim H. Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr 1998; 18: 39-61.

[102] Hinton BT, Snoswell AM, Setchell BP. The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals. J Reprod Fertil 1979; 56(1): 105-11.

[103] Gulcin I. Antioxidant and antiradical activities of L-carnitine. Life Sci 2006; 78(8): 803-11.

[104] Jeulin C, Lewin LM. Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Hum Reprod Update 1996; 2(2): 87-102.

[105] Lenzi A, Lombardo F, Sgro P, *et al.* Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. Fertil Steril 2003; 79(2): 292-300.

[106] Deana R, Rigoni F, Francesconi M, Cavallini L, Arslan P, Siliprandi N. Effect of L-carnitine and L-aminocarnitine on calcium transport, motility, and enzyme release from ejaculated bovine spermatozoa. Biol Reprod 1989; 41(5): 949-55.

[107] Costa M, Canale D, Filicori M, D'Lddio S, Lenzi A. L-carnitine in idiopathic asthenozoospermia: a multicenter study. Italian Study Group on Carnitine and Male Infertility. Andrologia 1994; 26(3): 155-9.

[108] Kanter M, Topcu-Tarladacalisir Y, Parlar S. Antiapoptotic effect of L-carnitine on testicular irradiation in rats. J Mol Histol 2010; 41(2-3): 121-8.

[109] Okada FK, Stumpp T, Miraglia SM. Carnitine reduces testicular damage in rats treated with etoposide in the prepubertal phase. Cell Tissue Res 2009; 337(2): 269-80.

[110] Dokmeci D, Inan M, Basaran UN, *et al.* Protective effect of L-carnitine on testicular ischaemia-reperfusion injury in rats. Cell Biochem Funct 2007; 25(6): 611-8.

[111] Ahmed MM, Ibrahim ZS, Alkafafy M, El-Shazly SA. L-carnitine protects against testicular dysfunction caused by gamma irradiation in mice. Acta Histochem 2014; 116(6): 1046-55.

[112] Amendola R, Cordelli E, Mauro F, Spano M. Effects of L-acetylcarnitine (LAC) on the post-injury recovery of mouse spermatogenesis monitored by flow cytometry. 2. Recovery after hyperthermic treatment. Andrologia 1991; 23(2): 135-40.

[113] Amendola R, Bartoleschi C, Cordelli E, Mauro F, Uccelli R, Spano M. Effects of L-acetylcarnitine (LAC) on the post-injury recovery of mouse spermatogenesis monitored by flow cytometry. 1. Recovery after X-irradiation. Andrologia 1989; 21(6): 568-75.

[114] Topcu-Tarladacalisir Y, Kanter M, Uzal MC. Role of L-carnitine in the prevention of seminiferous tubules damage induced by gamma radiation: a light and electron microscopic study. Arch Toxicol 2009; 83(8): 735-46.

[115] Ramadan LA, Abd-Allah AR, Aly HA, Saad-el-Din AA. Testicular toxicity effects of magnetic field exposure and prophylactic role of coenzyme Q10 and L-carnitine in mice. Pharmacol Res 2002; 46(4): 363-70.

[116] Yuncu M, Bukucu N, Bayat N, Sencar L, Tarakcioglu M. The effect of vitamin E and L-carnitine against methotrexate-induced injury in rat testis. Turk J Med Sci 2015; 45(3): 517-25.

[117] Hakovirta H, Parvinen M, Lähdetie J. Effects of etoposide on stage-specific DNA synthesis during rat spermatogenesis. Mutat Res 1993; 301(3): 189-93.

[118] Stumpp T, Freymuller E, Miraglia SM. Sertoli cell function in albino rats treated with etoposide during prepubertal phase. Histochem Cell Biol 2006; 126(3): 353-61.

[119] Stumpp T, Freymuller E, Miraglia SM. Sertoli cell morphological alterations in albino rats treated with etoposide during prepubertal phase. Microsc Microanal 2008; 14(3): 225-35.

[120] Oh SY, Sohn YW, Park JW, *et al.* Selective cell death of oncogenic Akt-transduced brain cancer cells by etoposide through reactive oxygen species mediated damage. Mol Cancer Ther 2007; 6(8): 2178-87.

[121] Saxena AK, Dhungel S, Bhattacharya S, Jha CB, Srivastava AK. Effect of chronic low dose of methotrexate on cellular proliferation during spermatogenesis in rats. Arch Androl 2004; 50(1): 33-5.

[122] Sener G, Eksioglu-Demiralp E, Cetiner M, *et al.* L-Carnitine ameliorates methotrexate-induced oxidative organ injury and inhibits leukocyte death. Cell Biol Toxicol 2006; 22(1): 47-60.

[123] Palamanda JR, Kehrer JP. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. Lipids 1993; 28(5): 427-31.

[124] Maneesh M, Dutta S, Chakrabarti A, Vasudevan DM. Experimental therapeutic intervention with alpha tocopherol in ethanol induced testicular injuries in rats. Indian J Clin Biochem 2007; 22(1): 138-42.

[125] Moradi M, Moradi A, Alemi M, *et al.* Safety and efficacy of clomiphene citrate and L-carnitine in idiopathic male infertility: a comparative study. Urol J 2010; 7(3): 188-93.

[126] Cavallini G, Ferraretti AP, Gianaroli L, Biagiotti G, Vitali G. Cinnoxicam and L-carnitine/acetyl-L-carnitine treatment for idiopathic and varicocele-associated oligoasthenospermia. J Androl 2004; 25(5): 761-70; discussion 71-2.

[127] Kutluyer F, Kayim M, Ogretmen F, Buyukleblebici S, Tuncer PB. Cryopreservation of rainbow trout Oncorhynchus mykiss spermatozoa: effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility. Cryobiology 2014; 69(3): 462-6.

[128] Bucak MN, Sarıözkan S, Tuncer PB, *et al.* The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Res 2010; 89(1): 24-30.

[129] Sariozkan S, Ozdamar S, Turk G, Canturk F, Yay A. In vitro effects of l-carnitine and glutamine on motility, acrosomal abnormality, and plasma membrane integrity of rabbit sperm during liquid-storage. Cryobiology 2014; 68(3): 349-53.

[130] Fujiwara Y, Satoh M. Protective role of metallothionein in chemical and radiation carcinogenesis. Curr Pharm Biotechnol 2013; 14(4): 394-9.

[131] Miura N, Satoh M, Imura N, Naganuma A. Protective effect of bismuth nitrate against injury to the bone marrow by γ-irradiation in mice: possible involvement of induction of metallothionein synthesis. J Pharmacol Exp Ther 1998; 286(3): 1427-30.

[132] Shibuya K, Suzuki JS, Kito H, Naganuma A, Tohyama C, Satoh M. Protective role of metallothionein in bone marrow injury caused by X-irradiation. J Toxicol Sci 2008; 33(4): 479-84.

[133] Saito T, Tezuka T, Konno R, Fujii N. Protective effects of metallothionein I and II against metal- and ultraviolet radiation-induced damage in cultured lens epithelial cells. Jpn J Ophthalmol 2010; 54(5): 486-93.

[134] Goossens L. Effects of gamma-ray-induced free radicals on the metal content and amino acid composition of human metallothionein-1. J Biosci 2011; 36(2): 235-41.

[135] McGee HM, Woods GM, Bennett B, Chung RS. The two faces of metallothionein in carcinogenesis: photoprotection against UVR-induced cancer and promotion of tumour survival. Photochem Photobiol Sci 2010; 9(4): 586-96.

[136] Sato M, Kondoh M. Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals. Tohoku J Exp Med 2002; 196(1): 9-22.

[137] Pedersen MO, Larsen A, Stoltenberg M, Penkowa M. The role of metallothionein in oncogenesis and cancer prognosis. Prog Histochem Cytochem 2009; 44(1): 29-64.

[138] Theocharis SE, Margeli AP, Klijanienko JT, Kouraklis GP. Metallothionein expression in human neoplasia. Histopathology 2004; 45(2): 103-18.

[139] Waalkes MP, Liu J, Kasprzak KS, Diwan BA. Hypersusceptibility to cisplatin carcinogenicity in metallothionein-I/II double knockout mice: production of hepatocellular carcinoma at clinically relevant doses. Int J Cancer 2006; 119(1): 28-32.

[140] Kondo Y, Himeno S, Endo W, *et al.* Metallothionein modulates the carcinogenicity of N-butyl-N-(4-hydroxybutyl)nitrosamine in mice. Carcinogenesis 1999; 20(8): 1625-7.

[141] Suzuki JS, Nishimura N, Zhang B, *et al.* Metallothionein deficiency enhances skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate in metallothionein-null mice. Carcinogenesis 2003; 24(6): 1123-32.

[142] Takaishi M, Sawada M, Shimada A, Suzuki JS, Satoh M, Nagase H. Protective role of metallothionein in benzo[a]pyrene-induced DNA damage. J Toxicol Sci 2009; 34(5): 449-58.

[143] Palmiter RD, Findley SD, Whitmore TE, Durnam DM. MT-III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci U S A 1992; 89(14): 6333-7.

[144] Kramer KK, Liu J, Choudhuri S, Klaassen CD. Induction of metallothionein mRNA and protein in murine astrocyte cultures. Toxicol Appl Pharmacol 1996; 136(1): 94-100.

[145] Honda A, Komuro H, Shimada A, *et al.* Attenuation of cadmium-induced testicular injury in metallothionein-III null mice. Life Sci 2010; 87(17-18): 545-50.

[146] Kheradmand F, Nourmohammadi I, Ahmadi-Faghih MA, Firoozrai M, Modarressi MH. Zinc and low-dose of cadmium protect sertoli cells against toxic-dose of cadmium: The role of metallothionein. Iran J Reprod Med 2013; 11(6): 487-94.

[147] Maremanda KP, Khan S, Jena G. Zinc protects cyclophosphamide-induced testicular damage in rat: involvement of metallothionein, tesmin and Nrf2. Biochem Biophys Res Commun 2014; 445(3): 591-6.

[148] Diaz-Gil J, Gavilanes J, Sanchez G, *et al.* Identification of a liver growth factor as an albumin-bilirubin complex. Biochem J 1987; 243: 443-8.

[149] Diaz-Gil JJ, Munoz J, Albillos A, *et al.* Improvement in liver fibrosis, functionality and hemodynamics in CCl4-cirrhotic rats after injection of the Liver Growth Factor. J Hepatol 1999; 30(6): 1065-72.

[150] Perez-Crespo M, Pericuesta E, Perez-Cerezales S, *et al.* Effect of liver growth factor on both testicular regeneration and recovery of spermatogenesis in busulfan-treated mice. Reprod Biol Endocrinol 2011; 9: 21.

[151] Lobo MV, Arenas MI, Huerta L, *et al.* Liver growth factor induces testicular regeneration in EDS-treated rats and increases protein levels of class B scavenger receptors. Am J Physiol Endocrinol Metab 2015; 308(2): E111-21.

[152] Gonzalo-Gobernado R, Calatrava-Ferreras L, Perucho J, *et al.* Liver Growth Factor as a tissue regenerating factor in neurodegenerative diseases. Recent Pat CNS Drug Discov 2014; 9(3): 173-80.

[153] Reimers D, Osuna C, Gonzalo-Gobernado R, *et al.* Liver growth factor promotes the survival of grafted neural stem cells in a rat model of Parkinson's disease. Curr Stem Cell Res Ther 2012; 7(1): 15-25.

[154] Calatrava-Ferreras L, Gonzalo-Gobernado R, Reimers D, *et al.* Neuroprotective role of liver growth factor "LGF" in an experimental model of cerebellar ataxia. Int J Mol Sci 2014; 15(10): 19056-73.

[155] Condezo-Hoyos L, Arribas SM, Abderrahim F, *et al.* Liver growth factor treatment reverses vascular and plasmatic oxidative stress in spontaneously hypertensive rats. J Hypertens 2012; 30(6): 1185-94.

[156] Diaz-Gil JJ, Garcia-Monzon C, Rua C, *et al.* Liver growth factor antifibrotic activity in vivo is associated with a decrease in activation of hepatic stellate cells. Histol Histopathol 2009; 24(4): 473-9.

[157] Catizone A, Ricci G, Del Bravo J, Galdieri M. Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. J Endocrinol 2006; 189(1): 137-46.

[158] Bakalska M, Atanassova N, Koeva Y, Nikolov B, Davidoff M. Induction of male germ cell apoptosis by testosterone withdrawal after ethane dimethanesulfonate treatment in adult rats. Endocr Regul 2004; 38(3): 103-10.

[159] Ariyaratne S, Kim I, Mills N, Mason I, Mendis-Handagama C. Effects of ethane dimethane sulfonate on the functional structure of the adult rat testis. Arch Androl 2003; 49(4): 313-26.

[160] Martin-Hidalgo A, Lobo MVT, Sacristán S, *et al.* Rat testicular regeneration after EDS administration is stimulated by the liver growth factor (LGF). FEBS J 2007; 274(F1-126): 296.

[161] Martin-Hidalgo A, Arenas MI, Sacristán S, *et al.* Rat testis localization of VEGFs and VEGF Receptors in control and testicular regeneration stimulated by the liver growth factor (LGF). FEBS J 2007; 274(F1-125): 296.

[162] Galaup A, Paci A. Pharmacology of dimethanesulfonate alkylating agents: busulfan and treosulfan. Expert Opin Drug Metab Toxicol 2013; 9(3): 333-47.

[163] Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem 1990; 265(14): 7709-12.

[164] Narayanan R, Kim HN, Narayanan NK, Nargi D, Narayanan B. Epidermal growth factor-stimulated human cervical cancer cell growth is associated with EGFR and cyclin D1 activation, independent of COX-2 expression levels. Int J Oncol 2012; 40(1): 13-20.

[165] Tarcic G, Avraham R, Pines G, *et al.* EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. FASEB J 2012; 26(4): 1582-92.

[166] Cai B, Tomida A, Mikami K, Nagata K, Tsuruo T. Down-regulation of epidermal growth factor receptor-signaling pathway by binding of GRP78/BiP to the receptor under glucose-starved stress conditions. J Cell Physiol 1998; 177(2): 282-8.

[167] Zhang L, Jope RS. Oxidative stress differentially modulates phosphorylation of ERK, p38 and CREB induced by NGF or EGF in PC12 cells. Neurobiol Aging 1999; 20(3): 271-8.

[168] Murshid A, Theriault J, Gong J, Calderwood SK. Investigating receptors for extracellular heat shock proteins. Methods Mol Biol 2011; 787: 289-302.

[169] Liu A, Flores C, Kinkead T, Carboni AA, Menon M, Seethalakshmi L. Effects of sialoadenectomy and epidermal growth factor on testicular function of sexually mature male mice. J Urol 1994; 152(2 Pt 1): 554-61.

[170] Wong RW, Kwan RW, Mak PH, Mak KK, Sham MH, Chan SY. Overexpression of epidermal growth factor induced hypospermatogenesis in transgenic mice. J Biol Chem 2000; 275(24): 18297-301.

[171] Radhakrishnan B, Oke BO, Papadopoulos V, DiAugustine RP, Suarez-Quian CA. Characterization of epidermal growth factor in mouse testis. Endocrinology 1992; 131(6): 3091-9.

[172] Bai S, Wilson EM. Epidermal-growth-factor-dependent phosphorylation and ubiquitinylation of MAGE-11 regulates its interaction with the androgen receptor. Mol Cell Biol 2008; 28(6): 1947-63.

[173] Kuijk EW, Colenbrander B, Roelen BA. The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 2009; 138(4): 721-31.

[174] Wahab-Wahlgren A, Martinelle N, Holst M, Jahnukainen K, Parvinen M, Soder O. EGF stimulates rat spermatogonial DNA synthesis in seminiferous tubule segments in vitro. Mol Cell Endocrinol 2003; 201(1-2): 39-46.

[175] Uguralp S, Bay Karabulut A, Mizrak B, Kaymaz F, Kiziltay A, Hasirci N. The effect of sustained and local administration of epidermal growth factor on improving bilateral testicular tissue after torsion. Urol Res 2004; 32(5): 323-31.

[176] Kurokawa S, Kojima Y, Mizuno K, Nakane A, Hayashi Y, Kohri K. Effect of epidermal growth factor on spermatogenesis in the cryptorchid rat. J Urol 2005; 174(6): 2415-9.

[177] Zhao X, Dai W, Zhu H, *et al.* Epidermal growth factor (EGF) induces apoptosis in a transfected cell line expressing EGF receptor on its membrane. Cell Biol Int 2006; 30(8): 653-8.

[178] Maeda H, Rajesh KG, Maeda H, Suzuki R, Sasaguri S. Epidermal growth factor and insulin inhibit cell death in pancreatic beta cells by activation of PI3-kinase/AKT signaling pathway under oxidative stress. Transplant Proc 2004; 36(4): 1163-5.

[179] Hussein Ael A, Shokeir AA, Sarhan ME, *et al.* Effects of combined erythropoietin and epidermal growth factor on renal ischaemia/reperfusion injury: a randomized experimental controlled study. BJU Int 2011; 107(2): 323-8.

[180] Zhang HY, James I, Chen CL, Besner GE. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) preserves gut barrier function by blocking neutrophil-endothelial cell adhesion after hemorrhagic shock and resuscitation in mice. Surgery 2012; 151(4): 594-605.

[181] Zhang S, Zeng Y, Qu J, Luo Y, Wang X, Li W. Endogenous EGF maintains Sertoli germ cell anchoring junction integrity and is required for early recovery from acute testicular ischemia/reperfusion injury. Reproduction 2013; 145(2): 177-89.

[182] Richburg JH, Boekelheide K. Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol 1996; 137(1): 42-50.

[183] Siu MK, Wong CH, Lee WM, Cheng CY. Sertoli-germ cell anchoring junction dynamics in the testis are regulated by an interplay of lipid and protein kinases. J Biol Chem 2005; 280(26): 25029-47.

[184] Taskara E, Gor A, Kutlu O, *et al.* Does propofol prevent testicular ischemia-reperfusion injury due to torsion in the long term? Pediatr Surg Int 2011; 27(9): 1003-7.

[185] Kanter M. Protective effects of Ginkgo biloba (EGb 761) on testicular torsion/detorsion-induced ischemia-reperfusion injury in rats. Exp Mol Pathol 2011; 91(3): 708-13.

[186] Tronchon V, Vialard F, El Sirkasi M, *et al.* Tumor necrosis factor-alpha -308 polymorphism in infertile men with altered sperm production or motility. Hum Reprod 2008; 23(12): 2858-66.

[187] Shukla KK, Agnihotri S, Gupta A, *et al.* Significant association of TNFalpha and IL-6 gene with male infertility--an explorative study in Indian populations of Uttar Pradesh. Immunol Lett 2013; 156(1-2): 30-7.

[188] Legué F, Guitton N, Brouazin-Jousseaume V, Colleu-Durel S, Nourgalieva K, Chenal C. IL-6 a key cytokine in in vitro and in vivo response of Sertoli cells to external gamma irradiation. Cytokine 2001; 16(6): 232-8.

[189] Van Der Meeren A, Squiban C, Gourmelon P, Lafont H, Gaugler MH. Differential regulation by IL-4 and IL-10 of radiation-induced IL-6 and IL-8 production and ICAM-1 expression by human endothelial cells. Cytokine 1999; 11(11): 831-8.

[190] Kennedy M, Kim KH, Harten B, *et al.* Ultraviolet irradiation induces the production of multiple cytokines by human corneal cells. Invest Ophthalmol Vis Sci 1997; 38(12): 2483-91.

[191] Neta R, Perlstein R, Vogel SN, Ledney GD, Abrams J. Role of interleukin 6 (IL-6) in protection from lethal irradiation and in endocrine responses to IL-1 and tumor necrosis factor. J Exp Med 1992; 175(3): 689-94.

[192] Neta R, Douches S, Oppenheim JJ. Interleukin 1 is a radioprotector. J Immunol 1986; 136(7): 2483-5.

[193] Neta R, Vogel SN, Sipe JD, Wong GG, Nordan RP. Comparison of in vivo effects of human recombinant IL 1 and human recombinant IL 6 in mice. Lymphokine Res 1988; 7(4): 403-12.

[194] Patchen ML, MacVittie TJ, Williams JL, Schwartz GN, Souza LM. Administration of interleukin-6 stimulates multilineage hematopoiesis and accelerates recovery from radiation-induced hematopoietic depression. Blood 1991; 77(3): 472-80.

[195] Gerard N, Syed V, Jegou B. Lipopolysaccharide, latex beads and residual bodies are potent activators of Sertoli cell interleukin-1 alpha production. Biochem Biophys Res Commun 1992; 185(1): 154-61.

[196] Syed V, Gerard N, Kaipia A, Bardin CW, Parvinen M, Jegou B. Identification, ontogeny, and regulation of an interleukin-6-like factor in the rat seminiferous tubule. Endocrinology 1993; 132(1): 293-9.

[197] Guitton N, Brouazin-Jousseaume V, Dupaix A, Jegou B, Chenal C. Radiation effect on rat Sertoli cell function in vitro and in vivo. Int J Radiat Biol 1999; 75(3): 327-33.

[198] Brouazin-Jousseaume V, Guitton N, Legue F, Chenal C. GSH level and IL-6 production increased in Sertoli cells and astrocytes after gamma irradiation. Anticancer Res 2002; 22(1a): 257-62.

[199] Okuda Y, Morris P. Identification of interleukin-6 receptor (IL-6R) mRNA in isolated Sertoli and Leydig cells: regulation by gonadotropin and interleukins in vitro. Endocrine 1994; 2: 1163-8.

[200] Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood 1991; 78(11): 2791-808.

[201] Shimamura M, Kobayashi Y, Yuo A, *et al.* Effect of human recombinant granulocyte colony-stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood 1987; 69(1): 353-5.

[202] Uckun FM, Souza L, Waddick KG, Wick M, Song CW. In vivo radioprotective effects of recombinant human granulocyte colony-stimulating factor in lethally irradiated mice. Blood 1990; 75(3): 638-45.

[203] Schmidberger H, Hess CF, Hoffmann W, Reuss-Borst MA, Bamberg M. Granulocyte colony-stimulating factor treatment of leucopenia during fractionated radiotherapy. Eur J Cancer 1993; 29A(14): 1927-31.

[204] Herodin F, Drouet M. Cytokine-based treatment of accidentally irradiated victims and new approaches. Exp Hematol 2005; 33(10): 1071-80.

[205] Lan X, Qu H, Yao W, Zhang C. Granulocyte-colony stimulating factor inhibits neuronal apoptosis in a rat model of diabetic cerebral ischemia. Tohoku J Exp Med 2008; 216(2): 117-26.

[206] Kudo T, Matsumoto T, Nakamichi I, *et al.* Recombinant human granulocyte colony-stimulating factor reduces colonic epithelial cell apoptosis and ameliorates murine dextran sulfate sodium-induced colitis. Scand J Gastroenterol 2008; 43(6): 689-97.

[207] Hiraumi Y, Iwai-Kanai E, Baba S, *et al.* Granulocyte colony-stimulating factor protects cardiac mitochondria in the early phase of cardiac injury. Am J Physiol Heart Circ Physiol 2009; 296(3): H823-32.

[208] Kim J, Lee S, Jeon B, Jang W, Moon C, Kim S. Protection of spermatogenesis against gamma ray-induced damage by granulocyte colony-stimulating factor in mice. Andrologia 2011; 43(2): 87-93.

[209] Kim JS, Yang M, Lee CG, Kim SD, Kim JK, Yang K. In vitro and in vivo protective effects of granulocyte colony-stimulating factor against radiation-induced intestinal injury. Arch Pharm Res 2013; 36(10): 1252-61.

[210] Marques R, Maia CJ, Vaz C, Correia S, Socorro S. The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease. Cell Mol Life Sci 2014; 71(1): 93-111.

[211] Maia CJ, Santos CR, Schmitt F, Socorro S. Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17beta-estradiol. Mol Cell Biochem 2008; 311(1-2): 81-6.

[212] Laurentino SS, Correia S, Cavaco JE, *et al.* Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction 2011; 142(3): 447-56.

[213] Vaz CV, Maia CJ, Marques R, *et al.* Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways. Prostate 2014; 74(12): 1189-98.

[214] Marques R, Vaz CV, Maia CJ, *et al.* Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis. Exp Cell Res 2015; 330(2): 325-35.

[215] Nakagawa T, Yamaguchi M. Overexpression of regucalcin suppresses apoptotic cell death in cloned normal rat kidney proximal tubular epithelial NRK52E cells: change in apoptosis-related gene expression. J Cell Biochem 2005; 96(6): 1274-85.
[216] Izumi T, Yamaguchi M. Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. J Cell Biochem 2004; 92(2): 296-306.
[217] Yamaguchi M. Role of regucalcin in cell nuclear regulation: involvement as a transcription factor. Cell Tissue Res 2013; 354(2): 331-41.
[218] Yamaguchi M. The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis 2013; 18(10): 1145-53.
[219] Yamaguchi M. Regucalcin and cell regulation: role as a suppressor protein in signal transduction. Mol Cell Biochem 2011; 353(1-2): 101-37.
[220] Laurentino SS, Correia S, Cavaco JE, *et al*. Regucalcin, a calcium-binding protein with a role in male reproduction? Mol Hum Reprod 2012; 18(4): 161-70.
[221] Correia S, Oliveira PF, Guerreiro PM, *et al*. Sperm parameters and epididymis function in transgenic rats overexpressing the Ca2+-binding protein regucalcin: a hidden role for Ca2+ in sperm maturation? Mol Hum Reprod 2013; 19(9): 581-9.
[222] Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis. J Appl Toxicol 2016; doi: 10.1002/jat.3333. [Epub ahead of print].
[223] Handa S, Maruyama N, Ishigami A. Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. Biol Pharm Bull 2009; 32(10): 1645-8.
[224] Ichikawa E, Yamaguchi M. Regucalcin increases superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats. Int J Mol Med 2004; 14(4): 691-5.
[225] Fukaya Y, Yamaguchi M. Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull 2004; 27(9): 1444-6.
[226] Yamaguchi M, Kobayashi M, Uchiyama S. Suppressive effect of regucalcin on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells. J Cell Biochem 2005; 96(3): 543-54.
[227] Ma ZJ, Yamaguchi M. Regulatory effect of regucalcin on nitric oxide synthase activity in rat kidney cortex cytosol: Role of endogenous regucalcin in transgenic rats. Int J Mol Med 2003; 12(2): 201-6.
[228] Yamaguchi M, Takahashi H, Tsurusaki Y. Suppressive role of endogenous regucalcin in the enhancement of nitric oxide synthase activity in liver cytosol of normal and regucalcin transgenic rats. J Cell Biochem 2003; 88(6): 1226-34.
[229] Ma ZJ, Yamaguchi M. Suppressive role of endogenous regucalcin in the regulation of nitric oxide synthase activity in heart muscle cytosol of normal and regucalcin transgenic rats. Int J Mol Med 2002; 10(6): 761-6.
[230] Vaz CV, Marques R, Maia CJ, Socorro S. Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin. Transl Res 2015; 166(6): 693-705.
[231] Sato T, Seyama K, Sato Y, *et al*. Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. Am J Respir Crit Care Med 2006; 174(5): 530-7.
[232] Kondo Y, Sasaki T, Sato Y, *et al*. Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice. Biochem Biophys Res Commun 2008; 377(1): 291-6.
[233] Sato Y, Kajiyama S, Amano A, *et al*. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. Biochem Biophys Res Commun 2008; 375(3): 346-50.
[234] Son TG, Zou Y, Jung KJ, *et al*. SMP30 deficiency causes increased oxidative stress in brain. Mech Ageing Dev 2006; 127(5): 451-7.
[235] Son TG, Kim SJ, Kim K, Kim MS, Chung HY, Lee J. Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress. Arch Pharm Res 2008; 31(7): 872-7.

Appendix III

Chapter

THE HORIZONS OF REGUCALCIN IN MALE REPRODUCTION

S. Correia[1,#], H. Pillai[2,#], A. M. S. Silva[1], V. M. Santos[1] and S. Socorro[1,*]

[1]CICS-UBI - Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

[2]Animal Husbandry Department, Government of Kerala, IndiaName, City, State

ABSTRACT

Regucalcin (RGN) is a highly conserved calcium (Ca^{2+})-binding protein, present throughout the evolution line, from prokaryotes to eukaryotes. It is highly expressed in the liver and kidney of mammalian vertebrates, but its broad expression in a panoply of other tissues has been reported. Over the years, the RGN role in the regulation of several biological processes apart from Ca^{2+} homeostasis also was being disclosed. RGN was shown to be a multifunctional protein involved in the regulation of intracellular signaling pathways, oxidative stress, cell proliferation, apoptosis, and also energetic metabolism. All these are crucial processes for the successful production of male gametes, which propelled researchers investigating the RGN role in male reproduction. The present chapter systematically describes the existent literature on the actions of RGN supporting spermatogenesis and sperm function, and also discusses the recent experimental advances that sustain the usefulness of this protein in reproductive technology.

* Corresponding Author address: CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal
Email: ssocorro@fcsaude.ubi.pt

Keywords: regucalcin, reproduction, male fertility, spermatogenesis, sperm capacitation, cryopreservation, sex-steroid target gene, calcium, oxidative stress, apoptosis

INTRODUCTION

Regucalcin (RGN) is a calcium (Ca^{2+})-binding protein highly conserved throughout evolution, being present from prokaryotes to the different levels of complexity of eukaryotes (Misawa et al. 2000; Shimokawa et al. 1995a; Goto 2000; Fujita et al. 1996b; Fujita et al. 1992; Maruyama et al. 2010; Nikapitiya et al. 2008; Wu et al. 2008; Nakajima et al. 2000; Gomi et al. 2002). Its role in the regulation of intracellular Ca^{2+} homeostasis has been shown to occur through the modulation Ca^{2+}-pumps activity at plasma membrane, endoplasmic reticulum and mitochondria (Yamaguchi 2000, 2005). However, RGN ability to bind other divalent cations also has been reported (Shimokawa et al. 1993; Chakraborti et al. 2010).

The full-length RGN protein has 299 amino acid residues and an approximate molecular weight of 33-34 kDa, which together with a characteristic downregulation with aging led some authors naming it Senescence Marker Protein-30 (SMP-30) (Shimokawa et al. 1993; Fujita et al. 1992; Fujita et al. 1996a).

Although being highly expressed in the liver and kidney cortex, RGN expression has been detected in a broad range of tissues and body fluids of several vertebrate and invertebrate species (Marques et al. 2014). Since 2008, this has encompassed the characterization of RGN expression in the male reproductive tract, including, the gonads, accessory glands, excurrent ducts fluids and spermatozoa (Maia et al. 2008; Laurentino et al. 2011b; Pillai et al. 2017c; Pillai et al. 2017b; Correia et al. 2013).

Also, a panoply of factors has been shown to regulate RGN tissue expression levels, namely, Ca^{2+}, glucose levels, oxidative stress, insulin, thyroid and parathyroid hormones, and steroid hormones (Vaz et al. 2016a; Marques et al. 2014). Moreover, RGN expression is modulated in response to different physiological conditions and diseases states (Son et al. 2009; Doran et al. 2006; Lv et al. 2008; Jung et al. 2006; van Dijk et al. 2012; Jeong et al. 2008).

The last decade has witnessed the emergence of RGN as a multifunctional protein regulating distinct biological processes besides the maintenance of Ca^{2+} homeostasis. For example, RGN has been shown to regulate intracellular signaling pathways by influencing the activity of kinases, phosphatases, phosphodiesterase, and proteases (Yamaguchi 2005; Fukaya et al. 2004). Several reports have been presented concerning the antioxidant properties of RGN, which described its influence diminishing oxidative stress, increasing the antioxidant defense, and

protecting from age deterioration of cell function (Marques et al. 2014; Vaz et al. 2015b). Interestingly, accumulating evidence has placed RGN as an important regulator of tissues homeostasis by its dual action controlling proliferation and apoptosis (Yamaguchi 2013; Vaz et al. 2015b; Vaz et al. 2014; Marques et al. 2015). Therefore, its role as a cytoprotector and anti-tumor protein has been proposed (Vaz et al. 2016a; Silva et al. 2016a; Yamaguchi 2015). More recently, the properties of RGN modulating cell metabolism started being disclosed and have been related to the control of glycolytic metabolism and lipid handling (Yamaguchi 2010; Vaz et al. 2016b).

The strict control of oxidative stress, the delicate balance between cell death and proliferation, the protection from damaging exogenous factors, as well, as the proper metabolic support to ensure successful germ cell development, are determinant issues for successful spermatogenesis and, thus, male fertility. Therefore, in the last years, the study of RGN in the context of male reproduction has deserved the attention of some research groups.

THE EVOLUTIONARY HISTORY OF RGN

The RGN protein has been identified in a large number of species, being present in invertebrates, mammalian and nonmammalian vertebrates, as well as in fungi and bacteria (Misawa et al. 2000; Shimokawa et al. 1995a; Goto 2000; Fujita et al. 1996b; Fujita et al. 1992; Maruyama et al. 2010; Nikapitiya et al. 2008; Wu et al. 2008; Nakajima et al. 2000; Gomi et al. 2002). To disclose the peculiarities of RGN protein structure and its functional domains, researchers used protein sequence alignment tools and determined the amino acid identities among RGN's sequences from several species (Laurentino et al. 2012; Marques et al. 2014; Shimokawa et al. 1993). The RGN gene is highly conserved from eukaryotes to prokaryotes, and 18 % of residues in RGN protein are conserved in all species (Laurentino et al. 2012). Overall, the amino acid sequence of the human RGN protein (299 residues long, 34 kDa) shows 98% of similarity with other primates, 93–96% with other mammalian species and 79–85% with non-mammalian vertebrates (reviewed by (Marques et al. 2014)). Moreover, human RGN shows 43 to 47% of similarity with invertebrates, namely bacteria and fungi (Marques et al. 2014), which is amazing considering the extremely high difference of organisms' complexity being compared. These findings represent irrefutable evidence about the importance of RGN since the very beginning of life and reinforced the idea that

it should play a relevant basic cellular function well-conserved throughout evolution (Marques et al. 2014).

Motif analysis showed that RGN do not has the typical EF-hand Ca^{2+}-binding sequence but the crystal structure of the human protein, solved by X-ray diffraction (Chakraborti et al. 2010), UV difference, fluorescent emission and circular dichroism studies (Yamaguchi 1988; Harikrishna et al. 2017) confirmed binding of Ca^{2+} ions. Nevertheless, X-ray diffraction together with mutagenesis analysis, also showed that RGN could also bind other divalent cations such as zinc, manganese and magnesium (Shimokawa et al. 1993; Chakraborti et al. 2010). In addition, RGN was shown to have enzymatic activity functioning as a gluconolactonase, an enzyme that catalyzes the penultimate step in the biosynthesis of L-ascorbic acid (Kondo et al. 2006).

RGN is a protein with a wide tissue distribution including the male reproductive tract (discussed in sections 4 and 5). Also, it has a vast distribution within the distinct cell compartments, being detected in the cytoplasm (Ishigami et al. 2003; Maia et al. 2008; Tsurusaki et al. 2000; Morooka et al. 2002; Tobisawa et al. 2003; Ichikawa et al. 2004a; Arun et al. 2011; Laurentino et al. 2011b), mitochondrial fractions (Arun et al. 2011) and peri-nuclear space, as well as, in the nucleus (Omura et al. 1999; Ishigami et al. 2003; Maia et al. 2008; Tsurusaki et al. 2000; Morooka et al. 2002; Tobisawa et al. 2003; Arun et al. 2011; Nakagawa et al. 2006; Nakagawa et al. 2008; Maia et al. 2009; Vaz et al. 2015a; Morooka et al. 2001; Laurentino et al. 2011b). Although several reports have shown the presence of RGN in the nucleus, no study has demonstrated the existence of a functional nuclear localization signal (NLS) in RGN protein sequence. Nevertheless, analysis of the first 60 N-terminal amino acid residues of mammalian RGN proteins (Fig. 1) allowed to identify an importin α-dependent putative NLS. Noteworthy, ~72% (23 of 32) of the residues of the putative NLS (Fig. 1) are 100% conserved in all species analyzed.

The RGN gene is localized in the q11.1-12 and p11.3-q11.2 segments of the rat and human X chromosomes, respectively (Shimokawa et al. 1995b; Fujita et al. 1995). Besides the full-length mRNA encoding the 299 residue RGN protein, two alternatively spliced mRNA variants, originated by an exon skipping mechanism, have been described. RGNΔ4 transcript has a deletion of exon 4, and the RGNΔ4,5 transcript lacks exons 4 and 5, which would likely correspond to the RGN lower molecular protein variants that have been identified (28 and 24 kDa) (Arun et al. 2011). These mRNA transcripts have been identified in several human tissues, namely, breast, prostate and testis (Maia et al. 2009; Laurentino et al. 2012), which suggests that they are functionally relevant, but their biological role remains to be identified.

The fact that the RGN protein is encoded by an X-linked gene is very interesting when envisaging its liaison with male reproduction. Y chromosome was initially thought as playing the major role in the regulation of male fertility. However, the enthusiastic efforts of scientific community allowed to discover that several genes located on X chromosome can modulate fertility (Shamsi et al. 2011). This is, for example, the emblematic case of androgen receptor (Brown et al. 1989; Ferlin et al. 2006; L. et al. 2017). In fact, over the years it has been unrevealed that the maternal X chromosome is enriched in genes with high testicular expression, which play important roles in the regulation of spermatogenesis (Stouffs et al. 2009; Zheng et al. 2010). Genetic studies have shown that several X-linked genes are implicated in the control of meiosis, playing critical functions in male germ cell maturation (Zheng et al. 2010). In fact, the X chromosome has been considered of particular interest in the study of human infertility attributed to the male factor (Zheng et al. 2010). Since males possess only one X chromosome, mutations in the single-copy of an X-linked gene would not be camouflaged by the normal allele and, therefore, would manifest. For this reason, X-linked genes, such as the case of RGN, would be especially important in male fertility and could give useful information into the clinical practice.

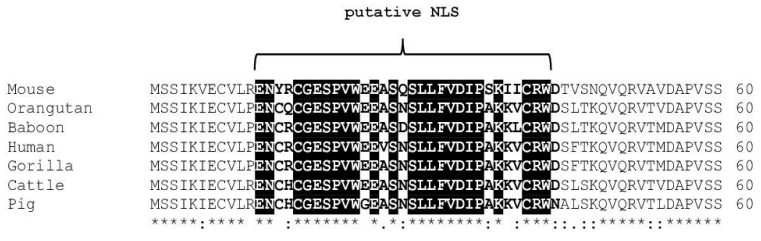

Figure 1. Multiple sequence alignment of the first 60 N-terminal amino acid residues of mammalian RGN proteins showing a putative importin α-dependent nuclear localization signal (NLS) predicted using the cNLS Mapper (Kosugi et al. 2009). The putative NLS was obtained with a score of 4 that is typical of a protein localized to both the nucleus and the cytoplasm. Alignment was produced with Clustal Omega alignment tool (Sievers et al. 2011). Black shading indicates residues of the putative NLS 100% conserved in all species. Latin names and Genbank accession numbers for corresponding sequences were as follows: mouse, *Mus musculus* (NP_033086.1); orangutan, *Pongo abelii* (NP_001127502.1); baboon, *Papio anubis* (XP_003917675.1); human, *Homo sapiens* (NP_004674.1); gorilla, *Gorilla gorilla* (XP_018874621); cattle, *Bos taurus* (NP_776382.1); pig, *Sus scrofa* (NP_001070688.1).

EVIDENCE OF RGN AS A SEX STEROID TARGET GENE

Hormone regulation and the complex interaction between hypothalamic, pituitary and gonadal hormones are crucial for mammalian reproductive function (Holdcraft et al. 2004). The steroid hormones produced by the gonads are normally referred to as sex steroid hormones because of their primary role in sex differentiation, gonadal function and germ cell development (Holdcraft et al. 2004). Androgens are recognized as the main regulators of spermatogenesis with their actions being fundamental for germ cells survival, maturation and sperm production (Walker 2009; Walker et al. 2005; Zhou 2010) whereas also exerting negative feedback on the hypothalamus and pituitary regulating the spermatogenic output (Verhoeven et al. 2010). Though estrogens' roles in spermatogenesis are not so clear, it is widely known that both somatic and germ cells express nuclear and membrane estrogens receptors (Oliveira et al. 2014; Cavaco et al. 2009), and that testicular cells are capable of synthesizing estrogens (Correia et al. 2015). Moreover, a physiological role for estrogens in male fertility has been proposed as regulators of germ cell survival and apoptosis (Correia et al. 2015; Correia et al. 2014b).

Sex steroid hormones, androgens and estrogens, exert their biological actions by interaction with androgen and estrogen receptors, respectively. This class of hormone receptors belong to the superfamily of nuclear transcription factors regulating gene expression network in cells and tissues dependently on environmental conditions and developmental stage (Hewitt et al. 2018; Pinto et al. 2014). Since the emergence of modern molecular biology techniques in the 80s-90s years, the structure and function of steroid receptors, as well as, their molecular targets and tissue expression patterns, have been characterized (e.g. (Figueira et al. 2018; Socorro et al. 2000; Correia et al. 2015; Oliveira et al. 2014)). In the reproduction field, the identification of new steroid target genes helped to clarify the molecular basis of spermatogenesis and the relevance of specific players for male fertility (e.g. (Laurentino et al. 2011b; Laurentino et al. 2011a; Correia et al. 2014b; Correia et al. 2015; Correia et al. 2017)). With this rationale, several studies aimed to investigate the role of steroid hormones regulating RGN expression levels in reproductive tissues. First evidence showed that RGN is a target of estrogens. Subcutaneous administration of 17β-estradiol (E_2) augmented RGN mRNA expression in the rat liver (Yamaguchi et al. 1995), whereas producing the opposite effect in the kidney (Kurota et al. 1996), mammary gland and prostate (Maia et al. 2008). Also, in bovine bulbourethral glands and prostate, E_2 administration lead to decreased RGN expression (Starvaggi Cucuzza et al. 2014). Interestingly, E_2-

treated MCF-7 breast cancer cells displayed up-regulated expression of RGN for 6 and 12 h of stimulation, but longer exposure times lead to a diminished expression of RGN (Maia et al. 2009), in accordance with the results in the rat mammary gland in vivo after 7 days of treatment (Maia et al. 2008).

The action of estrogens regulating RGN expression in the testis was assessed using the ex vivo culture of rat seminiferous tubules. E_2-treatment (100 nM) for 24 hours augmented RGN expression in intratubular testicular cells, which was suggested to be a response mechanism to counteract the enhanced apoptosis driven by estrogens (Correia 2014). Contrastingly, a study in bovines demonstrated that in vivo treatment (190 mg E_2/animal/day) caused down-regulation of RGN expression in the testis (58).

Concerning the regulation by androgens, orchidectomized rats displayed reduced RGN expression in the kidney that was increased after testosterone replacement (Lin et al. 2016). However, regarding reproductive tissues, orchidectomy increased RGN expression in rat prostate that was down-regulated after 5α-dihydrotestosterone (DHT) administration (Vaz et al. 2014). Accordingly, treatment of LNCaP prostate cancer cells with DHT diminished RGN expression, an effect that seemed to be mediated by the androgen receptor involving de novo protein synthesis (Maia et al. 2009). The androgen receptor was also implicated on the effect of DHT (10-7 M) up-regulating RGN expression in rat seminiferous tubules cultured ex vivo (Laurentino et al. 2011b). This was the first evidence confirming RGN as an androgen target gene. However, it was not followed by in vivo findings. In bovines, testosterone administration (1.050 g/animal/day) decreased RGN expression in the testis (Starvaggi Cucuzza et al. 2014).

Overall, the existent literature has established RGN both as an estrogen- and androgen-target gene. However, sex steroid hormones actions modulating RGN expression levels seem to be tissue- and species-specific, and time-dependent, which might be due to specificities in the interaction with the DNA or differential recruitment of estrogens and androgen receptors co-regulators. Moreover, changes in RGN expression in response to steroids were suggested to be related to the suppression of germ cells apoptosis with RGN mediating these hormones' functions as survival factors in spermatogenesis.

Androgens and estrogens have been proposed to increase intracellular Ca^{2+} levels in several cell types (Gorczynska et al. 1995; Audy et al. 1996; Picotto et al. 1999; Azenabor et al. 2001; Sun et al. 2006). Thus, considering the influence of RGN over Ca^{2+} handling (Correia et al. 2013; Yamaguchi 2000) and the importance of this ion in spermatogenesis and sperm function it would be relevant to explore the relationship between sex steroid hormones, RGN and intracellular Ca^{2+} levels in reproductive tissues.

EXPRESSION PATTERN OF RGN IN MALE REPRODUCTIVE TRACT AND SPERMATOZOA

The first indication about the association of RGN with reproduction came in 2004 when the RGN gene was shown to be expressed in the bovine ovarian follicle and reported to be associated with follicular growth and dominance. An 8-fold higher expression level was described in dominant follicles compared to that of small follicles (Fayad et al. 2004), which was the very early indication of the supportive role of RGN for germ cells. Later, *RGN* mRNA was identified in mice testis (Mori et al. 2004), and the first evidence of RGN protein expression in a broad range of male reproductive organs was reported for rat and human by Sílvia Socorro group in 2011 (Laurentino et al. 2011b). These findings were followed by the characterization of RGN expression in the testis, epididymis, prostate and seminal vesicles of other mammalian species, namely, buffalo and bovines. Table 1 summarizes the outcomes from the main studies reporting RGN expression in male reproductive organs and spermatozoa. In the testis, RGN was shown to be localized in the endocrine Leydig cells, in the intratubular somatic cells, the Sertoli cells, as well as in the germline (Laurentino et al. 2011b).

Pillai et al. (2017c) were pioneer describing the presence of RGN in spermatozoa by localizing the protein to the acrosome region of buffalo sperm. Acrosome is the major Ca^{2+}-storage organelle of spermatozoa, and the localization of RGN in the acrosomal region propelled researches to focus on finding out this protein role in Ca^{2+}-related functions like capacitation, acrosomal reaction and membrane fusion (to be detailed in section 7). Apart from the three reported isoforms of RGN (34 kDa, 28 kDa and 24 kDa) (Arun et al. 2011), two new isoforms of 48 kDa and 44 kDa were reported in sperm (Pillai et al. 2017c). The same study also showed the association of RGN to spermatozoa membrane and its relocation from the cytosol to the acrosomal region during maturation, i.e. from testicular spermatozoa to ejaculated spermatozoa.

Moreover, RGN has been identified as a secreted protein being detected in several fluids of male reproductive tract, namely, in seminiferous tubules, epididymis, and seminal vesicles fluids (Lin et al. 2016; Pillai et al. 2017b; Correia et al. 2013).

Table 1. RGN expression in tissues and cells of male reproductive tract.

Tissue	Species	Localization	Biological form	Reference
Testis	Human	Cytoplasm and some nuclei of Leydig cells, Sertoli cells and germ cells	mRNA and Protein	(Laurentino et al. 2011b)
	Bovine	Cytoplasm of Leydig cells and weak staining of nuclei of some spermatogonia	mRNA and Protein	(Starvaggi Cucuzza et al. 2014)
	Buffalo	Cytoplasm and nucleus of Sertoli cells and germ cells	mRNA and Protein	(Pillai et al. 2017b)
	Rat	Cytoplasm and some nuclei of Leydig cells, Sertoli cells and germ cells	mRNA and Protein	(Laurentino et al. 2011b)
	Mice	-	mRNA	(Mori et al. 2004)
Seminal vesicles	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	(Pillai et al. 2017b)
	Rat	Epithelium	mRNA and Protein	(Laurentino et al. 2011b)
Epididymis	Buffalo	Cytoplasm and nuclei of glandular epithelial cells, luminal secretion and interstitial tissue	mRNA and Protein	(Pillai et al. 2017b)
	Rat	Epithelium, smooth muscles and connective tissue	mRNA and Protein	(Laurentino et al. 2011b)
Prostate	Bovine	Cytoplasm of glandular epithelial cells		(Starvaggi Cucuzza et al. 2014)
	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	(Pillai et al. 2017b)
	Rat	Epithelium	mRNA and Protein	(Laurentino et al. 2011b)
Bulbo-urethral glands	Bovine	Nuclei of glandular cells	mRNA and Protein	(Starvaggi Cucuzza et al. 2014)
	Buffalo	Cytoplasm and nuclei of glandular epithelial cells	mRNA and Protein	(Pillai et al. 2017b)
Ampulla of vas deferens	Buffalo	Nuclei of glandular epithelium and interstitial tissue	mRNA and Protein	(Pillai et al. 2017b)
Spermatozoa	Buffalo	Acrosome	mRNA and Protein	(Pillai et al. 2017c)

RGN Expression in Distinct Phenotypes of Human Spermatogenesis

As a clue to understand the liaison of RGN to male reproduction, its expression pattern in human testis with normal (Table 1) and abnormal phenotypes of spermatogenesis was investigated. RGN expression levels were analyzed in cases of hypospermatognesis (HP) and Sertoli cell-only syndrome in comparison with those of testis with conserved spermatogenesis. Increased expression of RGN was found in the testis of men with HP comparatively with the cases of obstructive azoospermia with conserved spermatogenesis and Sertoli cell-only syndrome (Laurentino et al. 2012).

The aetiology of HP is complex and not entirely known, but it has been established that the development and maintenance of successful spermatogenesis depend on a delicate balance between germ cell proliferation and apoptosis (Print et al. 2000). Indeed, augmented rates of apoptosis and altered expression patterns of a panoply of cell death regulators have been detected in the testes of subfertile and infertile men (Lin et al. 1997; Takagi et al. 2001; Bozec et al. 2008). Moreover, accelerated apoptosis has been implicated as a cause for the decreased number of spermatogonia in HP cases, rather than proliferative dysfunction in the mitotic phase (Takagi et al. 2001).

Presently, no definitive conclusion can be drawn for the increased expression levels of RGN in HP cases. However, it is liable to speculate that RGN may be acting as a protective molecule counteracting the augmented rates of apoptosis associated with disrupted spermatogenesis. A dual role for RGN controlling cell cycle and apoptosis has been proposed (Marques et al. 2014; Vaz et al. 2016a), with RGN expression being increased in response to the induction of apoptosis by chemical or physical agents (Correia et al. 2017; Correia et al. 2014a; Silva et al. 2016b). Thus, the equilibrium of RGN expression levels would be determinant for the maintenance of the appropriate germ cell lineage number and, consequently, for successful spermatogenesis. The RGN' roles modulating apoptosis and as a protective molecule for male germ cells have been investigated in recent years and will be discussed further in the following sections.

RGN'S INFLUENCE IN MALE REPRODUCTION: WHAT WE HAVE LEARNED FROM THE TRANSGENIC RAT MODEL

After the description of Ca^{2+}-binding protein RGN in several tissues of male reproductive tract, including testis (Laurentino et al. 2011b), and its identification as an estrogen- and androgen-target gene (Maia et al. 2008; Maia et al. 2009; Laurentino et al. 2011b), the potential role of this protein in testicular function has been exploited. A substantial body of evidence has been produced using a transgenic rat model overexpressing RGN (Tg-RGN), which was originally generated by Yamaguchi M (2002) by means of oocyte transgene pronuclear injection. These animals are fertile and their spermatogenic output was first investigated by Correia et al. (2013). Quantitative and qualitative sperm parameters, as well as the morphology and function of epididymis, were compared between Tg-RGN and their wild-type (Wt) littermates. Although able to breed, Tg-RGN animals displayed lower sperm counts and reduced sperm motility (Table 2). However, these features seem to be counterbalanced by the higher viability and diminished incidence of morphological defects (Table 2) found in the epididymal sperm of Tg-RGN rats (Correia et al. 2013).

Table 2. Sperm parameters in the Tg-RGN rats in comparison with their Wt littermates.

*- Published in (Correia et al. 2013); Values represent mean ± SEM.

Sperm parameters*	Tg-RGN	Wt	p-value
Sperm counts	$1.28x10^8 \pm 9.24x10^6$	$1.72x10^8 \pm 1.57x10^7$	*P<0.05*
Motility	47.88% ± 3.67	64.60% ± 5.66	*P<0.05*
Viability	38.75% ± 2.36	28.00% ± 3.84	*P<0.05*
Normal morphology	74.13% ± 3.74	57.58 % ± 1.76	P<*0.01*
Tail defects	18.60 % ± 2.60	36.18 % ± 2.04	P<*0.001*

It is through the passage in the epididymis, specifically in the order *caput*, *corpus* and *cauda*, and before being released in *vas deferens*, that sperm acquire the ability to move progressively and fertilize an oocyte (Gervasi et al. 2017). The unique microenvironment of the epididymal lumen is mainly maintained by the secretory activity of the *caput* region (Dacheux et al. 2009). The *corpus*' secretory

activity is lower, playing a role in late sperm maturation events whereas *cauda* essentially stores the functionally mature sperm (Robaire et al. 2006). RGN expression was two-fold higher in the *corpus* relatively to *caput* and *cauda* regions, which emphasizes its importance for sperm maturation.

Interestingly, an altered morphology of *caput* was found in the epididymis of Tg-RGN rats. Although there were no differences in the tubule area, boundwidth, boundheight, and perimeter between Wt and Tg-RGN groups, the epithelial cell height of this region was significantly decreased in Tg-RGN (Correia et al. 2013). The altered morphology indicated important alterations in the reabsorptive/secretory activity of this epididymis region that could have an impact in sperm parameters. Indeed, diminished capacity of Ca^{2+} influx was detected in the epididymis of Tg-RGN rats, which suggested that Ca^{2+} concentrations are augmented in the epididymal fluid and that might be responsible for the reduced sperm motility observed in these animals (Correia et al. 2013).

Moreover, epididymal tissues of Tg-RGN animals showed a higher antioxidant potential compared with Wt (Correia et al. 2013). Oxidative damage is one of the main factors leading to male germ cells death and increased incidence of defects (Aitken et al. 2011). It is also known that RGN has been linked with the decreased generation of reactive oxygen species and increased activity of antioxidant defense systems in several cell types, including liver, lung, heart and brain cells (Fukaya et al. 2004; Handa et al. 2009; Ichikawa et al. 2004b; Son et al. 2006; Sato et al. 2006). This led the authors to assume that the higher sperm viability and the diminished incidence of morphological defects exhibited by Tg-RGN animals (Table 2) may be a consequence of the RGN' role protecting sperm from oxidative stress.

In this way, the antioxidant role of RGN in testicular cells was deepened in further research by exposing seminiferous tubules of Tg-RGN rats and controls to pro-oxidant stimuli, namely, tert-butyl hydroperoxide and cadmium chloride (Correia et al. 2017). As hypothesized, Tg-RGN animals displayed increased protection against oxidative damage, exhibiting lower levels of oxidative stress demonstrated by diminished lipid peroxidation levels (Correia et al. 2017). Also, antioxidant defenses, like glutathione S-transferase, were augmented in the Tg-RGN when exposed to oxidant conditions (Correia et al. 2017). As expected, the lower oxidative damage in the seminiferous tubules of Tg-RGN rats was translated in reduced rates of apoptotic cell death (Correia et al. 2017).

Germ cells are highly sensitive to endogenous and exogenous damaging factors and, as discussed previously in section 5, increased apoptosis has been indicated as a cause of male infertility. The anti-apoptotic effect of RGN has been suggested in different cell lines and *in vivo* models involving the activation of multiple pathways and molecular targets, such as Akt, p53, Fas, transforming growth factor-β, tumor

necrosis factor-α, caspases and several Bcl-2 family members (reviewed in (Marques et al. 2014; Vaz et al. 2016a; Yamaguchi 2013). The overexpression of RGN was shown to suppress thapsigargin- and actinomycin D-induced apoptosis in rat seminiferous tubules cultured *ex vivo* by modulating the expression and activity of key apoptotic and antiapoptotic factors (Correia et al. 2014a). Reduced expression and activity of the executioner of apoptosis caspase-3 were observed together with increased expression of p53 and Bcl-2 (Correia et al. 2014a).

In vivo findings also demonstrated the protective role of RGN over apoptosis of the germ line. Tg-RGN animals shown to be resistant to radiation-induced testicular damage displaying lower rates of apoptosis after irradiation (reduced activity of caspase-3, lower levels of caspase-8, and increased Bcl-2/Bax ratio) (Silva et al. 2016b). Moreover, suppressed apoptosis was concomitant with higher testis volume, augmented sperm viability and motility, as well as a higher frequency of normal sperm morphology and diminished incidence of head-defects relatively to Wt counterparts.

Sertoli cells are the somatic cells responsible for providing the germ cells with physical support, as well as, with the adequate supply of growth factors and nutrients, namely, lactate that has been indicated as the preferred energy source for germ cells (Alves et al. 2013). Very recently, the metabolic status of primary Sertoli cells cultures obtained from the testis of Tg-RGN rats was characterized. These cells, though consuming less glucose, produced high levels of lactate, and displayed increased expression of alanine transaminase, and augmented glutamine consumption indicating high plasticity of metabolic routes in response to RGN overexpression (Mateus et al. 2018). Moreover, the lactate produced seems to be consumed by the germ cells with a consequent diminution of apoptotic rate.

In sum, the Tg-RGN model contributed to establish the basis of the RGN' role in male reproduction. Also, it allowed a better understanding of the molecular regulation of spermatogenesis and sperm function opening new avenues of research, as well as, novel perspectives for the development of infertility treatments and contraceptive methods.

PROTECTIVE ROLES OF RGN FOR THE FERTILIZATION CAPACITY OF MAMMALIAN SPERMATOZOA

Anti-oxidant effect

Mammalian spermatozoa are highly susceptible to oxidative damage due to their higher content of polyunsaturated fatty acids. Therefore, the presence of antioxidants in the seminal fluid is very important to maintain the highly sensitive redox equilibrium of spermatozoa and their functionality. Superoxide dismutase, catalase and glutathione peroxidase are the major antioxidant enzymes present in semen playing a relevant role to reduce oxidative stress (Maneesh et al. 2006).

RGN is broadly present in male reproductive tract tissues (Table 1) and secretions till ejaculation (Laurentino et al. 2011b; Pillai et al. 2017b; Correia et al. 2013). After ejaculation RGN is removed from seminal plasma whereas spermatozoa maintain RGN expression localized at the acrosomal region (Pillai et al. 2017b; Pillai et al. 2017c). The antioxidant properties of RGN have been demonstrated by many studies in different cell lines and *in vivo* models (Sato et al. 2008; Kondo et al. 2008; Kim et al. 2013). RGN has been shown to reduce intracellular levels of oxidative stress through modulation of enzymes involved in the generation of free radicals, as well as, in the antioxidant defense (Son et al. 2008; Fukaya et al. 2004; Handa et al. 2009; Ichikawa et al. 2004b). It has been demonstrated that superoxide dismutase activity was enhanced in normal rat liver and heart in the presence of exogenous RGN (Fukaya et al. 2004; Ichikawa et al. 2004b) and the glutathione peroxidase activity was reduced in animals without RGN (Kondo et al. 2008; Sato et al. 2008). Also, RGN overexpression *in vivo* was capable of counteracting aging-associated changes in rat prostate, maintaining low levels of oxidative stress, reducing lipid peroxidation and sustaining high activity levels of glutathione-S-transferase (Vaz et al. 2015b).

Furthermore, RGN has been identified as a gluconolactonase (Kondo et al. 2006), an enzyme involved in the penultimate step of L-ascorbic acid synthesis in the liver. Ascorbic acid, a cofactor in metal-dependent oxygenases (Linster et al. 2007), has been indicated as an important antioxidant in semen that protects spermatozoa during cryopreservation (Paudel et al. 2010). As discussed earlier, a higher antioxidant potential was reported in the epididymis of Tg-RGN rats, which was linked to the higher sperm viability, higher percentage of normal morphology and diminished incidence of tail defects compared to Wt counterparts (Correia et al. 2013). Moreover, the testis of Tg-RGN animals was shown to display increased antioxidant capacity and enhanced activity of glutathione-S-transferase (Correia et

al. 2017). Future research efforts are warranted to disclose the role of RGN as a gluconolactonase and modulating the activity of superoxide dismutase and glutathione peroxidase in the testis and epididymis, as well as, in sperm storage and cryopreservation.

Anti-capacitatory effect

The capacitation process represents the set of biochemical, structural and physiological changes that sperm undergo in the female reproductive tract rendering them able to fertilize an oocyte (reviewed recently in (Puga Molina et al. 2018)). Seminal plasma and its components help to prevent the premature onset of sperm capacitation till ejaculation. Several decapacitation factors like phosphatidylcholine-binding proteins (Desnoyers et al. 1992), semenogelin (de Lamirande et al. 2001), cholesterol (Cross 1996) and zinc (Andrews et al. 1994) are present in seminal plasma. Moreover, the capacitation-associated tyrosine phosphorylation of sperm proteins is also shown to be inhibited by seminal plasma (Tomes et al. 1998).

The decapacitation factors should be removed after ejaculation for the occurrence of capacitation and the subsequent acrosomal reaction, which ultimately allows spermatozoa to fertilize the ovum. The proteolytic enzymes released upon acrosome reaction enable the fusion of sperm and oocyte membranes (Hirohashi et al. 2018). RGN is present in the testis, sperm and throughout male excurrent ducts, including epididymal and vesicular fluids, and disappearing in the ejaculated seminal plasma. These findings indicated the potential anti-capacitatory role of RGN, which was investigated by Pillai et al. (2017b). Co-incubation of recombinant RGN with buffalo seminal plasma confirmed the degradation of protein in the presence of seminal components (Pillai et al. 2017b). Another evidence for the anti-capacitatory property of RGN is that the epididymal spermatozoa of Tg-RGN showed a reduction in motility compared to their Wt counterparts (Correia et al. 2013).

The suppressive effect of RGN on *in vivo* capacitation was further studied in buffalo using the fluorescent cholortetracyclin assay to detect capacitated spermatozoa (Pillai et al. 2017b). Addition of recombinant RGN to capacitating media significantly reduced the percentage of capacitated spermatozoa compared to the untreated group. Overall, gathered information indicating the anti-capacitory role of RGN raises the curiosity about its action in human sperm and the benefits of this protein in assisted reproduction techniques.

Cryoprotective role of RGN in spermatozoa

Cryopreservation of semen and artificial insemination are important procedures that allowed significant benefits to the livestock industry (Bailey et al. 2000), as well as, introduced important advances in human reproduction technology (Hezavehei et al. 2018). Cryopreservation of human sperm started in the 1960s, and over the years it has been noted that the fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm (Said et al. 2005). Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease their fertility potential (Chatterjee et al. 2001). Factors as sudden temperature changes, ice formation and osmotic stress have been proposed as the main reasons for poor sperm quality after cryopreservation and thawing (Hezavehei et al. 2018). In addition, the procedures of cryopreservation induce premature capacitation of spermatozoa, which is known as cryo-capacitation (Bailey et al. 2000). These alterations may not affect motility but reduce viability, the ability to interact with the female reproductive tract and sperm fertility. For example, 8 times more cryopreserved bovine spermatozoa were required to achieve equivalent fertilization rates *in vivo* compared to fresh sperm (reviewed in (Bailey et al. 2000)).

A cytoprotective role has been proposed for RGN by its actions counteracting deregulated proliferation and apoptosis, as well as, minimizing Ca^{2+}-related stress and oxidative damage (Son et al. 2008; Marques et al. 2014). Noteworthy, deregulation of Ca^{2+} levels and abnormal levels of reactive oxidant species are the main cyto-damaging factors during sperm cryopreservation. Most importantly, RGN was reported as a putative cold tolerance gene in *Drosophila montana* (Vesala et al. 2012), which supported the advantageous role of this protein in sperm cryopreservation. Indeed, the cryoprotective role of recombinant RGN in buffalo spermatozoa was recently reported. Supplementation of buffalo semen extenders with 1 µM of recombinant RGN during freezing resulted in significant increases in the post-thaw progressive motility, acrosome integrity, and zona pellucida binding of spermatozoa compared to control conditions without RGN (Pillai et al. 2017a). The ability of RGN to counteract the excessive production of reactive oxygen species and Ca^{2+}stress may be accountable to its cryoprotective effect in spermatozoa.

CONCLUSION

Reports of the anti-apoptotic and anti-proliferative functions, together with the androgenic regulation of RGN expression, first pointed out the role of this protein

in spermatogenesis (Fig. 2). The anti-oxidative properties of RGN, and its capacity suppressing oxidative damage, also have been described and may account for the maintenance of viability and fertility potential of spermatozoa from spermatogenesis until fertilization of ovum, as well as, in sperm cryopreservation (Fig. 2).

Another aspect of RGN' biological actions is related to the control of Ca^{2+} homeostasis, an ion that has a crucial role in sperm function. RGN is reported to reduce intracellular Ca^{2+} concentrations in somatic cells by activation of Ca^{2+}-ATPases in mitochondrial and endoplasmic reticular membranes (Takahashi et al. 1999; Yamaguchi et al. 1989) and Ca^{2+}/Mg^{2+}-ATPase in the plasma membrane (Takahashi et al. 1993). The presence of RGN in spermatozoa cytoplasm and nucleus, as well as, in the acrosome along with its membrane association suggest it might be involved in the efflux of Ca^{2+} thereby reducing intracellular Ca^{2+} levels. Therefore, the anti-capacitatory function indicated for RGN may be due to Ca^{2+}-efflux , as many studies have shown that elevation of sperm intracellular Ca^{2+} (Ca^{2+} influx) is required for hyperactivation, capacitation, and acrosome reaction (Mannowetz et al. 2013; Kwon et al. 2013; Lishko et al. 2010; Suarez 2008; Foresta et al. 1997).

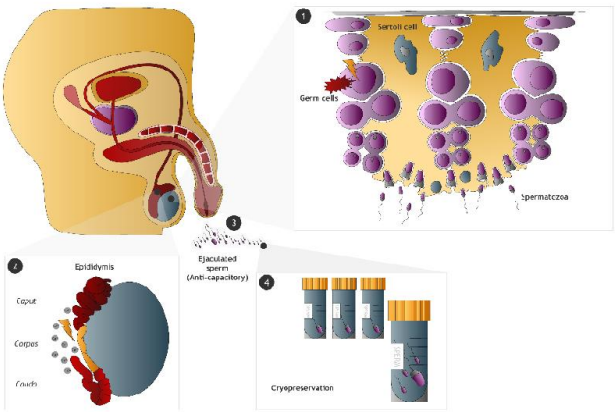

Figure 2. The broad range of RGN actions in the male reproductive tract from spermatogenesis to the fertility potential of spermatozoa. (1) RGN is expressed in testicular cells (both in germ and somatic Sertoli cells) exerting a protective function over apoptosis and oxidative damage of the germline; it also contributes to the high plasticity of Sertoli cells metabolism ensuring the supply of lactate for germ cells. As an epididymal protein (2), RGN is differentially expressed in the caput, corpus and cauda regions, which is related to the protection against oxidative stress and maintenance of Ca2+-levels in the epididymal lumen contributing for

sperm maturation. RGN also is a protein detected in the seminiferous tubules, epididymis, and seminal vesicles fluids, though it is removed from the seminal plasma after ejaculation being maintained in the acrosomal region of ejaculated sperm (3). Moreover, RGN seems to be an anti-capacitory agent having a beneficial role in sperm cryopreservation, preventing premature capacitation/ cryo-capacitation, Ca2+-related stress, and oxidative damage.

Similar to somatic cells, Ca^{2+} entry from the extracellular space as well as its release from intracellular stores are responsible for the increase of intracellular Ca^{2+} concentrations in spermatozoa (Benoff et al. 2007; Breitbart 2002; Darszon et al. 2006; Florman et al. 2008; Publicover et al. 1999). The internal storage of Ca^{2+} in sperm is low as mature cells do not contain endoplasmic reticulum, the major Ca^{2+} storing organelle, and potential areas for Ca^{2+} storing include the acrosome, a redundant nuclear envelope and the mitochondria packed in the midpiece (Costello et al. 2009). Thus, the primary source of Ca^{2+} for spermatozoa is the external environment (Foresta et al. 1997). Moreover, low basal levels of intracellular Ca^{2+} are maintained by Ca^{2+} absorption by mitochondria and active Ca^{2+} extrusion by the Ca^{2+}-pump at plasma membrane (Wennemuth et al. 2003).

In addition, the factors that regulate the function of numerous Ca^{2+}-permeable channels are known to affect male fertility (Lishko et al. 2010; Ren et al. 2010). Disrupted Ca^{2+}-channel or –pump activity can be occurring in sperm cryopreservation, given that thawed bull and human sperm have shown to contain increased intracellular Ca^{2+} levels compared to those before cryopreservation (Bailey et al. 1993; McLaughlin et al. 1994). These findings reflect impaired membrane-selective permeability and/or an inability to maintain physiological concentrations of Ca^{2+} (Bailey et al. 1993; McLaughlin et al. 1994). Moreover, this disruption has consequences, as elevated Ca^{2+} can lead to premature acrosome reaction as well as poor fertility outcomes for the post-thaw sperm. Conversely, the proper regulation of sperm Ca^{2+} channel function can reduce the rate of premature acrosome reaction (Florman et al. 1998). Therefore, it is likely to assume that RGN by regulating Ca^{2+} handling proteins can reduce the intracellular Ca^{2+} and minimize Ca^{2+} stress thereby protecting the spermatozoa from premature capacitation in reproductive ducts as well as from cryo-capacitation during cryopreservation.

Another possibility in preventing premature capacitation/ cryo-capacitation may be due to the Ca^{2+}-binding ability of RGN. Extracellular RGN can bind Ca^{2+} ions and thus reduce intracellular Ca^{2+} by limiting the availability of extracellular Ca^{2+} that would otherwise enter the sperm through the compromised membrane pumps or cation channels. In turn, the degradation of RGN in ejaculated seminal plasma may release the bound Ca^{2+} that can be further used for capacitation. Altogether, it can be concluded that RGN has very important roles in male

reproduction from spermatogenesis to fertilization. Further research would help to clarify the mechanisms underlying RGN actions in sperm physiology.

Although RGN has been assigned to many physiological functions of utmost importance in germ cell development and sperm function, no studies have been reported yet about its properties in the fertilizing ability and cryopreservation of human semen. Understanding of the mechanisms underlying the anti-capacitatory/cryoprotective effects of RGN together with the disclosure of other interacting proteins will help to define the beneficial use of RGN in assisted reproductive technologies.

REFERENCES

Aitken, Robert J, and Benjamin J Curry. 2011. "Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line." *Antioxid Redo Signal* 14 (3):367-381.

Alves, M. G., L. Rato, R. A. Carvalho, P. I. Moreira, S. Socorro, and P. F. Oliveira. 2013. "Hormonal control of Sertoli cell metabolism regulates spermatogenesis." *Cell Mol Life Sci* 70 (5):777-93. doi: 10.1007/s00018-012-1079-1.

Andrews, J. C., J. P. Nolan, R. H. Hammerstedt, and B. D. Bavister. 1994. "Role of zinc during hamster sperm capacitation." *Biol Reprod* 51 (6):1238-47.

Arun, Peethambaran, Vineela Aleti, Kalpana Parikh, Veeraswamy Manne, and Nageswararao Chilukuri. 2011. "Senescence marker protein 30 (SMP30) expression in eukaryotic cells: existence of multiple species and membrane localization." *PLoS ONE* 6 (2):e16545.

Audy, M. C., P. Vacher, and B. Duly. 1996. "17 beta-estradiol stimulates a rapid Ca^{2+} influx in LNCaP human prostate cancer cells." *Eur J Endocrinol* 135 (3):367-73.

Azenabor, A. A., and L. Hoffman-Goetz. 2001. "17 beta-estradiol increases $Ca(^{2+})$ influx and down regulates interleukin-2 receptor in mouse thymocytes." *Biochem Biophys Res Commun* 281 (2):277-81. doi: 10.1006/bbrc.2001.4341.

Bailey, J. L., J. F. Bilodeau, and N. Cormier. 2000. "Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon." *J Androl* 21 (1):1-7.

Bailey, J. L., and M. M. Buhr. 1993. "Ca^{2+} regulation by cryopreserved bull spermatozoa in response to A23187." *Cryobiology* 30 (5):470-81. doi: 10.1006/cryo.1993.1048.

Benoff, S., C. C. Chu, J. L. Marmar, R. Z. Sokol, L. O. Goodwin, and I. R. Hurley. 2007. "Voltage-dependent calcium channels in mammalian spermatozoa revisited." *Front Biosci* 12:1420-49.

Bozec, Aline, Souheila Amara, Basma Guarmit, Jacqueline Selva, Martine Albert, Jacques Rollet, Mohamed El Sirkasi, Francois Vialard, Marc Bailly, and Mohamed Benahmed. 2008. "Status of the executioner step of apoptosis in human with normal spermatogenesis and azoospermia." *Fertil Steril* 90 (5):1723-1731.

Breitbart, Haim. 2002. "Intracellular calcium regulation in sperm capacitation and acrosomal reaction." *Mol Cell Endocrinol* 187 (1):139-144.

Brown, C. J., S. J. Goss, D. B. Lubahn, D. R. Joseph, E. M. Wilson, F. S. French, and H. F. Willard. 1989. "Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism." *Am J Hum Genet* 44 (2):264-9.

Cavaco, Jose Eduardo B, Sandra S Laurentino, Alberto Barros, Mario Sousa, and Silvia Socorro. 2009. "Estrogen receptors α and β in human testis: both isoforms are expressed." *Syst Biol Reprod Med* 55 (4):137-144.

Chakraborti, S., and B. J. Bahnson. 2010. "Crystal structure of human senescence marker protein 30: insights linking structural, enzymatic, and physiological functions." *Biochemistry* 49 (16):3436-44. doi: 10.1021/bi9022297.

Chatterjee, S., E. de Lamirande, and C. Gagnon. 2001. "Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione." *Mol Reprod Dev* 60 (4):498-506. doi: 10.1002/mrd.1115.

Correia, S, PF Oliveira, PM Guerreiro, G Lopes, MG Alves, AVM Canário, JE Cavaco, and Sílvia Socorro. 2013. "Sperm parameters and epididymis function in transgenic rats overexpressing the Ca^{2+}-binding protein regucalcin: a hidden role for Ca^{2+} in sperm maturation?" *Mol Hum Reprod* 19 (9):581-589.

Correia, S., M. G. Alves, P. F. Oliveira, M. R. Alves, A. M. van Pelt, J. E. Cavaco, and S. Socorro. 2014a. "Transgenic overexpression of regucalcin leads to suppression of thapsigargin- and actinomycin D-induced apoptosis in the testis by modulation of apoptotic pathways." *Andrology* 2 (2):290-298. doi: 10.1111/j.2047-2927.2014.00186.x.

Correia, Sara. 2014. "Estrogens and regucalcin in testicular apoptosis and sperm function: “a matter of life and death"." University of Beira Interior.

Correia, Sara, Mário R Alves, José E Cavaco, Pedro F Oliveira, and Sílvia Socorro. 2014b. "Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility." *Fertil Steril* 102 (1):299-306.

Correia, Sara, Henrique J Cardoso, José E Cavaco, and Sílvia Socorro. 2015. "Oestrogens as apoptosis regulators in mammalian testis: angels or devils?" *Expert Rev Mol Med* 17:e2.

Correia, Sara, Cátia V Vaz, Ana Silva, José E Cavaco, and Sílvia Socorro. 2017. "Regucalcin counteracts tert-butyl hydroperoxide and cadmium-induced oxidative stress in rat testis." *J Appl Toxicol* 37(2):159-166. doi: 10.1002/jat.3333.

Costello, S., F. Michelangeli, K. Nash, L. Lefievre, J. Morris, G. Machado-Oliveira, C. Barratt, J. Kirkman-Brown, and S. Publicover. 2009. "Ca^{2+}-stores in sperm: their identities and functions." *Reproduction* 138 (3):425-37. doi: 10.1530/rep-09-0134.

Cross, N. L. 1996. "Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor." *Biol Reprod* 54 (1):138-45.

Dacheux, Jean-Louis, Clémence Belleannée, Russell Jones, Valérie Labas, Maya Belghazi, Benoît Guyonnet, Xavier Druart, Jean Luc Gatti, and Françoise Dacheux. 2009. "Mammalian epididymal proteome." *Mol Cell Endocrinol* 306 (1):45-50.

Darszon, A., P. Lopez-Martinez, J. J. Acevedo, A. Hernandez-Cruz, and C. L. Trevino. 2006. "T-type Ca2+ channels in sperm function." *Cell Calcium* 40 (2):241-52. doi: 10.1016/j.ceca.2006.04.028.

de Lamirande, E., K. Yoshida, T. M. Yoshiike, T. Iwamoto, and C. Gagnon. 2001. "Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process." *J Androl* 22 (4):672-9.

Desnoyers, L., and P. Manjunath. 1992. "Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid." *J Biol Chem* 267 (14):10149-55.

Doran, P., P. Dowling, P. Donoghue, M. Buffini, and K. Ohlendieck. 2006. "Reduced expression of regucalcin in young and aged mdx diaphragm indicates abnormal cytosolic calcium handling in dystrophin-deficient muscle." *Biochim Biophys Acta* 1764 (4):773-85. doi: 10.1016/j.bbapap.2006.01.007.

Fayad, Tania, Valérie Lévesque, Jean Sirois, David W Silversides, and Jacques G Lussier. 2004. "Gene expression profiling of differentially expressed genes in granulosa cells of bovine dominant follicles using suppression subtractive hybridization." *Biol Reprod* 70 (2):523-533.

Ferlin, A., C. Vinanzi, A. Garolla, R. Selice, D. Zuccarello, C. Cazzadore, and C. Foresta. 2006. "Male infertility and androgen receptor gene mutations: clinical features and identification of seven novel mutations." *Clin Endocrinol (Oxf)* 65 (5):606-10. doi: 10.1111/j.1365-2265.2006.02635.x.

Figueira, Marília, Henrique Cardoso, and Silvia Socorro. 2018. "The Role of GPER Signaling in Carcinogenesis: a Focus on Prostate Cancer." In *Recent Trends in Cancer Biology: Spotlight on Signaling Cascades and microRNAs*, 59-117. New York, USA: Springer.

Florman, H. M., C. Arnoult, I. G. Kazam, C. Li, and C. M. O'Toole. 1998. "A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels." *Biol Reprod* 59 (1):12-6. doi: 10.1095/biolreprod59.1.12.

Florman, H. M., M. K. Jungnickel, and K. A. Sutton. 2008. "Regulating the acrosome reaction." *Int J Dev Biol* 52 (5-6):503-10. doi: 10.1387/ijdb.082696hf.

Foresta, C., and M. Rossato. 1997. "Calcium influx pathways in human spermatozoa." *Mol Hum Reprod* 3 (1):1-4. doi: 10.1093/molehr/3.1.1.

Fujita, T., J. L. Mandel, T. Shirasawa, O. Hino, T. Shirai, and N. Maruyama. 1995. "Isolation of cDNA clone encoding human homologue of senescence marker protein-30 (SMP30) and its location on the X chromosome." *Biochim Biophys Acta* 1263 (3):249-52.

Fujita, T., T. Shirasawa, K. Uchida, and N. Maruyama. 1996a. "Gene regulation of senescence marker protein-30 (SMP30): coordinated up-regulation with tissue maturation and gradual down-regulation with aging." *Mech Ageing Dev* 87 (3):219-29.

Fujita, Toshiko, Takuji Shirasawa, and Naoki Maruyama. 1996b. "Isolation and characterization of genomic and cDNA clones encoding mouse senescence marker protein-30 (SMP30)." *Biochim Biophys Acta (BBA)-Gene Structure and Expression* 1308 (1):49-57.

Fujita, Toshiko, Takuji Shirasawa, Kazumi Uchida, and Naoki Maruyama. 1992. "Isolation of cDNA clone encoding rat senescence marker protein-30 (SMP30) and its tissue distribution." *Biochim Biophys Acta (BBA)-Gene Structure and Expression* 1132 (3):297-305.

Fukaya, Yuko, and Masayoshi Yamaguchi. 2004. "Regucalcin increases superoxide dismutase activity in rat liver cytosol." *Biol Pharm Bull* 27 (9):1444-1446.

Gervasi, M. G., and P. E. Visconti. 2017. "Molecular changes and signaling events occurring in spermatozoa during epididymal maturation." *Andrology* 5 (2):204-218. doi: 10.1111/andr.12320.

Gomi, K., K. Hirokawa, and N. Kajiyama. 2002. "Molecular cloning and expression of the cDNAs encoding luciferin-regenerating enzyme from Luciola cruciata and Luciola lateralis." *Gene* 294 (1-2):157-66.

Gorczynska, E., and D. J. Handelsman. 1995. "Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells." *Endocrinology* 136 (5):2052-2059. doi: 10.1210/endo.136.5.7720654.

Goto, S. G. 2000. "Expression of Drosophila homologue of senescence marker protein-30 during cold acclimation." *J Insect Physiol* 46 (7):1111-1120.

Handa, Setsuko, Naoki Maruyama, and Akihito Ishigami. 2009. "Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells." *Biol Pharm Bull* 32 (10):1645-1648.

Harikrishna, P., J. Thomas, A. M. Shende, and S. K. Bhure. 2017. "Calcium Binding Ability of Recombinant Buffalo Regucalcin: A Study Using Circular Dichroism Spectroscopy." *Protein J* 36 (2):108-111. doi: 10.1007/s10930-017-9701-6.

Hewitt, S. C., and K. S. Korach. 2018. "Estrogen Receptors: New Directions in the New Millennium." *Endocr Rev* 39 (5):664-675. doi: 10.1210/er.2018-00087.

Hezavehei, M., M. Sharafi, H. M. Kouchesfahani, R. Henkel, A. Agarwal, V. Esmaeili, and A. Shahverdi. 2018. "Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches." *Reprod Biomed Online* 37 (3):327-339. doi: 10.1016/j.rbmo.2018.05.012.

Hirohashi, N., and R. Yanagimachi. 2018. "Sperm acrosome reaction: its site and role in fertilization." *Biol Reprod* 99 (1):127-133. doi: 10.1093/biolre/ioy045.

Holdcraft, R. W., and R. E. Braun. 2004. "Hormonal regulation of spermatogenesis." *Int J Androl* 27 (6):335-342. doi: 10.1111/j.1365-2605.2004.00502.x.

Ichikawa, E., Y. Tsurusaki, and M. Yamaguchi. 2004a. "Suppressive effect of regucalcin on protein phosphatase activity in the heart cytosol of normal and regucalcin transgenic rats." *Int J Mol Med* 13 (2):289-93.

Ichikawa, Emiko, and Masayoshi Yamaguchi. 2004b. "Regucalcin increases superoxide dismutase activity in the heart cytosol of normal and regucalcin transgenic rats." *Int J Mol Med* 14:691-696.

Ishigami, A., S. Handa, N. Maruyama, and P. C. Supakar. 2003. "Nuclear localization of senescence marker protein-30, SMP30, in cultured mouse hepatocytes and its similarity to RNA polymerase." *Biosci Biotechnol Biochem* 67 (1):158-60.

Jeong, D. H., M. J. Goo, I. H. Hong, H. J. Yang, M. R. Ki, S. H. Do, J. H. Ha, S. S. Lee, J. K. Park, and K. S. Jeong. 2008. "Inhibition of radiation-induced apoptosis via overexpression of SMP30 in Smad3-knockout mice liver." *J Radiat Res* 49 (6):653-60.

Jung, K. J., N. Maruyama, A. Ishigami, B. P. Yu, and H. Y. Chung. 2006. "The redox-sensitive DNA binding sites responsible for age-related downregulation of SMP30 by ERK pathway and reversal by calorie restriction." *Antioxid Redox Signal* 8 (3-4):671-80. doi: 10.1089/ars.2006.8.671.

Kim, H. S., T. G. Son, H. R. Park, Y. Lee, Y. Jung, A. Ishigami, and J. Lee. 2013. "Senescence marker protein 30 deficiency increases Parkinson's pathology by impairing astrocyte activation." *Neurobiol Aging* 34 (4):1177-83. doi: 10.1016/j.neurobiolaging.2012.10.008.

Kondo, Yoshitaka, Yoko Inai, Yasunori Sato, Setsuko Handa, Sachiho Kubo, Kentaro Shimokado, Sataro Goto, Morimitsu Nishikimi, Naoki Maruyama, and Akihito Ishigami. 2006. "Senescence marker protein 30

functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy." *Proc Natl Acad Sci U S A* 103 (15):5723-5728.

Kondo, Yoshitaka, Toru Sasaki, Yasunori Sato, Akiko Amano, Shingo Aizawa, Mizuki Iwama, Setsuko Handa, Nobuko Shimada, Mitsugu Fukuda, and Masumi Akita. 2008. "Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice." *Biochem Biophys Res Commun* 377 (1):291-296.

Kosugi, S., M. Hasebe, M. Tomita, and H. Yanagawa. 2009. "Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs." *Proc Natl Acad Sci U S A* 106 (25):10171-6. doi: 10.1073/pnas.0900604106.

Kurota, Hideyuki, and Masayoshi Yamaguchi. 1996. "Steroid hormonal regulation of calcium-binding protein regucalcin mRNA expression in the kidney cortex of rats." *Mol Cell Biochem* 155 (2):105-111.

Kwon, W. S., Y. J. Park, S. A. Mohamed el, and M. G. Pang. 2013. "Voltage-dependent anion channels are a key factor of male fertility." *Fertil Steril* 99 (2):354-61. doi: 10.1016/j.fertnstert.2012.09.021.

L., O'Hara, and Smith L.B. 2017. "The Genetics of Androgen Receptor Signalling in Male Fertility." In *Genetics of Human Infertility*, edited by P.H. Vogt, 86-100. Basel, Karger.

Laurentino, Sandra, João Gonçalves, José Eduardo Cavaco, Pedro Fontes Oliveira, Marco G Alves, Mário de Sousa, Alberto Barros, and Sílvia Socorro. 2011a. "Apoptosis-inhibitor Aven is downregulated in defective spermatogenesis and a novel estrogen target gene in mammalian testis." *Fertil Steril* 96 (3):745-750.

Laurentino, Sandra S, Sara Correia, Jose E Cavaco, Pedro Fontes Oliveira, Luis Rato, Mario Sousa, Alberto Barros, and Sílvia Socorro. 2011b. "Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis." *Reproduction* 142 (3):447-456.

Laurentino, Sandra S, Sara Correia, José Eduardo Cavaco, Pedro Fontes Oliveira, Mário de Sousa, Alberto Barros, and Sílvia Socorro. 2012. "Regucalcin, a calcium-binding protein with a role in male reproduction?" *Mol Hum Reprod* 18 (4):161-170.

Lin, P. H., C. Y. Jian, J. C. Chou, C. W. Chen, C. C. Chen, C. Soong, S. Hu, F. K. Lieu, P. S. Wang, and S. W. Wang. 2016. "Induction of renal senescence marker protein-30 (SMP30) expression by testosterone and its contribution to urinary calcium absorption in male rats." *Sci Rep* 6:32085. doi: 10.1038/srep32085.

Lin, William W, Dolores J Lamb, Thomas M Wheeler, Jacki Abrams, Larry I Lipshultz, and Edward D Kim. 1997. "Apoptotic frequency is increased in

spermatogenic maturation arrest and hypospermatogenic states." *J Urol* 158 (5):1791-1793.

Linster, C. L., and E. Van Schaftingen. 2007. "Vitamin C. Biosynthesis, recycling and degradation in mammals." *Febs J* 274 (1):1-22. doi: 10.1111/j.1742-4658.2006.05607.x.

Lishko, P. V., and Y. Kirichok. 2010. "The role of Hv1 and CatSper channels in sperm activation." *J Physiol* 588 (Pt 23):4667-72. doi: 10.1113/jphysiol.2010.194142.

Lv, Sa, Jiang-hua Wang, Feng Liu, Yan Gao, Ran Fei, Shao-cai Du, and Lai Wei. 2008. "Senescence marker protein 30 in acute liver failure: validation of a mass spectrometry proteomics assay." *BMC Gastroenterol* 8 (1):17.

Maia, Claudio JB, Cecilia R Santos, Fernando Schmitt, and Sílvia Socorro. 2008. "Regucalcin is expressed in rat mammary gland and prostate and down-regulated by 17β-estradiol." *Mol Cell Biochem* 311 (1-2):81-86.

Maia, Cláudio, Cecília Santos, Fernando Schmitt, and Silvia Socorro. 2009. "Regucalcin is under-expressed in human breast and prostate cancers: Effect of sex steroid hormones." *J Cell Biochem* 107 (4):667-676.

Maneesh, M., and H. Jayalekshmi. 2006. "Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction." *Indian J Clin Biochem* 21 (2):80-9. doi: 10.1007/bf02912918.

Mannowetz, N., N. M. Naidoo, S. A. Choo, J. F. Smith, and P. V. Lishko. 2013. "Slo1 is the principal potassium channel of human spermatozoa." *Elife* 2:e01009. doi: 10.7554/eLife.01009.

Marques, Ricardo, Cláudio J Maia, Cátia Vaz, Sara Correia, and Sílvia Socorro. 2014. "The diverse roles of calcium-binding protein regucalcin in cell biology: from tissue expression and signalling to disease." *Cell Mol Life Sci* 71 (1):93-111.

Marques, Ricardo, Cátia V Vaz, Cláudio J Maia, Madalena Gomes, Adelina Gama, Gilberto Alves, Cecília R Santos, Fernando Schmitt, and Sílvia Socorro. 2015. "Histopathological and in vivo evidence of regucalcin as a protective molecule in mammary gland carcinogenesis." *Exp Cell Res* 330 (2):325-335.

Maruyama, N., A. Ishigami, and Y. Kondo. 2010. "Pathophysiological significance of senescence marker protein-30." *Geriatr Gerontol Int* 10 Suppl 1:S88-98. doi: 10.1111/j.1447-0594.2010.00586.x.

Mateus, I., M. Feijo, L. M. Espinola, C. V. Vaz, S. Correia, and S. Socorro. 2018. "Glucose and glutamine handling in the Sertoli cells of transgenic rats overexpressing regucalcin: plasticity towards lactate production." *Sci Rep* 8 (1):10321. doi: 10.1038/s41598-018-28668-4.

McLaughlin, E. A., and W. C. Ford. 1994. "Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone." *Mol Reprod Dev* 37 (2):241-6. doi: 10.1002/mrd.1080370216.

Misawa, H., and M. Yamaguchi. 2000. "The gene of Ca^{2+}-binding protein regucalcin is highly conserved in vertebrate species." *Int J Mol Med* 6 (2):191-6.

Mori, Takanori, Akihito Ishigami, Kuniaki Seyama, Reiko Onai, Sachiho Kubo, Kazue Shimizu, Naoki Maruyama, and Yoshinosuke Fukuchi. 2004. "Senescence marker protein-30 knockout mouse as a novel murine model of senile lung." *Pathol Int* 54 (3):167-173.

Morooka, Y., and M. Yamaguchi. 2001. "Inhibitory effect of regucalcin on protein phosphatase activity in the nuclei of rat kidney cortex." *J Cell Biochem* 83 (1):111-20.

Morooka, Y., and M. Yamaguchi. 2002. "Endogenous regucalcin suppresses the enhancement of protein phosphatase activity in the cytosol and nucleus of kidney cortex in calcium-administered rats." *J Cell Biochem* 85 (3):553-60. doi: 10.1002/jcb.10157.

Nakagawa, T., and M. Yamaguchi. 2008. "Nuclear localization of regucalcin is enhanced in culture with protein kinase C activation in cloned normal rat kidney proximal tubular epithelial NRK52E cells." *Int J Mol Med* 21 (5):605-10.

Nakagawa, Taeko, and Masayoshi Yamaguchi. 2006. "Overexpression of regucalcin enhances its nuclear localization and suppresses L-type Ca^{2+} channel and calcium-sensing receptor mRNA expressions in cloned normal rat kidney proximal tubular epithelial NRK52E cells." *J Cell Biochem* 99 (4):1064-1077.

Nakajima, Y., and S. Natori. 2000. "Identification and characterization of an anterior fat body protein in an insect." *J Biochem* 127 (5):901-8.

Nikapitiya, C., M. De Zoysa, H. S. Kang, C. Oh, I. Whang, and J. Lee. 2008. "Molecular characterization and expression analysis of regucalcin in disk abalone (Haliotis discus discus): intramuscular calcium administration stimulates the regucalcin mRNA expression." *Comp Biochem Physiol B Biochem Mol Biol* 150 (1):117-24. doi: 10.1016/j.cbpb.2008.02.004.

Oliveira, Pedro F, Marco G Alves, Ana D Martins, Sara Correia, Raquel L Bernardino, Joaquina Silva, Alberto Barros, Mário Sousa, José E Cavaco, and Sílvia Socorro. 2014. "Expression pattern of G protein-coupled receptor 30 in human seminiferous tubular cells." *Gen Comp Endocrinol* 201:16-20.

Omura, Masami, and Masayoshi Yamaguchi. 1999. "Regulation of protein phosphatase activity by regucalcin localization in rat liver nuclei." *J Cell Biochem* 75 (3):437-445.

Paudel, K. P., S. Kumar, S. K. Meur, and A. Kumaresan. 2010. "Ascorbic acid, catalase and chlorpromazine reduce cryopreservation-induced damages to crossbred bull spermatozoa." *Reprod Domest Anim* 45 (2):256-62. doi: 10.1111/j.1439-0531.2008.01278.x.

Picotto, G., G. Vazquez, and R. Boland. 1999. "17beta-oestradiol increases intracellular Ca^{2+} concentration in rat enterocytes. Potential role of phospholipase C-dependent store-operated Ca2+ influx." *Biochem J* 339 (Pt 1):71-7.

Pillai, H., M. S. Parmar, A. M. Shende, J. Thomas, H. S. Sharma, G. T. Sharma, S. K. Ghosh, A. Kumaresan, and S. K. Bhure. 2017a. "Effect of supplementation of recombinant Regucalcin in extender on cryopreservation of spermatozoa of water buffalo (Bubalus bubalis)." *Mol Reprod Dev* 84 (11):1133-1139. doi: 10.1002/mrd.22873.

Pillai, H., A. M. Shende, M. S. Parmar, A. A, S. L, A. Kumaresan, T. S. G, and S. K. Bhure. 2017b. "Regucalcin is widely distributed in the male reproductive tract and exerts a suppressive effect on in vitro sperm capacitation in the water buffalo (Bubalus bubalis)." *Mol Reprod Dev* 84 (3):212-221. doi: 10.1002/mrd.22767.

Pillai, H., A. M. Shende, M. S. Parmar, J. Thomas, H. S. Kartha, G. Taru Sharma, S. K. Ghosh, and S. K. Bhure. 2017c. "Detection and localization of regucalcin in spermatozoa of water buffalo (Bubalus bubalis): A calcium-regulating multifunctional protein." *Reprod Domest Anim* 52 (5):865-872. doi: 10.1111/rda.12991.

Pinto, Patrícia, Maria Estêvão, and Silvia Socorro. 2014. "Androgen receptors in non-mammalian vertebrates: Structure and function." In *Androgen Receptor: Structural Biology, Genetics and Molecular Defects*, 1-26. New York, USA: Nova Science Publishers, Inc.

Print, Cristin G, and Kate Lakoski Loveland. 2000. "Germ cell suicide: new insights into apoptosis during spermatogenesis." *Bioessays* 22 (5):423-430.

Publicover, S. J., and C. L. Barratt. 1999. "Voltage-operated Ca2+ channels and the acrosome reaction: which channels are present and what do they do?" *Hum Reprod* 14 (4):873-9.

Puga Molina, L. C., G. M. Luque, P. A. Balestrini, C. I. Marin-Briggiler, A. Romarowski, and M. G. Buffone. 2018. "Molecular Basis of Human Sperm Capacitation." *Front Cell Dev Biol* 6:72. doi: 10.3389/fcell.2018.00072.

Ren, Dejian, and Jingsheng Xia. 2010. "Calcium signaling through CatSper channels in mammalian fertilization." *Physiology* 25 (3):165-175.

Robaire, Bernard, Barry T Hinton, and Marie-Claire Orgebin-Crist. 2006. "The epididymis." In *Knobil and Neil´s Physiology of Reproduction*, 1071-1148. San Diego, CA: Elsevier.

Said, T. M., S. Grunewald, U. Paasch, M. Rasch, A. Agarwal, and H. J. Glander. 2005. "Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates." *Fertil Steril* 83 (5):1442-6. doi: 10.1016/j.fertnstert.2004.11.052.

Sato, T., K. Seyama, Y. Sato, H. Mori, S. Souma, T. Akiyoshi, Y. Kodama, T. Mori, S. Goto, K. Takahashi, Y. Fukuchi, N. Maruyama, and A. Ishigami. 2006.

"Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking." *Am J Respir Crit Care Med* 174 (5):530-537. doi: 10.1164/rccm.200511-1816OC.

Sato, Y., S. Kajiyama, A. Amano, Y. Kondo, T. Sasaki, S. Handa, R. Takahashi, M. Fukui, G. Hasegawa, N. Nakamura, H. Fujinawa, T. Mori, M. Ohta, H. Obayashi, N. Maruyama, and A. Ishigami. 2008. "Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice." *Biochem Biophys Res Commun* 375 (3):346-50. doi: 10.1016/j.bbrc.2008.08.020.

Shamsi, M. B., K. Kumar, and R. Dada. 2011. "Genetic and epigenetic factors: Role in male infertility." *Indian J Urol* 27 (1):110-20. doi: 10.4103/0970-1591.78436.

Shimokawa, N., M. Isogai, and M. Yamaguchi. 1995a. "Specific species and tissue differences for the gene expression of calcium-binding protein regucalcin." *Mol Cell Biochem* 143 (1):67-71.

Shimokawa, Noriaki, Yoichi Matsuda, and Masayoshi Yamaguchi. 1995b. "Genomic cloning and chromosomal assignment of rat regucalcin gene." *Mol Cell Biochem* 151 (2):157-163.

Shimokawa, Noriaki, and Masayoshi Yamaguchi. 1993. "Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver." *FEBS letters* 327 (3):251-255.

Sievers, F., A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J. D. Thompson, and D. G. Higgins. 2011. "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega." *Mol Syst Biol* 7:539. doi: 10.1038/msb.2011.75.

Silva, A. M., S. Correia, S. Socorro, and C. J. Maia. 2016a. "Endogenous Factors in the Recovery of Reproductive Function After Testicular Injury and Cancer." *Curr Mol Med* 16 (7):631-649.

Silva, Ana MS, Sara Correia, João E Casalta-Lopes, Ana C Mamede, José EB Cavaco, Maria F Botelho, Sílvia Socorro, and Cláudio J Maia. 2016b. "The protective effect of regucalcin against radiation-induced damage in testicular cells." *Life Sciences* 164:31-41.

Socorro, S., D. M. Power, P. E. Olsson, and A. V. Canario. 2000. "Two estrogen receptors expressed in the teleost fish, Sparus aurata: cDNA cloning, characterization and tissue distribution." *J Endocrinol* 166 (2):293-306.

Son, T. G., S. J. Kim, K. Kim, M. S. Kim, H. Y. Chung, and J. Lee. 2008. "Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress." *Arch Pharm Res* 31 (7):872-877. doi: 10.1007/s12272-001-1240-3.

Son, T. G., H. R. Park, S. J. Kim, K. Kim, M. S. Kim, A. Ishigami, S. Handa, N. Maruyama, H. Y. Chung, and J. Lee. 2009. "Senescence marker protein 30 is up-regulated in kainate-induced hippocampal damage through ERK-

mediated astrocytosis." *J Neurosci Res* 87 (13):2890-7. doi: 10.1002/jnr.22122.

Son, Tae Gen, Yani Zou, Kyung Jin Jung, Byung Pal Yu, Akihito Ishigami, Naoki Maruyama, and Jaewon Lee. 2006. "SMP30 deficiency causes increased oxidative stress in brain." *Mech Ageing Dev* 127 (5):451-457.

Starvaggi Cucuzza, L., S. Divari, C. Mulasso, B. Biolatti, and F. T. Cannizzo. 2014. "Regucalcin expression in bovine tissues and its regulation by sex steroid hormones in accessory sex glands." *PLoS One* 9 (11):e113950. doi: 10.1371/journal.pone.0113950.

Stouffs, Katrien, Herman Tournaye, Inge Liebaers, and Willy Lissens. 2009. "Male infertility and the involvement of the X chromosome." *Hum Reprod Update* 15 (6):623-637.

Suarez, S. S. 2008. "Control of hyperactivation in sperm." *Hum Reprod Update* 14 (6):647-57. doi: 10.1093/humupd/dmn029.

Sun, Y. H., X. Gao, Y. J. Tang, C. L. Xu, and L. H. Wang. 2006. "Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells." *J Androl* 27 (5):671-8. doi: 10.2164/jandrol.106.000554.

Takagi, Seiji, Naoki Itoh, Makoto Kimura, Takumi Sasao, and Taiji Tsukamoto. 2001. "Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia." *Fertil Steril* 76 (5):901-907.

Takahashi, Hiroko, and Masayoshi Yamaguchi. 1993. "Regulatory effect of regucalcin on (Ca^{2+}– Mg^{2+})-ATPase in rat liver plasma membranes: comparison with the activation by Mn^{2+} and Co^{2+}." *Molecular and Cellular Biochemistry* 124 (2):169-174.

Takahashi, Hiroko, and Masayoshi Yamaguchi. 1999. "Role of regucalcin as an activator of Ca^{2+}-ATPase activity in rat liver microsomes." *Journal of Cellular Biochemistry* 74 (4):663-669.

Tobisawa, M., and M. Yamaguchi. 2003. "Role of endogenous regucalcin in brain function: suppression of cytosolic nitric oxide synthase and nuclear protein tyrosine phosphatase activities in brain tissue of transgenic rats." *Int J Mol Med* 12 (4):581-5.

Tomes, C. N., R. Carballada, D. F. Moses, D. F. Katz, and P. M. Saling. 1998. "Treatment of human spermatozoa with seminal plasma inhibits protein tyrosine phosphorylation." *Mol Hum Reprod* 4 (1):17-25.

Tsurusaki, Y, H Misawa, and M Yamaguchi. 2000. "Translocation of regucalcin to rat liver nucleus: involvement of nuclear protein kinase and protein phosphatase regulation." *Int J Mol Med* 6 (6):655-715.

van Dijk, K. D., H. W. Berendse, B. Drukarch, S. A. Fratantoni, T. V. Pham, S. R. Piersma, E. Huisman, J. J. Breve, H. J. Groenewegen, C. R. Jimenez, and W. D. van de Berg. 2012. "The proteome of the locus ceruleus in

Parkinson's disease: relevance to pathogenesis." *Brain Pathol* 22 (4):485-98. doi: 10.1111/j.1750-3639.2011.00540.x.

Vaz, C. V., S. Correia, H. J. Cardoso, M. I. Figueira, R. Marques, C. J. Maia, and S. Socorro. 2016a. "The Emerging Role of Regucalcin as a Tumor Suppressor: Facts and Views." *Curr Mol Med* 16 (7):607-619.

Vaz, C. V., D. B. Rodrigues, S. Socorro, and C. J. Maia. 2015a. "Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells." *Biochim Biophys Acta* 1853 (10 Pt A):2621-8. doi: 10.1016/j.bbamcr.2015.07.006.

Vaz, Cátia V, Cláudio J Maia, Ricardo Marques, Inês M Gomes, Sara Correia, Marco G Alves, José E Cavaco, Pedro F Oliveira, and Sílvia Socorro. 2014. "Regucalcin is an androgen-target gene in the rat prostate modulating cell-cycle and apoptotic pathways." *Prostate* 74 (12):1189-1198.

Vaz, Cátia V, Ricardo Marques, Henrique J Cardoso, Cláudio J Maia, and Sílvia Socorro. 2016b. "Suppressed glycolytic metabolism in the prostate of transgenic rats overexpressing calcium-binding protein regucalcin underpins reduced cell proliferation." *Transgenic Res* 25 (2):139-148.

Vaz, Cátia V, Ricardo Marques, Cláudio J Maia, and Sílvia Socorro. 2015b. "Aging-associated changes in oxidative stress, cell proliferation, and apoptosis are prevented in the prostate of transgenic rats overexpressing regucalcin." *Transl Res* 166(6):693-705. doi: 10.1016/j.trsl.2015.08.009.

Verhoeven, G., A. Willems, E. Denolet, J. V. Swinnen, and K. De Gendt. 2010. "Androgens and spermatogenesis: lessons from transgenic mouse models." *Philos Trans R Soc Lond B Biol Sci* 365 (1546):1537-56. doi: 10.1098/rstb.2009.0117.

Vesala, L., T. S. Salminen, M. Kankare, and A. Hoikkala. 2012. "Photoperiodic regulation of cold tolerance and expression levels of regucalcin gene in Drosophila montana." *J Insect Physiol* 58 (5):704-9. doi: 10.1016/j.jinsphys.2012.02.004.

Walker, William H. 2009. "Molecular mechanisms of testosterone action in spermatogenesis." *Steroids* 74 (7):602-607.

Walker, William H, and Jing Cheng. 2005. "FSH and testosterone signaling in Sertoli cells." *Reproduction* 130 (1):15-28.

Wennemuth, Gunther, Donner F Babcock, and Bertil Hille. 2003. "Calcium clearance mechanisms of mouse sperm." *J Gen Physiol* 122 (1):115-128.

Wu, Y. D., L. Jiang, Z. Zhou, M. H. Zheng, J. Zhang, and Y. Liang. 2008. "CYP1A/regucalcin gene expression and edema formation in zebrafish embryos exposed to 2,3,7,8-Tetrachlorodibenzo-p-dioxin." *Bull Environ Contam Toxicol* 80 (6):482-6. doi: 10.1007/s00128-008-9395-8.

Yamaguchi, M, and S Mori. 1989. "Activation of hepatic microsomal Ca^{2+}-adenosine triphosphatase by calcium-binding protein regucalcin." *Chemical and Pharmaceutical Bulletin* 37 (4):1031-1034.

Yamaguchi, M. 1988. "Physicochemical properties of calcium-binding protein isolated from rat liver cytosol: Ca2+-induced conformational changes." *Chem Pharm Bull (Tokyo)* 36 (1):286-90.

Yamaguchi, M. 2010. "Regucalcin and metabolic disorders: osteoporosis and hyperlipidemia are induced in regucalcin transgenic rats." *Mol Cell Biochem* 341 (1-2):119-33. doi: 10.1007/s11010-010-0443-4.

Yamaguchi, M. 2015. "Involvement of regucalcin as a suppressor protein in human carcinogenesis: insight into the gene therapy." *J Cancer Res Clin Oncol* 141 (8):1333-41. doi: 10.1007/s00432-014-1831-z.

Yamaguchi, M., and K. Oishi. 1995. "17 beta-Estradiol stimulates the expression of hepatic calcium-binding protein regucalcin mRNA in rats." *Mol Cell Biochem* 143 (2):137-141.

Yamaguchi, Masayoshi. 2000. "Role of regucalcin in calcium signaling." *Life Sci* 66 (19):1769-1780.

Yamaguchi, Masayoshi. 2005. "Role of regucalcin in maintaining cell homeostasis and function (review)." *Int J Mol Med* 15 (3):371-390.

Yamaguchi, Masayoshi. 2013. "The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways." *Apoptosis* 18 (10):1145-1153.

Yamaguchi, Masayoshi, Yoshiko Morooka, Hiroyuki Misawa, Yoshinori Tsurusaki, and Rie Nakajima. 2002. "Role of endogenous regucalcin in transgenic rats: Suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal Ca^{2+}-ATPase activity." *J Cell Biochem* 86 (3):520-529.

Zheng, Ke, Fang Yang, and Peijing Jeremy Wang. 2010. "Regulation of Male Fertility by X-Linked Genes." *J Androl* 31 (1):79-85.

Zhou, Xinchang. 2010. "Roles of Androgen Receptor in Male and Female Reproduction: Lessons From Global and Cell-Specific Androgen Receptor Knockout (ARKO) Mice." *J Androl* 31 (3):235-243.